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Modeling anxiety using adult zebrafish: A conceptual review

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Abstract

Zebrafish (*Danio rerio*) are rapidly emerging as a useful animal model in neurobehavioral research. Mounting evidence shows the suitability of zebrafish to model various aspects of anxiety-related states. Here, we evaluate established and novel approaches to uncover the molecular substrates, genetic pathways and neural circuits of anxiety using adult zebrafish. Experimental approaches to modeling anxiety in zebrafish include novelty-based paradigms, pharmacological and genetic manipulations, as well as innovative video-tracking, 3D-reconstructions and bioinformatics-based searchable databases and *omics*-based tools. Complementing traditional rodent models of anxiety, we provide a conceptual framework for the wider application of zebrafish and other aquatic models in anxiety research.

Keywords

zebrafish; anxiety; novelty-based paradigms; pharmacological and genetic manipulations; bioinformatics; omics-based tools

1. Introduction

One of the central questions in biological psychiatry is how genes, molecular pathways and patterns of connectivity in the brain produce and modulate anxiety behavior (Bishop 2007; Olivier et al. 1998; Landgraf and Wigger 2002; Suveg et al. 2010; Burgess and Granato 2008). Through the use of numerous behavioral paradigms, genetic/pharmacological screens and neuroimaging, animals have been extensively used to model anxiety pathogenesis (Clement et al. 2002; Conti et al. 2004; de Angelis 1996; Ditzen et al. 2006; Kalueff et al. 2007). The zebrafish (*Danio rerio*) has emerged as a useful new model for studying the behavioral and molecular mechanisms of brain disorders (Cachat et al. 2010a; Cachat et al. 2011; Stewart et al. 2010a; Stewart et al. 2010b; Blaser et al. 2010; Maximino et al. 2010a; Sackerman et al. 2010).

Although fish behavior was initially presumed to be mostly primitive and instinctively driven (Burt de Perera 2004a; Laland et al. 2003); recent studies have revealed the

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complexity of zebrafish behavior, and its relevance to modeling fear- and anxiety-like states (Stewart et al. 2010b; Cachat et al. 2011; Bencan et al. 2009; Blaser et al. 2010; Speedie and Gerlai 2008). Mounting evidence also indicates that zebrafish anxiety-like behavior is driven by similar environmental factors as that of rodents (Champagne et al. 2010; Stewart et al. 2010a), and involves evolutionarily conserved circuits (Lee et al. 2010; Amo et al. 2010) that regulate aversive learning and emotionality (Jesuthasan 2011 (in press); Agetsuma et al. 2010). Furthermore, robust anxiogenic endocrine and genomic responses, similar to those seen in humans (Schulkin et al. 1998; Yehuda et al. 1993; Boulom et al. 2002; Zhang et al. 1992) and rodents (Yau et al. 1997; Roy et al. 2001; Hale et al. 2008; Kung et al. 2010), have also been established in zebrafish (Cachat et al. 2010a; Williams et al. 2011 (in press); Wong et al. 2010a; Baraban et al. 2005; Barcellos et al. 2007). Additionally, like rodents (Dvorkin et al. 2008; Eilam and Golani 1989), fish form spatial memories (Burt de Perera 2004b; Riedel 1998), and use them to orient in novel environments, establishing “safe zones” (homebases) where they frequently dwell and return to (Stewart et al. 2010c; Stewart et al. 2010d; Rosemberg et al. 2011). Moreover, zebrafish also display a robust habituation responses (Wong et al. 2010b), which in addition to cognitive map formation (O’keefe 1978), may represent deep neurobiological constructs, such as adaptive processing of sensory information (Eisenstein and Eisenstein 2006).

While affective disorders research has traditionally utilized rodent paradigms (Blanchard et al. 2003; de Mooij-van Malsen et al. 2010 (in press); Hefner and Holmes 2007), interest is growing in the use of zebrafish as a novel and high-throughput model of anxiety (Champagne et al. 2010; Stewart et al. 2010a; Stewart et al. 2010b; Blaser et al. 2010). For example, numerous paradigms have been adapted from rodents and applied to zebrafish (Table 1), showing a striking similarity in reduced exploration, and increased thigmotaxis or freezing (Cachat et al. 2010b; Maximino et al. 2010a; Maximino et al. 2010b; Gaikwad et al. 2011; Levin et al. 2007). In addition, zebrafish anxiety research has the advantage of using both adults and larvae, complementing the throughput of larval models with the complexity of neural phenotypes of adult animals (Norton and Bally-Cuif 2010a; Stewart et al. 2010a; Egan et al. 2009; Grossman et al. 2010; Burne et al. 2011; Cachat et al. 2010b; Webb et al. 2009). Zebrafish also display robust behavioral phenotypes, which are exhibited through overt and easily quantified behavioral endpoints. Finally, the high-throughput nature of zebrafish models also makes them an excellent species for studying various experimental, genetic, and pharmacological factors in anxiety (Stewart et al. 2010a; Chakraborty et al. 2009; Burgess and Granato 2008; Dlugos and Rabin 2003). Here, we discuss several current and emerging approaches that use zebrafish to uncover the neurobiological mechanisms that control animal anxiety behavior.

2. Behavioral modulation and analyses of zebrafish anxiety

Among several zebrafish anxiety paradigms (Table 1), the novel tank test has become one of the most popular tests (Cachat et al. 2010c; Stewart et al. 2010b; Wong et al. 2010b; Egan et al. 2009). Anxiety in this test is reflected in reduced exploration (i.e., longer latency to reach the top, fewer entries to the top, longer and more frequent freezing) together with elevated erratic movements and freezing (Levin et al. 2007; Barcellos et al. 2007; Egan et al. 2009; Cachat et al. 2010a; Stewart et al. 2010b).

While the link between anxiety and memory is well-recognized, the neurobiology of their interplay remains poorly understood, and has only recently been examined in zebrafish (de Castro et al. 2009; Gaikwad et al. 2011; Piato et al. 2011). Notably, the memory-impairing effects of acute stress on zebrafish strikingly parallels both rodent and clinical data (Morrow et al. 2000; Yun et al. 2010; Chen et al. 2010; Wright and Conrad 2005; Hu and Wang 2006; Hardison and Purcell 1959; Gaikwad et al. 2011; Sandi et al. 2005; Diamond et al. 2006;

Diamond et al. 1999; Park et al. 2008; El Hage et al. 2006). Specifically, chronic restraint stress (Yun et al. 2010; Chen et al. 2010; Wright and Conrad 2005; Hu and Wang 2006) and exposure to a predator or its odors (Sandi et al. 2005; Diamond et al. 1999; Morrow et al. 2000; Park et al. 2008; Diamond et al. 2006; El Hage et al. 2006; Cohen et al. 2009; Kozlovsky et al. 2008; Woodson et al. 2003) impairs memory in rodents, while acute psychological stress affects cognitive functions in humans (Hardison and Purcell 1959). In such paradigms, ecologically relevant stressors (e.g., predator exposure) are especially useful for eliciting an innate anxiety response, and have also been applied to zebrafish (Gerlai et al. 2009; Bass and Gerlai 2008; Egan et al. 2009). For example, examining the effects of 24- and 72-h exposure to the Indian leaf fish on zebrafish exploration, we found that novel tank testing does not reflect the traditional indices of anxiety *per se*, but triggers the apparent escape-like hypervigilance, as evidenced by an observed increase in locomotion and erratic movements (own unpublished data) similar to those evoked in rodents by predator stress (Blanchard et al. 1990; Blanchard et al. 2001). In contrast, chronic unpredictable mild stress reduced zebrafish exploratory behavior, but decreased shoal cohesion, suggesting a lower energy and/or decreased novelty seeking associated with an extended stress period (Piato et al. 2011) – a phenotype resembling rodent chronic unpredictable stress, with anhedonia and reduced self-care behavior and exploration (Isingrini et al. 2010; Pandey et al. 2010; Kazlauckas et al. 2011).

In addition to the high sensitivity of zebrafish anxiety-related behaviors, they benefit from being three-dimensional (3D) due to an additional *vertical* dimension (Cachat et al. 2010c; Cachat et al. 2011; Grossman et al. 2010; Rosemberg et al. 2011). Thus, while rodent models are traditionally studied in 2D coordinates, zebrafish paradigms offer an enhanced dimensionality for phenotyping anxiety (Grossman et al. 2010; Cachat et al. 2011) profiles. As a more “realistic” assessment of zebrafish behavior, 3D analyses can also be used to globally assess behavioral profiles while mapping individual endpoints to the spatiotemporal reconstructions. Cluster analyses may also complement 3D reconstructions to identify informative subgroups within a large data set, categorize experimental manipulations or behavioral endpoints based on the similarity of their alterations, consolidate behavioral data and increased their density (Cachat et al. 2011).

3. Pharmacological modulation of zebrafish anxiety

In addition to behavioral manipulations, zebrafish anxiety can be challenged pharmacologically, as recently comprehensively evaluated in (Stewart et al. 2010b). To assess pharmacogenic anxiety, fish are usually treated with an anxiogenic or anxiolytic agent in exposure beakers (acute treatment) or home tanks (chronic treatment) prior to testing (Cachat et al. 2010a; Cachat et al. 2011; Grossman et al. 2010; Stewart et al. 2010b; Stewart et al. 2011a). Several compounds recently tested in adult zebrafish will be used as examples here, to illustrate fish sensitivity to various anxiotropic drugs. Tranylcypromine (TCP) blocks the degradation of serotonin (Jie et al. 2009), lysergic acid diethylamide (LSD) is a potent hallucinogen that acts on several serotonin receptors (Backstrom et al. 1999; Wing et al. 1990), and dizocilpine (MK-801) is an antagonist of N-methyl-D-aspartate (NMDA) receptors (Del Pozo et al. 1996; Layer et al. 1993).

Monoamine oxidase inhibitors (MAOIs) are widely used clinically to treat anxiety (Ballenger 1999; Mallinger et al. 2009) and yield similar results in rodents for both chronic (Crawley 1985; Maki et al. 2000; Takamori et al. 2001) and acute (Maki et al. 2000; de Angelis 1996; Freund et al. 1979) treatments. Similar to rodents, the MAOI agent TCP reduced anxiety in the novel tank test in zebrafish both acutely (as assessed by shorter latency to enter the top, increased number of top transitions and reduced freezing duration) (Stewart et al. 2010b) and chronically (as reflected in fewer erratic movements; Fig. 1). LSD

has been extensively tested in rodents, and exhibits a biphasic action with initial anxiety/hypoactivity followed by hyperlocomotion (Mittman and Geyer 1991; Adams and Geyer 1985; Adams and Geyer 1982; Marona-Lewicka et al. 2005). The effects of LSD have recently been studied in zebrafish, demonstrating anxiolytic-like action for both acute administration (e.g., shorter latency to enter the top, increased number of top transitions, top duration, and reduced freezing duration in the novel tank, as well as more center entries in the open field test) (Grossman et al. 2010; Stewart et al. 2010b) and repeated treatment (e.g., increased top duration in the novel tank test; Fig. 2).

In rodents, the NMDA receptor antagonist MK-801 evokes hyperlocomotion, place preference, reduced predator avoidance, and increased exploratory activity (Layer et al. 1993; Del Pozo et al. 1996; Adamec et al. 1999; Jessa et al. 1996; Sharma and Kulkarni 1991; Rikuko and Akemi 1998). Similarly, MK-801 also produces hyperlocomotion and circling behavior in zebrafish (Seibt et al. 2010; Swain et al. 2004). With the growing use of MK-801 in modeling behavioral disorders, we have further examined its behavioral and endocrine effects, showing anxiolytic-like effects such as increased top duration, decreased latency to top entry and lower cortisol levels in the novel tank test (Fig. 3). With the growing use of MK-801 in modeling behavioral disorders, we have further examined its behavioral and endocrine effects, showing anxiolytic-like effects such as increased top duration and decreased latency to top entry (Fig. 3) as well as lower cortisol levels in the novel tank test (data not shown). Interestingly, an increase in erratic movements was also observed here. While heightened erratic behavior can reflect increased anxiety in zebrafish (Egan et al. 2009; Cachat et al. 2010a), its appearance together with the other anxiolytic behaviors is in line with the hyperlocomotion, demonstrated for MK-801 in previous rodent_studies (Layer et al. 1993; Martin et al. 1997; Mathe et al. 1996) as well in some zebrafish models (Ewald 2009).

Notably, studies outlined here were performed at different times, using different cohorts, and evaluated by different experimenters, thereby leading to some data variability (e.g., Fig. 1–3) not uncommon for both zebrafish (Sackerman et al. 2010; Bencan et al. 2009; Echevarria et al. 2008; Levin et al. 2007) and rodent (Cryan et al. 2003; Klenerova et al. 2009; Eckerman et al. 1980) models. While similar baseline behaviors are the ideal situation in psychopharmacology research, this is not always the case since behavioral endpoints are highly sensitive to procedural/environmental factors. However, it is important to ensure that the controls and experimental fish are always tested under the same conditions within each experiment. In this situation, while a variance among controls could lead to variable numerical values for a specific endpoint, the overall relationship between groups should be maintained (Fig. 1–3).

While conventional assays for assessing zebrafish behavior like novel tank test remain prevalent, new measures of anxiety-like behavior are emerging. For example, paradigms for screening classic anxiolytic agents through evaluating color, shoal cohesion, and position relative to tank height have recently been developed (Gebauer et al. 2011 (in press); Miller and Gerlai 2007; Saverino and Gerlai 2008) (also see Table 1 for details). Taken together, this confirms the high sensitivity of adult zebrafish to various pharmacological manipulations that affect clinical or rodent anxiety-like behavior. In combination with high throughput, phenotypical robustness, low cost and enhanced behavioral dimensionality, this makes zebrafish an excellent model for anxiolytic drug screening.

4. Genetic manipulation and zebrafish anxiety

The ease of genetic manipulations in zebrafish contributes to their utility for studying anxiety disorders. The isolation of multiple zebrafish behavioral mutants has allowed

researchers to uncover the genetic pathways and neural circuits underlying behaviors (Norton and Bally-Cuif 2010b). For example, as the habenula area appears to play a role in experience-dependent fear-like behaviors (Hauptman 2011), the genetic disruption of its afferents in zebrafish prevents normal stress avoidance responses (Lee et al. 2010). Moreover, transgenic zebrafish lines have been developed to differentially express colored fluorescent proteins in the neurons of habenula subnuclei to help confirm their putative targeting (Hauptman 2011). Overall, research using zebrafish anxiety models has increasingly incorporated mutant zebrafish models to assess the genetic factors that precipitate abnormal neurobiological, physiological and behavioral phenotypes (Gerlai et al. 2000; Hogan et al. 2008; Key and Devine 2003) (see (Bergner et al. 2009) for a recent review).

5. Modeling zebrafish anxiety in the *age of omics*

One of the key challenges in neuroscience is to decipher the functional and structural layout of the brain at the neuronal level (Stewart et al. 2011b). The *connectome*, reflecting the development of a highly organized connection matrix of the brain (DeFelipe 2010), offers the possibility to elucidate the pathogenesis of anxiety disorders based on identified quantitative or qualitative defects in circuitry (Lichtman et al. 2008). While efforts have been primarily focused on the human brain, animal models of anxiety also benefit from functional connectivity mapping. With a relatively limited number axons, the zebrafish brain is a good object to investigate its connectivity (Friedrich et al. 2010). For example, synaptic output has been suppressed in zebrafish by tetanus toxin light chain (TeTxLC), a permanent blocker of synaptic vesicle release, which can be used to identify subsets of neurons involved in various behaviors (Friedrich et al. 2010; Asakawa et al. 2008; Koide et al. 2009; Hauptman 2011). Brainbow technology is another promising *connectomic* approach, in which neurons are labeled in varying hues, and used for the multicolor labeling and axonal tracing of the zebrafish sensory system (Lichtman et al. 2008; Pan et al. 2011). Since Brainbow labeling facilitates the surveying of quantitative and qualitative aspects of circuitry in diverse brain regions, it may be a useful approach for assessing the "connectopathology" of anxiety in zebrafish.

Notable advances have also been achieved at a higher level – specifically with the recent comprehensive mapping of all zebrafish dopaminergic axon projections. For example, injected anterograde and retrograde tracers to examine the projections and subnuclei of the habenula and associated brain regions, enable using pathway-specific manipulations to examine their involvement in affective behaviors (Hauptman 2011; Agetsuma et al. 2010). Furthermore, by combining the selective genetic marking of individual nerve cells with high resolution microscopy, researchers have assembled a 3D *projectome* map for zebrafish (Tay et al. 2011).

Another approach in modeling affective disorders is assessing the differential engagement of neuronal pathways. For example, mapping neuronal activity by assessing the expression of immediate early gene (e.g., *c-fos)* is increasingly used in zebrafish (Lau et al. 2011; Baraban et al. 2005; Wong et al. 2010a; Williams et al. 2011 (in press); Stewart et al. 2011a). Genetically encoded calcium indicators also show promise of monitoring neuronal activity in zebrafish (Fetcho 2007; Higashijima et al. 2003; Muto et al. 2011). Further research is needed to harness the noninvasiveness of *in vivo* analysis, while combining it with the single cell resolution offered by other techniques.

Efforts to dissect interconnected physiological pathways underlying anxiety also apply innovative bioinformatics tools to identify behavioral patterns and phenotypes. For example, while the Zebrafish Information Network (ZFIN (Zebrafish_Information_Network_(ZFIN)

2011)) is the main searchable database of zebrafish genetic, genomic and developmental data (Sprague et al. 2008), a recently launched Zebrafish Neurophenome Project (ZNP (Zebrafish_Neuroscience_Research_Consortium_(ZNRC) 2011)) is an interactive searchable database of behavioral and related phenotypes in zebrafish. It allows investigators to rapidly search previously published zebrafish data, refine their research using these models and share their findings. Further ZNP development, based on published information and curated data deposited by established zebrafish investigators, will enable sophisticated data-mining and complex data analyses, to foster our understanding of affective pathogenesis in zebrafish.

6. Further directions: using other aquatic models and cross-species comparisons

Comparison between various species is crucial for uncovering conserved and divergent mechanisms underlying pathogenic mechanisms (Signore et al. 2009; Furutani-Seiki and Wittbrodt 2004). As one such step, comparing related fish species (e.g., zebrafish and medaka, provides an excellent means to unravel the similarities and differences in their behavioral phenotypes. Other aquatic species, such as guppies, are used to model pharmacogenic anxiety, and demonstrate some similarity to zebrafish responses (Hallgren et al. 2011 (in press)). Clearly, a widening of spectrum of model species to study anxiety behavior by including zebrafish and other aquatic models remains an important strategy for translational biological psychiatry (Kalueff et al. 2007). For example, the role of sex determinants in behavior has emerged as significant area of investigation (Lopez Patino et al. 2008; Ruhl and McRobert 2005; Piyapong et al. 2010). While zebrafish have little sex-linked genetic markers, medaka have an XX, XY sex-determination system, like mammals (Furutani-Seiki and Wittbrodt 2004). Therefore, complementary use of zebrafish and other aquatic model species may be particularly beneficial in the field of affective research.

Furthermore, while complex behavioral phenotypes may be closely connected with the genotype, they are not necessarily dependent upon it alone. Gene expression profiles instead represent the primary level of integration between environmental factors and the genome, ultimately guiding complex trait behaviors such as anxiety. Thus, in order to elucidate the molecular basis of phenotypic variation, cross-species comparisons of gene expression profiles are necessary (Renn et al. 2004).

In conclusion, anxiety is a complex and multifaceted neurobehavioral disorder, and its full understanding can only be achieved through different coordinated approaches. Zebrafish models strongly parallel animal and clinical evidence, further supporting their validity and translatability for identifying pathways involved in anxiety regulation, and discovering potential new classes of psychotropic drugs. Conceptual innovations in this field, including sophisticated video-imaging and bioinformatics/*omics* tools (Stewart et al. 2011b; Kalueff et al. 2007), will further foster pre-clinical anxiety research using zebrafish and other aquatic models.

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Highlights

- Zebrafish are useful for studying the underpinnings of neurobehavioral disorders
- Current and emerging approaches used to uncover anxiety mechanisms are discussed
- Pharmacological and genetic manipulations have validated zebrafish anxiety models
- New and innovative bioinformatic approaches are emerging tools in anxiety research
- Inter-species comparisons are key for uncovering pathogenic mechanisms

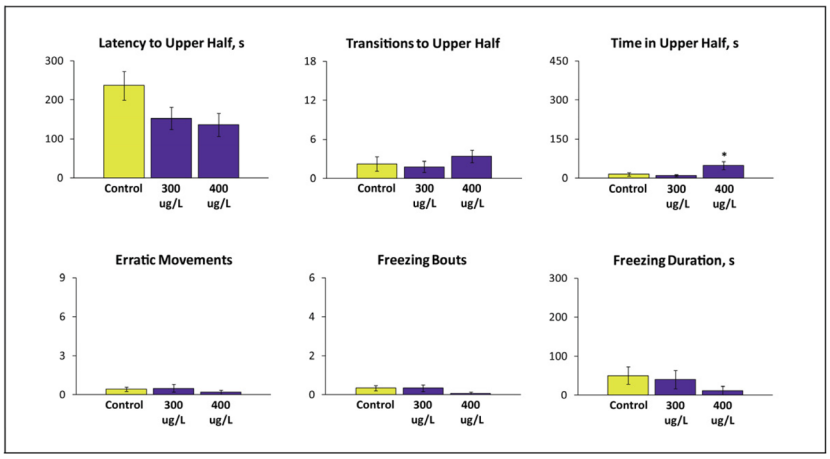

Figure 1.

Effects of tranylcypromine (TCP) (300–400 μg/L) exposure on adult wild type (short-fin) zebrafish behavior in the 6 min novel tank test. TCP was administered for 30 min followed by testing 1 week later. A one-way ANOVA test (factor: dose) revealed that the drug significantly affects the number of erratic movements ($F_{(2, 41)} = 5.699$, $P < 0.05$) in adult wild type (short-fin) zebrafish. Data are presented as mean ± SEM (n = 14 per group), *P<0.05 vs. control; post-hoc Tukey test for significant ANOVA data.

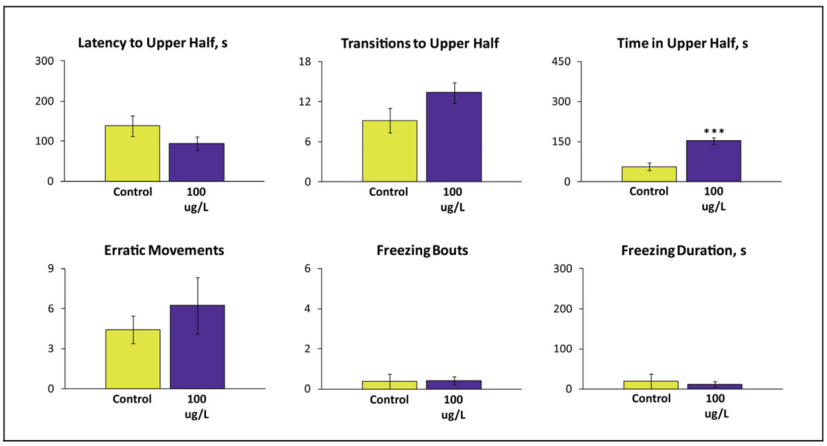

Figure 2.

Effects of repeated lysergic acid diethylamide (LSD, 100 μg/L) exposure on adult wild type (short-fin) zebrafish behavior in the 6 min novel tank test. Zebrafish were exposed to LSD twice a day for one week. Wilcoxon U-test revealed that the drug significantly affects the time spent in the top in adult wild type (short-fin) zebrafish. Data are presented as mean ± SEM (n = 14 per group), ***P<0.005 vs. control, U-test.

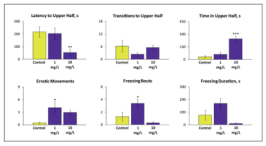

Figure 3.

Effects of acute 20-min dizocilpine (MK-801, 1–10 mg/L) exposure on adult wild type (short-fin) zebrafish behavior in the 6 min novel tank test. A one-way ANOVA test (factor: dose) revealed that the drug significantly affects the latency to enter the top ($F_{(3, 55)} = 9.315$, $P < 0.005$), time spent in the top ($F_{(3, 55)} = 20.834$, $P < 0.005$), number of erratic movement ($F_{(3, 55)} = 4.563$, $P < 0.005$), and number of freezing bouts ($F_{(3, 55)} = 8.255$, $P < 0.005$) in adult wild type (short-fin) zebrafish. Data are presented as mean ± SEM (n = 14 per group), *P<0.05, **P < 0.01, ***P < 0.005 vs. control; post-hoc Tukey test for significant ANOVA data.

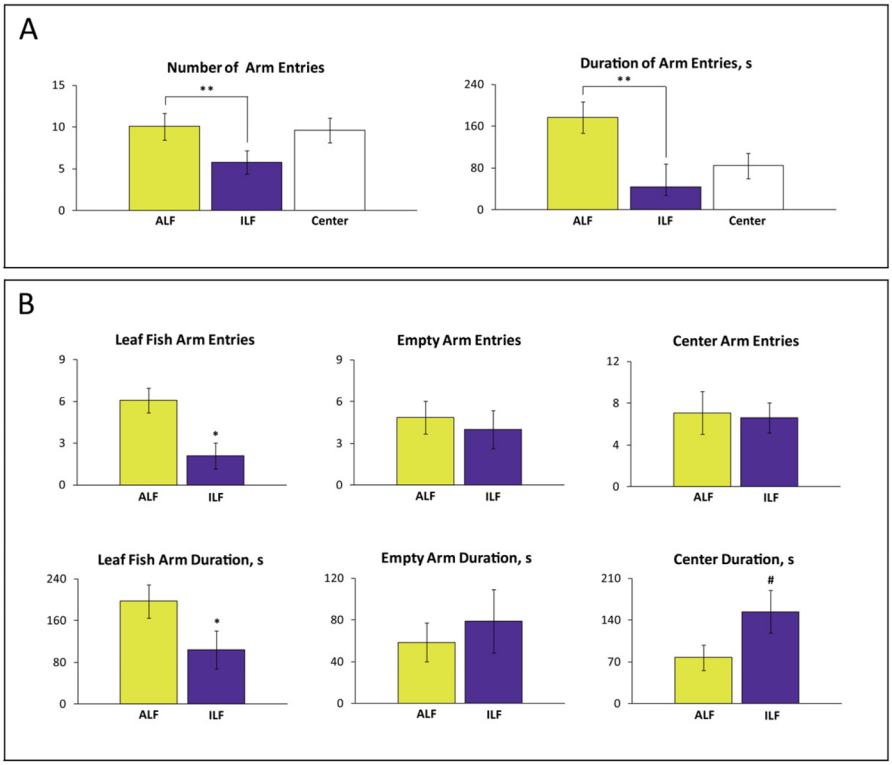

Figure 4.

Behavioral effects of the predator avoidance in adult wild type (short-fin) zebrafish in the two-compartment choice test. Zebrafish were placed in the center of the tank and the exposed to a predator (placed in an adjacent arm) for 6 min. The number or entries to, and the time spent in, the predator vs. non-predator containing arms was assessed. (A) In this experiment, zebrafish were exposed to two different predators simultaneously, the Indian leaf fish (ILF) and African leaf fish (ALF), with each placed in separate, opposite arms of the tank. Zebrafish generally exhibit stronger avoidance toward the open center arm and the ILF (indigenous to their natural environment in the wild) compared to the ALF. (B) In this experiment, zebrafish were exposed to either the ILF or ALF on alternating trials, during which the single predator was placed into one arm of the test apparatus. The number of arm entries and duration is significantly affected by ILF vs. ALF predator exposure. Data are presented as mean ± SEM (n = 15 per group), *P<0.05, **P < 0.005, #P = 0.05–0.09 vs. control, U-test

Table 1

Summary of several commonly used paradigms for assessing anxiety-related behavior in adult zebrafish.

Test rationale	Test description and major endpoints	Key references
Novel tank test*		
Exposure to a novel arena, similar to rodent open field test*	After a period of acclimation, zebrafish are placed individually in a narrow tank (e.g., 1.5-L) maximally filled with water and divided into two (or three) equal virtual horizontal sections, demarcated with a line on the outside walls. The following endpoints are typically recorded in the novel tank test for 5–6 min: the latency to reach the upper portion of the tank (s), time spent in the upper portion of the tank (s), number of transitions (entries) into the upper portion of the tank, number of erratic movements, number of freezing bouts and time spent frozen (s). Increased anxiety is typically accompanied with reduced exploratory behavior, thigmotaxis and geotaxis. In addition to manual recording, video-recording can be used in this test, assessing distance traveled, average velocity, turning angle, and angular velocity.	(Egan et al. 2009; Wong et al. 2010b; Stewart et al. 2010e; Levin et al. 2007; Bencan et al. 2009)
Open field test*		
Exposure to a novel arena, similar to the rodent open field test*	After a period of acclimation, zebrafish are placed individually in a novel arena filled with water to the level of about 12 cm. Apparatuses of differing size, color, shape, and texture can be used. Due to the nature of the experimental set-up, manual observation may be precluded. However, video-tracking can be used to measure relevant endpoints, including those associated with thigmotaxis and exploration. Other analyses of anxiety may include assessments of spatiotemporal patterning and/or lateral swimming in response to a stressor. Parameters may be defined to divide the arena into separate zones to record behavior in specific regions of the tank. The following endpoints are typically recorded in the tank for 6 or 30 min: time spent in the periphery or center of the tank (s), distance traveled in each zone in the tank (m), velocity in each zone of the tank (m/s), number of transitions (entries) between zones, number of freezing bouts and time spent frozen (s).	(Stewart et al. 2010c; Stewart et al. 2010d; Grossman et al. 2010)
Light-dark box		
Rodent light-dark box as a measurement of scototaxis	After a period of acclimation, zebrafish are placed individually in the light-dark box filled with water (e.g., to a height of 12 cm) and representing a rectangular tank divided into two equal vertical portions by black and white coloration. The following endpoints are typically recorded in this test: latency to enter the white half (s), time spent in the white half (s), the number of entries to the white half of the apparatus. White:total time spent ratios can be calculated to assess scototactic behavior. In addition to manual recording, video-recording can be used in this test, assessing distance traveled, average velocity, turning angle, and angular velocity in the white compartment.	(Maximino et al. 2010b; Maximino et al. 2010c; Stewart et al. 2010f; Serra et al. 1999)
Social preference test		
Measures social behavior	After a series of habituation and training trials, zebrafish are placed in groups in a tank (e.g., 40-L) resting on level surface and maximally filled with water. The paradigm has been use to assess how zebrafish respond to conspecific, heterospecific, or changes in coloration and patterning. Prospective shoaling partners can be separated by transparent Plexiglass, with variation in preference exhibited by the subject fish then observed. With the addition of an anxiety-inducing stressor (e.g., anxiogenic or anxiolytic drug, predator) or memory modulating agent, social preference may also be altered.	(Saverino and Gerlai 2008; Engeszer et al. 2004)
Shoaling		
Measures the effects of anxiety on social behavior	Zebrafish are placed in groups in a tank (e.g., 40-L) resting on level surface and maximally filled with water. The fish may be exposed to pharmacological manipulation or other type of stressor to evoke anxiety. The cohesion of their shoal is then assessed, with an anxiogenic response leading to an increase in shoal cohesion. Alternatively, with lower anxiety, fish have a greater tendency to break away from the group, and shoal cohesion is lessened. To assess shoaling, still images are obtained for every 10 s via video- recording, and the average distance among all members of the experimental zebrafish shoal, the average distance among all members of the stimulus group, and the average distance between all experimental and stimulus fish can be quantified. The fish coloration and the “nearest neighbor” distance can also be assessed in this test	(Engeszer et al. 2004; Miller and Gerlai 2007; Wright et al. 2003; Ruhl and McRobert 2005) [May be add some m ore refs on fish coloration and nearest neighbor distance in the shoaling test – can you find them?]
Boldness and novel object approaching		

Test rationale	Test description and major endpoints	Key references
A novelty-based paradigm that can also be adapted to assess predator avoidance or social behavior	Boldness has been assessed in zebrafish, such as through latency to feed after a disturbance and biting to a mirror stimulus. The novel object test is based on placing fish in a cylindrical tank devoid of visual cues, either individually or in a group, and after a period of acclimation, a novel stimulus in introduced. Video-aided analysis can be used to segregate the tank into concentric rings centered around the object, with the following endpoints typically recorded for 10 min: latency to approach the object (s), frequency of approach, time spent near the object (s), number of freezing bouts, and time spent frozen (s).	(Wright et al. 2003; Wright et al. 2006; Ogwang 2008; Moretz et al. 2007)
Predator avoidance		
Assesses fear- and anxiety-like behavior in the presence of a natural stressor	The tendency for zebrafish to avoid an inherent stressor, such as the natural predator Indian leaf fish, is assessed. Zebrafish are place in the center of a two-compartment choice test with a predator placed in one of the apparatus arms. Avoidance or willingness to approach the predator is then measured. The following endpoints are typically recorded: the latency to enter the same arm as the predator (s), time spent in the same arm as the predator (s), time spent in the arms without the predator (s) number of transitions (entries) into the same arm as the predator, and number of transitions (entries) into the arms without the predator.	(Bass and Gerlai 2008; Gerlai 2010)

*While the zebrafish novel tank and open field tests are both based conceptually on the rodent open field paradigm, they differ in several key aspects, as the novel tank test mainly assesses geotaxic “vertical” behavior, and the zebrafish open field test mainly measures thigmotaxis and locomotion in the horizontal direction.