Chapter 3

Modeling Stress and Anxiety in Zebrafish

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Abstract

While zebrafish (*Danio rerio*) are widely utilized as a model species for neuroscience research, they also possess several qualities that make them particularly useful for studying stress and anxiety-related behaviors. Zebrafish neuroendocrine responses are robust, and correlate strongly with behavioral endpoints. These fish are also highly sensitive to various environmental challenges, including novelty stress, exposure to predators, alarm pheromone, anxiogenic drugs, and drug withdrawal. In addition, varying levels of baseline anxiety can be observed in different strains of zebrafish. Collectively, this supports the validity and efficacy of the adult zebrafish model for studying both acute and chronic anxiety.

Key words: Novel environment, video-aided analysis, stress, anxiety, fear, affective behavior, predator stress, endocrine response, endocrine signaling, behavioral phenotyping, drug withdrawal, novel tank test, genetic differences.

1. Introduction

As summarized in several chapters of this book, the zebrafish is commonly used as a model species in biomedical research (1, 2). A vast array of genetic knowledge and a complete genome sequence is available for zebrafish, placing our genetic understanding of this species on par with the fruit fly and mouse (3). Although these studies have predominantly examined genetic and embryological phenomena (4), zebrafish are increasingly used in

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neuroscience research (5–10). Importantly, zebrafish possess all of the "classical" neurotransmitters found in vertebrates (11), suggesting their potential for studying disorders such as Parkinson's, Alzheimer's, anxiety, and depression (12). While complex neuropsychiatric disorders are difficult to reproduce in zebrafish, analogous brain mechanisms may be investigated using such models (11).

Stress and anxiety have been studied extensively using various animal (primarily murine) models (13–15). Recently, zebrafish have emerged as a promising new organism for anxiety research due to their robust cortisol stress response (16), behavioral strain differences (17) and sensitivity to drug treatment (7, 18–20), as well as to various stressors, such as exposure to predators (6) and alarm substance (21). This chapter outlines several aspects of zebrafish behavior that are relevant to the study of fear and anxiety-related states.

2. The Novel Tank Diving Paradigm: A Fish "Open Field"?

Zebrafish behavioral assays are currently used for high-throughput phenotyping and testing various psychotropic drugs (8, 22, 23). A popular method of behavioral analysis in zebrafish is the novel tank diving paradigm (**Fig. 3.1**), conceptually similar to the open field test used for rodents (**Table 3.1**). In the open field test, mice exposed to a novel environment initially exhibit anxiety-like behavior by staying close to the walls (thigmotaxis), but begin to display increased exploration as they become acclimated to the new setting (24). Similarly, exposure of zebrafish to a novel environment evokes a robust anxiety response (8), as the

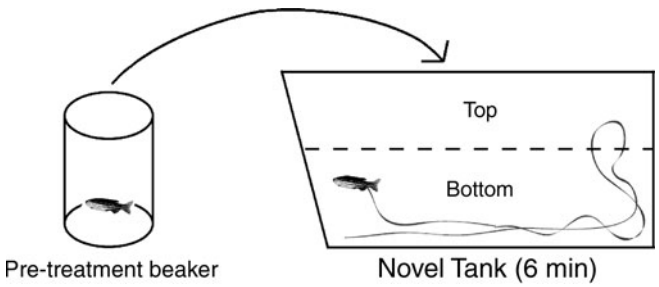

Fig. 3.1. The novel tank diving test (also referred to as the novel tank test) examines novelty-evoked anxiety. When a zebrafish is exposed to a novel (potentially dangerous) environment, it initially dives to the bottom, and then gradually explores the top. Inhibited exploration, reduced speed, and increased frequency of escape-like erratic behaviors are usually associated with higher levels of anxiety elicited by different stressors (see **Table 3.2** for details).

Table 3.1
Comparison of behavioral endpoints in mouse and zebrafish novelty-based paradigms, such as the open field and novel tank tests

Mice		Zebrafish	
Open field	**References**	**Novel tank**	**References**
Thigmotaxis (staying close to the walls)	Heisler et al. (50)	Preference of the bottom of the tank	Levin et al. (22)
Center:periphery ratio	Kalueff et al. (51)	Top:bottom ratio	Own unpublished observations, 2008
Vertical and horizontal exploratory activity	Prut and Belzung (52)	Exploratory swimming	Panula et al. (12) and Egan et al. (17)
Freezing	Heisler et al. (50)	Freezing	Gerlai et al. (19)
Risk assessment	Choleris et al. (53)	Short visits to top of the tank	Own unpublished observations, 2008
Hyperarousal in dangerous situations	Siegmund and Wotjak (54)	Erratic movements	Egan et al. (17) and Gerlai et al. (9)
Locomotor activity (e.g., total distance travelled)	Eilam (55)	Locomotor activity (e.g., total distance swam)	Levin et al. (22)
Vegetative behavior (e.g., defecation, urination)	Fernandez-Teruel et al. (56) and Flint et al. (57)	–	–

animals dive to the bottom and limit exploration until they feel safe to swim in the upper regions of the tank (**Table 3.1**).

Until recently, quantification of zebrafish behavior was mostly performed manually, making it vulnerable to human error and incorrect data interpretation. In contrast, automated video-tracking technologies can analyze animal behavior to provide standardized observation of behavioral endpoints and reduce subjective influence (17). Another advantage of using the video-tracking approach in zebrafish research is the ability to store, replay, and reanalyze videos. Finally, during the novel tank diving test, video-tracking programs can calculate additional behavioral endpoints not available through manual observation, such as distance traveled in top/bottom, velocity, meandering, and angular velocity (**Table 3.2**). Comparison of data produced by the video-tracking system with that recorded manually shows significant (>80–90%) correlation between the two (17), confirming that the video-tracking approach is a reliable method of analysis in zebrafish neurobehavioral research.

3. Analyzing Endocrine Responses to Stress

Physiological phenotypes contribute markedly to the utility of zebrafish models for anxiety research. The zebrafish hypothalamus-pituitary-interrenal (HPI) axis is homologous to the human hypothalamus-pituitary-adrenal (HPA) axis, with cortisol being the primary stress hormone in both species (25, 26) (**Fig. 3.2**). Following animal exposure to stressful stimuli, the hypothalamus secretes corticotropin releasing hormone (CRH), which activates the pituitary gland and signals the pituitary to release andrenocorticotropic hormone (ACTH). Stimulated by ACTH, the adrenal (mammals) or interrenal (zebrafish) glands synthesize glucocorticoid hormones from a cholesterol precursor (26, 27). Increased levels of glucocorticoids initiate metabolic effects that modulate the stress reaction (26, 28), including gluconeogenesis, anti-inflammatory effects, and immune system suppression (29). The effects of the stress reaction are harmful in excess and are alleviated through a negative feedback to the hypothalamus and pituitary, which suppresses CRH and ACTH release (30, 31). This evolutionarily conserved stress response between zebrafish and humans makes zebrafish a valid model to study cortisol-mediated stress responses (16, 32).

Analysis of the physiological (neuroendocrine) responses to stress in zebrafish is a valuable tool complementing behavioral studies. The cortisol assay in zebrafish (5, 17) is relatively simple, inexpensive, can be easily adopted in a variety

Table 3.2
Summary of behavioral endpoints measured in the novel tank diving test

Endpoint (units)	Registration	Definition	Interpretation
Latency to enter the top (s)	m, a	The amount of time to first cross (by the center of mass of the body) from the defined bottom portion to the top of the novel tank	When introduced to a novel environment, zebrafish naturally dive to the bottom of the tank and gradually explore as it habituates to the test apparatus. The longer latency indicates higher anxiety levels
Time spent in top (s)	m, a	Total time spent in the top portion of the novel tank	A longer duration in the top of the tank indicates lower anxiety levels
Time spent top:bottom ratio	c	The ratio of the time spent on top over bottom	Lower ratio indicates higher anxiety level
Number of entries to the top	m, a	The number of crosses from the defined bottom portion to the top of the novel tank	More top entries indicate lower anxiety levels
Entries top:bottom ratio	c	The ratio of the number of entries to the top over bottom	Lower ratio indicates higher anxiety level
Average entry duration (s)	c	The amount of time spent at the top of the novel tank during each crossing	Calculated as time spent in the top divided by the number of entries to the top. Shorter average entry duration indicates higher anxiety level
Distance traveled in the top (m)	a	Total distance traveled in the defined top portion	Zebrafish with high anxiety would travel more distance at the bottom of the tank
Distance traveled top:bottom (m)	c	A ratio of the total distance traveled in the defined top portion versus the defined bottom	A lower top:bottom ratio indicates a higher stressed fish
Total distance traveled (m)	a	Total distance the zebrafish traveled within the novel tank	Reflects general motor/neurological phenotypes. Zebrafish are generally quite sensitive to nonspecific motor impairments and sedative drug effects (see troubleshooting section)

(continued)

Table 3.2 (continued)

Endpoint (units)	Registration	Definition	Interpretation
The number of erratic movements	m, a	Sharp or sudden changes in direction of movement or repeated darting behavior	Indicates increased fear/anxiety, and are generally higher in stressed zebrafish
Average velocity (m/s)	a	Magnitude and direction of zebrafish speed	Reflects motor aspects of zebrafish swimming, may be increased or decreased depending on the nature of behavioral test
Freezing bouts (frequency)	m, a	Total immobility(>1 s), except for the eyes and gills	Indicate increased anxiety and are generally higher in stressed zebrafish
Freezing duration (s)	m, a	Total duration of all freezing bouts	Indicates increased anxiety and is generally higher in stressed zebrafish
Meandering (°/m)	a	The degree of turning (vs. straight locomotion)	Reflects motor aspects of zebrafish swimming, may be increased or decreased depending on the nature of behavioral test
Turning angle (°)	a	Total turning angle	Reflects motor aspects of zebrafish swimming, may be increased or decreased depending on the nature of behavioral test
Angular velocity (°/s)	a	Magnitude and direction of zebrafish angular speed	Reflects motor aspects of zebrafish swimming, may be increased or decreased depending on the nature of behavioral test

a, Automatic observation; m, manual observation; c, calculations based on manually or automatically recorded data.

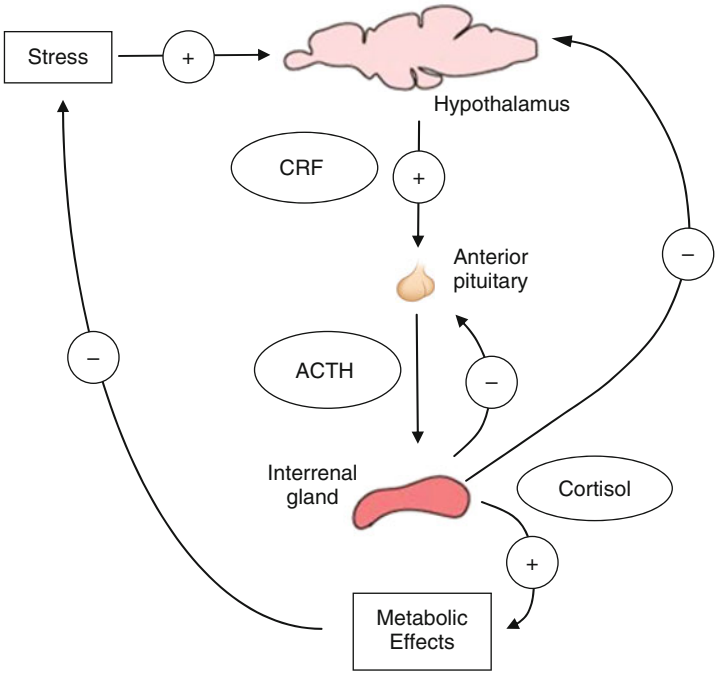

Fig. 3.2. Zebrafish endocrine stress axis. "+" or "−" signs indicate activation or inhibition of activity or secretion, respectively. CRH – corticotropin releasing hormone; ACTH: adrenocorticotropic hormone.

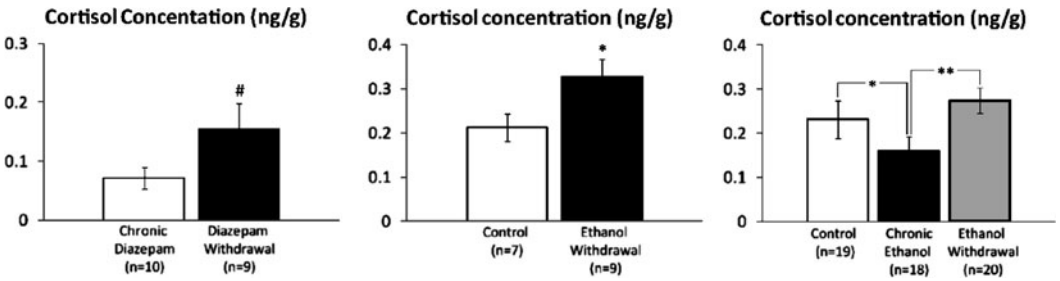

Fig. 3.3. Zebrafish endocrine responses (whole-body cortisol, ng/g fish) to anxiogenic behavioral effects produced by withdrawal from diazepam and ethanol. Data are presented as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, #p = 0.05–0.09 (trend) vs. controls, *U*-test (modified from Egan et al. (17)).

of laboratory settings, and strikingly parallels observed anxiety behavior (**Fig. 3.3**). Statistical analysis of correlation between behavior and endocrine response may further assist in data interpretation. For example, the Spearman's rank correlation coefficient, used to assess the relationship between two variables, can determine the level of correlation between behavioral data and cortisol concentration values.

4. Behavioral Responses to Experimental Stressors: Predator and Alarm Pheromone Exposure

The presence of a predator is a universal stressor for animals. Zebrafish have demonstrated significant behavioral responses to their natural predator, the Indian Leaf Fish (*Nandus nandus*), and to foreign predators (6) (**Fig. 3.4**). Zebrafish also show an increase in whole body cortisol levels after visual contact with a predator fish, confirming their increased stress response (5). In general, two possible explanations for predator-avoidance behavior include learned antipredatory responses (following exposure to a harmful predator), or instinctive avoidance behavior.

Mounting evidence supports the importance of learning in the development of animal predatory responses. For example, while visual predator recognition skills seem to be based on unlearned predispositions, antipredatory behavior using olfactory stimuli can be modified with experience, particularly during the

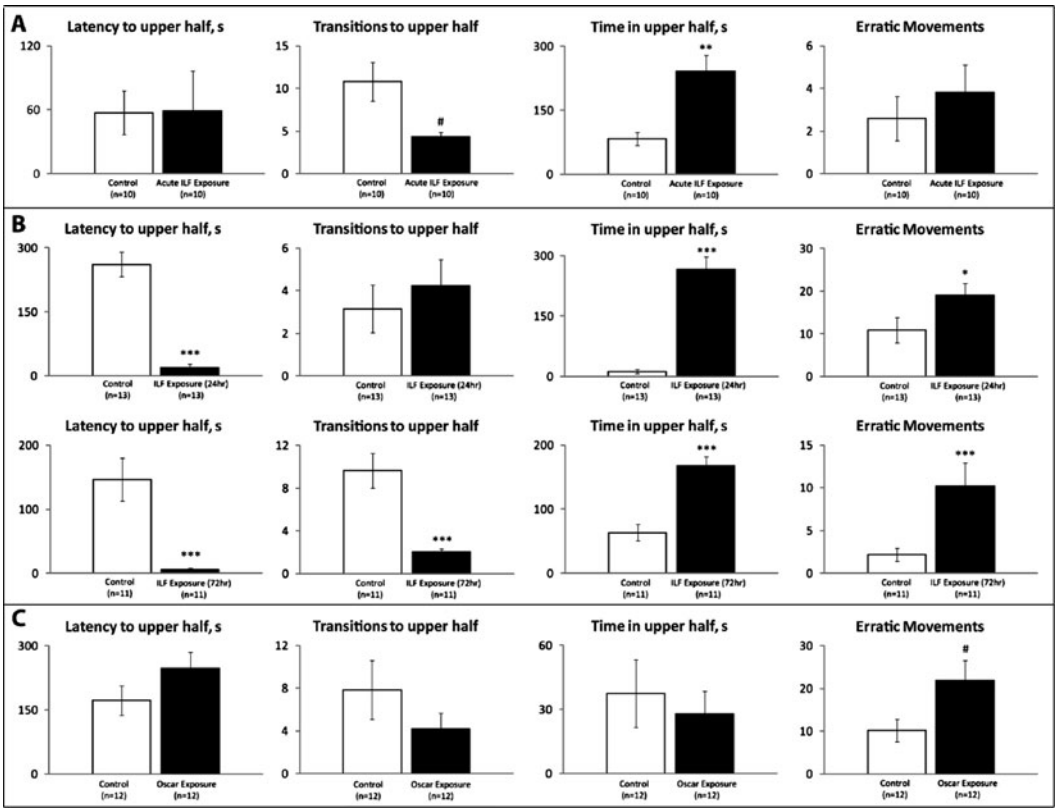

Fig. 3.4. Anxiogenic effects of predator exposure on zebrafish behavior. **a** and **b** Exposure to the sympatric predator Indian Leaf fish (ILF): **a** – acute 5-min exposure; **b** – chronic 24-h (*top*) and 72-h (*bottom*) exposure. **c** Acute 10-min exposure to the allopatric predator Oscar fish. Data are presented as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $\#p = 0.05$–0.09 (trend) vs. controls, *U*-test.

initial stages of the predator-prey interaction (33). Olfactory cues enable zebrafish to recognize predators following a single exposure to the predator fish (34). However, experimentally naïve zebrafish respond significantly stronger to their natural predator than to an allopatric predator, suggesting a genetically based predator anxiety (6).

Our laboratory has recently examined zebrafish stress responses to the Indian Leaf fish, a natural sympatric predator, and the Oscar fish (*Astronotus ocellatus*), an allopatric predator native to South America. Using experimentally naïve zebrafish, we conducted acute and chronic predator exposure tests using the novel tank diving paradigm. As can be seen in **Fig. 3.4a, b**, both acute and chronic predator exposure produced similar behavioral responses to the Indian Leaf fish. Notably, although the zebrafish displayed a typical response to stress with an increase of erratic movements, they also displayed shorter latency to enter the upper half and more time spent in the upper half, which are not characteristics associated with stress in the novel tank paradigm (**Tables 3.1** and **3.2**). However, as the predator fish spent the majority of the time in the bottom of the tank, it appears that the zebrafish displayed a distinct learned avoidance behavior by moving to the area least likely to be occupied by a predator. In contrast, typical anxiety-like behavior was only significant in the erratic movement endpoint during Oscar fish exposure, indicating weaker responses as compared to the Indian Leaf fish experiment (**Fig. 3.4c**). Although zebrafish were noticeably stressed by the Oscar fish, these findings indicate a greater fear of sympatric (compared to allopatric) predators. This suggests the importance of a genetic, innate influence on the zebrafish fear response.

In line with this, we have also examined the effect of alarm pheromone exposure in zebrafish. As will be mentioned in this book, the zebrafish olfactory system detects alarm pheromone released by injured skin cells, and has been shown to cause behavioral responses. While behavioral alterations in zebrafish could, in theory, be affected by alarm pheromone, the composition of this molecule is not completely understood. Therefore, exact concentrations and dosing cannot be determined when using nonquantifiable extraction from zebrafish skin (35). After extracting alarm pheromone from the epidermal cells of euthanized zebrafish (21), we exposed naïve fish to water containing the alarm pheromone, and measured behavioral responses again through the novel tank paradigm. Acute alarm pheromone exposure (**Fig. 3.5a**) resulted in a robust anxiety-like behavioral response, notably represented through significantly decreased exploration and increased erratic movements and freezing bouts (17). A recent study found that hypoxanthine 3-N-oxide, a molecule common to the alarm substances secreted by several fish species, elicits more erratic movements and jumps when zebrafish were acutely exposed to its

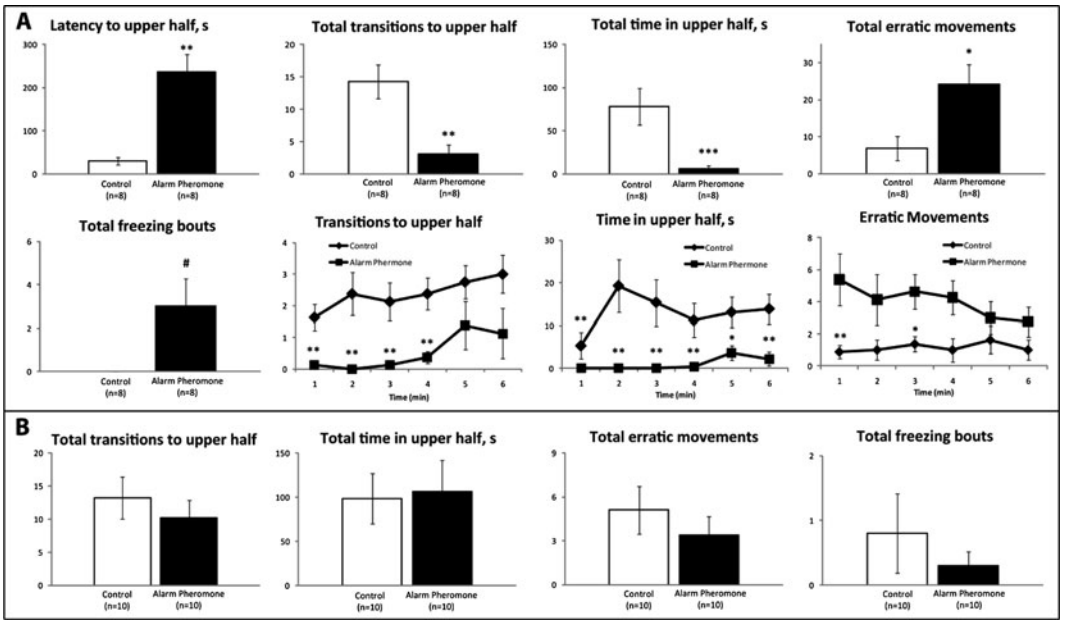

Fig. 3.5. Anxiogenic effects of alarm pheromone on zebrafish behavior in the novel tank diving test: **a** acute alarm pheromone exposure (6 min). **b** Prolonged alarm pheromone (30 min). Data are presented as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $\#P = 0.05$–0.09 (trend) vs. control, *U*-test (modified from Egan et al. (17)).

increasing doses (35). In contrast, chronic alarm pheromone exposure in our studies produced no significant change from the control cohort (**Fig. 3.5b**), suggesting that alarm pheromone is only effective acutely, most likely reflecting its natural use as a fast-acting danger signal to nearby shoals.

5. Pharmacogenic and Withdrawal-Associated Anxiety

Past zebrafish studies demonstrated robust behavioral phenotypes following acute and chronic exposure to psychotropic agents such as diazepam, caffeine, ethanol, morphine, cocaine, nicotine, barbiturates, and hallucinogens (17, 18, 22, 36–38). The observed predictable bidirectional behavioral responses to known anxiolytic or anxiogenic drugs indicate that zebrafish demonstrate high translation value in stress- and anxiety-related pharmacological research.

Anxiety symptoms are commonly seen in patients withdrawing from chronic drug therapy (39–41). Increasing interest in the underlying neurobiological mechanisms of withdrawal syndrome necessitates the development of appropriate animal models. Robust anxiety phenotypes have been elicited in zebrafish

Table 3.3
Comparison of robustness of zebrafish behavioral phenotypes elicited by different stressors

Type of stress	Stressor	Phenotype
	Alarm pheromone	+++
Acute	Sympatric predator	+++
	Allopatric predator	+
	Alarm pheromone	0
Chronic	Sympatric predator	++
	Strain differences	++
	Drug withdrawal	++

+++, Robust; ++, mild; +, weak effects; 0, no effects; also see Fig. 3.7.

through discontinuation of chronic drug exposure, suggesting the existence of withdrawal syndrome in this species. For example, drug-evoked anxiogenic effects were reported following abrupt cessation of chronic cocaine administration (23), confirming zebrafish as a valid animal model of withdrawal syndrome-associated anxiety (**Table 3.3**).

6. Strain Differences in Zebrafish Behavior

As with other species, genetic differences in zebrafish may lead to varying behavioral phenotypes. One study found that chronic ethanol exposure decreased shoaling behavior in wild-type short-fin zebrafish, but increased shoaling behavior in long-fin striped strain (42). Our group investigated baseline anxiety levels in short-fin and leopard strains, reporting that the leopard zebrafish generally display higher levels of anxiety in the novel tank test (**Fig. 3.6**). Interestingly, using automated video-tracking software, we found no significant differences in swimming velocity or total distance traveled between these two strains (**Fig. 3.6**), indicating that these differences in anxiety were not due to motor/neurological deficits.

Understanding the behavioral differences between zebrafish strains is crucial for expanding this animal model to investigate population differences in humans and their susceptibility to stressors. In addition, selection of a certain strain could optimize data generated in screening of anxiolytic or anxiogenic drugs. For example, due to floor/ceiling effects, choosing a more anxious (e.g., leopard) strain may provide more robust results if examining the behavioral effects of an anxiolytic compound, while the use of

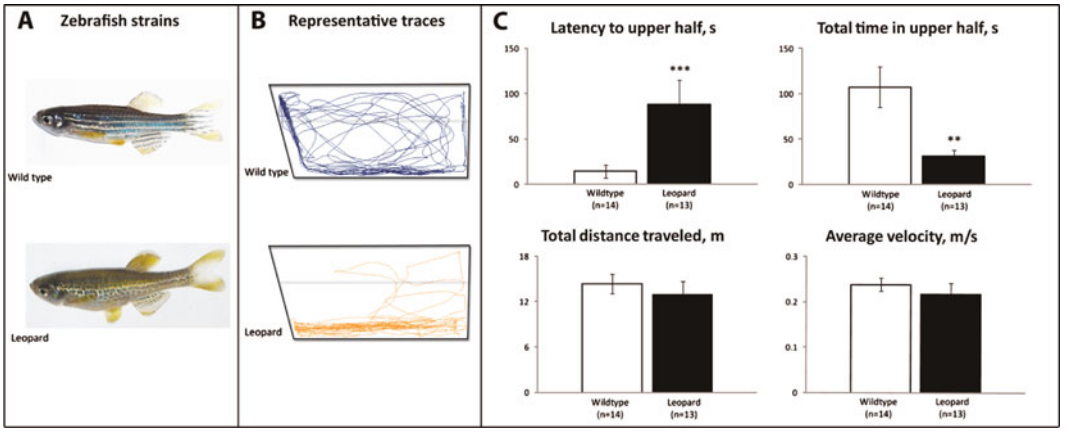

Fig. 3.6. Strain differences in zebrafish novel tank diving test behavior. Two different strains display strain-distinct patterns of their exploratory behavior, as illustrated by representative swimming traces and selected behavioral endpoints analyzed using video-tracking software (CleverSys Inc). Data are presented as mean ± SEM, $^{**}p < 0.01$, $^{***}p < 0.005$ vs. wild type, *U*-test (modified from Egan et al. (17)).

a less anxious strain (such as short-fin zebrafish) could yield more clear-cut phenotypes while testing anxiogenic drugs and manipulations.

7. Mutant and Transgenic Zebrafish

Ease of genetic manipulation, high fecundity, and rapid development make zebrafish a useful tool to study the genetic factors involved in pathogenesis (43). Applied to zebrafish, mutagenesis, transgenesis, and mapping approaches enable the researchers to use invertebrate-style forward genetics on a vertebrate organism (43). There are also certain drawbacks to the use of zebrafish in genetic research, as they have a duplicate genome, and not all duplicated genes have been retained through time (44). For example, it is frequently argued that further comprehension of zebrafish gene function will only uncover invalid redundant and species-specific information (44). However, duplicate genes can also provide significant advantages when zebrafish co-orthologs represent selected expression patterns and developmental functions of mouse orthologs. Thus, restricted expression of zebrafish genes, in comparison to the corresponding mouse orthologs, may lead to an improved comprehension of developmental relations in cell lineage or tissue patterning in mice (44).

Furthermore, several transgenic zebrafish exhibit robust aberrant behavioral phenotypes linked to the knockout of specific target genes. For example, *nevermind* (nev) gene mutant zebrafish display severe disruption of optic nerve innervation (45). While their muscular morphology is normal, *nev* dorsal

retinotectal axon projections terminate on both the dorsal and ventral side of the tectum, resulting in atypical locomotion, such as corkscrew swimming, in which zebrafish rotate around their long body axis. Similarly, sphingosylphosphorylcholine knockout zebrafish perform spontaneous erratic movements and escape behaviors (e.g., rapid turning) without provocation from stressful stimuli (46).

Some of the transgenic zebrafish models focus on abnormal developmental patterns that prevent proper innervations between nuclei and in turn disrupt neurophysiology. One example of this is the mutation of the Lhx2 homolog, *bel*, a transcription factor involved in retinotectal axonal growth. In zebrafish, achiasmatic-induced oculomotor deficits generate spontaneous eye oscillations that may model congenital nystagmus in humans, in addition to causing reversed perception of visual stimuli, misappropriated eye movements, and circling swimming behavior (47). It is possible to expect that numerous other zebrafish mutations may lead to interesting motor- and anxiety-related behavioral phenotypes that will be revealed in future studies.

8. Conclusion

Although anxiety-related disorders continue to be one of the most prevalent neuropsychiatric conditions, their pathological mechanisms are poorly understood. One hypothesis stipulates that

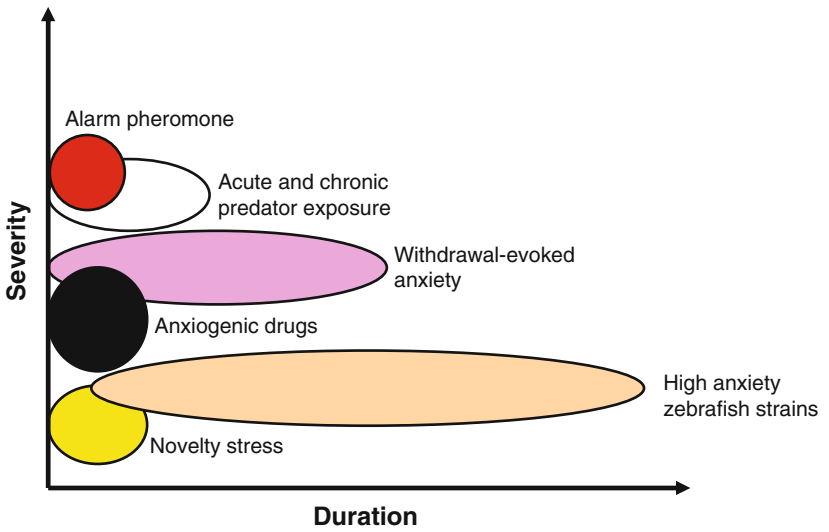

Fig. 3.7. A summary of different forms of stress used in zebrafish neurobehavioral research. Fear-like responses are more likely to occur following alarm pheromone and predator exposure, anticipatory generalized "trait" anxiety is more likely to occur following anxiogenic drug treatment or novelty stress, whereas chronic long-term "state" anxiety can be seen following withdrawal, or in more anxious zebrafish strains (genetic differences).

these disorders are most likely caused by abnormally functioning biological mechanisms of danger avoidance (35). Current challenges to phenotype-based drug discovery include expensive mammalian animal models that require ample physical space and large quantities of compounds for use in experiments. Mammalian animal models also exhibit complex behavioral phenotypes that are sometimes too difficult to characterize and interpret (4). Using zebrafish as an alternative animal model (**Table 3.3**, **Fig. 3.7**) effectively reduces these limitations, and together with computer-aided video tracking technology, endocrine correlates, and genetic manipulation makes high-throughput behavioral phenotyping and pharmacological screens a promising possibility (2, 4, 17, 48, 49).

Acknowledgments

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