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Research report

Zebrafish (*Danio rerio*) responds differentially to stimulus fish: The effects of sympatric and allopatric predators and harmless fish

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Abstract

The zebrafish has been an excellent model organism of developmental biology and genetics. Studying its behavior will add to the already strong knowledge of its biology and will strengthen the use of this species in behavior genetics and neuroscience. Anxiety is one of the most problematic human psychiatric conditions. Arguably, it arises as a result of abnormally exaggerated natural fear responses. The zebrafish may be an appropriate model to investigate the biology of fear and anxiety. Fear responses are expressed by animals when exposed to predators, and these responses can be learned or innate. Here we investigated whether zebrafish respond differentially to a natural predator or other fish species upon their first exposure to these fish. Naïve zebrafish were shown four species of fish chosen based on predatory status (predatory or harmless) and geographical origin (allopatric or sympatric). Our results suggest that naïve zebrafish respond differentially to the stimulus fish. Particularly interesting is the antipredatory response elicited by the zebrafish's sympatric predator, the Indian Leaf Fish, and the fact that this latter species exhibited almost no predatory attacks. The findings obtained open a new avenue of research into what zebrafish perceive as "dangerous" or fear inducing. They will also allow us to develop fear and anxiety related behavioral test methods with which the contribution of genes to, or the effects of novel anxiolytic substances on these behaviors may be analyzed.

Keywords: Antipredatory behavior; Anxiety; Fear; Genetic predisposition; Zebrafish

1. Introduction

Zebrafish, or zebra danio (*Danio rerio*), have been a popular subject of developmental biology and as a result numerous genetic tools have already been developed for this species [23]. Due to the accumulation of genetic knowledge and techniques, other disciplines that could utilize genetics have also taken notice of zebrafish. Among these disciplines are behavioral neuroscience and behavior genetics (e.g. [17]). Given the speed with which a large number of offspring may be generated (a single female zebrafish can spawn 200 eggs every other day) and given the small size and ease of maintenance of this species [40], some suggest that the zebrafish is an ideal vertebrate model system with which large scale genetic and pharmacological screens may be conducted (e.g. [37]). A significant drawback of this species, however, is the lack of understanding of its brain function and behavior [37]. The foundation of genetic and pharmacological screens is phenotypical characterization. Perhaps one of the best ways to analyze brain function is to measure the output of this organ, i.e. behavior (e.g. [21,18], see also [7]). Thus understanding of the behavior of zebrafish is of crucial importance.

One of the most prevalent human psychiatric diseases is anxiety or pervasive phobias [38,11]. It is likely that these psychiatric conditions are due to abnormally functioning neurobiological processes (pathways, circuits, connections, and/or molecular mechanisms) that originally evolved to support adaptive fear responses (e.g. [6]). An important adaptive fear response is predator avoidance, or avoidance of harmful species [6]. For example, being afraid of the dark or of snakes and spiders may have been adaptive features of our own species that led to differentially increased survival of genes that predisposed their carriers to the appropriate avoidance behaviors [9,28]. It is likely that pathological alterations in the mechanisms that developed in response to natural selection pressures underlie numerous

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forms of anxiety disorders (e.g. [26,28]). Understanding the mechanisms of antipredatory responses may therefore have clinical relevance, and thus predation models have been proposed in anxiety research [26]. One way to investigate such mechanisms is with the use of model organisms. Given the features of zebrafish discussed above, we suggest that this species will be an appropriate model organism for this purpose.

Little is known about the fear reactions or antipredatory behavior of zebrafish. However, being able to properly respond to a dangerous predator has been shown to be highly adaptive in numerous species including cephalopods [1], fish [19,34], amphibians [30], reptiles [10], birds [33], and mammals (e.g. [6]), including primates [36]. The appropriate responding can be achieved via learning. After multiple exposures to the predator and after having experienced its harmful effects, members of a prey species may develop avoidance. Alternatively, a prey species may evolve genetic predispositions that would "instruct" the prey to avoid the predator even at the first encounter. It appears that evolution has favored this latter solution to learning in a number of species. Numerous examples exist for this phenomenon in fish. Paradise fish exhibit differential responses towards a natural (sympatric) predator in comparison to a foreign (allopatric) predator without prior exposure to these species [19]. Notably, the differential responses could be elicited both by visual and olfactory cues. Hawkins et al. [5] found that newly hatched Atlantic salmon reacted differently to the smell of a pike, their natural predator, in comparison to the smell of an omnivorous fish [25]. Berejikian et al. discovered that naïve Chinook salmon also display an innate differential response to a natural predator, the northern pikeminnow [5]. Bleakley et al. demonstrated innate behavioral responses in various populations of guppies towards a predator, the Midas Cichlid (*Amphilophus citrinellus*) [8].

Based on the above it is reasonable to propose that zebrafish may also be able to differentiate predatory and non-predatory species and perhaps even sympatric and allopatric predators. The current study was conducted to investigate this question. We analyzed how zebrafish responded to four different fish species, i.e. the stimulus fish: a sympatric predator, an allopatric predator, a sympatric harmless species, and an allopatric harmless species.

2. Materials and methods

2.1. Experimental subjects: zebrafish

Three hundred and six zebrafish were tested in this study. The fish were obtained from a local pet store, Big Al's Aquarium Warehouse (Mississauga, Ontario, Canada), and were of a genetically mixed origin. The rationale for choosing this fish population is as follows: strains kept in scientific breeding facilities have a limited effective population size (number of breeding individuals), which leads to random fixation of alleles, i.e. genetic drift. Such populations may thus develop unique heritable characteristics. Big Al's Aquarium Warehouses import their zebrafish from commercial breeding facilities located near or at the natural habitat of zebrafish (e.g. Singapore, India, Thailand). The effective population size in these facilities is enormous given that they supply aquarium fish to multiple continents. Thus the zebrafish population we chose is expected to have a high level of genetic variability and should better represent the natural, genetically heterogeneous, wild population, and the behavior of the chosen population should better approximate what is characteristic of the species in general. The experimental fish were housed in 3 l transparent acrylic tanks (15 fish per tank) that were part of a special zebrafish rack system (Aquaneering Inc., San Diego, CA, USA) with multistage filtration that contained a mechanical filter, a fluidized glass bed biological filter and an activated carbon filter, as well as a fluorescent UV light sterilizing unit. Every day 10% of the water was replaced with fresh system water (deionized water supplemented with 60 mg/l Instant Ocean Sea Salt [Big Al's Pet Store, Mississauga, Ontario, Canada]). The water temperature was maintained at 27 °C. Illumination was provided by fluorescent light tubes from the ceiling with lights turned on at 08:00 h and off at 19:00 h. Fish were fed a mixture of ground freeze-dried krill and flake food (Tetramin Tropical Flakes, Tetra, USA).

Zebrafish were tested in groups of five at their age of 8 months (3.6–4.0 cm long). The rationale for using multiple test subjects at a time is that the zebrafish is a shoaling species that forms groups in which fish swim close to one another. At this point it is unknown how shoal size may affect the behavior of zebrafish in the current behavioral tests. Nevertheless, by allowing the experimental fish to shoal, we hoped our subjects would behave in a more natural way. Furthermore, shoaling is also believed to reduce fear associated with handling the fish by the experimenter and allowed us to better quantify fear reactions induced by the stimulus fish. It is also notable that pharmacological screening is feasible with groups of zebrafish (see, e.g. Daniolabs, http://www.daniolabs.com/).

2.2. Stimulus fish

Four species of fish were selected as stimulus fish (fish to which the zebrafish would respond in the test). The selection criteria were as follows: one, predatory status (predatory or harmless), and two, geographical origin (sympatric or allopatric). The sympatric predator was the Leaf Fish (*Nandus nandus*) and the sympatric harmless fish was the Giant Danio (*Danio malabaricus*) ([27,12], see also [15]). The allopatric predator was the Compressed Cichlid (*Nimbochromis compressiceps*), a species that lives in Lake Malawi (Africa) [29]. The allopatric harmless fish was an artificially bred strain of Swordtail Fish (*Xiphophorus helleri*) whose natural variety lives in Central America [4]. All stimulus fish subjects were of the same size (7 cm long). Notably, a Leaf Fish and Compressed Cichlid of this size could eat adult sized (3–4 cm long) zebrafish, but the harmless fish species could not. The stimulus fish were housed in 40 l glass tanks (four individuals of the same species per tank). Only a pair of stimulus fish per species was used throughout the experiments, i.e. the experimental zebrafish assigned to a particular stimulus group viewed the same stimulus fish individuals. Although this set-up does not allow characterization of the stimulus fish species *per se*, it provides a consistent and reliable stimulus set to which our experimental zebrafish respond. The conditions of maintenance were identical to what has been described for zebrafish, except that the Compressed Cichlid was kept in hard tap water. The tanks were filtered using Eheim external canister filters with mechanical, biological, and chemical filtration components.

2.3. Apparatus

The experimental zebrafish were monitored in 37 l observation tanks (50 cm × 25 cm × 30 cm, length × width × height) illuminated by a fluorescent light tube Eclipse 13 W placed directly above the observation tank. A digital video-camera (Sony DCR-HC20, Sony Corporation, Japan) placed in front of the observation tank recorded the behavior of zebrafish. The video-recordings were later replayed and were analyzed. A 10 l tank (30 cm × 15 cm × 20 cm, length × width × height), the stimulus tank, was placed on each side of the observation tank. One of these stimulus tanks contained the stimulus fish and the other was empty, except for water. Due to the fact that the stimulus fish and experimental zebrafish were in separate tanks, only visual stimuli may be perceived by the test fish and olfactory or lateral line stimuli were obstructed. The rationale for such a set up, i.e. for focusing on the effects of visual stimuli alone, is that we are hoping to develop computer animations in the future and use precisely controlled computer images in high throughput screening applications.

Removable visual barriers (white plastic sheets) were placed in between the observation and stimulus tanks and were removed for the duration of the observation sessions. Water temperature and quality were maintained as described above.

2.4. Procedure

Experimental zebrafish were randomly assigned to five groups: zebrafish in group 1 were exposed to no stimulus fish, zebrafish in group 2 were exposed to the Giant Danio (sympatric harmless fish), zebrafish in group 3 were exposed to the Swordtail fish (allopatric harmless fish), zebrafish in group 4 were exposed to the Indian Leaf Fish (sympatric predator), and zebrafish in group five were exposed to the Compressed Cichlid (allopatric predator). For zebrafish groups 2–5, two stimulus fish of the same species were placed into one of the stimulus tanks 1 h before the recording session started. The other stimulus tank was left empty. For the no stimulus group, both stimulus tanks were empty. Stimulus fish were presented on either the left or right side of the observation tank and the presentation side varied from session to session in a random manner. The rationale for using two stimulus fish at a time is that when in pairs these fish would habituate to their stimulus tank faster and exhibit their natural behavioral repertoire thus providing a more naturalistic stimulus for the experimental zebrafish.

Zebrafish were tested in the observation tank in groups of five. They were netted out from their holding tank and while in the net were placed in a 500 ml beaker that contained oxygenated system water. Fish were moved to the observation tank with the beaker and were netted into it. As explained before, zebrafish is a shoaling species that forms small groups, or shoals, in nature and thus is expected to exhibit its natural behavioral repertoire better when in groups. Solitary fish may experience elevated fear unrelated to the stimulus fish provided. Each experimental zebrafish group was only tested once and only with a single species of stimulus fish; a between subject design. After a 10 min acclimation period the white boards covering the stimulus fish tank were removed and the recording of fish behavior began. The recording session lasted for 10 min during which the behavior of both the experimental zebrafish and the stimulus fish was video recorded. During the habituation and testing periods the experimenter was not present in the testing room. Experimental zebrafish groups exposed to different stimulus fish species were tested in a randomized order.

2.5. Quantification of behavior

A custom software application developed in our laboratory [31] was used to measure the average distance of zebrafish from the stimulus tank. Briefly, the horizontal distance between each zebrafish and the side of their tank closest to that of the stimulus fish was measured every 10 s for three 1 min intervals, the 1st, 5th, and 9th min of the observation. All of the measurements for each interval were averaged for the five fish of the given group resulting in three distance values per group that we statistically analyzed. In addition, the software also allowed us to precisely measure the distance among all fish within a shoal (group of experimental fish in a session). The distances between every possible pair of fish of the shoal are calculated and averaged for each image sample (taken once every 10 s). The high resolution and averaging allowed us to reduce variation associated with potential recording error.

Importantly, we also quantified motor and posture patterns using Observer ColorPro, an event recording software (Noldus, Wageningen, The Netherlands). Video-recordings were replayed and the following behaviors were quantified: *Freezing*: this behavior is associated with complete cessation of movement, only the opercula and the eyes may move. *Swimming*: locomotion with the use of the caudal fin. *Thrashing*: forceful swimming movement against the glass wall of the tank. This latter behavior was performed against the glass of the tank facing the camera, the stimulus tank, or the empty tank. Thus we differentiated *thrashing towards camera*, *thrashing towards stimulus*, and *thrashing away from stimulus*, respectively. *Erratic movement*: a series of quick, zig-zagging movements. *Jumping*: a quick single leap. According to our preliminary observations most experimental fish of a given group performed the particular motor or posture pattern simultaneously and thus we did not measure the above behaviors for every single experimental zebrafish separately, but rather recorded the occurrence of behavior in at least one of the fish of the group as follows: erratic movement was measured separately from all other behaviors as a percentage of test time spent moving erratically. If at least one of the fish of the group of five performed erratic movement, erratic movement was recorded. Freezing, swimming, and the three thrashing behaviors were also measured as a percentage of time of the observation session and were mutually exclusive. If at least one of the fish showed freezing, freezing was recorded. If freezing was not performed by any of the experimental fish but at least one of them showed thrashing, thrashing was recorded. Given the shoaling nature of zebrafish, thrashing always occurred on one glass wall only and thus only one of the above three thrashing measures could be recorded. If no freezing and no thrashing occurred, but the fish were moving actively, swimming was recorded. Jumping appeared to have a well-defined and very short bout. Thus this behavior was quantified using the number of times it occurred, i.e. its frequency. Frequency is defined as the number of episodes during which the behavior was performed by any fish. For example, if three fish performed jumping simultaneously, a single jumping episode was recorded.

Importantly, we recorded the behaviors of the stimulus fish as well. This is not always done in studies analyzing antipredatory behaviors despite that such analysis may shed light on what aspects of the stimulus fish the potential prey, zebrafish in this case, may be responding to. The following stimulus fish behaviors were recorded: *Swimming*, active and fast (2 cm s^{-1} or faster) locomotion with the use of the caudal fin. *Moving*, slow locomotion without the use of the caudal fin. *Stationary behavior*, a lack of locomotory movements (note that we do not call this behavior freezing because here not only the opercula and the eyes but also the pectoral and/or dorsal and anal fins may move, a behavior previously described as "floating", see e.g. [22]). *Approach*, a slow movement similar to "Moving" but in the direction of the zebrafish. *Thrashing toward*, a forceful swimming against the glass of the tank facing the zebrafish observation tank. *Thrashing* is similar to the previous behavior but on glass walls other than the one adjacent to the zebrafish observation tank. *Attack*, a quick, darting movement towards the zebrafish. All behaviors but attack were measured as a percentage of observation time and were mutually exclusive, whereas attack was quantified as frequency, i.e. the number of times it occurred. Similarly to the experimental zebrafish, the pair of stimulus fish presented to the test fish also performed most behaviors in synchrony. Therefore, the behavior of the two stimulus fish was not recorded separately, but instead a hierarchical recording procedure similar to the methods employed for the quantification of zebrafish behavior was followed. Only if both stimulus fish were stationary, stationary behavior was recorded. If one of the stimulus fish performed thrashing, thrashing was recorded. If one of them was swimming but neither showed thrashing, swimming was recorded. If the stimulus fish showed slow movement, moving was recorded. When an attack was performed by either stimulus fish, an attack episode was recorded. The number of such episodes (frequency) was counted and later statistically analyzed.

2.6. Statistical analysis

Data analysis was carried out using SPSS 14.01 for the PC. The unit of analysis was the fish shoal (the group of five experimental zebrafish observed and quantified in each session). Thus sample sizes (n) indicated in the figure legends represent the number of five-fish shoals. One-way analysis of variance (ANOVA) was conducted to investigate the effect of stimulus fish (four stimulus fish and one control = five levels). Two-way repeated measures ANOVA (with time as repeated measure factor and stimulus treatment condition as between subject factor) was also employed when appropriate. In case of significant effect, *post hoc* multiple comparisons Tukey honestly significant difference test was performed. In case of significant variance inhomogeneity, appropriate scale transformation was sought before ANOVA as indicated in the results section. The correlation between stimulus fish behavior and zebrafish behavior was conducted using Pearson product moment correlation separately for each stimulus fish species.

3. Results

3.1. Behavioral responses of zebrafish to the stimulus fish

Fig. 1 shows the distance of zebrafish from the stimulus side of the observation tank, or in case of the control (no stimulus), the distance from the same side where the previous stimulus fish treatment was administered. Repeated measures ANOVA

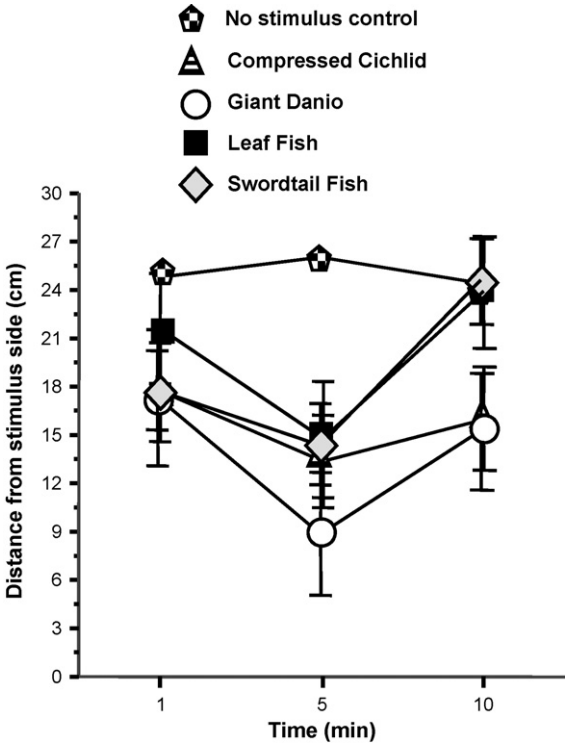

Fig. 1. Temporal changes of the location of experimental zebrafish relative to the designated stimulus presentation side. Mean ± S.E.M. shown. Sample sizes of zebrafish shoals exposed to the different stimulus fish treatments were as follows (stimulus treatment indicated in brackets): n (Compressed Cichlid) = 12, n (Giant Danio) = 11, n (Leaf Fish) = 13, n (Swordtail Fish) = 12, n (Control, no stimulus) = 9. Note that the distance was measured during three 1 min intervals of the recording session and that a significant time effect was found with the values smallest in the middle of the session for zebrafish exposed to stimulus fish but not for zebrafish exposed to no stimulus fish. For procedural and analysis details see Sections 2 and 3.

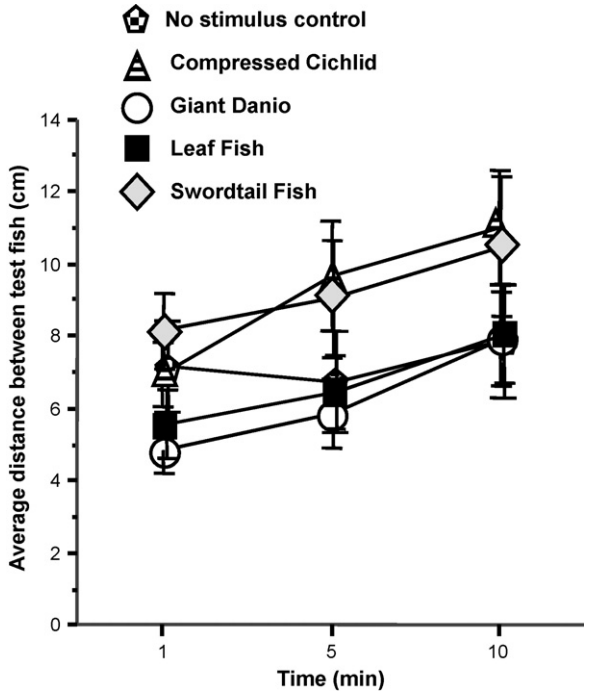

Fig. 2. Average distance between experimental zebrafish (shoal cohesion) was unaffected by stimulus treatment. Mean ± S.E.M. shown. Sample sizes of zebrafish shoals exposed to the different stimulus treatments were as follows (stimulus treatment indicated in brackets): n (Compressed Cichlid) = 12, n (Giant Danio) = 11, n (Leaf Fish) = 13, n (Swordtail Fish) = 12, n (Control, no stimulus) = 9. Note that the distance between all possible pairs of zebrafish within the shoal was measured and averaged during three 1 min time intervals of the recording session and that a significant time-dependent increase of distance between shoal mates was found but stimulus treatment had no significant effect.

revealed a significant time effect ($F(2, 104) = 5.46$, $p < 0.01$), but the effect of stimulus fish treatment was non-significant (ANOVA, $F(4, 54) = 1.37$, $p > 0.05$), and the interaction between time and stimulus fish bordered significance (ANOVA, $F(8, 104) = 2.01$, $p = 0.06$). The latter effect is apparent on Fig. 1 that shows that the distance from stimulus was smallest in the middle of the session (5th min) compared to the beginning (1st min) and the end (10th min) of the session in zebrafish that were presented with stimulus fish, but the control fish (no stimulus) remained unchanged across these periods. Notably, the pattern of temporal changes did not differ among zebrafish groups presented with different stimulus fish species.

Repeated measures ANOVA also showed that the distance between members of the zebrafish shoal significantly increased ($F(2, 104) = 18.08$, $p < 0.001$) with time during the observation session (Fig. 2). However, neither the effect of stimulus fish treatment (ANOVA, $F(4, 54) = 1.37$, $p > 0.05$) nor the interaction between time and stimulus fish treatment was significant (ANOVA, $F(8, 104) = 1.00$, $p > 0.05$). The results reveal a stimulus independent decrease of shoal cohesion, a phenomenon that is likely associated with habituation to, i.e. decreasing fear of, the novel test environment [31].

Analysis of the motor and posture patterns exhibited by zebrafish revealed significant stimulus fish treatment effects. Fig. 3 shows the results obtained for freezing. To homogenize variances among groups, we employed logarithm transformation. Analysis of the logarithm transformed data demonstrated that stimulus fish treatment had a significant effect on freezing in zebrafish (ANOVA, $F(4, 54) = 3.26$, $p < 0.05$). Tukey HSD *post hoc* multiple comparison test revealed that in response to the Swordtail Fish (the allopatric harmless species) the zebrafish froze more ($p < 0.05$) as compared to in response to the Giant Danio (the sympatric harmless species). The control (no stimulus) group exhibited no freezing and also significantly ($p < 0.05$) differed from the zebrafish exposed to the Swordtail Fish. Analysis of swimming (Fig. 4) also showed that the type of stimulus fish used affected the behavior of zebrafish significantly (ANOVA, $F(4, 54) = 13.69$, $p < 0.001$). Tukey HSD *post hoc* multiple comparison test revealed that in response to the Giant Danio zebrafish swam less ($p < 0.05$) as compared to the other fish. Notably, zebrafish that were exposed to no stimulus fish exhibited the highest amount of swimming and were significantly ($p < 0.05$) different from all other groups. This appeared to be due to the fact that zebrafish exposed to stimulus fish engaged in performing a number of behaviors other than swimming. For example, the reduced swimming and reduced freezing response shown towards the Giant Danio was accompanied by a significantly increased Thrashing towards (Fig. 5) this stim-

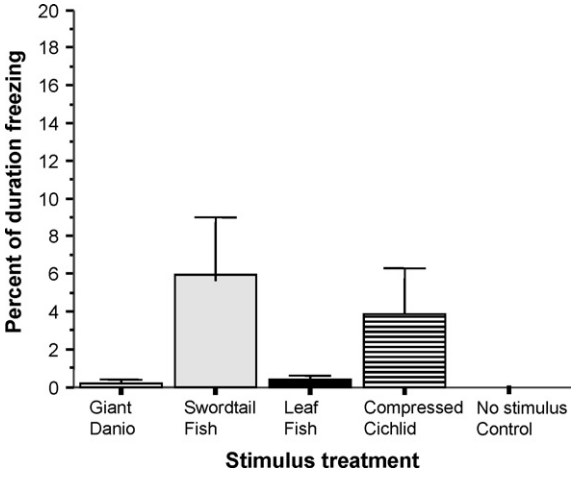

Fig. 3. Zebrafish showed a significantly different amount of freezing (complete immobility) dependent upon stimulus fish treatment. Mean ± S.E.M. shown. Sample sizes of zebrafish shoals exposed to the different stimulus fish treatment were as follows (stimulus treatment indicated in brackets): n (Compressed Cichlid) = 12, n (Giant Danio) = 11, n (Leaf Fish) = 13, n (Swordtail Fish) = 12, n (Control, no stimulus) = 9. Note that although it appears zebrafish responded with highest amount of freezing to the two allopatric species (the Swordtail Fish and the Compressed Cichlid), only the difference between the Swordtail Fish and the Giant Danio turned out to be statistically significant. Also note that the no stimulus control group showed no freezing and is also significantly different from the Swordtail Fish treatment group.

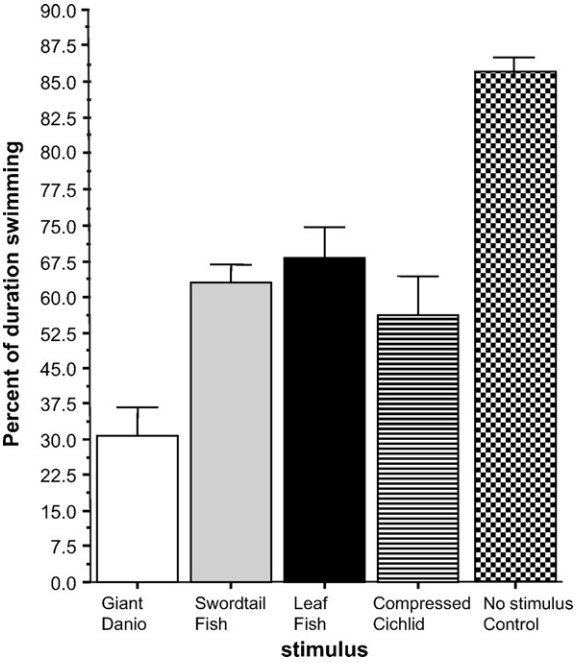

Fig. 4. Zebrafish's active swimming was significantly affected by stimulus fish treatment. Mean ± S.E.M. shown. Sample sizes of zebrafish shoals exposed to the different stimulus fish treatment were as follows (stimulus treatment indicated in brackets): n (Compressed Cichlid) = 12, n (Giant Danio) = 11, n (Leaf Fish) = 13, n (Swordtail Fish) = 12, n (Control, no stimulus) = 9. Note that the amount of swimming zebrafish performed was significantly smaller in response to the Giant Danio as compared to the other three stimulus fish species, and the effect of these latter species did not differ from one another. Also note that the no stimulus control group performed significantly higher amount of swimming compared to all other groups.

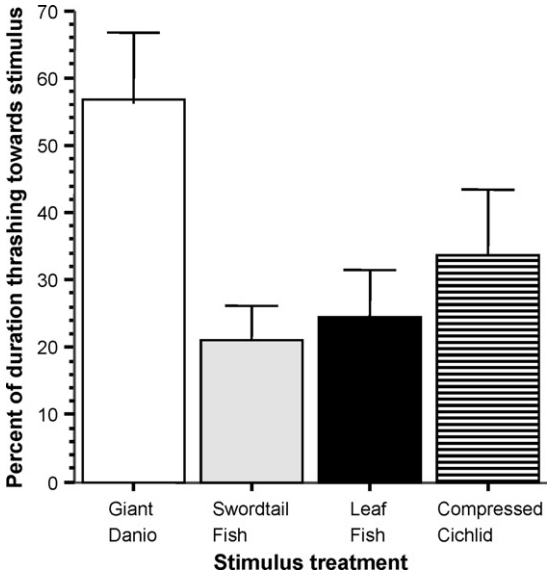

Fig. 5. Zebrafish responded with significantly more thrashing towards the Giant Danio than towards any other stimulus fish (attempting to swim through the glass wall of the tank adjacent with that of the tank holding the Giant Danio). Mean ± S.E.M. shown. Sample sizes of zebrafish shoals exposed to the different stimulus fish species were as follows (stimulus treatment indicated in brackets): n (Compressed Cichlid) = 12, n (Giant Danio) = 11, n (Leaf Fish) = 13, n (Swordtail Fish) = 12, n (Control, no stimulus) = 9. Note that this behavior is defined in relation to the stimulus fish and is thus not recorded in the no stimulus control group.

ulus fish species (ANOVA, $F(3, 46) = 3.99$, $p = 0.01$). Tukey HSD *post hoc* multiple comparison test confirmed the significantly ($p < 0.05$) higher thrashing towards the Giant Danio than towards any other stimulus fish. The latter results suggest that the experimental zebrafish were trying to swim through (thrashing towards) the glass wall that separated them from the Giant Danio, a response likely representing an attempt to shoal with this stimulus fish species.

The effect of stimulus fish on the appearance of fear associated behaviors erratic movement and Jumping [20,22] is shown in Figs. 6 and 7 respectively. Although it appears the highest amount of erratic movement was elicited by the Leaf Fish, the sympatric predator, ANOVA found no significant stimulus fish treatment effect for this behavior ($F(4, 54) = 0.66$, $p > 0.05$). However, analysis of the frequency of jumping demonstrated that stimulus fish treatment had a significant effect (ANOVA, $F(4, 54) = 6.48$, $p < 0.001$) and Tukey HSD *post hoc* multiple comparison test confirmed that in response to the Leaf Fish, zebrafish jumped significantly ($p < 0.05$) more frequently than in response to any other fish species or in response to no stimulus fish.

3.2. Behavior of stimulus fish

Experimental zebrafish and the stimulus fish were physically isolated from each other (were in separate tanks) and the former could only obtain visual information on the latter. Visual information includes the motor and posture patterns of the stimulus fish. Figs. 8 and 9 show the different motor and posture patterns

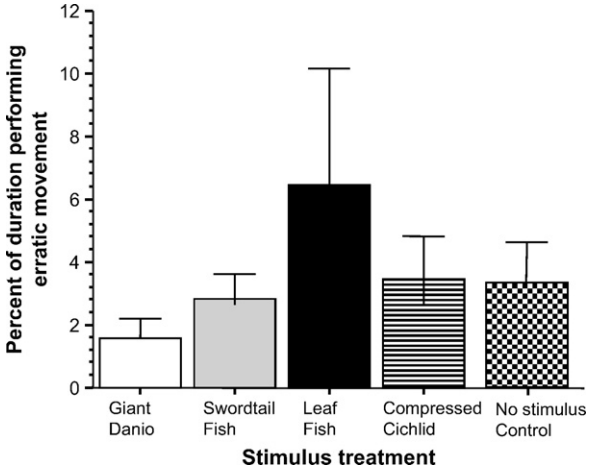

Fig. 6. Effects of stimulus fish treatment on erratic movement in zebrafish. Mean ± S.E.M. shown. Sample sizes of zebrafish shoals exposed to the different stimulus fish treatment were as follows (stimulus treatment indicated in brackets): n (Compressed Cichlid) = 12, n (Giant Danio) = 11, n (Leaf Fish) = 13, n (Swordtail Fish) = 12, n (Control, no stimulus) = 9. Note that no significant stimulus fish treatment effect was found.

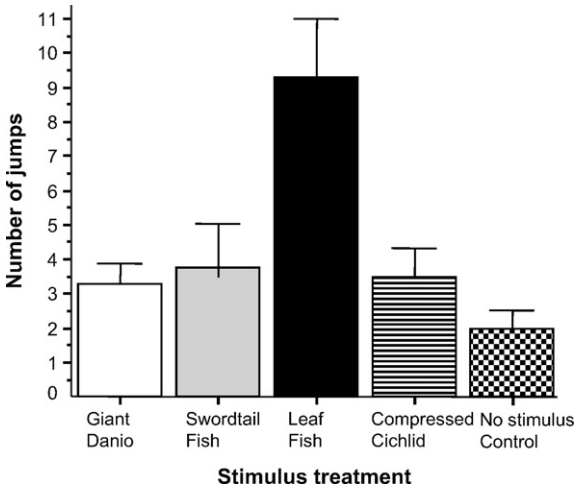

Fig. 7. Zebrafish responded to the Leaf Fish, its sympatric predator, with a significantly increased number of jumps. Mean ± S.E.M. shown. Sample sizes of zebrafish shoals exposed to the different stimulus fish treatment were as follows (stimulus treatment indicated in brackets): n (Compressed Cichlid) = 12, n (Giant Danio) = 11, n (Leaf Fish) = 13, n (Swordtail Fish) = 12, n (Control, no stimulus) = 9. Note that the number of jumps in response to the Leaf Fish is significantly higher than in response to the other stimulus fish species or in comparison with the no stimulus control and that the effect of these other stimulus treatments did not differ from each other.

that characterized the stimulus fish. Given that we used a single pair of stimulus fish for each species throughout the experiments the behavior of these stimulus fish species was not statistically compared. Nevertheless, the pattern of behavioral differences among the species was striking. Thus the effect of these stimulus fish on the experimental zebrafish may be attributable to the different motor patterns exhibited by the stimulus fish, to the different visual characteristics (shape, color, patterns) of the stimulus fish, or to both. The Compressed Cichlid performed the highest amount of thrashing towards the experimental zebrafish.

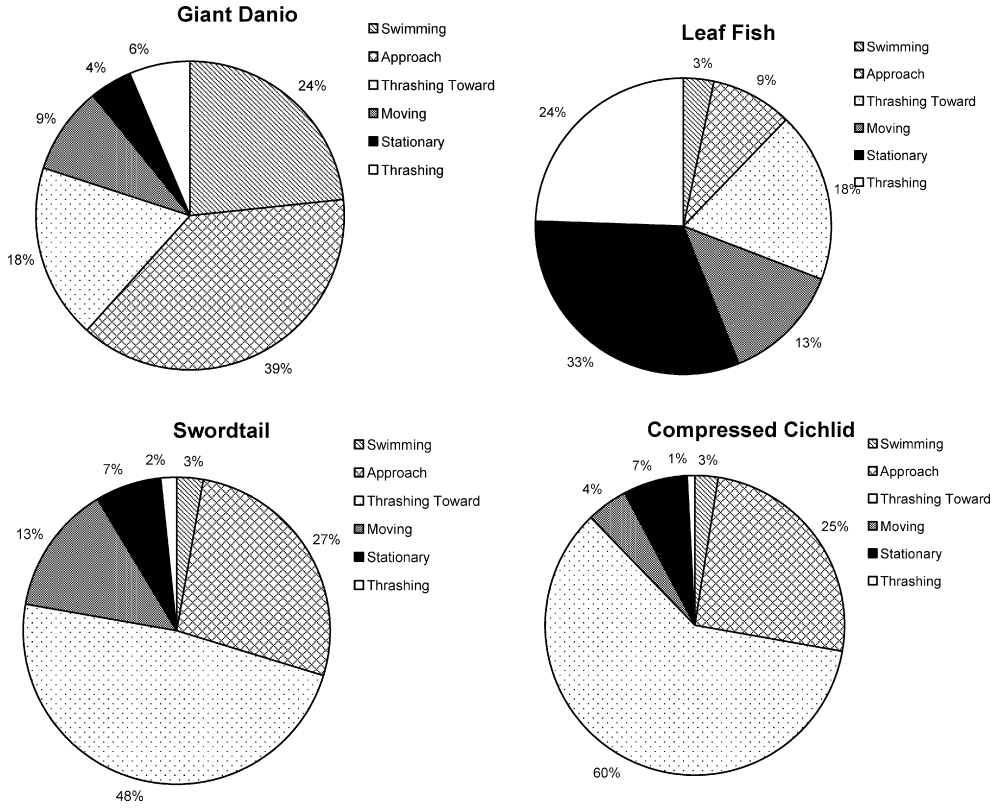

Fig. 8. Characteristic behavioral patterns of the four stimulus fish species. The proportion of duration of time each stimulus fish species exhibited the behavioral motor patterns is shown. Note the high amount of active swimming and approach in the Giant Danio, the large amount of thrashing towards the zebrafish in the Compressed Cichlid and the Swordtail Fish, and the substantial amount of stationary (immobile) behavior in the Leaf Fish.

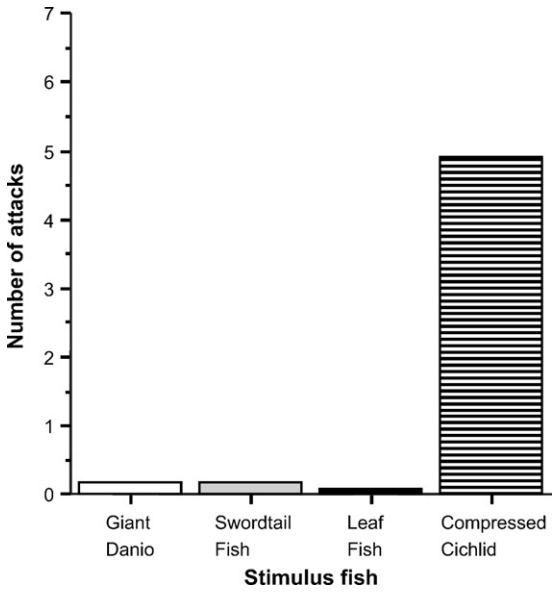

Fig. 9. Characteristic differences in the number of attacks performed by the stimulus fish. Note the substantial number of attacks by the Compressed Cichlid, a behavior that was practically absent in the other stimulus fish species.

It also performed the largest number of predatory attacks (Fig. 9) as well as the slow orienting movement, Approach, which often preceded the attacks (Fig. 8). This predatory species, allopatric to zebrafish, appeared to be highly motivated to catch the zebrafish. Contrary to our expectations, the Leaf Fish, the sympatric predator of zebrafish, performed the fewest number of predatory attacks among all stimulus fish used (Fig. 9), and stayed stationary for the longest time. These fish also showed the least amount of Thrashing toward or Approach behaviors as compared to the other stimulus fish. On the other hand, as expected of a typical shoaling fish similar to zebrafish, the Giant Danio was the most active (Swimming) and also showed the highest amount of Approach behavior. Last, the most prevalent motor pattern performed by the Swordtail Fish was thrashing toward the zebrafish.

3.3. Correlation between stimulus fish and experimental zebrafish behavior

Although the stimulus fish provided a fairly consistent stimulus pattern across multiple trials, it is possible that variation in the behavior of stimulus fish across trials occurred and may have co-varied with zebrafish responses. For example, a predator exhibiting elevated activity at a given trial may elicit increased antipredatory responses in the corresponding experimental zebrafish. To investigate this possibility we calculated Pearson product moment correlation coefficients between stimulus fish and zebrafish behaviors. Notably, we calculated these coefficients separately for each stimulus fish species. The rationale for this is twofold. First, the effect of different stimulus fish presentation on zebrafish behavior is accounted for by the analysis of variance but variation of stimulus fish behavior and the resulting covariation within a stimulus fish treatment group is not. Second, and more importantly, as explained above, a particular stimulus fish behavior may have highly different effects on zebrafish behavior depending on which stimulus fish species emitted it.

The results of the correlation analyses are shown in Tables 1–4. They demonstrate that certain zebrafish behaviors significantly correlated with particular stimulus fish behaviors and the correlation was stimulus fish species-specific. For example, thrashing toward the zebrafish, exhibited by the Compressed Cichlid, was negatively correlated with the zebrafish behaviors thrashing towards the camera and thrashing away from the stimulus fish, and moving and stationary behavior by the Compressed Cichlid positively correlated with the above zebrafish behaviors (Table 1). As suspected, the pattern of correlations between Leaf Fish and zebrafish behaviors was markedly different from the above. Table 2 shows that the only significant correlations are between the stimulus fish thrashing toward the zebrafish and the zebrafish behaviors thrashing towards camera and erratic movement. The correlation coefficients are positive, suggesting that the more the sympatric predator performed the above behavior the stronger the escape and fear reaction it elicited from the zebrafish.

The correlation matrix obtained for the behaviors of the Giant Danio and zebrafish is again different from that of the other stimulus fish. Thrashing performed by the Giant Danio in directions other than towards the zebrafish was found to positively correlate with swimming and erratic movement of zebrafish and to negatively correlate with thrashing in other directions of zebrafish. Importantly, the Giant Danio, when thrashing towards the zebrafish or when stationary, did not elicit fear or escape related behaviors in the zebrafish, i.e. no significant correlations were found between these behaviors.

Table 1
Compressed Cichlid

Stimulus fish behaviors	Zebrafish freezing	Zebrafish swimming	Zebrafish thrashing towards camera	Zebrafish thrashing other directions	Zebrafish away from stimulus fish	Zebrafish erratic	Zebrafish Jump
Swimming	−.338	−.318	−.322	.451	−.330	−.506	.075
Approach	−.404	−.355	−.269	.483	−.273	−.532	.047
Thrashing toward	.446	.231	−.846**	−.109	−.686*	.426	.354
Moving	−.257	−.148	.929**	−.022	.731**	−.189	−.333
Stationary	−.194	.027	.971**	−.222	.852**	−.140	−.420
Thrashing	−.146	−.151	−.200	.240	−.256	.370	.476
Attack	.027	−.074	−.296	.164	−.372	−.179	.547

Correlations (Pearson product moment correlation, r) between zebrafish and stimulus fish (Compressed Cichlid) behaviors. Significant correlations are denoted by asterisks: **$p < 0.01$ level (two-tailed), *$p < 0.05$ level (two-tailed). Note the differences in the correlation pattern among the four stimulus fish treatments.

Table 2
Leaf Fish

Stimulus fish behaviors	Zebrafish freezing	Zebrafish swimming	Zebrafish thrashing towards camera	Zebrafish thrashing other directions	Zebrafish away from stimulus fish	Zebrafish erratic	Zebrafish jump
Swimming	.004	.006	.210	.070	−.168	.142	.521
Approach	.077	.114	−.030	.186	−.550	.080	−.160
Thrashing toward	−.317	−.202	−.429	.348	−.225	−.122	.054
Moving	.264	.292	−.235	−.019	−.464	−.334	−.108
Stationary	−.154	−.171	−.263	−.090	.512	−.394	−.252
Thrashing	.277	.208	.657*	−.151	−.181	.649*	.269
Attack	−.121	−.218	−.107	.290	−.140	−.118	.181

Correlations (Pearson product moment correlation, *r*) between zebrafish and stimulus fish (Leaf Fish) behaviors. Significant correlations are denoted by asterisks: ** $p < 0.01$ level (two-tailed), * $p < 0.05$ level (two-tailed). Note the differences in the correlation pattern among the four stimulus fish treatments.

Table 3
Giant Danio

Stimulus fish behaviors	Zebrafish freezing	Zebrafish swimming	Zebrafish thrashing towards camera	Zebrafish thrashing other directions	Zebrafish away from stimulus fish	Zebrafish erratic	Zebrafish jump
Swimming	.126	−.081	.228	−.077	.125	.136	−.056
Approach	.516	−.196	.474	−.217	.368	−.152	−.265
Thrashing toward	−.379	−.287	−.399	.528	−.438	−.177	.274
Moving	.044	−.122	−.247	.168	−.080	−.459	−.029
Stationary	.147	−.332	−.209	.200	.051	−.357	−.131
Thrashing	−.152	.822**	.264	−.644*	.181	.792**	.017
Attack	−.091	.192	−.096	−.007	−.136	−.188	−.360

Correlations (Pearson product moment correlation, *r*) between zebrafish and stimulus fish (Giant Danio) behaviors. Significant correlations are denoted by asterisks: ** $p < 0.01$ level (two-tailed), * $p < 0.05$ level (two-tailed). Note the differences in the correlation pattern among the four stimulus fish treatments.

Table 4
Swordtail Fish

Stimulus fish behaviors	Zebrafish freezing	Zebrafish swimming	Zebrafish thrashing towards camera	Zebrafish thrashing other directions	Zebrafish away from stimulus fish	Zebrafish erratic	Zebrafish jump
Swimming	−.056	−.273	.544	.341	.196	−.590	.138
Approach	.200	.563	.243	−.711*	.094	.131	.049
Thrashing toward	−.362	−.464	−.457	.816**	−.011	−.050	.172
Moving	−.105	.477	.472	−.634*	.221	−.256	−.193
Stationary	.637*	−.047	−.272	−.503	−.356	.550	−.236
Thrashing	.125	.373	.985**	−.298	.025	−.343	−.252
Attack	−.289	−.371	−.212	.123	−.489	−.034	−.230

Correlations (Pearson product moment correlation, *r*) between zebrafish and stimulus fish (Swordtail fish) behaviors. Significant correlations are denoted by asterisks: ** $p < 0.01$ level (two-tailed), * $p < 0.05$ level (two-tailed). Note the differences in the correlation pattern among the four stimulus fish treatments.

The pattern of correlations obtained between the behaviors of the Swordtail fish and that of the zebrafish are again unique. Approach and moving in the Swordtail fish negatively correlated with the zebrafish thrashing towards the stimulus fish, while thrashing toward the zebrafish by the Swordtail positively correlated with zebrafish's thrashing in directions other than toward the camera or away from the stimulus fish. Furthermore, the Swordtail Fish's stationary behavior positively correlated with zebrafish freezing and the Swordtail fish's thrashing positively correlated with zebrafish thrashing towards the camera.

4. Discussion

On the basis of previously published studies conducted with a wide variety of species representing all major taxa [1,19,34,30,10,33,6,36], we expected zebrafish to respond differentially to stimulus fish. Particularly, we assumed that zebrafish would respond differentially to predatory species with fear reactions and perhaps especially strongly if the predator is sympatric. Our expectations were partially correct. Contrary to our assumption, zebrafish did not show increased shoal cohesion in response to predators; in fact no changes were observed among groups exposed to any of the stimulus fish. Similarly, the distance of zebrafish from the stimulus fish was independent of what species the stimulus fish were. It is possible that in the small test tank and with only a small shoal size (five fish per shoal) such effects could not manifest.

Nevertheless, the analysis of motor and posture patterns did reveal interesting stimulus fish species-dependent effects. Zebrafish exhibited a significantly elevated fear response (increased number of jumps) to its natural predator, the Leaf Fish. Interestingly, however, such a fear reaction was not elicited

in the zebrafish by the sight of the allopatric predator, the Compressed Cichlid. Also noteworthy is the finding showing the differential response of the zebrafish towards the Giant Danio. This species elicited an increase in the amount of thrashing in the direction of the stimulus fish in the zebrafish, a response we interpreted as shoaling behavior. Importantly, such a response by the zebrafish (thrashing toward stimulus) was the smallest towards the Swordtail fish, indicating that it is not the harmless nature of the stimulus fish alone, but perhaps the combination of being sympatric and harmless that elicits the response in zebrafish.

It is important to note that the experimental zebrafish used in the current study have never been exposed to the stimulus fish before and thus their differential responses to these fish are not experience dependent but likely due to genetic predisposition, i.e. instinct, that developed during evolution and has now become characteristic of this species. What is the nature of such genetic predispositions? Do these genetic predispositions allow zebrafish to respond to all predator-like fish or do they facilitate the antipredatory response of zebrafish to only a very unique set of stimuli characterizing the sympatric predator alone?

One could hypothesize that predators share certain characteristics and these characteristics tell them apart from non-predatory fish and thus recognition of these general features is what evolution may have worked on. A prey fish could detect the proportions of the head, e.g. the relative size of the mouth, size of eyes, and relative positioning of such structures on the body of the predator. A prey fish could also be responsive to certain general behavioral patterns, e.g. fast approaching larger fish should elicit an escape reaction. The ability to detect such common features associated with predators may be evolutionarily adaptive if numerous predatory species have co inhabited the natural environment with the prey species and have co-evolved there with the prey. The second, alternative hypothesis is that the prey may have evolved with only a very limited number of predatory species present in its environment and thus an ability to detect "generalized predatory" features may not be necessary for survival. Instead, detection of the specific features associated with the particular sympatric predatory species could evolve in the prey. Although the current study was not powered to distinguish between these two hypotheses conclusively (response to general predatory features versus to features unique to the sympatric predator) as only two predatory stimulus species were used, our data are compatible with the second hypothesis. Despite the predatory features of the Compressed Cichlid that are obvious to the human observer, zebrafish responded with fear reactions only to the sympatric predator, the Leaf fish. Also notable is that although the allopatric predator exhibited the largest number of attacks compared to the other stimulus fish and the sympatric predator showed the smallest, zebrafish showed elevated fear reactions only towards the sympatric predator. Thus, surprisingly, a relatively large predatory fish with a large mouth fast approaching the zebrafish does not appear to represent the stimulus set that elicits a fear response in zebrafish. It is thus likely that the zebrafish does not generalize, but rather it responds to some specific features unique to the sympatric predator.

The question as to what features may define the sympatric predator for the zebrafish will be investigated in the future by using computer animated images that will be systematically manipulated and precisely controlled (for a similar application see [35]). It is possible that physical characteristics including the body shape, color and pattern, or the behavior of the Leaf fish, or the combination of these cues allowed zebrafish to respond differentially to this stimulus fish. Our results are compatible with the latter possibility, i.e. the zebrafish is likely to attend to a combination of cues that include the physical characteristics of the stimulus fish as well as its behavior. The analysis of stimulus fish behavior and its correlation with zebrafish behavior supports this argument. Briefly, what we found is that behavioral responses that were the same in appearance, i.e. in form, but were exhibited by different stimulus fish species correlated with different zebrafish responses. For example, thrashing towards the zebrafish when performed by the allopatric predator was associated with reduced amount of thrashing towards the camera and reduced amount of thrashing away from the stimulus performed by zebrafish, which we interpret as reduced escape responses. Again, it appears that a fast moving allopatric predator orienting towards the zebrafish was not perceived as dangerous by zebrafish. A slow moving or stationary allopatric predator, contrary to our expectations, did, however, elicit the active escape reaction in zebrafish (positive correlations between these behaviors and zebrafish's thrashing away or towards the camera). Given that the sympatric predator, which was the least active among the stimulus fish, also elicited the strongest fear reactions in zebrafish, one may hypothesize that being still, a strategy of ambush piscivores, is one aspect of the predatory stimulus set to which the zebrafish is naturally sensitive. It is also notable that elevated activity levels of the sympatric predator manifesting as increased thrashing behavior (not in the direction of zebrafish) was associated with elevated zebrafish erratic behavior and thrashing towards the camera. These responses we interpret as elevated escape and fear reactions. Erratic movement, which was almost always performed on the bottom, would lead to stirring up debris in the natural environment of zebrafish and thus may be an effective camouflage eliciting behavior in nature. Thrashing towards the camera may represent an effective response by which zebrafish moves away from the line of sight of the predator (moving in the opposite direction, i.e. thrashing away from the predator, may not be as effective). Also notable is that the allopatric harmless fish, when exhibiting thrashing, also elicited the thrashing towards the camera response in zebrafish but without the erratic movement, and that the sympatric harmless fish performing the same thrashing behavior did not. Instead, this latter stimulus induced active swimming in zebrafish, a response likely associated with shoaling. In summary, seemingly similar behavioral patterns emitted by different stimulus fish species were associated with distinct zebrafish behaviors, which suggest that both the physical appearance and the behavior of stimulus fish are important factors influencing the response of zebrafish.

A point we would like to return to now concerns the generalizability of our findings. Our experimental design included a single representative of sympatric predatory and harmless, and allopatric predatory and harmless fish species. Thus, our results only allow us to conclude how zebrafish respond to each of

these specific stimulus fish species, but not how zebrafish would generally respond to sympatric versus allopatric or predatory versus harmless species. To be able to draw general conclusions one would need to examine the responses of zebrafish to numerous species representing each of the above categories. The natural habitat of zebrafish and the fish fauna of the corresponding geographical region is somewhat underexplored (e.g. [39,12]), but recently interest in these questions has increased [14] and thus it is likely that such analyses will become possible in the near future. It would also be interesting to investigate whether the zebrafish is capable of responding to different stimulus fish using perceptual modalities other than vision. Although zebrafish, as well as the stimulus fish used in the current study, are diurnal species that mainly rely on their vision, examples from the literature (see [19]) suggest that diurnal fish species can detect predatory fish using other modalities, e.g. olfaction. Olfaction has been shown to contribute to antipredatory behavior of zebrafish (e.g. [24]). Furthermore, there are other predators, e.g. catfish species, which possibly prey upon zebrafish in nature [14]. These sympatric catfishes are mostly nocturnal and may rely upon lateral line or olfactory detection. It is unknown whether zebrafish use non-visual modalities necessary in the dark to avoid these predators.

The last point we would like to consider is the most general aspect of our research, which perhaps will have broader implications in the future. It concerns the utility of zebrafish as a model organism in general and the use of the current behavioral paradigms in translational research in particular. Zebrafish have been proposed to be an excellent model organism for the analysis of mechanisms of biological phenomena and for the understanding of human diseases (e.g. [13]). In the past, the diseases investigated with the use of zebrafish mainly involved developmental abnormalities [3]. Currently, however, interest in the use of zebrafish in the analysis of CNS disorders has increased [32]. One of the major CNS dysfunctions is anxiety disorders [2]. These diseases represent a broad spectrum of clinical conditions that may have diverse underlying neurobiological mechanisms (for a recent review see ref. [16]). Nevertheless, it is plausible that many of these abnormalities are due to aberrantly functioning neurobiological mechanisms that evolved to serve adaptive avoidance responses under physiological conditions in nature (e.g. [26]). One of the most important such adaptive responses is antipredatory behavior [6]. With appropriate behavioral paradigms tapping into antipredatory responses, one may be able to investigate the mechanistic details of this brain function (e.g. [26]). By understanding the mechanisms of this natural behavior, one may be able to shed light onto the neurobiology of the abnormalities associated with it. It is also possible that one may use the behavioral paradigm testing antipredatory behavior for drug screening and identify fear reducing or anxiolytic compounds [26]. For example, previously we have found that intermediate doses of ethyl alcohol (EtOH) significantly reduced antipredatory responses in zebrafish elicited by a predator model [20]. We propose that the use of visual stimuli closely matching the characteristics of the sympatric predator employed in the current study, and the recording of the frequency of zebrafish jumps these stimuli elicit will be a simple and effective method with which anxiolytic drugs could be screened. In order for such a paradigm to be successful one has to generate a computer image whose movement patterns and physical characteristics are realistic and closely match those of the sympatric predator. Generation of this image and the custom software application associated with it are under development in our laboratory. In addition, it will be important to pharmacologically validate the paradigm for zebrafish, i.e. to test anxiolytic and anxiogenic compounds that have known effects in mammals; experiments that are being planned in our laboratory.

In summary, our current study demonstrated that zebrafish, a simple vertebrate, is capable of selectively responding to different stimulus fish species without any prior exposure to these species. These results suggest that perhaps genetic factors may underlie the zebrafish's responses. Particularly useful and interesting will be to study whether genetic variability (strain differences or mutagenesis induced genetic changes) can be found in antipredatory responses of zebrafish elicited by the sympatric predator, and whether such responses may be modified by anxiolytic or anxiogenic compounds previously developed using, and clinically employed for, mammalian species.

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