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**Abstract**

Modeling of stress and anxiety in adult zebrafish *(Danio rerio)* is increasingly utilized in neuroscience research and CNS drug discovery. Representing the most commonly used zebrafish anxiety models, the novel tank test (NTT) focuses on zebrafish diving in response to potentially threatening stimuli, whereas the light-dark test (LDT) is based on fish scototaxis (innate preference for dark vs. bright areas). Here, we systematically evaluate the utility of these two tests, combining meta-analyses of published literature with comparative in-vivo behavioral and whole-body endocrine (cortisol) testing. Overall, the NTT and LDT behaviors demonstrate a generally good cross-test correlation in-vivo, whereas meta-analyses of published literature shows that both tests have similar sensitivity to zebrafish anxiety-like states. Finally, NTT evokes higher levels of cortisol, likely representing a more stressful procedure than LDT. Collectively, our study reappraises NTT and LDT for studying anxiety-like states in zebrafish, and emphasizes their developing utility for neurobehavioral research. These finding can help optimize drug screening procedures by choosing more appropriate models for testing anxiolytic or anxiogenic drugs.

**Keywords:** zebrafish; anxiety-like behavior; behavioral phenotyping; the novel tank test; the light-dark test

## 1. Introduction

Evoked in response to novel, potentially dangerous situations, anxiety is a natural emotion which is critical for the organism to survive [1, 2]. However, anxiety may also be pathological, appearing in inappropriate contexts and reaching high levels that disrupt normal life [3]. Anxiety disorders (ADs) are a diverse group of mental diseases defined by excessive worries or fears [4]. Widespread globally [5], ADs represent the most prevalent psychiatric conditions, and may trigger other psychiatric disorders, such as depression and addiction [6]. Pathological anxiety is caused by aberrant 'emotionality' brain circuits, including the limbic system and cortex [3, 7]. However, neural mechanisms of ADs remain poorly understood, necessitating novel experimental approaches and theoretical concepts [8-11].

Animal experimental models, especially rodent paradigms, are an indispensable tool for understanding the basic neurobiology of ADs [10, 12]. Reflecting the importance of translational, *cross-species* analyses of neural phenotypes [8, 13-15], there is also a growing interest in widening the spectrum of model species in neurobehavioral research [9, 16-18]. For example, adult zebrafish (*Danio rerio*) are rapidly emerging as a promising model organism to study anxiety- and other stress-related conditions [9, 19, 20]. Among multiple behavioral tests and models [21-25] (Table 1), the novel tank (NTT) and the light-dark (LDT) tests are the two most popular experimental paradigms of zebrafish anxiety [26, 27].

### *1.1. The novel tank test (NTT)*

Based on geotaxis - an innate escape 'diving' behavior of fish in novel environments – the novel tank test (NTT) has long been used to assess adult zebrafish behaviors [21] and drug responses [20]. Representing a conceptual analog of the rodent open field (OF) paradigm, NTT evokes motivational conflict between the 'protective' diving behavior and subsequent vertical exploration [28]. Indeed, when placed in novel environments, zebrafish initially spent more time at the bottom, reduce 'top' swimming, and exhibit more erratic movements and freezing/immobility episodes [29]. Later, due to habituation to the NTT novelty, zebrafish gradually explore the top area (potentially more dangerous for zebrafish in their natural habitats due to the presence of fish or bird predators). Stress and pharmacological agents can modulate these zebrafish NTT behaviors [30, 31], as anxiolytic drugs

(e.g., buspirone [32], chronic fluoxetine or diazepam [33, 34]) tend to increase time spent in top, whereas anxiogenics promote diving, immobility and erratic movements [31]. Successfully applied to NTT, modern video-tracking techniques have markedly improved testing zebrafish behavior by reducing data processing time, increasing the ability to register more diverse parameters of locomotion as well as to analyze them off-line, objectively and simultaneously [29, 35-38].

Typically, the NTT apparatus consists of transparent narrow trapezoidal or rectangular tank divided into two equal halves either virtually [39] or with horizontal line marked directly on the wall [40]. The parameters analyzed in NTT target two major phenotypic domains - the exploration and locomotion [41]. The main NTT anxiety-related endpoints are time spent in the upper/bottom zone, the latency to enter the top, the number of crossings between the zones, as well as the number and duration of freezing (a total absence of movement, except for the gills and eyes) and erratic movements (sharp changes in direction and velocity) [40, 42]. Locomotor phenotypes can be assessed by recording various automated zebrafish NTT endpoints, including distance traveled, absolute or average turn angle, average and maximal swimming speed, meandering or the number of 360$^{o}$ rotations [29]. Reduced vertical NTT exploration may also represent inhibited swimming activity in the presence of sedative agents (e.g., high sedative concentrations of ethanol) and not necessarily reflect increased anxiety-like states [43]. Conversely, a greater exploration of the top can be due to enhanced locomotion, a phenotype usually observed after exposure to psychostimulant or hallucinogenic drugs [44-46]. Therefore, evaluating more than one endpoint is needed to judge whether the effect is locomotor or anxio-tropic. Thus, there is a great value in assessing locomotor parameters of zebrafish during novelty stress in NTT using automated video tracking software and 3D reconstruction plots of behavior as neurophenotyping tools [35, 47].

Zebrafish also demonstrate behavioral differences in the NTT depending on their strain, sex, age [27, 48] and housing conditions [49]. For example, fish exposed to ethanol show mild hyperlocomotion in the home tank water, but display increased anxiety-like behavior with unaltered locomotion in newly replaced water [50]. The apparatus size and shape also modulate fish behavioral responses in this model, as zebrafish constantly housed in a narrow tank identical to the NTT show no NTT-specific

behaviors during testing, including absent diving response or changes in swimming speed [32]. However, the main stimuli controlling zebrafish NTT diving responses remain unclear [51]. In the wild, zebrafish feeds in the water column, and therefore their vertical distribution is a trade-off between feeding and predator avoidance [52]. While exposure to fish predators or their models may unalter zebrafish bottom-dwelling [35, 53-55], presenting a computer-animated image of a bird silhouette strongly increases their diving response [56]. Moreover, when fish are exposed to a tank with two compartments differing in real and perceived depth, the preference is observed for the side allowing further escape from the surface, but not the side with closer proximity to the substrate [57], collectively suggesting that NTT diving is an *escape* from the water surface rather than *approaching* the bottom.

### *1.2. The light-dark test (LDT)*

The LDT is also widely used in zebrafish, since adult fish avoid brightly lit areas and spend more time in the dark [22, 58]. This behavior is associated with the natural tendency of wild zebrafish to show overt scototaxis, thereby facilitating *crypsis* (avoidance the detection by other animals). Since adequate response to external stimuli is crucial for animal survival, zebrafish light avoidance rises from morning to evening, but decreases at night [59]. Various stressors (e.g., [60]) or anxiogenic substances (e.g., [26, 61]) predictably increase time spent in dark, allowing the LDT to evaluate anxiety-related behaviors and drugs. Other main endpoints used in this test are the number of total transitions between the two compartments, latency to enter the white area, the number of risk assessment episodes (fast entries to the lit area followed by re-entries to the dark, or as partial entries to the white), thigmotaxis in the white area, and other behavioral endpoints (e.g., erratic swimming and freezing) similar to those recorded in the NTT [62].

The predominantly accepted interpretation of LDT is that adult zebrafish show robust dark preference in the test (note, however, that zebrafish larvae have reversed light preference [63, 64]). A typical LDT apparatus is a rectangular glass or acryl tank consisting of two equal vertical portions: black and white or black and transparent chambers [44, 65, 66]. The color of the lighter portion (white vs. transparent) can change preference, as animals tend to prefer the transparent vs. the black compartment [65, 67]. Another modification of the preference paradigm apparatus is the light dark plus

maze with transparent walls and black or white arms' floor coloration [68]. Size of the light/dark box also usually differs markedly between the laboratories. Parts of the preference tank can be divided with a grey divider (after habituation, fish are able to swim between light and dark areas freely without a sliding door) [69, 70] or have no physical barrier [71, 72]. Considering that the currently available software tools do not properly detect animal in the black compartments, their behaviors are usually recorded in the light chamber, or using a gray floor (which may *per se* influence the behavior). Like for the NTT, the stimulus control is not fully established in the LDT [51]. For example, varying light levels or color of the white compartment and intra-/inter-session habituation data suggest that zebrafish LDT behavior is not driven solely by white aversion (photophobia) or scototaxis, but is based on approach-avoidance conflict [51]. As a result, it remains unclear whether the LDT and NTT may target different aspects of anxiety-like behavior, or different levels of anxiety-like states, or both. Here, we systematically compare these commonly used aquatic tests and evaluate their utility in characterizing zebrafish behavioral syndromes.

## 2. Materials and Methods

### *2.1. Behavioral meta-analyses*

Although the question of data comparison and testing priority was already raised for NTT and LDT [51, 73], their systematic comparative analyses have not been performed. Addressing this knowledge gap here, we first analyzed the literature, focusing on NTT and LDT studies of various anxiolytic, anxiogenic and toxic substances (Table 2). The inclusion criteria required 1) both tests be used in the same study available in PubMed; 2) testing the same concentrations of the drug(s); and 3) group size, mean and SEM/SD values be indicated for each group. Because of multiple parameters measured in NTT and LDT, we chose two most frequently measured endpoints that can also be considered functionally analogous: the time spent in, and the number of transitions to, the top half of NTT or the lit half of the LDT, respectively. For these endpoints, we calculated Standardized Mean Difference (SMD, Fig. 1) commonly used for the effect size estimation in meta-analyses [74]. As some mean and SEM for SMD calculations were represented in the graphs in the original publications, we performed their accurate quantification using the ImageJ software [75]. Statistical analysis was

conducted using the metafor package [76] for R version 3.2.5 [77]. We used a mixed-effects model using assay as a moderator to indicate a possible advantage of using the NTT or the LDT. While it is possible that treatment differences (i.e., drug, concentration/dose, etc.) are important moderators, we were interested in the main effect of any treatment on the behavioral endpoints, and in differences between the NTT and LDT. Therefore, using assay as a moderator, such as in our present analyses, was expected to be able to uncover differences in sensitivity between the two tests (see further).

### *2.2. Cortisol responses to NTT and LDT procedures*

Complementing behavioral endpoints, various physiological biomarkers are indispensable for clinical and preclinical AD research [78-80]. In a separate *in-vivo* experiment we directly compared physiological consequences of the two tests, using ELISA assays to access whole-body cortisol levels in adult zebrafish following their acute single NTT or LDT exposure [40]. Briefly, a total of 45 adult zebrafish (~50/50 male/female ratio) of the wild-type short-fin strain were housed 1 fish/L in 20-L tanks equipped with biological filters at the University of Passo Fundo (Passo Fundo, Brazil), under constant aeration and a 14-h light:10 h dark photoperiod. Water temperature was maintained at 27 ± 0.3 $^{o}$C; with pH kept at 7.0 ± 0.05, dissolved oxygen kept at 6.0 ± 0.05 mg/L, total ammonia at <0.01 mg/L, total hardness at 6 mg/L, and alkalinity at 22 mg/L $CaCO_3$. The experiment utilized three groups of fish: controls (experimentally naïve, unexposed fish), NTT- and LDT-exposed fish (which remained in their tests for 10 min, and were then immediately sacrificed for cortisol analysis using ELISA). Fish were gently transferred individually (using the net) from their hometanks to the testing apparatus (NTT or LDT) for 10 min. The NTT represented a glass tank 24 × 8 × 20 cm (width × depth × height) [60]. The LDT apparatus consisted of a glass tank (18 × 9 × 7 cm; width × depth × high) divided by a sliding guillotine-type partition (9 × 7 cm) in two equally sized dark and white compartments, filled with water [60]. Fish were individually placed in the light zone of the apparatus, and evaluated for 10 min. Behavioral results were subjected to one-way ANOVA, and were further analyzed by Tukey post-hoc test to compare all three groups (Fig. 3). The two tests were further compared between themselves by the U-test (Fig. 3).

### *2.3. Correlational analyses of behaviors generated in NTT and LDT*

Commonly used in neurobehavioral research, correlational analyses were next applied to the NTT and LDT data collected from a large cohort of male and female wild-type zebrafish housed in Federal University of Sul e Sudeste do Para (Maraba, Brazil). While the test battery effects were reported as minimal for zebrafish [27], the testing order was randomized. Since animals were kept in single groups, no sequence generation method was used. The animals came from different tanks, and were randomly drawn. Similar to [81], correlations were taken between measurements of time and transitions for each test. Time on top and time on white constituted a first set of "operational definitions" of anxiety-like behavior in theses assays, and transitions to top and transitions to white constituted the second set of operational definitions. Overall, we found convergence for the first set of operational definitions ("monotrait-heteromethod", marked by A in Table 3), as the measures moderately but significantly correlated. The second set of operational definitions revealed a strong significant correlation, supporting the possibility that both measures in the two tests reflect the similar/overlapping behavioral trait. Another type of correlation ("heterotrait-monomethod", marked by B in Table 3) refers to the relationship between the different operational definitions of the same trait measured using the same method, and for both assays, were non-significant. The last type of correlation ("heterotrait-heteromethod", C in Table 3) examined to the relationship between activity in one assay and occupancy of the less protected area in the other.

## 3. Results

### *3.1. NTT and LDT sensitivity to stress and CNS drugs: behavioral meta-analyses*

The omnibus meta-analysis performed here indicated an effect of drugs/treatments on the time spent in the top/lit part (Fig. 1), as the intercept for the mixed-effects model was significant ($\beta$ = 1.0923, CI 95%[0.2241, 1.9604]; z = 2.446, p = 0.0137) but the test for the moderators was not ($QM_{(df = 1)}$ = 0.0204, p = 0.8865). This suggests that in the specific dataset of drugs and treatments acting on different targets, the NTT and the LDT remained equally sensitive to the treatments if the experimental conditions (e.g., concentrations) were the same ($\beta$ = -0.04, CI 95% [-0.5899, 0.5098]; z = -0.1428, p = 0.8865). The lack of significance was also characteristic for comparisons in the drug groups, although effects differed for LDT and NTT when transitions were considered (Fig. 1), since

treatments increased transitions to white in LDT ($\beta = 4.67$, CI 95% [2.44, 6.9], $z = 4.1051$, $p < 0.0001$) but not to top in NTT ($\beta = -3.85$, CI 95% [-9.11, 1.42], $z = -1.4325$, $p = 0.1520$). The omnibus test revealed a significant effect of the moderators ($QM_{(df = 1)} = 9.1854$, $p = 0.0024$), with a significant assay effect ($z = 3.0307$, $p = 0.0024$). Analyzing SMDs between the two endpoints themselves revealed some controversy in the effect direction (e.g., for fluoxetine or pCPA [34]). As expected for not including treatment as a moderator, the heterogeneity of the results was significantly high ($\tau^2 = 1.8160 \pm 0.2886$; $I^2 = 89.1\%$, $QE_{(df = 104)} = 747.6830$, $p < 0.0001$). Although the lack of significant SMD differences between the most frequently measured parameter (time in aversive portions) was unexpected, it suggests that none of the two tests produces higher effect sizes on these parameters, thus making the two tests complementary, rather interchangeable. Finally, funnel plot analysis (Fig. 2) also indicates considerable publication bias towards significant findings, which should be considered and, eventually, corrected in future research.

### *3.2. Cortisol responses to NTT and LDT*

Overall, while both tests were more stressful vs. control, NTT was significantly more stressful than LDT, based on test-evoked cortisol responses (Fig. 3). This result is important and predictable, given the rigorous nature of NTT diving responses vs. a more 'protective' LDT choice situation. Moreover, this also suggests that while the measures of bottom and dark preference in these two paradigms can be conceptually similar, they may also differ in the *levels* of evoked stress and/or in their ability to act as tests (i.e., to measure different behaviors). Clearly, further studies are needed to examine the underlying stress responses and their neural circuitry in both models.

### *3.3. Correlations between behaviors generated in NTT and LDT*

Overall, correlations between transitions to white and time on top were not significant in this analysis, whereas correlations between transitions to top and time in white were significant, but smaller than the monotrait-heteromethod correlations (Table 3). Thus, correlations in the "validity diagonal" A were higher than the heterotrait-heteromethod and heterotrait-monomethod correlations, which reflect convergent and discriminant validity [82]. Overall, these results support the notion that

the operational definitions of anxiety in LDT and NTT seem to converge on the same zebrafish behavioral trait.

## 4. Discussion

Despite the advantages of using fish for modeling mental disorders, all animal models are limited since they cannot fully recapitulate the complex repertoire of human behavior [17]. Furthermore, it can be difficult to distinguish between various subtypes of animal anxiety (e.g., generalized anxiety vs. fear), and they have not yet been dissected in zebrafish [51]. In addition, despite shared construct rationale and targeting similar *evolutionarily-conserved* traits in fish and rodents [19], test- and species-differences may further complicate data interpretation and analyses. One example is 3D tracking of zebrafish movement [35, 83] in NTT (which enables quantification of vertical geotaxic anxiety-like response), but is impossible to assess in 2D-based OF (focusing on thigmotaxis instead). On the one hand, LDT typically measures a limited number of behavioral endpoints, enabling a more focused characterization of anxiety-related phenotypes. However, LDT does not usually track zebrafish in 3D, and therefore misses endpoints related to angular velocity or turn angle. Thus, while NTT may have an added value of registering more behavioral endpoints and phenomena, LDT can be quite useful for its high-throughput and lower stress. Furthermore, as many drugs modifying anxiety display characteristic U-shaped dose-response curve for commonly used endpoints [84], the range of concentrations used for drug screening should be sufficient to account for possible effect increase and dropdown. Clearly, this may slow down the screening process, but will help minimize type II errors in CNS drug discovery.

Another aspect that merits further consideration is the extent of automation that can be achieved using these approaches, and whether this represents a drawback, especially given the unrivalled potential of zebrafish for high-throughput analysis. Indeed, while without the potential for automation, there may be strong between-lab reliability for these measures, other extraneous factors (such as housing/husbandry and testing procedures) may trigger such variance as well. Thus, we strongly support further automation in studies, and welcome their further inclusion in subsequent meta-analyses, in addition to offering a clear experimental advantage of assessing reliably a wider

range of behavioral analysis in a shorter period of time. Likewise, another factor that is largely overlooked in zebrafish literature is the differences in basal locomotor responses by individual fish, and how this may affect the typical responses behavioral phenotypes. While individual differences in zebrafish behaviors do exist, this aspect was not in the scope of our study, and was somewhat mitigated here by using relatively large n's in our cohorts. Nevertheless, we fully recognize the importance of individual differences in zebrafish NTT and LDT behaviors, and acknowledge the fact that automation of fish testing may not only reduce 'phenotypic noise' by better controlling the testing environments, but can also help better detect meaningful patterns in individual differences for multiple computer-generated parameters.

In summary, as NTT and LDT remain widely used tests of zebrafish anxiety, they are often considered 'similar' in their construct, face and predictive validity. Analyzing their effect sizes across multiple studies, including data from our own group, we found their comparable sensitivity, based on similar SMD values for their main endpoints. Thus, the two tests can be both needed for assessing zebrafish anxiety-like behaviors and drug screening, reinforcing Prof. Slava Lapin's famous notion "one experiment is not an experiment, and one behavioral test is not a test". However, although the cumulative responsivity of NTT and LDT to treatments appear similar, some drugs may affect one test more than the other. Respectively, this suggests that predictive validity of the two models may be somewhat different (Fig. 1). For example, as NTT also measures endpoints that are also relevant to other behavioral domains (e.g., locomotion or cognition/habituation), it has the advantage of versatility and the ability to characterize 3D locomotion [35, 71, 83]. At the same time, given our endocrine/cortisol data (Fig. 3), it is likely that NTT also represents a more stressful procedure than LDT. Accordingly, this may contribute to the two tests' differing predictive validity, as anxiogenic drugs may have floor effects in NTT, which can therefore be more useful for testing anxiolytics instead. In contrast, a seemingly 'less aversive' LDT may be more suitable for testing anxiogenics, but may exhibit more ceiling effects when screening anxiolytic drugs. Likewise, it is possible to suggest that the two models may differ in their ability to reflect decision-making behavior. For example, a more survival-driven innate 'diving' response may seem to be more forced upon the

animal by the NTT testing protocol, as compared to a less aversive LDT procedure, which affords the fish more time and a better control over their light-dark preference behaviors (e.g., see [85]). Quite interesting, this hypothesis merits further scrutiny, and may help develop novel behavioral models beyond affective domains (e.g., targeting zebrafish impulsivity based on their NTT vs. LDT responses).

Further analyses of the exact nature of stress in the two aquatic tests remains an important priority for zebrafish behavioral research [86], because they are both related to anxiety, but may differ in the domains of stress, fear, aversion, visual comprehension and decision-making. It may also require studies of brain activation patterns in one or another setup with paralleling the results to the mammalian brain studies. Such analyses have already been performed in LDT [85], implicating the medial zone of the dorsal telencephalic region and the dorsal nucleus of the ventral telencephalic area (the teleost homologs of the mammalian amygdala and striatum, respectively) in fish anxiety responses. It would therefore be interesting to conduct a similar study in NTT. Future dissection of the differences between brain activation patterns may necessitate sophisticated in-vivo imaging studies in freely moving animals, which can be particularly useful since the genetically encoded calcium reporters can be analyzed in transparent strains of zebrafish.

Finally, with a large number of behavioral endpoints resulting from zebrafish behavioral tests (e.g., [35, 87, 88]), the integral parameter that would consistently reflect the level of zebrafish anxiety, is still missing. Would it help to have such a biomarker? Clearly yes, since, for example, some of NTT and LDT endpoints are closely and directly related to anxiety and endocrine (cortisol) levels, and can have higher weight in the integral anxiety testing. Thus, the upcoming aim of zebrafish behavioral neuroscience can be to rank various endpoints for the overall effect appraisal, and to extract important biological information from those various *ranked* phenotypes accordingly. In other words, this may involve creating an integral index based on multiple factors and endpoints (somewhat similar to a credit score in financial world) to provide a rough estimate of fish anxiety levels, thereby empowering time/cost-efficient drug screening. One attempt to establish such an integral value has been made recently [89], using the concept of Integrated Biological Response that summarizes the range of

biomarkers into single star plot, that is then analyzed by square and shape. Such plots are rather visual and allow for input endpoints ranking and at the same time they can be expressed in the form of single number, enabling rapid comparison. However, a conceptually different (but equally fruitful) approach can be to continue to explore the potential complexity of zebrafish anxiety behavior, based on a theoretical possibility (not yet tested empirically) that zebrafish anxiety-like states may include subtypes, similar to clinical generalized anxiety- vs. fear/panic-like states, and the respective rodent analogous models that do distinguish these states [90-94]. At the same time, it may also be possible [17, 84] that zebrafish affective phenotypes (and their respective circuits) are merely not as complicated as in mammals, and may reflect broader (e.g., generalized anxiety-like) affective categories rather than target more specific AD subtypes, such as anxiety vs. fear/panic.

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**Figure 1. Meta-analysis results for effects of various drugs/treatments on zebrafish novel tank test (NTT) and light-dark test (LDT) behaviors, including the number of transitions (A) and time spent (B) in respective top or light areas. Data are presented as forest plots (based on 27 studies listed in Table 1 that use both behavioral tests) for the standardized mean differences (SMD) for treated vs. control groups with corresponding 95% confidence intervals in the individual studies, based on a mixed-effects model.**

**Figure 2. The funnel plot showing the relationships between residual values vs. standard errors (estimated by the mixed-effects model) in data presented in Fig. 1; bands refer to confidence intervals at the 90% (white area), 95% (light gray area), and 99% (dark grey area) levels.**

**Figure 3. Whole-body cortisol levels in control vs. NTT- and LDT-tested fish assessed immediately after behavior test procedure (n=15 in each group). There was significant treatment effect by one-way ANOVA ($F_{2, 41}$ = 13.14; P < 0.0001); *P<0.05, ****P<0.0001 for Tukey post-hoc test (left panel). The two tests also significantly differed between themselves (P < 0.05, U-test, right panel).**

**Table 1. Alternative tests and models available for studying zebrafish anxiety-like behaviors**

| Test/Model | Details | References |
|---|---|---|
| ***Tests (used to assess anxiety levels)*** | | |
| Open field test | A direct analog of rodent open field, this paradigm measures mainly horizontal locomotion and thigmotaxis. The apparatus can have diverse size, shape and color. Endpoints in this test include the time spent in the periphery or center of the tank (s), distance traveled in each zone in the tank (m), number of transitions between zones, velocity in each zone of the tank (m/s), number of freezing bouts and time spent frozen (s) | 24, 41, 44 |
| Social preference test | The test has been applied to evaluate zebrafish response to con- and heterospecifics. Zebrafish social preference may be altered because of the effect of anxiety-related pharmaceutical substances or environmental stressors. | 95, 96 |
| Shoaling | Fish are placed to the test apparatus in groups and impact of stressor or pharmacological manipulation are assessed measuring alterations in shoal cohesion (it is higher when zebrafish feel anxiety). | 23, 97 |
| Predator avoidance test | The test examines fear- and anxiety-like behavior in the presence of a natural stressor. Zebrafish individually or in group are placed in one part of apparatus with two separate compartments, another arm contains predator (e.g., Indian leaf fish, *Nandus nandus*). | 55, 98, 99 |
| Predator exposure test | The test is aimed at evaluation of fear- and anxiety-like behavior following a brief exposure to a natural predator (e.g. Indian leaf fish), its image or robotic models | 100 |
| Boldness and novel object approaching | During the novel object test zebrafish either individually or in groups are placed to a cylindric tank and, after period of acclimatization, the unfamiliar object is added to the apparatus. Main endpoints include the latency to approach the object (s), frequency of approach, time spent near (within 1.5 body-lengths) the object (s), number of freezing bouts, and time spent frozen (s). | 25, 101 |
| Emergence test | This test assesses anxiety behaviors by placing animals in a reassuring chamber, and measuring latency to exit the chamber. | 102 |
| Food neophobia | Avoidance of novel food, which can be assessed by measuring latency to approach novel food items, and frequency/duration of time spent interacting with novel food items | 103 |
| ***Models (used to evoke anxiety-like states)*** | | |
| Beaker stress | During beaker stressor protocols, an individual fish is separated from its shoal and confined in a 250-ml beaker filled with 100-ml of house tank water. This stressor markedly increases baseline cortisol levels after 15-min exposure. | 17 |
| Impoverished housing | Housing fish singly in a barren environment increases anxiety-like behaviors in the NTT and LDT | 49 |
| Chronic stress | Pathological anxiety is commonly seen in zebrafish exposed to various chronic stress models | 33, 104 |
| Social isolation | Chronic social isolation decreases, while acute isolation increases anxiety levels | 17, 51, 105, 106 |
| Genetic models | Various inbred and outbred zebrafish strains with different anxiety levels | 20, 86 |

**Table 2. List of treatments and publications used for the analysis.**

| Treatment | Dose (mg/L), other details | References |
|---|---|---|
| ***Anxiolytic-like treatments*** | | |
| 2,5-Dimethoxy-4-bromoamphetamine (DOB) | 0.05, 0.1, 0.5, 1 | 107 |
| *para*-Methoxyamphetamine (PMA) | 0.0005, 0.005, 0.05, 0.1, 0.25, 0.5 | 107 |
| Buspirone | 25, 50 | 34 |
| Fluoxetine | 2.5, 5, 10 | 34 |
| | 5 mg/kg long-fin and leopard strains | 108 |
| *para*-chlorophenylalanine (pCPA) | 300 twice | 34 |
| WAY 100635 | 0.003, 0.03 | 34 |
| Ethanol (acute) | 0.5, 1, 1.5% | 72 |
| | 0.5% | 68 |
| Desipramine | 25 | 68 |
| Nicotine | 25 | 68 |
| Chlordiazepoxide | 25 | 68 |
| Citalopram | 100 | 68 |
| Taurine | 42, 150, 400 | 89 |
| Piracetam | 200 (chronic, for 7 days) | 109 |
| N-acetylcysteine | 0.1, 1, 10 | 60 |
| ***Hallucinogenic-like treatments*** | | |
| 3,4-Methylenedioxymethamphetamine (MDMA)* | 2.5, 5, 10 | 107 |
| Lysergic acid diethylamide (LSD)* | 0.25 | 44 |
| Ibogaine* | 10, 20 | 71 |
| ***Anxiogenic-like treatments*** | | |
| SB 224289 | 2.5, 5 | 34 |
| 1% Ethanol (chronic) withdrawal | 2,6 days (1,7 days for LDT) | 72 |
| Alarm substance | Short-fin, leopard strains | 61 |
| Yohimbine | 25 | 68 |
| ***Toxic treatments*** | | |
| Paraquat | 20 | 110 |
| Methylmercury | 1, 5 µg/g | 62 |
| Dimethyl sulfoxide (DMSO)* | 0.05% | 68 |
| Copper | 0.006 | 111 |

* The drug also exerts anxiolytic-like effects in zebrafish

**Table 3. Cross-correlations between occupancy (time on white, time on top) and locomotion (entries on white, entries on top) measurements in 40 adult wild-type control zebrafish tested in the novel tank (NTT) and light-dark (LDT) tests at the Federal University do Sul e Sudeste do Pará, Maraba, Brazil. Values represent Pearson's correlation coefficients based on individual scores across all animals. (A) Convergence of operational definitions; (B) different operational definitions, same method; (C) different operational definitions, different methods (see text for details).**

| **Behaviors/Tests** | **NTT time** | **NTT transitions** | **LDT time** |
|---|---|---|---|
| NTT transitions | (B) 0.08 | | |
| LDT time | (A) **0.55** | (C) **0.43** | |
| LDT transitions | (C) 0.11 | (A) **0.75** | (B) 0.18 |

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