Three-dimensional scoring of zebrafish response to psychoactive drugs questions the predictive validity of two-dimensional analyses

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Abstract

Zebrafish (*Danio rerio*) have recently emerged as a valuable laboratory species in the field of behavioral pharmacology, where they afford rapid and precise high-throughput drug screening. Although the behavioral repertoire of this species manifests along three dimensions (3D), most of the efforts in behavioral pharmacology rely on 2D projections acquired from a single overhead or front camera. We recently showed that, compared to a 3D scoring approach, 2D analyses could lead to inaccurate claims regarding individual and social behavior of drug-free experimental subjects. Here, we examined whether this conclusion extended to the field of behavioral pharmacology by phenotyping adult zebrafish, acutely exposed to citalopram (30 mg/L, 50 mg/L, and 100 mg/L) or ethanol (0.25%, 0.50%, and 1.00%), in the novel tank diving test over a six-minute experimental session. We observed that both compounds modulated the time course of general locomotion and anxiety-related profiles, the latter being represented by specific behaviors (erratic movements and freezing) and avoidance of anxiety-eliciting areas of the test tank (top half and distance from the side walls). We observed that 2D projections of 3D trajectories (ground truth data) may introduce a source of unwanted variation in zebrafish behavioral phenotyping. Predictably, both 2D views underestimate absolute levels of general locomotion. Additionally, while data obtained from a camera positioned on top of the experimental tank are similar to those obtained from a 3D reconstruction, 2D front view data yield false negative findings.

Keywords: anxiety; automated tracking; citalopram; ethanol; novel tank diving test.

1. Introduction

Preclinical animal models constitute a central tool to detail the fundamental mechanisms underlying the expression of human emotions in physiological and pathological conditions [1]. Within this framework, several experimental models have been proposed to investigate the neurobiological processes underlying anxiety [2, 3], an evolutionarily preserved adaptive emotion, normally occurring as an anticipatory response to a potential threat [4]. The adaptive value of anxiety resides in the fact that it limits the negative outcomes associated with a potential threat [5]. Notwithstanding its adaptive nature, inappropriate (context-independent) or excess anxiety may often culminate in anxiety-related disorders that require medical attention [4].

In parallel with the aforementioned evolutionary roots, the underlying biological determinants of anxiety are very well conserved across different taxa. For example, the neuroendocrine machinery activated in response to external stressors exhibits striking homologies and analogies among fish [6], birds [7], rodents [8], monkeys [9], and humans [10]. Likewise, neurotransmitters such as serotonin have been associated with anxiety-related behaviors in species as diverse as fish [11], birds [12], humans [13], and sheep [14].

Although rodents have traditionally constituted the species of choice in this field [3, 15], zebrafish have recently emerged as an extremely promising experimental species [16-18]. The success of this freshwater species rests upon several advantages that range from genetic and neuroanatomic isomorphism between zebrafish and humans [19], to their small size and high reproductive rates favoring the execution of high-throughput studies [20]. In addition, the possibility to dissolve substances in water allows for the non-invasive administration of drugs readily absorbed through the gills [21]. These characteristics designate zebrafish as a fundamental tool in the field of psychopharmacology whereby they allow the preliminary screening of numerous drugs within spaces and time frames much smaller than those required by laboratory mammals [22].

High-throughput behavioral experiments on zebrafish generally share the following methodological structure: administration of water-soluble drugs, videorecording of observable phenotypes, offline scoring of video, coding of the observed behaviors, and data analysis [18].

Traditional behavioral phenotyping leveraged the use of a single camera positioned on top or in front of the experimental tank and the subsequent use of behavioral scoring software, in which the phenotype of interest had to be input by a trained observer [23, 24]. Albeit extremely productive, this approach was prone to observer bias and has been recently complemented by tracking algorithms capable of automatically coding and scoring zebrafish behavior with limited human supervision [25-28].

However, from the two-dimensional (2D) view offered by a single video-camera it is impossible to phenotype the 3D swimming pattern exhibited by zebrafish. This consideration prompted the design and development of experimental platforms capable of investigating zebrafish behavior adopting a 3D approach [29-32]. We recently demonstrated that the limitation of 2D scoring methods extends beyond the geometrical underestimation of swimming paths (3D trajectories being longer than their 2D projections by definition), and may result in numerous false positive and false negative findings [30]. Specifically, we first tested zebrafish in conventional binary choice behavioral assays, and then analyzed group differences based on 3D or 2D (top and front views) trajectories. This analysis demonstrated that 2D views generated approximately 20% of false findings, being represented by inappropriate reporting of significant inter-group differences in spite of undistinguishable ground truth data (false positives) or failure to detect significant results in instances in which experimental groups belonged to different populations (false negatives) [30].

In the present study, we aimed at prospectively investigating whether 3D scoring of zebrafish behavior may also benefit pharmacological research. To this aim, we exposed experimentally naïve zebrafish to drugs capable of modulating anxiety-related behaviors in both humans and zebrafish [23, 33], and then analyzed their phenotype in response to an anxiety-provoking test paradigm in 3D or in 2D (top and front views). Specifically, we investigated the behavior of zebrafish in a novel tank diving test in response to the administration of the selective serotonin reuptake inhibitor citalopram (30 mg/L, 50 mg/L, and 100 mg/L) or ethanol (0.25%, 0.50%, and 1.00%). The goal of this study was twofold: first, we sought to replicate existing findings indicating that ethanol [34] and citalopram [33] modulate anxiety in zebrafish (predictive validity of the assay), and then we aimed at testing whether the experimental advantages afforded by 3D scoring in drug-free states [30] also extend to zebrafish psychopharmacology.

The novel tank diving test has already been validated as a locomotion- and anxiety-related behavioral test [35]. Therein, anxiety is measured through the evaluation of fish position in the water column, swimming speed, erratic movements, and freezing, as functions of the time spent in the experimental tank from the initial release. In order to detail the specific information that can be potentially inferred from these measurements, we preliminarily conducted a principal component analysis (PCA) on nine behavioral measures, objectively scored from 3D trajectories (average speed, average peak speed, average angular speed, average peak angular speed, average acceleration, average peak acceleration, time spent freezing, time spent in the top half of the tank, and time spent in the vicinity of the walls). The PCA was aimed at detecting potential correlations among the variables and identifying underlying orthogonal factors associated with independent domains.

Grounded in our previous work, we anticipated 2D views to be characterized by reduced absolute locomotion values compared to 3D trajectories. Most importantly, in the light of the high rate of false findings observed in drug-free conditions [30], we expected the predictive validity of 2D trajectories to be potentially jeopardized. This hypothesis rests on the fact that, when exposed to psychoactive substances, fish may exhibit a series of responses that vary in space and time. For example, increased anxiety may reflect in a progressive reduction in general locomotion, increased freezing, erratic movements, and preference for the bottom of the experimental tank. These patterns manifest differentially depending on the time spent in the experimental apparatus (with preference for the bottom varying with the prolonged exposure), and on the view (i.e., top or front view). For example, while horizontal erratic movements are best detected through a top view, geotaxis can be appropriately scored only from a side view. Therefore, we hypothesized that the specific view may reflect into a bias in detecting time-dependent effects of psychoactive drugs, thereby potentially generating view × drug × experimental-progression effects.

2. Materials and methods

2.1 Animal care and maintenance

The experiments and analysis were performed and reported according to the ARRIVE guidelines [36]. A total of 112 wild-type adult zebrafish (*Danio rerio*), with a 1:1 male/female ratio were used in this study. The fish were purchased from Carolina Biological Supply Co. (Burlington, NC,

USA), and housed in 10 L (2.6 gallons) vivarium tanks (Pentair Aquatic Eco-Systems Locations, Cary, NC, USA), with a density of no more than 10 fish per tank. Fish were kept under a 12 h light/12 h dark photoperiod [37], and fed with commercial flake food (Hagen Corp. Nutrafin max, Mansfield, MA, USA) once a day, approximately at 7 PM. Water parameters of the holding tanks were regularly checked, and temperature and pH were maintained at 26 °C and 7.2 pH, respectively. Regular tap water was used with the addition of a stress coat to remove chlorine and chloramines. Prior to the beginning of the experiments, fish were acclimatized in the holding facility for a period of 12-15 days.

The number of fish used in the study – compatible with obtaining sufficiently reliable and biologically relevant data – was estimated through a power analysis. Briefly, we computed the minimum required sample size considering the two-tail Student t test for independent groups using the following values, based on the results of previous studies [23, 24, 38]: (i) standard deviation homogeneous among groups $s = 0.23$; (ii) Type I error probability $a = 0.05$ and power $1-b = 0.80$ (conventional values); and (iii) minimum difference between control and treatment group means $D = 0.17$. The sample size resulting from this calculation was 15 subjects per group. To promote the generalizability of our findings, we conducted experiments on both males and females. We thus increased the sample size to 16 per group (eight males and eight females). We estimated that a sample size of 16 subjects (per group) would have 80% power to detect a 0.60 effect size on the principal outcome measures with a 2-sided 0.05 significance level.

2.2 Experimental setup

To obtain 3D trajectories, we used two Flea 3 high resolution cameras (one overhead and one in front). The dimensions of the test tank were 29 cm (length) × 14 cm (height) × 8.5 cm (width) and water 13 cm deep, similar to tanks used in comparable studies [39]. To maximize the visual contrast and ease automatic tracking, the bottom of the tank was lined with white contact paper. The two short sides of the tank were covered with black contact paper to prevent reflection. On the other hand, the two long sides were kept transparent to allow frontal camera acquisition and avoid position bias (i.e., a potential side preference had one side been kept transparent for data acquisition and the other kept opaque). The experimental arena was surrounded by black curtains to prevent light reflection and visual disturbance from the outside.

2.3 Experimental procedure

Experiments, performed in June 2018, were conducted on seven groups, each consisting of 16 subjects (eight males and eight females). Specifically, the experimental design entailed one control group exposed to vehicle (water), three groups treated with citalopram (30 mg/L, 50 mg/L, 100 mg/L), and three groups treated with ethanol (0.25%, 0.50%, 1.00% ethanol/water solution in volume/volume %). The fish were randomly allocated to each of the seven conditions in the following way. The conditions were randomly distributed over several weeks, testing eight subjects per day (four in the morning and four in the afternoon). We balanced sex across conditions, and conditions across mornings and afternoons. Male and female fish were kept in separate tanks; in total, fish were housed in 12 tanks. At the beginning of each test session, we sampled one subject from a tank. Such a tank was different from that out of which we chose the previous subject tested in the same condition. This procedure guaranteed that potential tank effects were distributed evenly across all experimental groups.

Due to technical issues, four trials had to be discarded: this resulted in a slight reduction in the number of subjects in the 100 mg/L citalopram group (15 subjects instead of 16) and in both the 0.25% and 1.00% ethanol groups (14 and 15 subjects instead of 16, respectively). Following [33], we measured the effect of acute exposure to citalopram by treating the fish to the substance for five minutes before testing it. Following previous work on the effect of ethanol by our group [23], we measured the effect of exposure to ethanol over a one-hour period. In the interest of reducing the number of subjects used in animal experimentation, the same control subjects were used to test the effects of citalopram and ethanol. Fish were treated and tested in isolation.

Since these substances required a differential pre-exposure time (five minutes for citalopram and one hour for ethanol), we devised a common procedure for vehicle, citalopram and ethanol. Thus, one hour before testing, fish were placed in a 500 mL beaker filled with 450 mL of the following fluid: water for control and citalopram groups, or a solution of ethanol (0.25%, 0.50%, and 1.00%) for the other groups. Five minutes before testing, an additional 50 mL of fluid were slowly added to the beaker over a period of 20-30 seconds. These 50 mL were constituted by water for the control group, a solution of ethanol of the same concentration of that already present in the beaker for the ethanol groups, or a concentrated solution of citalopram that, when added to the 450 mL of water, resulted in a final concentration in the beaker of 30 mg/L, 50 mg/L, or 100

mg/L. Fish were left in the beaker for five minutes, at the end of which they were transferred to the test tank and filmed for six minutes.

Simultaneous recording from both cameras was initiated before transferring the fish into the test tank. In addition, at the beginning of the recording, a laser beam, visible from both cameras, was pointed into the test tank in order to ensure later synchronization of both video streams. At the end of the experiment, the fish was hand-netted into a separate tank.

2.4 Tracking and 3D reconstruction

Images recorded from the high-resolution cameras were processed through an in-house developed tracking software, see [40] for a detailed description. The top and front view cameras provided time series of the trajectory projected onto the x-y and x-z planes, respectively. Each pair of tracks were automatically synchronized using the common x coordinate along length of the tank. The time-series for each x coordinate of the pair were shifted relative to each other and the relative shift producing the smallest difference was selected. Once synchronized, the tracks from the top view and from the front views were combined to construct the x, y, and z coordinates of the trajectory in the three-dimensional space (see figure 1 for a representative trajectory exhibited by a control subject).

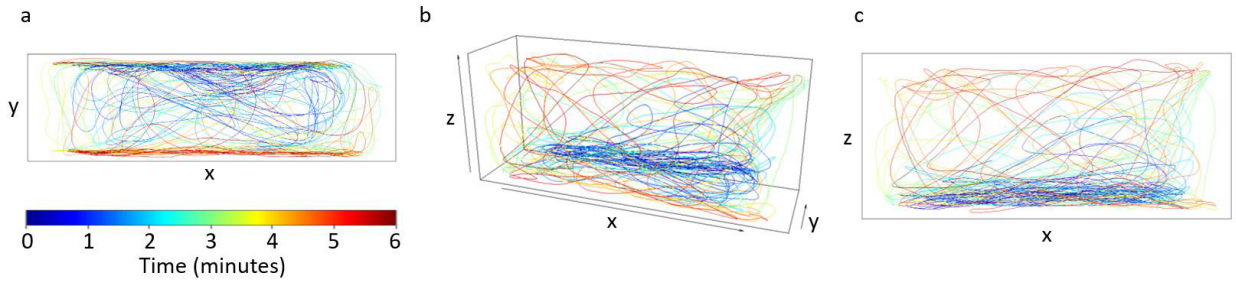

Figure 1. Trajectory for a single fish from a control trial from a) top view, b) 3D reconstructed trajectory obtained from synchronizing trajectories from top and front views, and c) front view. The color of the trajectory denotes the evolution of the position of the fish along the six-minute trial. The axes dimensions are 29 cm × 8.5 cm × 13 cm.

Reconstructed trajectories were used to quantify the following ethogram: time spent freezing (percentage of time that the fish moved less than 2 cm anywhere in the tank over a rolling period of 2 s), time spent wall following (percentage of time that the fish spent within 3 cm of any side wall or the bottom of the tank), average speed (time-average of the first-order numerical

differentiation of the position time series), average peak speed (time-average of the speed values greater than the 90th percentile), average acceleration (time-average of the magnitude of the first order numerical differentiation of the velocity time series), average peak acceleration (time-average of the acceleration values greater than the 90th percentile), average angular speed (time-computed on the basis of a finite difference approximation of the curvature of fish trajectories), average peak angular speed (time-average of the angular velocity values greater than the 90th percentile), and time spent in the top half of the water column. These nine measures were selected from the technical literature on zebrafish behavior in novel tank tests [35] and their objective scoring from 3D trajectories follows our previous work [30, 41].

2.5 Statistical analyses

Experiments with ethanol and citalopram were analyzed separately, but both were compared to the same control condition.

2.5.1 Principal component analysis on 3D data

Using raw data on all the nine measures of our ethogram identified from 3D trajectories, we conducted a PCA to identify correlation structure of behavior and potentially reduce the number of variables analyzed. Only principal components with eigenvalues larger than one were retained in the analysis. For each compound (citalopram or ethanol), the loadings were varimax-rotated, and the resulting scores for each principal component were used as dependent variables in a four (citalopram: vehicle, 30 mg/L, 50 mg/L, and 100 mg/L, or ethanol: vehicle, 0.25%, 0.50%, and 1.00%) × six (time bins, one minute each) × two (sex: male, female) repeated measures analysis of variance (ANOVA) for split-plot designs. Testing males and females served the aim to access a heterogeneous experimental population and therefore improve the generalizability of our findings.

PCA data, derived from 3D observations, have been used to test the efficacy of ethanol and citalopram in modifying zebrafish behavior. It was not possible to use PCA data to compare the different views since the variables loading on the principal components in 3D were more than those loading on principal components in 2D.

2.5.2 Statistical model to compare 2D and 3D analyses

To investigate whether 2D projections of 3D trajectories may introduce a confound in the predictive validity of behavioral data on each of the nine measures, we conducted another repeated measures ANOVA for split-plot designs. In this analysis, the two general models for citalopram and ethanol were, respectively: three (view: 3D, 2D top, 2D front) × four (treatment: vehicle, 30 mg/L, 50 mg/L, 100 mg/L) × six (time bins, one minute each) × two (sex: male, female), and three (view: 3D, 2D top, 2D front) × four (treatment: vehicle, 0.25%, 0.50%, 1.00% ethanol/water solution) × six (time bins, one minute each) × two (sex: male, female) repeated measures ANOVAs. Similar to the PCA analysis, predictions of the effect of sex were not considered.

For all ANOVAs, the distribution of the model residuals was visually inspected to verify that they were close to normality [42]. Statistical analyses were performed using R 3.5.0, with the aov function for ANOVAs, the prcomp function for the PCA, and the emmeans 1.3.0 package for post-hoc comparisons using the Dunnett's multiple comparisons test, comparing control to other conditions and first minute to other minutes.

This statistical model allowed testing the hypothesis that 2D views yielded spurious results compared to 3D data. While main effects of the view factor allowed assessing whether absolute values differed depending on the tracking method, significant interactions between view and any other factor suggested that the effects of the latter were moderated by the tracking method. For example, a significant view × treatment interaction would suggest that the effects of a given compound may vary as a function of how the behavior of the animal was scored (i.e., using 2D projections from top or front, or resorting to 3D trajectories). Upon detecting a significant interaction, we performed post-hoc comparisons, correcting for type-1 errors, to detail whether and which pairwise comparisons were significant. Among these comparisons, those contrasting 2D and 3D were germane to the key question of the study.

3. Results

3.1 Ethanol and citalopram alter individual habituation to the test

For citalopram and ethanol treatments, three principal components with eigenvalue larger than one were extracted by the PCA (table 1), accounting for 87% of the total variance. The first principal component, accounting for 47% of the variance, reflected locomotion, with positive loadings for average speed, average peak speed, average acceleration, and average peak acceleration, and a

modest negative loading for the time spent freezing. The second principal component, accounting for 26% of the variance, reflected anxiety-related behavioral patterns (behavioral anxiety) with positive loadings for average angular speed, average peak angular speed, and the time spent freezing. The third principal component, accounting for 11% of variance, reflected anxiety-related spatial preference (positional anxiety), with positive loadings for the time spent wall following, and negative loadings for the time spent in top half.

Table 1. Principal component analysis.

	Citalopram			Ethanol		
	Locomotion	Behavioral anxiety	Positional anxiety	Locomotion	Behavioral anxiety	Positional anxiety
Eigenvalues	4.29	2.34	1.19	4.29	2.47	1.06
Explained variance (%)	47.7	26.0	13.3	47.7	27.4	11.8
Cumulative variance (%)	47.7	73.6	86.9	47.7	75.1	86.9
Varimax-rotated loadings						
Speed	**0.938**	-0.261		**0.924**	-0.289	
Average peak speed	**0.948**	-0.172		**0.953**	-0.122	
Average angular speed		**0.939**			**0.946**	0.106
Average peak angular speed		**0.971**			**0.976**	0.113
Average acceleration	**0.977**			**0.976**		
Average peak acceleration	**0.942**			**0.969**		
Freezing	-0.542	**0.760**		-0.524	**0.746**	0.170
Wall following		0.176	**0.817**		0.309	0.670
Time in top half	-0.213		**-0.788**	-0.118		**-0.873**

Summary results from the principal component analysis for citalopram and ethanol conditions. Principal components with eigenvalue larger than 1 are shown. Loadings greater than 0.7 or smaller than -0.7 are emboldened; loadings smaller than 0.1 in magnitude are not displayed.

When analyzing the three factors identified by PCA, we observed that absolute levels of locomotion were indistinguishable between control and citalopram-treated subjects (condition: $F_{3,55} = 0.52$, $P = 0.668$) (figure 2a). Additionally, general locomotion steadily declined throughout the experimental session in all subjects (time: $F_{5,275} = 3.03$, $P = 0.011$; $t_{275} > 3.12$, $P < 0.009$), regardless of the specific experimental group (time bins × condition: $F_{15,275} = 1.13$, $P = 0.329$). Absolute values of behavioral anxiety did not significantly vary across citalopram conditions (condition: $F_{3,55} = 0.76$, $P = 0.524$) (figure 2b). Yet, it significantly decreased over the trial (time:

$F_{5,275} = 3.52$, $P = 0.004$; $t_{275} > 2.69$, $P < 0.033$), albeit at a different rate (time bins × condition: $F_{15,275} = 1.85$, $P = 0.029$). Specifically, while behavioral anxiety remained constant throughout the experimental session in citalopram 50 mg/L and 100 mg/L conditions, it significantly declined in control and citalopram 30 mg/L conditions ($t_{275} > 2.60$, $P < 0.043$). While positional anxiety did not significantly vary across citalopram conditions (condition: $F_{3,55} = 0.95$, $P = 0.421$) (figure 2c), it significantly increased over time (time: $F_{5,275} = 3.51$, $P = 0.004$; $t_{275} > 2.82$, $P < 0.023$). Such time-dependent profile varied depending on the experimental treatment (time bins × condition: $F_{15,275} = 1.79$, $P = 0.036$). Thus, while it remained constant in control and citalopram 100 mg/L, it was low at the beginning of the test session and steadily increased in citalopram 30 mg/L and citalopram 50 mg/L subjects ($t_{275} > 2.74$, $P < 0.029$).

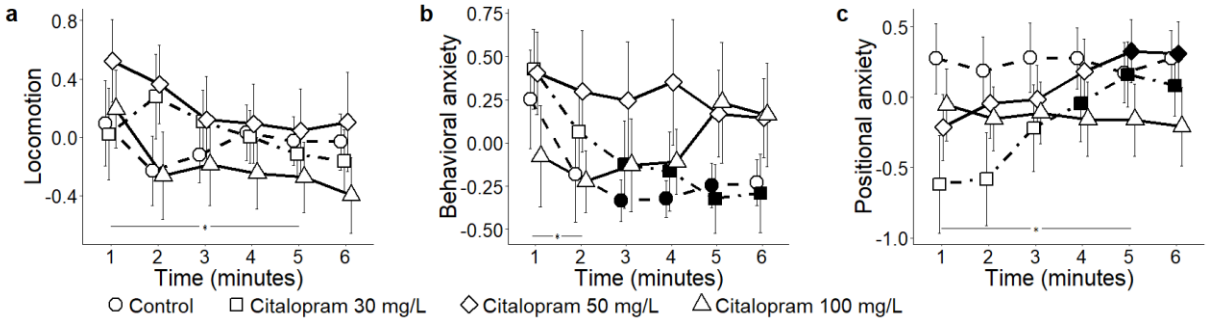

Figure 2. Mean ± standard error for a) locomotion, b) behavioral anxiety, and c) positional anxiety, over six-minute trials, showing overall variation, as well as for each concentration of citalopram (control 0 mg/L, 30 mg/L, 50 mg/L, and 100 mg/L) based on the reconstructed trajectories in 3D. Filled symbols denote a significant difference from the first minute within each condition. Horizontal bar denotes a significant overall difference in time.

In response to ethanol administration, absolute levels of locomotion failed to reach a statistically significant variation across experimental groups (condition: $F_{3,52} = 2.43$, $P = 0.072$) (figure 3a). When analyzing the time course of general locomotion, we observed that it significantly decreased over time (time: $F_{5,260} = 3.02$, $P = 0.011$; $t_{260} > 2.67$, $P < 0.035$), and that such a decrease was indistinguishable across all experimental groups (time bins × condition: $F_{15,260} = 1.50$, $P = 0.105$). Behavioral anxiety did not significantly vary across ethanol conditions (condition: $F_{3,52} = 0.80$, $P = 0.500$) (figure 3b), neither did it apparently change over time (time: $F_{5,260} = 1.66$, $P = 0.144$). However, we observed that the habituation profile varied depending on

the specific experimental group (time bins × condition: $F_{15,260} = 1.83$, $P = 0.031$). Specifically, while behavioral anxiety remained constant in most experimental groups, it significantly declined over time in the ethanol 0.5% condition ($P < 0.050$; $t_{260} = 2.89$; $P = 0.019$). Finally, positional anxiety failed to reach a statistically significant variation across ethanol conditions (condition: $F_{3,52} = 2.49$, $P = 0.071$) (figure 3c), although it significantly decreased over time (time: $F_{5,260} = 3.25$, $P = 0.007$; $t_{260} > 2.75$; $P < 0.029$). Specifically, it significantly decreased for the ethanol 1.0% condition (time bins × condition: $F_{15,260} = 2.33$, $P = 0.004$; $t_{260} > 3.76$; $P < 0.001$).

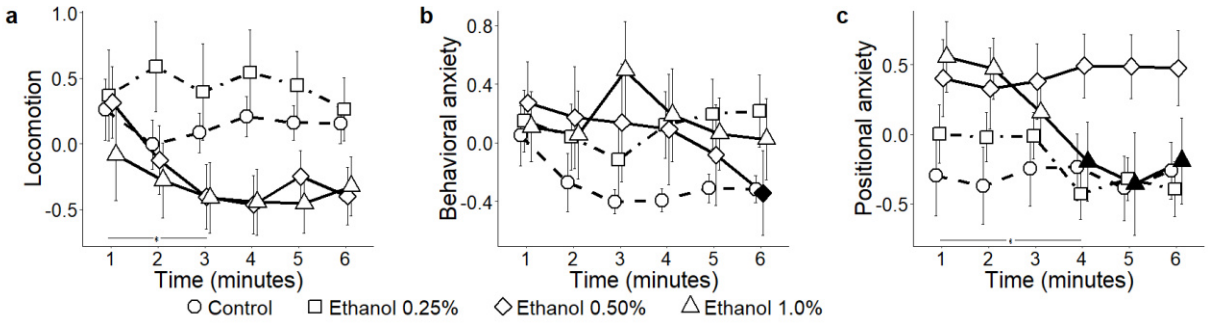

Figure 3. Mean ± standard error for a) locomotion, b) behavioral anxiety, and c) positional anxiety, over six-minute trials, showing overall variation, as well as for each concentration of ethanol (control 0%, 0.25%, 0.50%, and 1.0%) based on the reconstructed trajectories in 3D. Filled symbols denote a significant difference from the first minute within each condition. Horizontal bar denotes a significant overall difference in time.

3.2 The scoring view influences the validity of experimental outcomes

Herein, we report data concerning the effects of the views on all the experimental variables measured in the study. For the sake of clarity, in this section, we only report statistical findings associated with the scoring view (3D, 2D top, and 2D front) and its interactions with time or condition. Results concerning the main effects of condition, time, and their interaction irrespective of view are available in the supplementary material.

Before delving into detailed comparisons between the three different views for all the considered behavioral measures, we present an aggregated assessment of potentially inaccurate conclusions that would be drawn from 2D projections against 3D trajectories. Briefly, we identified that the specific view selected to quantify the behavioral repertoire reverberated in both false negative (erroneous reporting of absence of differences *in lieu* of significant findings in 3D)

and false positive (erroneous reporting of significant differences *in lieu* of non-significantly different findings in 3D) results. The rate of false negative and false positive findings is synoptically reported in Tables 2a and 2b.

Table 2a. Number of false positive and false negative findings for citalopram.

Citalopram	Differences between 3D and 2D top view			Differences between 3D and 2D front view		
Parameters	False positives	False negatives	Total	False positives	False negatives	Total
Average speed	1	0	1	0	0	0
Average peak speed	0	0	0	0	2	2
Average angular speed	0	0	0	0	3	3
Average peak angular speed	1	0	1	0	2	2
Average acceleration	0	0	0	0	3	3
Average peak acceleration	0	0	0	0	2	2
Wall following	0	0	0	0	2	2
Time in top half	-	-	-	0	0	0
Freezing	0	0	0	0	0	0
Total	2	0	2	0	14	14

Number of false positives and false negatives produced for each parameter when computed based on 2D top view and front view data, for the citalopram conditions. A false positive indicates that the 2D view (top or front) yields a significant result that is not supported by the 3D scoring approach. A false negative indicates that the 2D view (top or front) fails to detect a significant result that is instead evident from the 3D scoring approach.

Table 2b. Number of false positive and false negative findings for ethanol.

Ethanol	Differences between 3D and 2D top view			Differences between 3D and 2D front view		
Parameters	False positives	False negatives	Total	False positives	False negatives	Total
Average speed	0	0	0	0	0	0
Average peak speed	0	0	0	0	1	1
Average angular speed	1	0	1	0	1	1
Average peak angular speed	1	0	1	0	1	1
Average acceleration	0	0	0	3	0	3
Average peak acceleration	0	0	0	0	0	0
Wall following	0	0	0	3	0	3
Time in top half	-	-	-	0	0	0
Freezing	0	0	0	0	0	0
Total	2	0	2	6	3	9

Number of false positives and false negatives produced for each parameter when computed based on 2D top view and front view data, for the citalopram conditions. A false positive indicates that the 2D view (top or front) yields a significant result that is not supported by the 3D scoring approach. A false negative indicates that the 2D view (top or front) fails to detect a significant result that is instead evident from the 3D scoring approach.

3.3.1. Citalopram

Average speed: Predictably, average speed varied significantly depending on which view was used to compute it (view: $F_{2,110} = 118.46$, $P < 0.001$) (figure 4a). Specifically, both 2D front and top views underestimated absolute levels of locomotion compared to 3D data ($t_{291.7} = 9.87$, $P < 0.001$; and $t_{291.7} = 6.88$, $P < 0.001$, respectively); additionally, 2D front view resulted in reduced average speed compared to top view ($t_{291.7} = 2.98$, $P = 0.009$). Experimental subjects did not show a habituation profile to the test, yet 2D top projections indicated that the average speed decreased from the first to the last minute (time bins × view: $F_{10,550} = 14.08$, $P < 0.001$; $t_{317.5} = 3.03$, $P = 0.012$).

Average peak speed: Average peak speed was significantly underestimated in both 2D front and top views in comparison with 3D data (view: $F_{2,110} = 81.93$, $P < 0.001$; $t_{346.6} > 6.64$, $P < 0.001$)

(figure 4b). While the average peak speed decreased over time in all subjects (supplementary material), experimental groups apparently showed a differential habituation profile (time bins × view: $F_{10,550} = 15.64$, $P < 0.001$; $t_{286.4} > 2.73$, $P < 0.029$).

Average angular speed: Average angular speed was underestimated in the 2D front view compared to both 3D and 2D top views (view: $F_{2,110} = 88.45$, $P = 0.001$; $t_{295.3} > 5.33$, $P < 0.001$) (figure 4c). Furthermore, a decrease in average angular speed over time was observed in all views (time bins × view: $F_{10,550} = 7.40$, $P < 0.001$; $t_{275} > 2.69$, $P < 0.034$).

Average peak angular speed: Average peak angular speed was underestimated when scored from 2D top view (view: $F_{2,110} = 10.09$, $P < 0.001$; $t_{358.6} = 3.62$, $P = 0.001$) (figure 4d). A decrease in average peak angular speed over time was recorded from all views, but not at the same times (time bins × view: $F_{2,550} = 2.72$, $P = 0.003$; $t_{275} > 2.64$, $P < 0.038$).

Average acceleration: Average acceleration was underestimated in both front and top 2D views compared to 3D (view: $F_{2,110} = 126.62$, $P < 0.001$; $t_{284.4} = 9.24$, $P < 0.001$; and $t_{284.4} = 5.57$, $P < 0.001$, respectively); additionally, 2D front view underestimated average acceleration compared to 2D top view ($t_{284.4} = 3.67$, $P < 0.001$). Average acceleration varied over time depending on the view adopted to score fish behavior (time bins × view: $F_{10,550} = 13.40$, $P < 0.001$) (figure 4e). Specifically, although average acceleration steadily declined from the third minute in ground truth 3D data ($t_{371.0} = 2.57$, $P < 0.046$), such a decline was observable also from 2D top view ($t_{371.0} > 2.80$, $P < 0.025$), but only during the last minute in 2D front view ($t_{275.0} = 2.70$, $P < 0.033$).

Average peak acceleration: Average peak acceleration significantly decreased over time, regardless of the specific view adopted to compute this measure (time bins × view: $F_{10,550} = 4.42$, $P < 0.001$; $t_{344.7} > 3.15$, $P < 0.008$) (figure 4f). Yet, average peak acceleration was underestimated in 2D front and top views compared to 3D (view: $F_{2,110} = 79.67$, $P < 0.001$; $t_{402.3} = 6.05$, $P < 0.001$; and $t_{402.3} = 3.46$, $P = 0.002$, respectively). Additionally, 2D front view yielded a lower average peak acceleration compared to top view ($t_{402.3} = 2.59$, $P = 0.027$).

Wall following: Time spent wall following was significantly underestimated in both 2D front view compared to 3D data (view: $F_{2,110} = 237.90$, $P < 0.001$; $t_{234.4} = 17.55$; $P < 0.001$). Additionally, this metric was lower in 2D front view compared to 2D top view ($t_{234.4} = 15.95$, $P < 0.001$) (figure 4g). While 3D and 2D top view data indicated that wall following increased between the first and

fifth minute of the experimental session (time bins × view: $F_{10,550} = 2.16$, $P = 0.019$, $t_{824.7} > 2.56$, $P < 0.05$), 2D front view data failed to identify this time dependent pattern of thigmotaxis.

Position in the water column (proportion of time spent in the top half): Since this metric takes into account only the vertical position of the fish, it cannot be scored from 2D top view and there is no difference between values from 2D front view and 3D reconstructed trajectories (figures 4h and 5h).

Freezing: Although the time spent freezing seemed to vary depending on which view was used to compute it (view: $F_{2,110} = 4.19$, $P = 0.018$) (figure 4i) post-hoc tests revealed no pairwise difference. Similarly, although an interaction between view and time was registered (time bins × view: $F_{10,550} = 2.35$, $P = 0.010$), post-hoc comparisons did not indicate any specific difference.

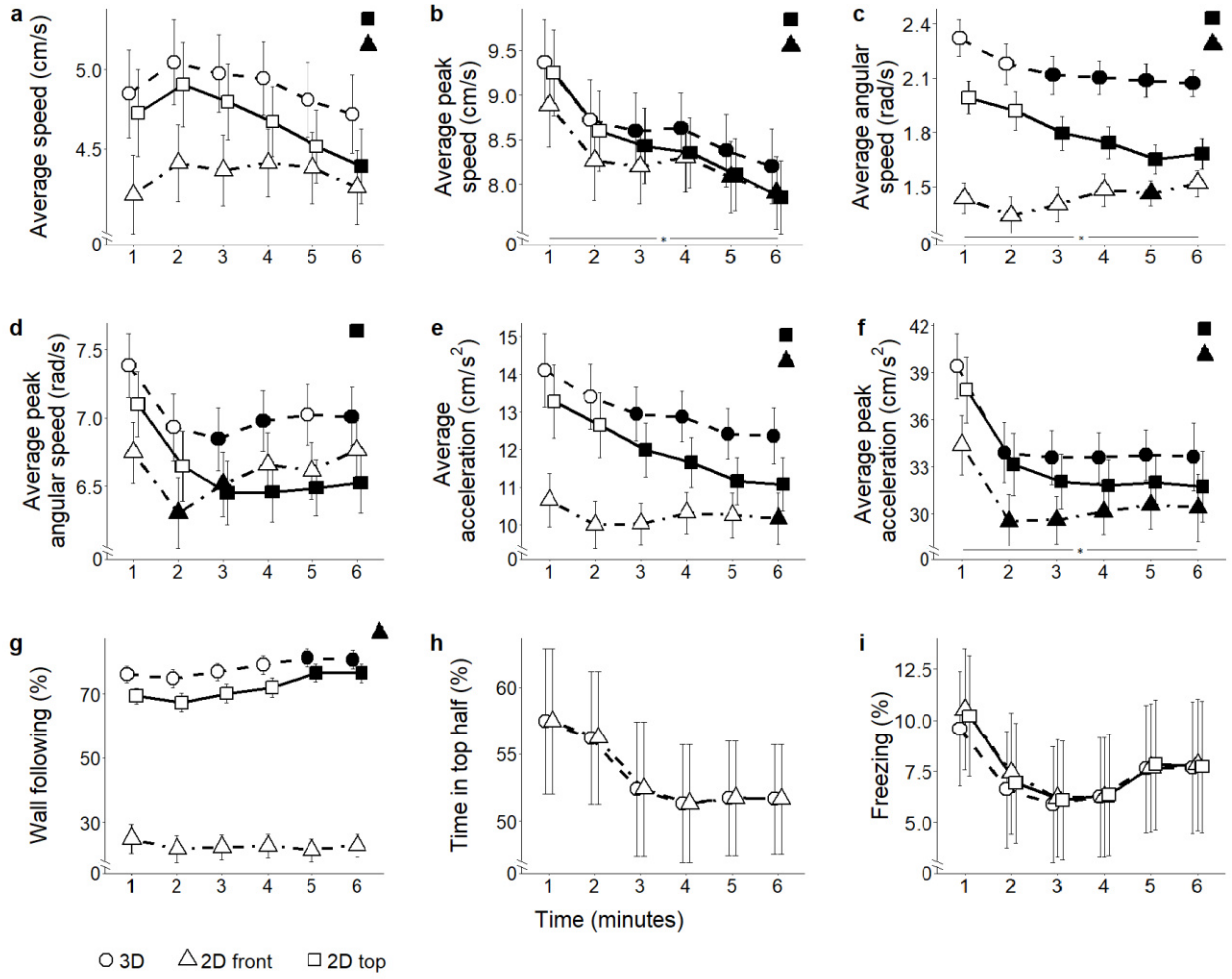

Figure 4. Mean ± standard error for a) average speed, b) average peak speed, c) average angular speed, d) average peak angular speed, e) average acceleration, f) average peak acceleration, g) proportion of time spent within 3 cm of walls, h) proportion of time spent in the top half of the tank, and i) proportion of time spent freezing, over six-minute trials aggregated for all citalopram conditions, computed from 2D front and top views, and 3D reconstructed trajectories. Filled symbols denote a significant difference from the first minute within each condition. Horizontal bar denotes a significant overall difference over time. Filled symbols in the top right corner of each panel indicate a significant overall difference with respect to 3D data.

3.3.2. Ethanol

Average speed: The different scoring views resulted in variable average speed values (view: $F_{2,104} = 90.45$, $P < 0.001$) (figure 5a). Both 2D top and front views underestimated average speed compared to 3D ($t_{174.3} = 3.79$; $P < 0.001$; and $t_{174.3} = 9.47$; $P < 0.001$, respectively). Additionally, average speed was lower in 2D front view compared to top view ($t_{174.3} = 5.69$; $P < 0.001$). While data inspection suggested that habituation profiles were skewed by the view adopted to score

individual behavior (time bins × view: $F_{10,520} = 10.14$, $P < 0.001$), post-hoc analyses failed to show significant view-dependent variations in this parameter.

Average peak speed: Average peak speed varied in all subjects, and this profile was apparently influenced by the view adopted to score individual trajectories (time bins × view: $F_{10,520} = 9.36$, $P < 0.001$; $t_{260.0} > 2.64$, $P < 0.039$). This variation was manifested as a robust decline in subjects treated with ethanol 0.50% concentration (time bins × condition × view: $F_{30,520} = 1.83$, $P = 0.005$; $t_{260.0} > 2.57$, $P < 0.047$, see figure S2, supplementary information). Furthermore, average peak speed was significantly underestimated in both 2D front and top views compared to 3D data (view: $F_{2,104} = 83.2$, $P < 0.001$; $t_{270.2} > 4.41$; $P < 0.001$) (figure 5b), as well as from the front view compared to the top view ($t_{260.0} = 2.60$; $P = 0.027$). Although ANOVA reported a significant interaction between view and condition (condition × view: $F_{6,104} = 2.33$, $P = 0.037$), post-hoc tests failed to reveal any significant pairwise difference.

Average angular speed: Predictably, both 2D front and top views underestimated average angular speed compared to the 3D view (view: $F_{2,104} = 63.33$, $P < 0.001$; $t_{265.7} = 7.73$, $P < 0.001$; and $t_{265.7} = 4.78$, $P < 0.001$, respectively). Additionally, average angular speed was less in 2D front view compared to 2D top view ($t_{265.7} = 2.95$, $P < 0.010$) (figure 5c). Furthermore, 3D data demonstrated that average angular speed declined between the first and the last minute of observation. Such a decline, observable in 2D top view data, was not detected in 2D front view (time bins × view: $F_{10,520} = 5.19$, $P < 0.001$, $t_{730.9} = 3.902$, $P < 0.005$).

Average peak angular speed: Although average peak angular speed appeared significantly different depending on which view was used (view: $F_{2,104} = 4.37$, $P = 0.015$), such a difference failed to emerge in post-hoc comparisons. From all views, average peak angular speed declined throughout the experimental session (time bins × view: $F_{10,520} = 3.53$, $P < 0.001$; $t_{707.1} > 2.66$, $P < 0.035$) (figure 5d).

Average acceleration: In line with most of the locomotion-related variables, average acceleration was underestimated in both 2D front and top views compared to 3D view (view: $F_{2,104} = 64.41$, $P < 0.001$; $t_{164.6} = 7.45$, $P < 0.001$; and $t_{164.6} = 3.20$, $P = 0.005$, respectively, see figure 5e). Furthermore, 2D front view yielded lower values of the average acceleration compared to the top view ($t_{164.6} = 4.25$, $P < 0.001$). Individual habituation profile was differentially expressed by

experimental subjects depending on the specific view (time bins × view: $F_{10,520} = 11.87$, $P < 0.001$). Specifically, while 3D and 2D top view data indicated a general decrease in average acceleration throughout the experimental session ($t_{312.7} = 2.94$, $P < 0.016$), such a profile was not visible in 2D front view, showing only a reduction during the third minute of the test ($t_{260.0} = 2.70$, $P = 0.033$). Data analysis suggested that the habituation profile varied depending on both the view and the ethanol treatment (time bins × condition × view: $F_{30,520} = 1.50$, $P = 0.046$). Specifically, we observed that the reduction in average acceleration was significant in ethanol 0.5% ($t_{260.0} > 2.80$, $P < 0.025$), and that this decrease occurred regardless of the specific view from which data were scored.

Average peak acceleration: Average peak acceleration varied depending on the specific scoring view (view: $F_{2,104} = 61.85$, $P < 0.001$); specifically it was underestimated in both 2D front and top views compared to 3D ($t_{273.9} = 5.69$, $P < 0.001$; and $t_{273.9} = 2.41$, $P = 0.044$, respectively) and was also less in 2D front view compared to 2D top view ($t_{273.9} = 3.28$, $P = 0.003$) (figure 5f). Furthermore, although data inspection suggested that the time-dependent habituation profile varied depending on the specific view (time bins × view: $F_{10,520} = 5.70$, $P < 0.001$; $t_{312.6} = 2.57$, $P < 0.047$), post-hoc tests did not support this suggestion. Thus, acceleration decreased with time in experimental subjects regardless of the specific view adopted.

Wall following: The time spent in the proximity of the walls significantly varied depending on the specific view used to compute it (view: $F_{2,104} = 56.09$, $P < 0.001$). Wall following was significantly underestimated in both 2D front and top views compared to 3D ($t_{178.8} = 9.96$; $P < 0.001$; and $t_{178.8} = 3.49$; $P = 0.002$, respectively), and this parameter was less in 2D front view compared to 2D top ($t_{178.8} = 6.47$, $P < 0.001$). The individual habituation profiles varied depending on the view (time bins × view: $F_{10,520} = 3.37$, $P = 0.001$) (figure 5g). Specifically, wall following remained constant in 3D and 2D top view, and decreased in 2D front view ($t_{260.0} = 2.79$; $P < 0.025$). While wall following was apparently differed between conditions depending on the scoring view (condition × view: $F_{2,104} = 5.54$, $P < 0.001$), such difference was not statistically significant in pairwise comparisons.

Freezing: While the time spent freezing seemed to vary depending on the specific scoring view, (view: $F_{2,104} = 5.35$, $P = 0.006$) (figure 5i), such a difference was not confirmed by post-hoc tests performed between the first and the sixth minute.

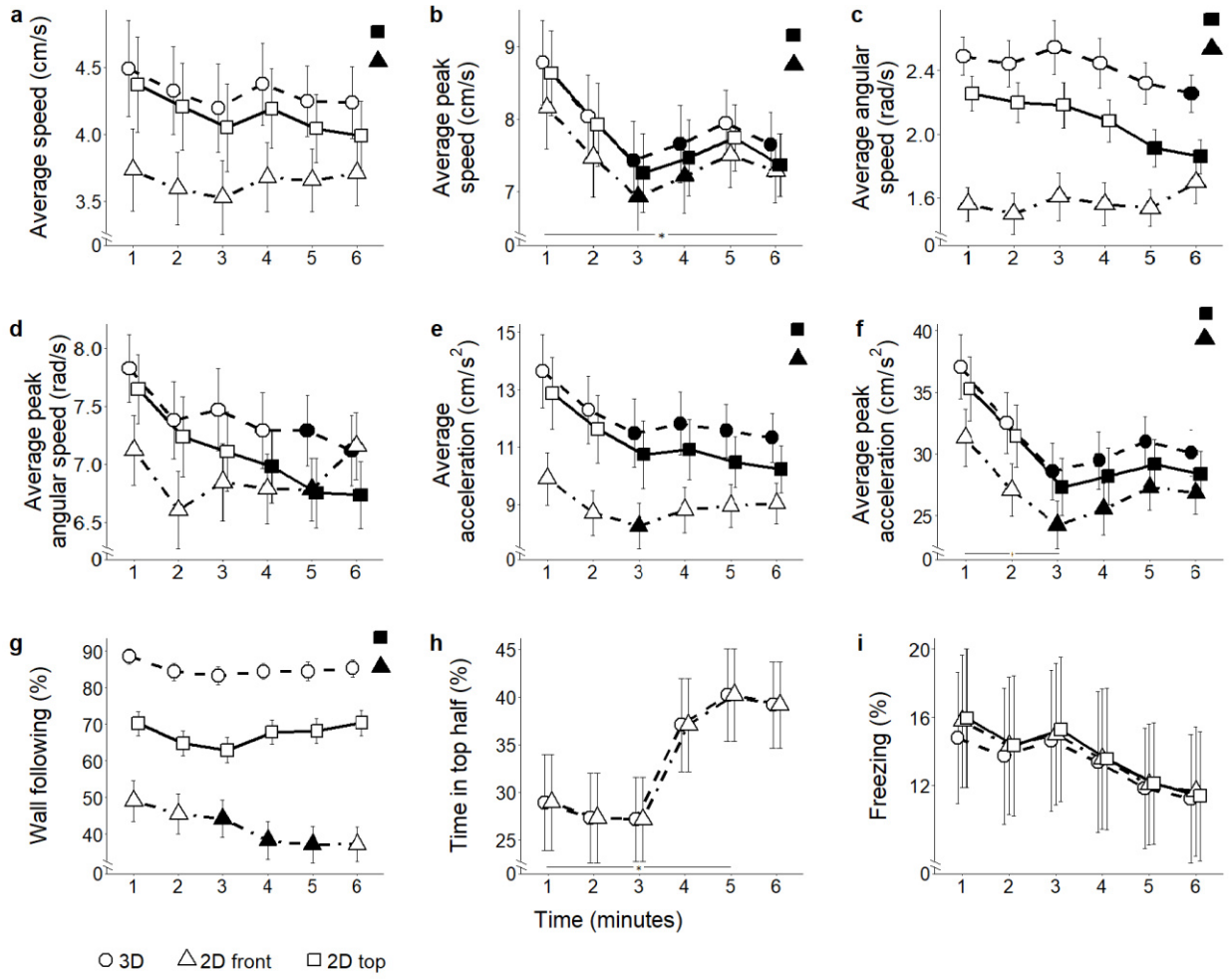

Figure 5. Mean ± standard error for a) average speed, b) average peak speed, c) average angular speed, d) average peak angular speed, e) average acceleration, f) average peak acceleration, g) proportion of time spent within 3 cm of walls, h) proportion of time spent in the top half of the tank, and i) proportion of time spent freezing, over six-minute trials aggregated for all ethanol conditions, computed from 2D front and top views, and 3D reconstructed trajectories. Filled symbols denote a significant difference from the first minute within each condition. Horizontal bar denotes a significant overall difference over time. Filled symbols in the top right corner of each panel indicate a significant overall difference with respect to 3D data.

4. Discussion

The methodological nature of the present study first reverberated in the systematic evaluation of the correlation among the variables that constitute the ethogram exhibited in the novel tank diving

test. The PCA revealed the presence of three orthogonal factors, reflecting general locomotion (average speed, average peak speed, average acceleration, and average peak acceleration), anxiety-related behavioral patterns (average angular speed, average angular peak speed, and freezing), and anxiety-related spatial preference (time spent close to the side walls and time spent in the upper half of the water column). The first principal component relates to the translational motion within the water tank. The behavioral patterns loading on the second principal component have been consistently associated with anxiety, in the form of erratic movements (zig-zagging) and freezing [43]. From the catalog of Kalueff and colleagues [43], anxiety-related behavior is also related to thigmotaxis and geotaxis, which are the two behavioral measures that load on the third principal component.

While this analysis aligns with previous evidence indicating that anxiety can be expressed through different modalities, it also points at potential pitfalls of common practice in the construction of the ethogram of the novel tank diving test from 2D views. Specifically, the fact that variables contributing to the same principal component require different perspectives further corroborates the need for a 3D approach. For example, while position in the water column requires a front camera, wall distance and erratic movements need an overhead camera.

The analysis conducted on the aforementioned principal components revealed that both citalopram and ethanol influenced anxiety-related behaviors, thus corroborating the predictive validity of the novel tank diving test. Importantly, while citalopram concentration-dependently reduced locomotion and predictably reduced anxiety, ethanol resulted in increased anxiety, but only at a medium concentration [44]. Higher and lower ethanol concentrations were apparently ineffective. Low and medium concentrations of citalopram did not influence general locomotion but were associated with the exhibition of reduced anxiety, selectively during the first three minutes of testing. High concentrations of citalopram were associated with reduced locomotion and reduced anxiety throughout the entire test session. The anxiolytic effects of citalopram have already been reported in several studies. For example, [33] reported that zebrafish treated with 100 mg/L citalopram spent significantly more time than control fish in the top two thirds of the tank, suggesting a decrease in anxiety compared to the control.

It is worth noticing that, when analyzing discrete parameters rather than focusing on the PCA, some anxiety-related behavioral parameters seemed unaffected by the anxiolytic treatments applied. Specifically, we failed to observe a significant effect of citalopram on the time spent in

the upper portion of the tank, a classical measure of anxiety. We note that such absence of a concentration-dependent behavioral response to anxiolytic compounds has also been reported in other studies. For example, [33] reported that acute exposure to 0.5% ethanol failed to alter the time spent in the upper portion of the test tank in zebrafish. Similarly, [45] failed to observe significant anxiety-related behavioral alterations in response to fluoxetine. Finally, in a previous study, we also observed that 0.25% and 0.5% ethanol did not modulate anxiety-related behaviors in the light-dark test [23]. These false negative findings further corroborate the potential heuristic value of conducting PCA in zebrafish behavioral pharmacology.

The anxiolytic effects of citalopram are likely related to its direct influence on serotonergic concentrations. For example, handling stress has been shown to increase anxiety-like behavior and reduce brain concentrations of the serotonin metabolite 5-HIAA [46]. Furthermore, [47] observed that acute administration of the 5-HT1a receptor agonist buspirone reduced behavioral anxiety in the light-dark test. Finally, in accordance with the present study, the acute administration of the selective serotonin reuptake inhibitor fluoxetine resulted in reduced anxiety in the geotaxis test [48].

With respect to ethanol, available literature [49] indicates that its effects vary depending on the concentration, administration schedule, and methodological issues. [50] reported that ethanol can have either anxiogenic or anxiolytic effects on zebrafish depending on whether the water in the test tank comes from the individual's holding tank or from a tank that did not hold any fish. Further, since ethanol influences general locomotion, some of its effects on anxiety may be spurious and potentially related to locomotor effects. For example, a lack of vertical exploration may reflect a decrease in swimming behavior due to the sedative effect of high concentration of ethanol, rather than an anxiety response [51]. In our previous study [23], we observed that high ethanol concentration resulted in reduced anxiety, associated with reduced motility and increased freezing. Likewise, [52] observed that ethanol administration resulted in reduced anxiety in the light/dark test, but not in the novel tank diving test. In contrast with these findings, Tran and collaborators [44] reported that acute exposure to high ethanol concentration resulted in increased preference for the bottom of the test tank, and that such a variation related to alterations in brain monoamines. Specifically, alcohol-treated subjects showed reduced concentrations of the dopamine metabolite DOPAC, of serotonin and its metabolite 5-IAA [44]. Thus, while the effects of ethanol on anxiety are more variable compared to those exerted by citalopram, they apparently

impinge on the same neurochemical pathways modulated by citalopram. Ultimately, the complementary use of these substances served the aim to address the validity of 2D approaches in zebrafish pharmacology of anxiety.

In order to compare 3D and 2D approaches, all experimental variables were also analyzed independently from one-another. This comparison was aimed at confirming the intuition that locomotion is underestimated when scoring the behavior in 2D and at assessing whether 2D views yielded incorrect conclusions regarding the effects of anxiolytics on individual behavior. Working with raw experimental variables rather than aggregated principal components allowed for a direct comparison of our findings with available literature, where the selected metrics are routinely assessed in pharmacological phenotyping of zebrafish [43]. With respect to absolute values of locomotion, predictably, they were higher in 3D than 2D, regardless of whether the latter referred to the frontal or the horizontal plane. This can be easily explained by recognizing that 2D trajectories correspond to the projection of the full 3D motion on independent views, which would, by definition, abolish movement along a third dimension. This evidence echoes our previous findings obtained in drug-free states [30].

The core objective of the present study was to evaluate whether 2D views may result in inaccurate rejection of null hypotheses or acceptance of alternative ones. We observed that the specific view consistently skewed the time course of the behavioral response to the novel tank. This was reflected in the presence of ubiquitous significant view × time bins interactions across most of the variables, and only few instances of view × condition interactions. Thus, these data could preliminarily suggest a relative robustness of current scoring methods in zebrafish pharmacology. Yet, in the light of the paucity of drug-dependent effects and of the nature of the statistical model required to test the suitability of the 2D approaches compared to 3D, we argue that this assessment only reflects a partial consideration of the observed results.

Specifically, in our previous study, we demonstrated that 2D experiments are underpowered compared to 3D and therefore more prone to false negative findings than false positive ones [30]. While in situations characterized by few significant main effects of a given variable the likelihood to observe false negatives is intrinsically limited, data with numerous significant main effects shall be amenable to the identification of numerous false reporting instances. Accordingly, in the present study, the sporadic main effects of the condition have apparently masked view-dependent false negatives; complementarily, the ubiquitous presence of

main effects of time bins allowed the detection of numerous view × time bins interactions. Thus, the specific view from which data were scored influenced the observed individual habituation patterns to the experimental paradigm. For example, while 3D data indicated that locomotion-related parameters (e.g., speed, angular speed, and acceleration) declined throughout the experimental session, 2D front view data failed to capture such a time-dependent habituation pattern. While this aspect may simply indicate the limited heuristic potential of the front view and advocate in favor of the use of a top view camera, we nonetheless note that a front camera is indispensable to quantify the position in the water column, which contributes to the anxiety-related phenotype.

These considerations extrapolate to zebrafish pharmacology, whereby our and others' data [35] indicate that anxiety-modulating compounds often alter habituation profiles rather than absolute values averaged across different time points [53]. For example, we reported that anxiety-related behaviors in control subjects appear relatively constant throughout the entire course of the experimental session. Conversely, experimental subjects treated with low and medium concentrations of citalopram exhibit reduced anxiety-related behaviors during the early stages of the task, which gradually rise to attain control values towards the end of the session. Similar to [54], we found that although 3D measures offer higher precision, the benefit of using 3D compared to a view from the top is limited regarding general behavioral pattern. The use of a front view remains necessary to capture specific behaviors linked to the position of the fish in the water column.

It is important to emphasize that in the present study we primarily focused on anxiolytic drugs and we thus cannot extrapolate our findings to the entire spectrum of anxiety-related behaviors. Future studies are needed to test whether the considerations outlined in this manuscript also extend to anxiogenic compounds (e.g., caffeine) and non-pharmacological anxiety-eliciting stimuli (e.g., predators).

Author contributions

SM and MP designed the research; RC and CS performed the experiments; RC and CS scored animal behavior; SM, RC, and MP performed statistical analyses; RC and CS provided a preliminary draft; SM and MP wrote the final draft; and all the authors reviewed the final draft and offered comments.

Acknowledgments

This work was supported by the National Institutes of Health, National Institute on Drug Abuse under grant number 1R21DA042558-01A1, the Office of Behavioral and Social Sciences Research that co-founded the National Institute on Drug Abuse grant, and by the National Science Foundation under grant number CMMI-1505832. The authors are grateful to Hannah Kurdila for her help in data analysis, Shinnosuke Nakayama for his help with the statistics, and Boris Arbuzov for his help in scoring fish behavior from the video recordings. Datasets and codes used in the analyses are stored at the authors' home institution and will be provided on request.

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