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Research report

Zebrafish (*Danio rerio*) responds to the animated image of a predator: Towards the development of an automated aversive task

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ABSTRACT

Zebrafish is gaining popularity in basic behavioral brain research, behavior genetics, and in translational studies because it offers a cheap and efficient alternative to rodents. Abnormally exaggerated fear and anxiety are some of the most prevalent neuropsychiatric diseases in the human society whose mechanisms are not well understood. These diseases still represent major unmet medical needs. Ethologically relevant fear paradigms involving the presentation of predators, or of stimuli characteristic of predators, have been proposed as appropriate for the modeling and the analysis of fear responses. Previously, we have shown that zebrafish respond specifically to visual stimuli of their sympatric predator, the Indian leaf fish. In the current paper we show that an animated (moving) image of this predator elicits significant behavioral responses and that these responses can be quantified using video-tracking. As stimulus presentation and behavioral response quantification are both computerized and automated, we suggest the paradigm is appropriate for high throughput screening and, once pharmacologically and behaviorally validated, may have utility in the detection of anxiolytic or anxiogenic properties of compounds or in the identification of fear altering mutations.

1. Introduction

Zebrafish has enjoyed much attention in developmental biology due to such characteristics as external fertilization and development and the transparency of its embryo [17]. These features coupled with its high fecundity (2–300 eggs per female per spawning every other day) and its ease of maintenance and small size (4 cm when adult) have also increased its popularity as a laboratory animal. Last, the fact that zebrafish is a vertebrate that shares basic structural as well as genetic properties with other vertebrate species including our own, has launched zebrafish to being one of the primary model organisms in modern day genetics [17]. Indeed, by now numerous genetic tools and a large amount of genetic information have been accumulated for zebrafish [17]. As a consequence other disciplines including behavioral neuroscience and behavior genetics have also taken notice of zebrafish. Zebrafish has been suggested as an appropriate model organism and/or research tool for the analysis of the effects of alcohol [15], drug addiction [8], learning and memory [1,4,24], aggression [11] and social behavior [22,23,26], to mention but a few research areas. This species has also been suggested for the analysis of fear and anxiety [3,20,27].

Chronically elevated levels of or abnormally directed fear, e.g. anxiety disorders and phobias, are among the most prevalent and devastating neuropsychiatric conditions in humans [7,28]. Although academic as well as major industry laboratories have been trying to develop appropriate treatments, these diseases still represent a large unmet medical need because their mechanisms remain poorly understood. Animal models have been suggested as an additional approach with which the understanding of mechanistic aspects of such diseases may be facilitated (e.g. [5]). Particularly promising are those approaches that intend to tap into natural behavioral reactions [5,17].

These ethologically oriented studies may have the potential to engage biological mechanisms (neural circuits, biochemical responses, genes, etc.) relevant from an evolutionary fitness perspective, but more importantly they may also allow us to better understand both the physiologically "normal" as well as the pathological, "aberrant" aspects of brain function (e.g. [18]). In this line of argument, it has been proposed that human anxiety disorders and phobias are the result of abnormally functioning fear mechanisms that originally have been shaped by natural selection and have been in place for danger (e.g. predator) avoidance in humans [18,19]. Similarly, it has also been argued that for translational research perhaps the most relevant fear paradigms using animal models should be those that engage such natural responses and, for example, use natural predators or stimuli typical of such predators [2].

Previously, we [27] and others [29] have shown that zebrafish, similar to several other fish species [10,25], respond to the alarm

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substance. This substance, which is released by epidermal club cells upon physical injury of the skin, induces species-specific behavioral responses in zebrafish, including erratic movement (fast zig-zagging) as well as tightening of the shoal (decreasing inter-individual distances among group members) [27]. Using an olfactory cue such as the alarm substance is appropriate from an ethological and biological viewpoint but it brings certain practical problems. The onset and offset of the administration of the cue is not as easy to control as stimuli of other modalities including visual stimuli. Furthermore, the alarm substance has to be extracted fresh from the skin of fish and thus its concentration can vary across experiments [27]. Ascertaining that no residual cues are left in the test tank for subsequent sessions is also problematic as it requires a meticulous and labor intensive cleaning procedure [27].

Given that zebrafish is a diurnal species with good visual acuity, we have explored the use of alternative cues, e.g. visual stimuli. We studied how zebrafish responded to the sight of heterospecific fish including allopatric and sympatric predators [3]. Our results suggested that zebrafish exhibited species-specific responses to its sympatric predator, the Indian leaf fish (*Nandus nandus*) including elevated frequency of jumping and somewhat increased amount of erratic movement [3]. Importantly, these responses were elicited just by the sight of the predator and additional information such as olfactory cues or lateral line responses (vibration) were not required for the responses to reach maximum levels. However, although the sight of the predator was a good alternative, a visual stimulus whose onset and offset was easy to control, the stimulus was a live fish whose behavior varied across experimental sessions again causing a potential inter-session variability in stimulus delivery. Last, in both the alarm substance and the predator avoidance tasks there were multiple experimental subjects present in the test tank and their behavior was observed and quantified manually [3,27].

In low throughput or pilot behavioral studies slight variability in stimulus delivery and/or the manual behavioral recording approach may not represent major drawbacks. But in a high throughput mode, these problems become significant. Briefly, in order for one to screen large number of zebrafish, a high throughput paradigm must have the following features. First, the stimulus presentation must be precisely controlled and must be consistent across all trials and subjects. Two, the behavior of the experimental subject must be quantified in an automated manner and should not require the presence of the experimenter.

To address these points, in the current paper we utilize a software application that allows us to present an animated (moving) image of the sympatric predator of zebrafish (the Indian leaf fish) in a controlled manner consistent across sessions. In addition, we now monitor the behavior of fish placed singly in the test tank and quantify its responses using video-tracking, an efficient and precise method allowing us to measure a number of parameters of the swim path of the experimental subject without the need to view the videotapes. Our results show that the predator-image induces significant behavioral changes in zebrafish and these responses can be quantified in an automated manner. Our findings imply that the paradigm will have utility in mutagenesis and/or drug screens aimed at the identification of anxiogenic and/or anxiolytic effects of mutations and/or pharmaceutical compounds.

2. Methods

2.1. Animals and housing

Adult zebrafish (*Danio rerio*) of the AB strain bred in our facility (University of Toronto Mississauga Vivarium, Mississauga ON, Canada) were used for obtaining fertilized eggs. The progenitors of this population were obtained from the ZFIN Center (Eugene, Oregon). AB is one of the most frequently studied zebrafish strains and is often used in forward genetic (mutagenesis) studies (e.g. [21]). The developing eggs were transferred to 1.3 l nursery tanks that were part of a nursery rack (Aquaneering Inc., San Diego California, USA) and were maintained there in system water (deionized and UV-light sterilized water supplemented with 60 mg/l Instant Ocean Sea Salt [Big Al's Pet Store, Mississauga, Ontario, Canada]). The developing fish were fed Larval Artificial Plankton 100 (particle size below 100 μm, ZeiglerBros.Inc., Gardners, Pennsylvania, USA). Three weeks later the fish were moved into 2.8 l rearing tanks (20 fish per tank) of a high density rack system (Aquaneering Inc., San Diego California, USA) designed specifically for zebrafish. The system had a multistage filtration that contained a mechanical filter, a fluidized glass bed biological filter, an activated carbon filter, and a fluorescent UV-light sterilizing unit. Every day 10% of the water was automatically replaced with fresh system water on the rack. The water temperature was maintained at 27 °C. Illumination was provided by fluorescent light tubes from the ceiling with lights turned on at 0800 h and off at 1900 h. While the fish were in the high density racks they received a mixture of dried fish food (4 parts of Nelson Silver Cup, Aquaneering Inc., San Diego California, USA) and powdered spirulina (1 part, Jehmco Inc., Lambertville, New Jersey, USA) three times a day. Behavioral experiments were conducted after the fish reached 6 months of age (fully developed sexually mature young adults).

2.2. Test apparatus

The experimental set up consisted of a standard 37 l tank (50 cm × 25 cm × 30 cm, length × width × height) with a flat LCD computer monitor (17 inch Samsung SyncMaster 732N) placed on the left and right side of the tank. Each monitor was connected to a Dell Vostro 1000 laptop running a custom made software application (first utilized in 25) that allowed the presentation of animated fish images. The experimental tank was illuminated by a 15 W fluorescent light tube from above. The back side and the bottom of the test tank were coated with white corrugated plastic sheets to increase the contrast and reduce glare and reflections for videotracking analysis. Two identical experimental set ups were used in parallel. The behavior of experimental fish was recorded onto the hard drive of two videocameras (JVC GZ -MG37u and GZ-MG50, recording 30 frames per second) and the digital recordings were transferred to the hard drive of a desktop computer (Dell, Dimension 8400) and later replayed and analyzed using the Ethovision Color Pro Videotracking software (Version 3, Noldus Info Tech, Wageningen, The Netherlands).

2.3. Test procedure

Adult experimental zebrafish were placed to the test tank singly and were allowed to habituate to the test environment for 20 min. During this habituation period the zebrafish were first presented with a blank (black) screen on both sides of the tank and subsequently, to further reduce potential fear responses they were shown moving (speed ranging between 1.5 cm/s and 4 cm/s) images of 6 zebrafish, an animated shoal.

Behavioral recording started after this habituation period. For the first minute of the recording session, a blank (black) screen was shown. Subsequently the experimental fish was presented with a slowly (0.3 cm/s) moving image of a single Indian leaf fish (Fig. 1). The predator image moved across the screen horizontally within a range of predetermined heights, i.e. 2–15 cm from the bottom always presenting a lateral view of the predator. During the 1 min predator presentation period, the exact length of time the image moved in one direction and the starting direction of movement of the image were randomized. In addition to the horizontal movement of the predator image, we also added small vertical movements mimicking potential wave action induced changes in the position of the predator in the water column. These vertical position changes occurred within the specified range, i.e. the predator image remained between 2 cm to 15 cm from the bottom of the experimental tank. The first 1 min predator presentation period was followed by a 3 min no stimulus interval, another 1 min predator presentation, another 3 min no stimulus interval, and finally the last 1 min predator interval, a 10 min recording session in total. The animated stimulus fish were presented only on one side of the tank for each experimental fish but the side of presentation alternated randomly across experimental subjects.

Fig. 1. Photograph of the predator (Indian leaf fish, *Nandus nandus*) that was used for the animated image presentation. For details of how the image was presented see Section 2.

2.4. Quantification of behavior and statistical analysis

The digital video files (AVI format) were later replayed and analyzed by Ethovision as described in detail in [14] (also see [6]). This tracking system allowed us to precisely quantify the swim path pattern of the experimental fish. The following measures were quantified and expressed for 1-min intervals of the 10 min session. Mean distance to stimulus screen: we expected the experimental zebrafish to move away from the stimulus screen (the computer monitor presenting the stimulus fish) upon presentation of the predator, an antipredatory escape reaction, and measured the mean distance between the experimental subject and the stimulus screen (the average of distance values sampled once every 0.1 s). Variability of distance to stimulus screen: we also quantified the variance of the distance values obtained for each fish for each 1-min interval, a measure that reflects how stable a distance the experimental fish maintained from the stimulus screen. Mean distance to bottom: we expected zebrafish to increase bottom dwell time in response to the appearance of the predator image [20] and quantified and averaged the distance values (sampled once every 0.1 s, i.e. a 10 Hz sampling rate) between the location of the experimental fish and the bottom of the tank. Variability of distance to bottom: we also calculated the variance of these distance values for each 1-min interval for each fish, a measure that quantifies how consistent a distance the fish maintained from the bottom. Mean velocity: previously, we have observed both increased and decreased swimming activity (depending on circumstances and the timing of quantification) in response to the appearance of the predator (also see [15]), and thus quantified the swim speed (in cm/s) of the experimental fish. Variability of velocity: our expectation was that swim speed will become variable (high speed escape episodes and low speed, or freezing bouts), and this measure allowed us to investigate how stable a swim speed experimental fish maintained in response to the appearance of the predator. Mean turn angle: previously we observed that upon presentation of a predator, zebrafish reduce the time spent with straight line locomotion and instead often exhibit fast changes in their swim direction (e.g. zig-zagging or erratic movement). We therefore expected mean turn angle to increase in response to the presentation of the predator image. Variability of turn angle: to examine how consistent the turning behavior of zebrafish is in response to the predator image, we also quantified the variance of the values of turn angle sampled once every 0.1 s for each 1-min interval for each fish.

In addition to video-tracking, we also conducted an observation-based event recording analysis to improve interpretation of results of the video-tracking analysis. We replayed the video-recordings previously analyzed by videotracking and using the Observer software (Noldus Info Tech, Wageningen, The Netherlands) we quantified species-specific motor patterns including the frequency of jumping and duration of erratic movements. These behaviors were previously shown to increase in response to fear inducing stimuli [14]. We also measured the relative duration (percent of time) of swimming. We expected this behavior to decrease in response to predator presentation. Jumping has been defined as a single forceful leap using the caudal fin [5]. Erratic movement is a characteristic zig-zagging response associated with fast (over 3 cm/s) swimming and quick direction changes [6]. This behavior is most often performed at the bottom of the tank but can occasionally occur in midwater as well [3]. Swimming is defined as fast locomotion mainly with the use of the caudal and pectoral fins during which the fish does not change its direction more than 45 degrees and does not physically touch the glass walls of the tank (also see ref [6]).

Data were analyzed using SPSS (version 14.1) for the PC. Repeated measure variance analysis (ANOVA) was used to investigate the effect of interval (ten 1 min intervals). To compare certain intervals paired *t*-tests were also performed.

3. Results

Unexpectedly, experimental zebrafish did not appear to move away from the stimulus screen upon the presentation of the predator image (Fig. 2). Variance Analysis of the mean distance to stimulus screen found no significant interval effect ($F(9, 279) = 1.581$, $p > 0.10$). Similarly, the variability of the distance to stimulus screen (Fig. 3) was also non-significantly affected by the appearance of the predator image (ANOVA interval $F(9, 279) = 1.830$, $p > 0.05$). Furthermore, the distance at which zebrafish swam from the bottom was also unaffected by the image of the predator (Fig. 4; ANOVA interval $F(9, 279) = 0.989$, $p > 0.40$). However, the variability of distance to bottom was significantly affected by the presentation of the predator image (Fig. 5, ANOVA interval $F(9, 279) = 5.495$, $p < 0.0001$). Paired *t*-tests showed that upon the presentation of the predator image, the variability of distance to bottom significantly decreased (interval of first predator image vs. prior interval $t = 5.926$, df = 31, $p < 0.0001$; interval of second predator image vs. prior interval $t = 2.417$, df = 31, $p < 0.05$; interval of third predator image vs. prior interval $t = 1.704$, df = 31, $p > 0.05$) as compared to the value obtained during the 1-min interval preceding

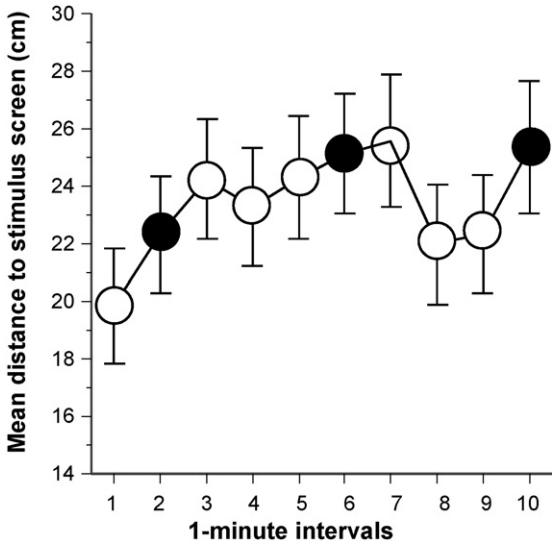

Fig. 2. Distance of experimental zebrafish to the stimulus screen is not affected by the presentation of the predator image. Mean +S.E.M. are shown. Sample size, $n = 32$. Each data point represents the average of responses during the corresponding 1-min interval. The intervals during which the predator image was shown are represented by the black circles.

the image presentation. The speed of experimental fish (velocity) was also significantly affected by the appearance of the image (ANOVA interval $F(9, 279) = 5.711$, $p < 0.0001$). Velocity significantly decreased each time the image appeared (Fig. 6; interval of first predator image vs. prior interval $t = 2.723$, df = 31, $p < 0.01$; interval of second predator image vs. prior interval $t = 2.733$, df = 31, $p < 0.01$; interval of third predator image vs. prior interval $t = 4.463$, df = 31, $p < 0.0001$). Interestingly, while mean velocity decreased, the variability of velocity significantly increased (ANOVA interval $F(9, 279) = 5.098$, $p > 0.0001$) upon presentation of the image of the predator (Fig. 7; interval of first predator image vs. prior interval $t = 1.347$, df = 31, $p > 0.05$; interval of second predator image vs. prior interval $t = 2.665$, df = 31, $p < 0.05$; interval of third predator image vs. prior interval $t = 2.238$, df = 31, $p < 0.05$). Mean turn

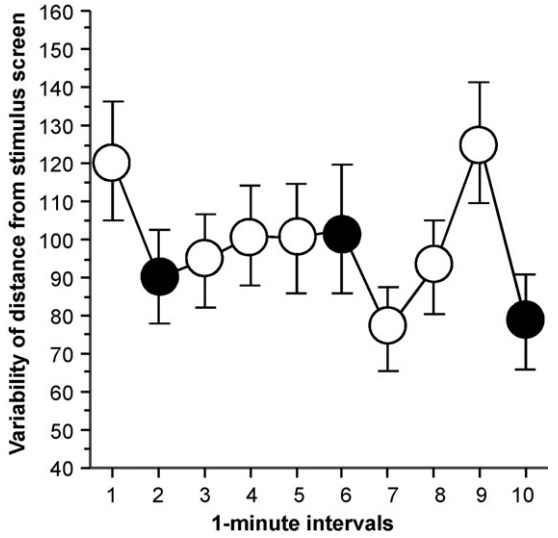

Fig. 3. The variability of distance of zebrafish to the stimulus screen is not significantly affected by the presentation of the predator image. Mean +S.E.M. are shown. Sample size, $n = 32$. Each data point represents the average of responses during the corresponding 1-min interval. The intervals during which the predator image was shown are represented by the black circles.

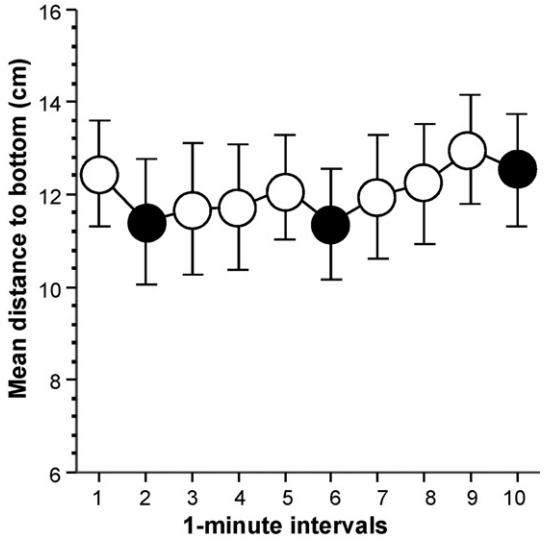

Fig. 4. The distance of zebrafish to the bottom of the tank is not significantly affected by the presentation of the predator image. Mean +S.E.M. are shown. Sample size, $n = 32$. Each data point represents the average of responses during the corresponding 1-min interval. The intervals during which the predator image was shown are represented by the black circles.

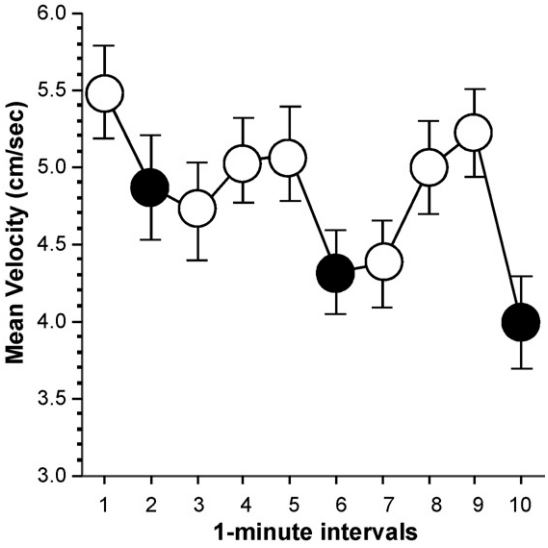

Fig. 6. Velocity of zebrafish is significantly reduced by the presentation of the predator image. Mean +S.E.M. are shown. Sample size, $n = 32$. Each data point represents the average of responses during the corresponding 1-min interval. The intervals during which the predator image was shown are represented by the black circles.

angle also significantly increased in response to the appearance of the predator (ANOVA interval $F(9, 279) = 2.757$, $p < 0.01$) and this increase became more pronounced with repeated presentations (Fig. 8; interval of first predator image vs. prior interval $t = 0.943$, df = 31, $p > 0.35$; interval of second predator image vs. prior interval $t = 2.919$, df = 31, $p < 0.01$; interval of third predator image vs. prior interval $t = 2.936$, df = 31, $p < 0.01$). Last, the variability of turn angle (Fig. 9) showed a very similar pattern to that of the mean turn angle, with significant increases in values (ANOVA interval $F(9, 279) = 4.365$, $p < 0.0001$) that became more pronounced with repeated exposure to the predator image (interval of first predator image vs. prior interval $t = 0.777$, df = 31, $p > 0.40$; interval of second predator image vs. prior interval $t = 2.935$, df = 31, $p < 0.01$; interval of third predator image vs. prior interval $t = 3.103$, df = 31, $p < 0.01$).

To enhance the interpretation of the results of the computer automated video-tracking we also analyzed data obtained using observation-based quantification of motor-posture patterns. This analysis showed that the percent of time fish performed erratic movement (zig-zagging) significantly increased (Fig. 10) in response to the predator image presentation (ANOVA $F(9, 270) = 3.080$, $p < 0.01$; interval of first predator image vs. prior interval $t = 1.901$, df = 30, $p = 0.087$; interval of second predator image vs. prior interval $t = 2.158$, df = 30, $p < 0.05$; interval of third predator image vs. prior interval $t = 3.276$, df = 30, $p < 0.01$). A similar pattern of results was obtained for the frequency of jumping (Fig. 11). ANOVA revealed a significant interval effect ($F(9, 270) = 4.643$, $p < 0.0001$) and paired t-tests showed that the predator presentation significantly increased this behavior (interval of first predator image vs. prior interval $t = 3.960$, df = 30, $p < 0.0001$;

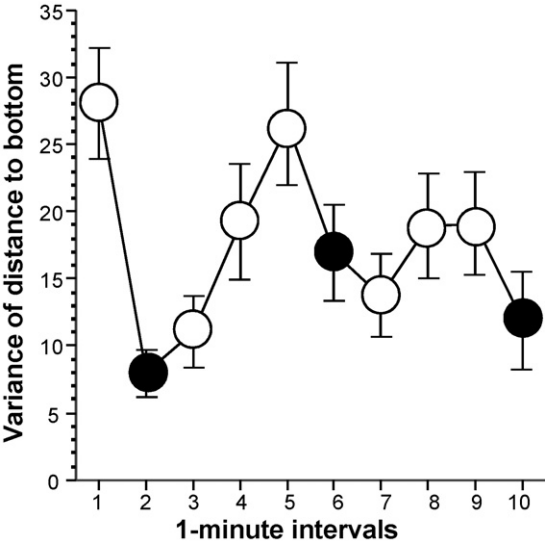

Fig. 5. The variability of distance of zebrafish to the bottom of the tank is significantly decreased by the presentation of the predator image. Mean +S.E.M. are shown. Sample size, $n = 32$. Each data point represents the average of responses during the corresponding 1-min interval. The intervals during which the predator image was shown are represented by the black circles.

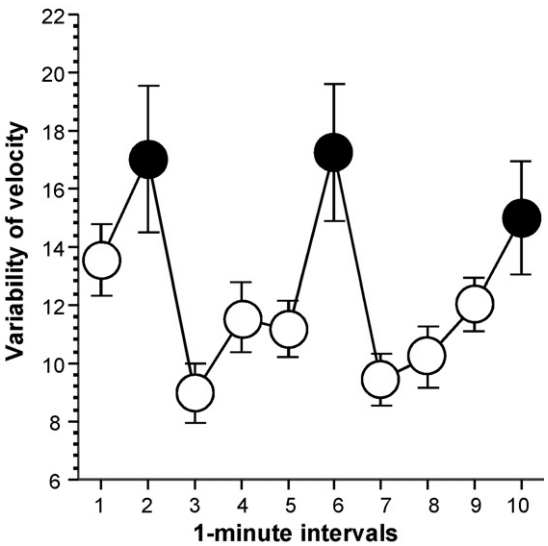

Fig. 7. The variability of velocity is significantly increased by the presentation of the predator image. Mean +S.E.M. are shown. Sample size, $n = 32$. Each data point represents the average of responses during the corresponding 1-min interval. The intervals during which the predator image was shown are represented by the black circles.

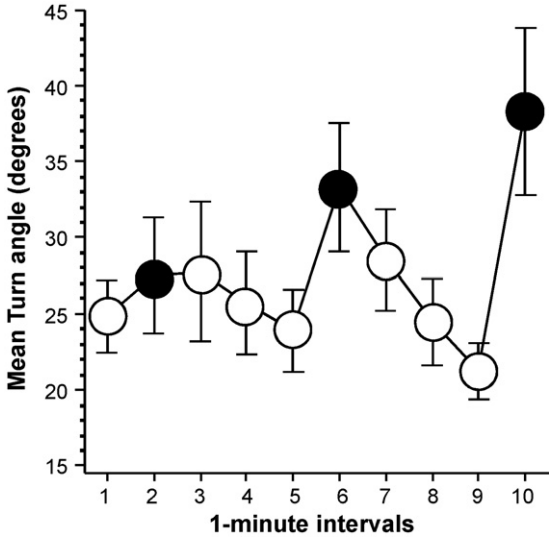

Fig. 8. The mean angle of turns is significantly increased by repeated presentation of the predator image. Mean +S.E.M. are shown. Sample size, $n = 32$. Each data point represents the average of responses during the corresponding 1-min interval. The intervals during which the predator image was shown are represented by the black circles.

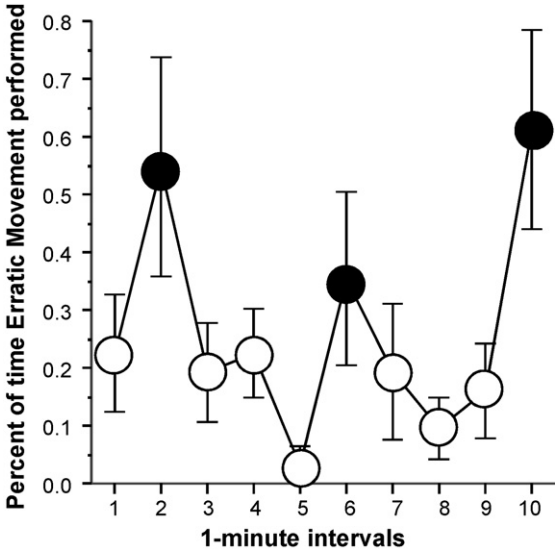

Fig. 10. Percent of time Erratic Movement was performed is significantly increased by the presentation of the predator image. Mean +S.E.M. are shown. Sample size, $n = 31$. Each data point represents the average of responses during the corresponding 1-min interval. The intervals during which the predator image was shown are represented by the black circles.

interval of second predator image vs. prior interval $t = 2.295$, df = 30, $p < 0.05$; interval of third predator image vs. prior interval $t = 2.572$, df = 30, $p < 0.05$). Last, we analyzed the percent of time the fish were swimming (Fig. 12) and changes in this behavior in response to the presentation of the predator image. ANOVA revealed a significant interval effect ($F(9, 270) = 2.401$, $p < 0.05$) and paired t-tests suggested that upon predator image presentation, zebrafish decreased swimming (interval of first predator image vs. prior interval $t = 1.840$, df = 30, $p = 0.076$; interval of second predator image vs. prior interval $t = 3.669$, df = 30, $p < 0.001$; interval of third predator image vs. prior interval $t = 1.825$, df = 30, $p = 0.078$).

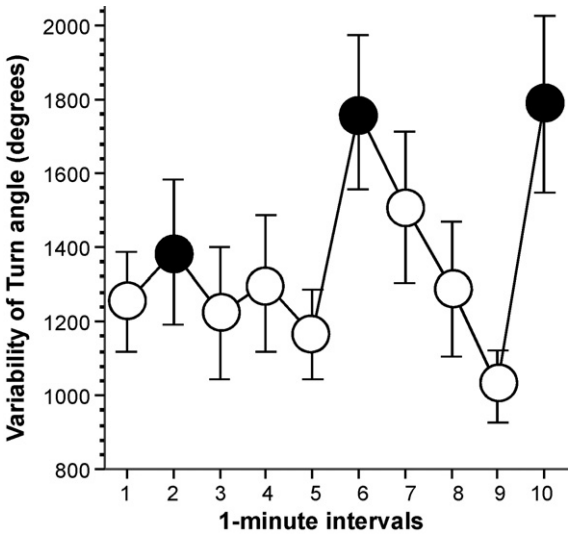

Fig. 9. Variability of turn angle is significantly increased by repeated presentation of the predator image. Mean +S.E.M. are shown. Sample size, $n = 32$. Each data point represents the average of responses during the corresponding 1-min interval. The intervals during which the predator image was shown are represented by the black circles.

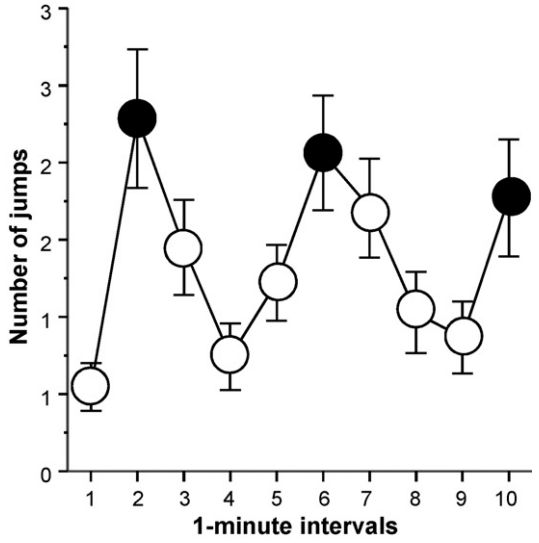

Fig. 11. The number of jumps is significantly increased by repeated presentation of the predator image. Mean +S.E.M. are shown. Sample size, $n = 31$. Each data point represents the average of responses during the corresponding 1-min interval. The intervals during which the predator image was shown are represented by the black circles.

4. Discussion

Briefly, our results demonstrate that the presentation of a moving image of the Indian leaf fish elicits significant behavioral responses in zebrafish and that these responses are quantifiable using video-tracking. The behavioral changes include decreased variability of distance from the bottom, decreased mean velocity and increased variability of velocity, as well as increased mean turn angle and increased variability of turn angle.

Importantly, variability in this context does not refer to inter-individual variability characteristic of the sampled group, but rather it represents the temporal variability of the behavioral response of each individual. Thus decreased variability of the distance from bottom in response to the predator image (but no change in the mean)

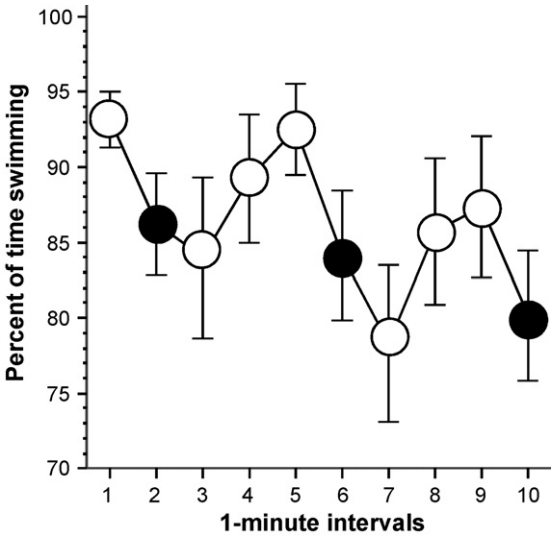

Fig. 12. The percent of time fish were swimming is significantly reduced by repeated presentation of the predator image. Mean +S.E.M. are shown. Sample size, $n = 31$. Each data point represents the average of responses during the corresponding 1-min interval. The intervals during which the predator image was shown are represented by the black circles.

reflects reduced changes in swimming along the vertical axis. It is possible that the experimental fish stopped moving around, reduced their exploratory activity aimed at their general environment, and focused their attention to the stimulus presented. This hypothesis is supported by finding significantly reduced swim speed in response to the predator image and is in accordance with previous results where zebrafish were presented with inanimate predator models [6] or live stimulus fish [3].

The significantly increased variability of swim speed is also noteworthy in this context. Again, this measure reflects the variability of swim speed within each individual, i.e. variability that arises as a result of temporal changes of velocity during the recording of individual fish. It appears that while overall activity levels decreased, zebrafish that were shown the predator image engaged in occasional fast swim bouts. We predicted that the bouts of increased swim speed were likely to be associated with fast zig-zagging (also called erratic movement) and/or leaps (also termed jump) often observed in response to aversive stimuli, including the presence of a live Indian leaf fish [3,15] or the delivery of alarm substance of zebrafish [27]. Our subsequent observation-based analyses confirmed this prediction and found that the percent of time erratic movement was performed and the number of jumps indeed increased in response to the predator image presentation while the percent of time the fish swam decreased. Furthermore, zig-zagging and jumping appear sporadically and are associated with abrupt swim direction changes therefore they are expected to increase both the variability and the mean of turn angle of the swim path and thus also explain why increases were indeed detected in these latter measures. In summary, the videotracking-based behavioral measures correlated well with the results obtained using the labor intensive observation-based quantification approach. Given that in the newly developed paradigm both stimulus delivery and behavior response quantification is computerized and does not require the constant presence of an experimenter, the paradigm is automated and multiple sessions may be run in parallel, an important consideration in high throughput screening.

The lack of changes in the distance from stimulus screen and distance from the bottom was unexpected. Previously, we found that when presented with a moving predator model, an 11.5 cm long and 3 cm diameter black falcon tube with artificial eyes, zebrafish moved away from the location of presentation (but not from the surface) [6]. Others reported that zebrafish swim to the bottom (diving) under aversive conditions [20]. We also found that, albeit in an inverted U-shaped manner, certain doses of the alarm pheromone increased bottom dwell time [27]. Interestingly, however, neither response was observed here: our zebrafish did not move away from the predator image nor did they increase their bottom dwell time in response to the image. Notably, the species-typical antipredatory responses induced by the live sympatric predator of zebrafish also did not include these responses [3]. It is notable that antipredatory responses may involve a range of behaviors that manifest in a context, i.e. environment-specific manner. For example, depending on the distance from which a prey detects the appearance of the predator, different antipredatory strategies may be adaptive (for review see [9]). A predator not detected in time, i.e. one that appears within striking distance, may not induce simple avoidance, and under these circumstances the prey may respond with behaviors other than moving away from the predator. For example, paradise fish (*Macropodus opercularis*) or the three-spined stickleback, (*Gasterosteus aculeatus*) respond with a fin erection display and perform a dance in front of the predator instead of swimming away (e.g. see [13] and references therein). It is possible that the length of the experimental tank was too short for zebrafish to stay outside of "striking distance" from the predator and thus our experimental fish chose an alternative strategy, e.g. jumping and erratic movement [3,27], a hypothesis that suggests we will need to further explore and optimize the parameters of our test paradigm.

A general question whether behavioral screening may have utility at all in the analysis of brain function has been extensively discussed elsewhere [12,16]. The more specific issue of how one can utilize behavioral methods to screen mutation induced changes in zebrafish has also been discussed [15]. Briefly, the issue is that behavioral phenotypes are variable and thus a mutation (or a drug) induced alteration must be detected in the backdrop of this variability. Although this is a valid concern, experimental examples show this problem not to be insurmountable to address. Pharmaceutical compound screens are routinely conducted using laboratory rodents and even such complex phenotypes as cocaine rewarded place preference have been successfully used as screening criteria for the identification of mutant zebrafish [8].

The last point we consider is the utility of zebrafish in translation research, i.e. the applicability of the above results for the analysis of human anxiety. First, it is important to distinguish fear and anxiety. In the current paradigm, we measured natural fear responses and did not attempt to induce prolonged or abnormally exaggerated fear, the latter features often regarded as defining anxiety. But, given the ethological relevance of the employed paradigm [3], and the argument that human anxiety is likely to be associated with exaggerated or abnormal activation of fear circuits that evolved under natural selection [5], one could argue that the newly developed paradigm does have some face validity for anxiety research. Whether the mechanisms underlying the observed fear responses are similar to those of other vertebrates including humans, i.e. whether the paradigm has construct validity, is difficult to answer at this point, given the paucity of such information in zebrafish and in human alike. Predictive validity, i.e. the question of whether the paradigm could detect the effects of anxiolytic, anxiogenic and/or fear altering substances, is also difficult to address at this point because very few studies have attempted to use pharmacological approaches with zebrafish and thus numerous issues including optimization of delivery methods, absorption, distribution, metabolism, and secretion of the drug, as well as dose response analyses must be conducted before predictive validity may be addressed.

It is clear therefore that the novel paradigm presented here is not a "model" of any human disorder. Nevertheless, the results suggest

that zebrafish is a promising tool with which the mechanisms of vertebrate fear responses may be studied. In summary, we view the fear paradigm and the results it generated as only the first step in the right direction towards the investigation and modeling of human anxiety and exaggerated fear.

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