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Research report

Constructing the habituome for phenotype-driven zebrafish research

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HIGHLIGHTS

- Habituation is an evolutionarily conserved behavior relevant to exploration.
- Numerous zebrafish behaviors demonstrate robust habituation in novelty-based tests.
- The *habituome* is a new conceptual approach to study zebrafish phenotypes.
- Multiple behaviors habituate independent of anxiolytic and anxiogenic states.
- Anxiety and habituation sensitivities show no correlation for multiple behaviors.

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ABSTRACT

Intra-session habituation to novelty reflects spatial working memory (related to exploration and cognition), and is observed in various species, including zebrafish (*Danio rerio*). With the growing understanding of complex zebrafish behaviors, the extent to which they habituate remains unclear. Here we perform a large-scale characterization of zebrafish novelty-evoked (novel tank and open field) behaviors, to establish their grouping based on intra-session habituation and sensitivity to anxiolytic or anxiogenic manipulations. We also assess multiple behaviors in high- and low-anxiety sub-cohorts of a large heterogeneous zebrafish population, comparing their habituation profiles. Overall, our analyses demonstrate that anxiety responsivity and the ability to habituate show little correlation for multiple zebrafish behaviors, suggesting that they most likely represent distinct behavioral phenomena in novel environments. Using these data, we also present the *habituome* – a new conceptual approach to study affective and cognitive responses in zebrafish by examining a big set of their habituation phenotypes. Given marked similarity in animal novelty exploration, this approach may also be used to construct *habituomes* in other model organisms, including rodents and humans.

1. Introduction

As a form of memory, habituation has long been used in neuroscience research to study cognition and its experimental modulation [1–4]. Representing a reduction in responses to novelty over time [5,6], within-trial (intra-session) habituation is observed in multiple species as an evolutionarily conserved, adaptive behavior relevant to exploration and cognition [1,7–16]. Possessing significant genetic and physiological homology to other vertebrates, zebrafish (*Danio rerio*) are becoming increasingly popular in neurobehavioral research of affective and cognitive phenotypes [17–22]. Zebrafish display robust anxiety-like behavior in various novelty-based paradigms, including the novel tank [23–25], light-dark box [26], open field (OFT) [27,28] and startle [29,30] tests. These behaviors also habituate well in novelty-based tests, demonstrating high sensitivity to experimental manipulations and confirming the utility of zebrafish models to study both affective and cognitive phenomena [10,24]. Since zebrafish swimming is also characterized by three-dimensional locomotion, they offer the additional value of an 'extra' (*vertical*) dimension of locomotion for in-depth behavioral analysis using this species [31–33]. Mounting evidence shows that zebrafish represent an excellent species to study various behavioral syndromes [34,35]. However, as our understanding of the complexity of zebrafish behavior grows [27,32,36,37], the extent to which their multiple behaviors habituate remains unclear. Here, we apply two paradigms – the novel

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tank and open field tests – to examine how zebrafish behavioral phenotypes can be grouped based on habituation and sensitivity to novelty stress.

From a theoretical point of view, the sensitivity to anxiety and the ability to habituate may reflect either inter-related or independent behavioral phenomena [38]. For example, a specific behavior can be highly sensitive to anxiogenic factors, but show low or unaltered habituation (e.g., habituate equally well in both control and experimental groups, or habituate in controls but not in experimental cohorts). Although human [39,40] and rodent [2,41] literature supports a complex interplay between anxiety and habituation, this aspect has not been analyzed in a systematic manner. Capitalizing on robust anxiety and habituation phenotypes in zebrafish, our study examined their behaviors in several anxiety paradigms, while also assessing their ability to habituate. Specifically, we studied whether behaviors that are highly sensitive to anxiety would also be those that habituate to the greatest extent. Developed here as a novel conceptual and methodological approach, the zebrafish *habituome* (a big set of their habituation phenotypes) may become a useful tool to understand complex affective and cognitive responses.

2. Methods

2.1. Animals, housing and behavioral testing

In Experiment 1, we analyzed raw 6 min novel tank data previously generated for 200 adult (4–7 month-old; ≈50:50 male:female ratio) wild type '*short-fin*' zebrafish in a previously published study on 3D video-tracking [32]. In Experiment 2, the 30 min novel tank test data were generated for this project using 40 adult (4–7 month-old; ≈50:50 male:female ratio) wild type '*short-fin*' zebrafish obtained from a local vendor (50 Fathoms, Metairie, LA). Two trial durations were chosen here as commonly used in zebrafish research [23,32,33,42], and also to assess the possibility that zebrafish habituation responses can be more robustly affected during the first minutes (e.g., 6 min) of novelty exposure vs. trials of longer duration (see [24] for details). Animals were housed in groups of 20–30 per 20 L tank, and given at least 10 days to acclimate to the laboratory environment. Tanks were filled with filtered facility water maintained at a temperature of 25–27 °C. Illumination was provided using fluorescent lights on a 12 h cycle (on 6:00 h; off 18:00 h), consistent with the standards of zebrafish care [43]. Fish were fed Tetramin Tropical Flakes (Tetra USA, Blacksburg, VA) twice daily. The novel tank protocol applied here used a 1.5 L trapezoidal tank (15 cm $h \times 7$ cm $w \times 28$ cm top $\times$ 23 cm bottom l; Aquatic Habitats, Apopka, FL) maximally filled with aquarium water and divided into two equal halves, demarcated by a virtual horizontal line [32], recorded manually and using video-tracking (see further) for 6 or 30 min (see Table 1, Fig. 1 and [32] for a detailed list of endpoints). All experimenters used in this study were highly trained and showed a high inter- and intra-rater reliability >85%, as assessed by Spearman correlation.

To modulate zebrafish anxiety in Experiment 1, several genetic, psychological and pharmacological manipulations used in the novel tank test [32] included anxiolytic drugs (chronic fluoxetine, 100 μg/L × 2 weeks; chronic ethanol, 0.3% × 1 week; chronic morphine, 1.5 mg/L × 2 weeks, and acute nicotine, 10 mg/L × 5 min) and anxiogenic treatments (acute caffeine, 250 mg/L × 20 min; chronic morphine exposure, for 3 h × 2/day × 1 week; acute alarm pheromone exposure for 5 min, and 'high-anxiety' leopard zebrafish strain [32]). The pharmacological manipulations and doses were chosen based on prior studies with these and other drugs [19,44,45].

The OFT data for Experiment 3 was generated using 80 naïve adult wild-type '*short-fin*' zebrafish (4–7 month-old; ≈50:50 male:female ratio) obtained from a local vendor (50 Fathoms, Metairie, LA). For this study, we utilized 6 and 30 min trials, exposing parallel cohorts of zebrafish ($n = 20$) to 'large' OFT1 (12 cm $h \times 39$ cm $w \times 47$ cm l) or 'small' OFT2 (14 cm $h \times 29$ cm $w \times 37$ cm l) with a 12 cm water level. Since the larger rectangular OFT tank was of similar size to that used in the rodent OFT studies [46], a smaller arena was also utilized in our study, to allow the results to be translatable between different model organisms (see Fig. 2 and [28] for a detailed list of endpoints).

Behavioral testing in all experiments was performed between 11:00 and 16:00 h. Each trial was recorded via auto-focusing 2.0 MP USB webcams placed 50 cm in front of the novel tank, and 1 m above the OFT. Automated data analysis was performed on the recorded videos using EthoVision XT7 (Noldus IT, Wageningen, Netherlands) suite, with detection settings selected to acquire 23 novel tank and 23 OFT endpoints, to the best of our knowledge representing the most detailed analyses of zebrafish behavior via currently available IT-based video-tracking tools.

In Experiment 4, focusing on the population validity of our study, we used data from a large cohort of 200 naïve adult (4–7 month-old; ≈50:50 male:female ratio) wild type '*short-fin*' zebrafish obtained from the local vendor and used as naïve controls in various other ongoing projects of our laboratory. Allowing us to capitalize on the availability of raw behavioral data from multiple control animals, this should not be perceived as the general requirement to have a large number of animals for habituation studies, since robust habituation was observed in smaller cohorts previously [24]. However, the fact that we maximize the use of raw data from other research to extract new information is consistent with the growing recognition of meta-analysis of raw clinical and biological data as critical for ethical biomedical research [47,48]. All animals used in this study were exposed to a standard 6 min novel tank test, and assessed using EthoVision XT7 software, as in Experiment 1. Using cumulative (6 min) top duration data as a primary measure of zebrafish anxiety [32,42], we grouped zebrafish into high- and low-anxiety sub-cohorts with each representing 10% of the overall 200 fish population, using low and high top duration, respectively. A large-scale evaluation of 23 novel tank behavioral endpoints (Table 2) was then performed, including analyzing their per-minute distribution and habituation (assessed by single-minute habituation ratio SHR, see further), to compare habituation profiles of the two sub-cohorts selected from a large population solely based on their anxiety differences. To eliminate locomotion as a potential confounding factor in this experiment, the average distance traveled was calculated for the entire 200 fish cohort (9.5 ± 5 m), and fish were finally selected for high- or low-anxiety sub-cohorts of 20 fish, based on their activity levels being similar to the population average, but with robust differences in top duration (used here as the primary anxiety measure; Table 2). All experimental procedures were in full compliance with National and Institutional guidelines on animal experimentation and care.

2.2. Statistical analysis

Analyzing anxiety responses in Experiment 1 and using raw data from [32], we compared cumulative 6 min values for each experimental endpoint to its control cohort by non-paired Wilcoxon–Mann–Whitley U-test ($P < 0.05$). Data were then analyzed for their per-minute distribution, computing the ratio of behaviors during the first:last minute (single-minute habituation ratio, SHR) of a 6 min (Experiment 1) or 30 min (Experiment 2) novel tank trial, as described previously [24], by the paired U-test ($P < 0.05$). The OFT 6 min/30 min anxiety and habituation data in Experiment 3, and 6 min novel tank test data in Experiment 4, were analyzed in a similar manner.

While this was not the main focus of this study, cluster analysis was first applied to Experiment 1 data to reconfirm subgroups of observed novel tank test behavioral endpoints [32]. To assess their sensitivity to anxiety, behavioral endpoints for each experimental manipulation were normalized (with the sum of min 1–6 values taken as 100%) and expressed as a percent change. Hierarchical clustering was performed across all behavioral endpoints and treatment groups with Hierarchal Clustering Explorer 3.0 (University of Maryland, College Park, MD) using Average Linkage as the linkage method and Euclidian Distance as the similarity metric. The habituation ability and anxiety responses (Table 1) were further evaluated for possible correlation across all experimental manipulations and behavioral endpoints. For this, habituation data were first normalized for min 1–6, and their SHR values expressed as percent change, calculated as $|(\text{Min } 1 - \text{Min } 6)|$. Sensitivity to anxiety was calculated by expressing mean non-normalized control value as 100%, the behavior of the experimental animals as % of average control group, and expressed as the percent change $|100\% - \text{experimental group\%}|$. Finally, Spearman correlation was applied to correlate anxiety and habituation data, and to assess inter- and intra-rater reliability for manual obersvers. In all experiments reported here, $P < 0.05$ was set as statistically significant.

3. Results

Assessing exploratory behavior of naïve zebrafish in the 6 min and 30 min novel tank tests, we observed an overt increase over time in transitions and time spent in the top of the novel tank, as well as decreased freezing bouts, but not erratic movements (Fig. 1, Experiments 1 and 2), as reported previously [24]. Similar profiles were observed in the 6 min and 30 min OFT trials, with an increase in mobility as the trial progressed (Fig. 2, Experiment 3). In the novel tank test (Experiment 1), anxiogenic manipulations predictably lowered top exploration while increasing freezing activity, while anxiolytic manipulations reduced erratic and freezing behavior but increased top exploration [32]. Correlating experimental manipulations with behavioral endpoints, a hierarchical cluster analysis in our earlier study [32] revealed two distinct groups – 'anxiogenic' Cluster 1 (alarm pheromone, caffeine and the leopard strain) and 'anxiolytic' Cluster 2 (chronic ethanol, morphine, fluoxetine and acute nicotine), which were reconfirmed here (data not shown) based on raw behavioral data from a project unrelated to habituation analyses.

Zebrafish habituation, which was the main focus of this study, was also assessed in relation to the anxiolytic or anxiogenic