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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

Table 1
Correlation of habituation and anxiety sensitivities across experimental manipulations and behavioral endpoints. Behavioral endpoints here are listed based on their clustering in Fig. 3 (only treatments and behaviors demonstrating significant correlation ($P < 0.05$) between single-minute habituation ratio and anxiety are highlighted). The strength of correlation is given as percentage, expressed as $R \times 100\%$ and classified based on positive (0–50%, italics; 50–100%, italics underlined) and negative (0% to −50%, bold; −50% to −100%, bold underlined) correlation. Other non-significant endpoints (not shown) include highly mobile duration (s), rapid moving frequency, rapid moving duration (s), mobile frequency, and immobile frequency. (Please refer to [72] for detailed descriptions of all behavioral endpoints.).

Behavioral endpoints	Anxiogenic manipulations			Anxiolytic manipulations			
	Alarm pheromone	Acute caffeine	Leopard strain	Chronic ethanol	Chronic fluoxetine	Acute nicotine	Chronic morphine
Highly mobile frequency	−7.6	1.1	15.2	*52.2*	13.6	**−73.1**	**−51.9**
Slow moving duration (s)	**−70.1**	−29.9	−10.7	27.8	−52.2	−29.3	−26.5
Immobile duration (s)	**−74.2**	−32.0	−39.3	−29.8	−47.6	**−70.7**	**−53.6**
Slow moving frequency	−48.2	−36.5	−3.1	*47.1*	−44.8	17.4	−9.1
Distance traveled (m)	−0.7	41.5	*82.3*	10.9	−7.5	*75.2*	41.1
Mobile duration (s)	57.2	*84.4*	*83.7*	17.1	−40.4	*84.2*	*73.2*
Vertical turn angle (°)	−50.6	**−61.7**	38.3	*52.3*	2.0	7.2	−34.2
Average velocity (m/s)	−19.1	25.6	−54.4	−18.3	5.5	25.3	**−67.5**
Vertical turn bias (°)	**−80.3**	−40.0	−52.8	−34.0	−47.4	0	**−56.4**
Vertical turn rate (°/s)	−50.6	**−60.1**	38.3	*52.3*	2.0	0	−34.2
Vertical meandering (°/m)	−41.0	42.9	38.8	*51.9*	42.9	52.9	*77.6*
Total vertical meandering (°/m)	−34.5	25.4	36.2	18.5	−27.5	50.0	*82.0*
Time in top (s)	*80.4*	−2.0	−3.2	0.8	−48.6	−24.6	−15.8
Transitions to top	**−99.5**	−41.6	−24.8	3.1	56.5	−50.5	−23.9

manipulations described above. As shown in Fig. 3, the ability of multiple behaviors to habituate was independent of anxiolytic and anxiogenic states, since various indices in Experiment 1 exhibited high sensitivity to anxiety, yet showing a low degree of habituation, and vice versa. For example, despite their considerable sensitivity to anxiety, some inter-related behaviors (e.g., top transitions and duration, or immobility frequency and duration) exhibited opposite alterations within the same (i.e., anxiogenic or anxiolytic) treatment cluster (Fig. 3). Importantly, the obtained habituation-based clustering differed markedly from clustering of zebrafish behaviors based on their sensitivity to anxiety (performed in a separate large-scale study [32]), lending further support to the notion that sensitivity to anxiety and the ability to habituate are generally independent for zebrafish phenotypes observed in novelty-based paradigms. The two distinct clusters observed here, while seemingly unrelated, may derive from spatio-temporal influences on zebrafish behavioral patterning. For example, zebrafish scale their locomotor activity depending on the size of the tank and exhibit an inherent behavioral organization in a new environment [28]. In turn, the habituation sensitivity of different behavioral indices may be modulated depending on their role in the spatio-temporal strategies of zebrafish exploration.

Table 1 shows correlation of habituation and anxiety data in the novel tank test performed across all experimental

Table 2
Comparison of anxiety-related behaviors and their habituation in high- and low-anxiety sub-cohorts of zebrafish selected from a large heterogenous population of 200 naïve adult wild-type zebrafish. Zebrafish sub-cohorts were selected based on top:bottom preference (assessed by top duration as the primary anxiety-related measure) and unaltered locomotor activity (mean distance traveled ± 1 SD from the mean for the large population; see Section 2 for details). Habituation was assessed as % change of single-minute habituation ratio. Sensitivity to anxiety was expressed as the mean of the cumulative min 1–6 data per for behavioral endpoint ($^{*}P < 0.05$; $^{\#}P = 0.05$–0.1, trend; unpaired *U*-test for anxiety or habituation data comparing the respective high- vs. low-anxiety sub-cohorts).

Behavioral endpoints	Low-anxiety sub-cohort ($n = 20$ each)		High-anxiety sub-cohort ($n = 20$ each)		P (*U*-test)
	Raw data (anxiety sensitivity)	Habituation (%)	Raw data (anxiety sensitivity)	Habituation (%)	
Selection criteria					
Distance traveled (m)	9.7 ± 0.2	43.5	9.5 ± 0.3	45.4	NS
Top duration (s)	56 ± 16*	99.8	1 ± 0.5*	327.06	*<0.015
Associated endpoints					
Bottom duration (s)	303 ± 16*	−12.6	358 ± 1*	−1.21	*<0.015
Bottom frequency	15 ± 2*	95.0	6.8 ± 0.4	4.2*	*<0.014
Number of top entries	11 ± 2*	191.7	0.85 ± 0.4*	0	*<0.014
Vertical meander (°/m × 10^6)	1.5 ± 0.5$^{\#}$	−81.2	1.7 ± 0.7$^{\#}$	−74.2	$^{\#}$0.058
Other endpoints					
Average velocity (m/s)	0.2 ± 0.0	44.6	0.2 ± 0.0	45.1	NS
Erratic movements	0.6 ± 0.2	−80.0	0.8 ± 0.5	−83.3	NS
Freezing bouts	0.9 ± 0.3	−81.8	0.8 ± 0.2	−88.9	NS
Freezing duration (s)	64 ± 22	−71.5	83 ± 27	−17.7	NS
Highly mobile duration (s)	0.7 ± 0.2	−50.3	0.9 ± 0.2	−64.3	NS
Highly mobile frequency	14 ± 3	−29.8	18 ± 3	−37.9	NS
Immobile duration (s)	337 ± 2	−3.8	335 ± 2	−1.2	NS
Immobile frequency	265 ± 24	82.1	294 ± 26	45.4	NS
Mobile duration (s)	22 ± 2	50.1	23 ± 2	16.0	NS
Mobile frequency	260 ± 27	69.3	297 ± 27	38.9	NS
Moving duration (s)	212 ± 9	71.1	198 ± 9	84.8	NS
Moving frequency	1016 ± 71	14.8	974 ± 57	38.8	NS
Not moving duration (s)	152 ± 8	−50.1	162 ± 10	−49.6	NS
Not moving frequency	1031 ± 65	17.3	971 ± 57	41.7	NS
Vertical turn angle (°)	205 ± 19	−48.3	218 ± 17	−48.4	NS
Vertical turn bias (°)	122 ± 37	−64.0	111 ± 31	−63.1	NS
Vertical turn rate (°/s)	6152 ± 583	−48.3	7313 ± 824	−43.1	NS

NS - nonsignificant (*U-test*).

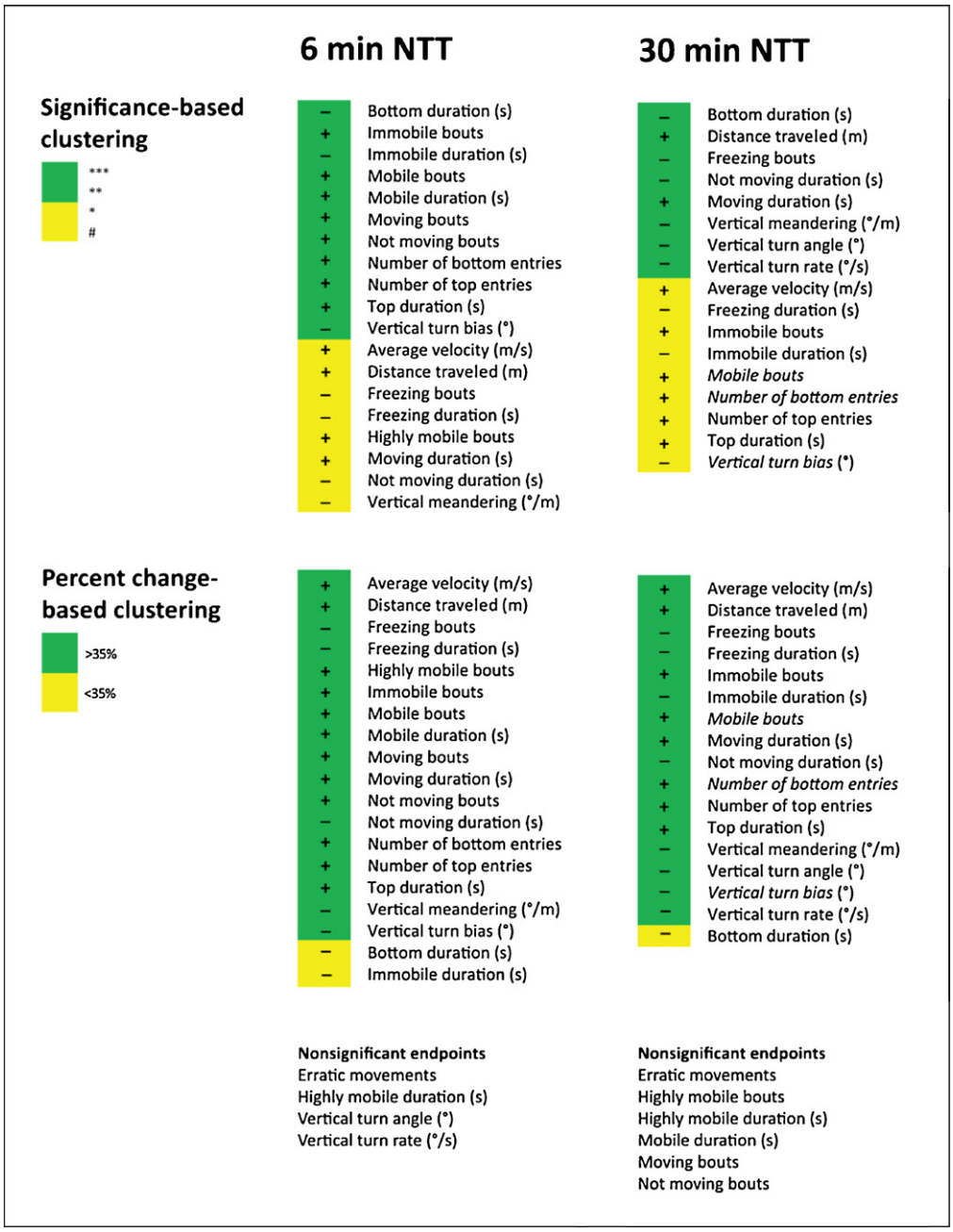

Fig. 1. Cluster analysis of endpoints recorded in the 6 min and 30 min novel tank tests (NTT) based on their ability to habituate (min 1 vs. min 6 or min 30) in naïve control fish. Data is presented based either on significance (top panel; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$; $^{\#}P=0.05$–0.1, trend; *U*-test) or percent change (bottom panel; last vs. first min of the test, expressed as absolute value vs. min 1 taken as 100%; high: >35%; low: <35%). Only behaviors demonstrating robust habituation are shown in this diagram, color-coded to denote degree of habituation for each endpoint (endpoints showing trends for their habituation ($P=0.05$–0.1, *U*-test) are denoted by italics). Habituation for significant endpoints is also denoted as either increasing (+) or decreasing (–) over the course of the 6 min or 30 min session.

manipulations and behavioral endpoints. Similar to Fig. 3, this analysis further revealed habituation and anxiety responsivity as independent of anxiolytic and anxiogenic states. This lack of correlation is again highlighted by the variability in the behaviors that are conventionally inter-related. For example, top transitions and duration showed a counter-intuitive relationship relative to one another (with a negative and positive correlation, respectively), and no correlation between SHR and anxiety for most experimental manipulations.

Finally, Experiment 4 provided another important confirmation to the independence of habituation and sensitivity to anxiety for zebrafish novel tank behaviors. As shown in Table 2, while highly significant differences were predictably detected for main anxiety measures in high- vs. low-anxiety sub-cohorts, these two groups did not show overt differences in the ability to habituate for most of the recorded behavioral endpoints.

4. Discussion

This study is the first large-scale analysis of adult zebrafish habituation using a wide spectrum of manual and computer-generated endpoints in various high- and low-anxiety situations, and in several popular zebrafish behavioral tests. We first

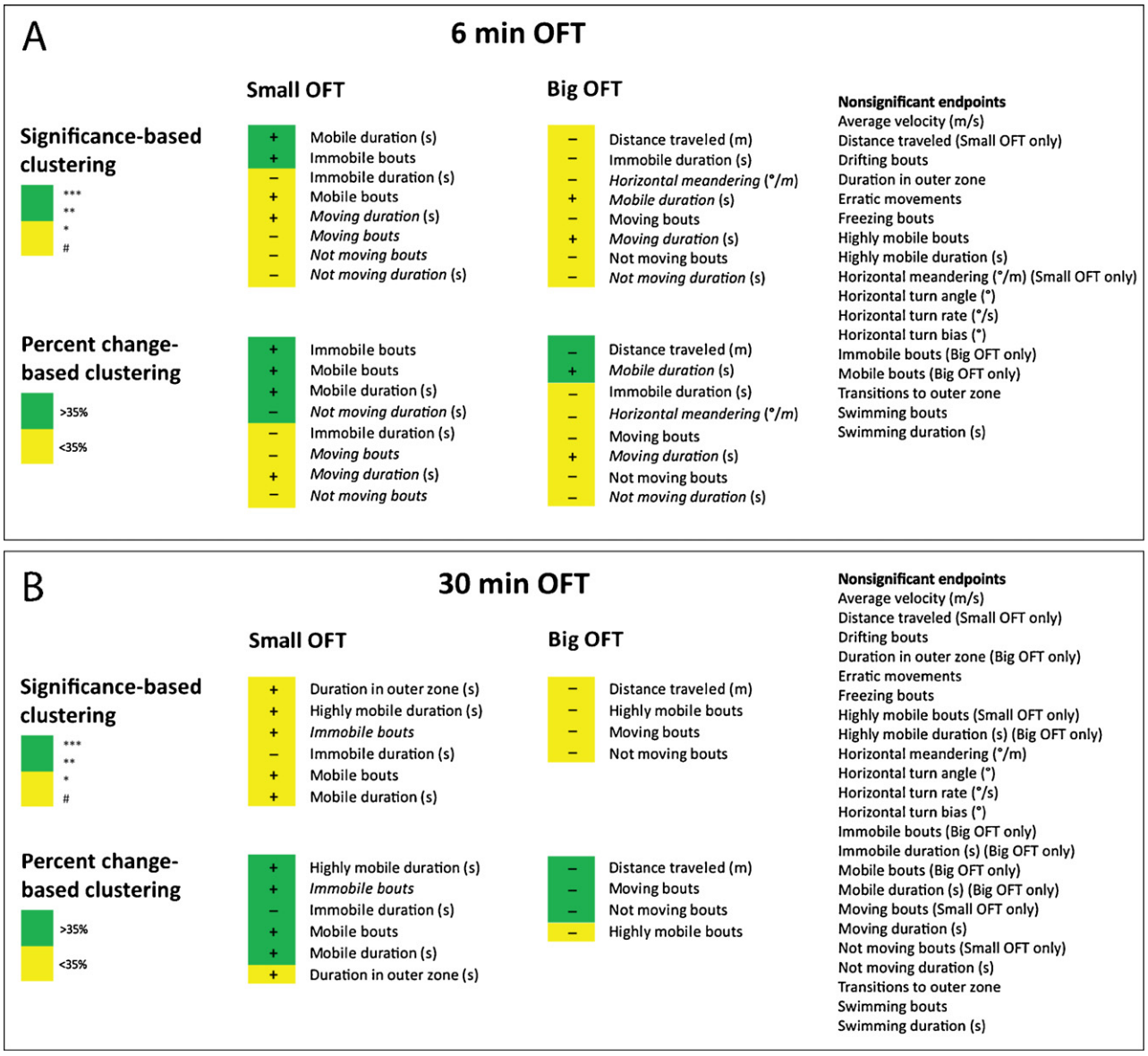

Fig. 2. Cluster analysis of endpoints recorded in the 6 min and 30 min open field tests (OFT) based on their ability to habituate (min 1 vs. min 6 or min 30) in naïve control fish. Data is presented based on either significance ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$; $^{\#}P<0.05$–0.1, trend; *U*-test) or percent change. Only behaviors demonstrating habituation are shown in this diagram, color-coded to denote degree of habituation for each endpoint. Non-significant endpoints are listed to the right, while 'trends' ($P=0.05$–0.1) are denoted by italics. Habituation for significant endpoints is also denoted as either increasing (+) or decreasing (–) over the course of the 6 min or 30 min session.

characterized intra-session habituation to novelty, using both short (6 min) and long (30 min) trials to cluster zebrafish behaviors based on their ability to habituate (Figs. 1 and 2). We then overlapped the identified clusters with known grouping of these behaviors based on their sensitivity to anxiety [32], assessing habituation profiles of these behaviors under high- and low-anxiety conditions. Completion of these tasks allowed us to construct the zebrafish *habituome* (Fig. 3), where treatments and behavioral phenotypes were organized based on the degree of their habituation.

Further validating the recently established zebrafish models of habituation [23,24], the *habituome* approach developed here presents an integrative concept for modeling zebrafish phenotypes. It globally assesses multiple behavioral endpoints based on their habituation, and provides several valuable insights into its relation to anxiety by showing how some behaviors may be highly sensitive to anxiety, yet show relatively low habituation. For example, while the distance traveled and average velocity are often modulated in zebrafish by exposure to stress [17,32], they exhibited no clear-cut habituation over the 6 min novel tank trial (Fig. 3). Therefore, this finding may provide a dissection between anxiety, activity and habituation phenotypes for some zebrafish behaviors, as can be suggested theoretically (see [38] for discussion).

Notably, zebrafish habituation in the novel tank and OFT tests seems to parallel some rodent habituation responses in the OFT paradigms. For example, zebrafish gradually increase mobility without significant alteration in distance traveled throughout the trials, and do not demonstrate the habituation of thigmotaxis (which rodents do in the OFT [27,49–51]) since their time spent near the walls remained relatively constant throughout the trial (Fig. 2). In contrast, the habituation of geotaxis (bottom preference) exhibited in the novel tank strikingly parallels rodent thigmotaxis, with the fish gradually entering the top as the trial progressed (Fig. 1). Such similarity in both anxiety and habituation profiles suggests that zebrafish geotaxis in the novel tank may be a better

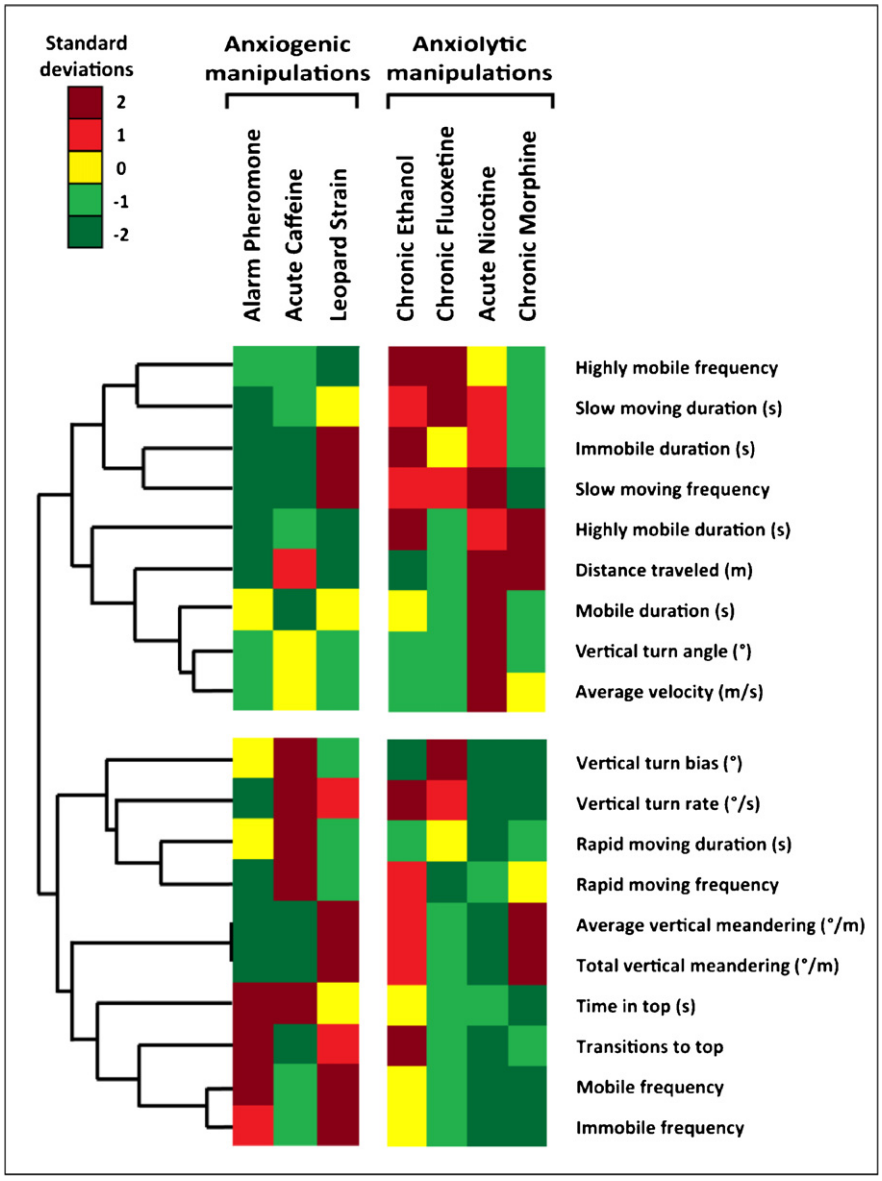

Fig. 3. The zebrafish *habituome* representing bi-directional cluster analysis of behavioral endpoints according to habituation sensitivity for zebrafish exposed to selected anxiolytic and anxiogenic experimental manipulations in the 6 min novel tank test, with min 1 vs. min 6 habituation data normalized and expressed as % change ratio. Note that habituation-based clustering of these endpoints differs markedly from clustering of the same endpoints based on their sensitivity to anxiety, performed in a separate large-scale study using the same raw data [32]. This finding further supports the notion that sensitivity to anxiety and the ability to habituate are independent phenomena/traits in zebrafish novelty-based paradigms.

measure of zebrafish novelty responses, with higher construct validity and similarity to rodent (than fish) thigmotaxis. Albeit not the main focus of this study, this observation also implies that geotaxis-based models, such as the novel tank test, may represent a more sensitive aquatic behavioral paradigm, compared to thigmotaxis-based anxiety tests like zebrafish OFT. The lower number of OFT behaviors able to habituate, as compared to the novel tank test (Figs. 1 and 2), further supports this notion. Finally, relatively similar habituation profiles between the small and large OFTs further extend the generality of behavioral observations made in this study (Fig. 2).

In addition to predictive, construct and face validity of animal models of brain disorders, the importance of population validity (the ability to reflect natural variance in phenotypes observed in general population) is becoming widely recognized in translational neuroscience research [52,53]. The covariation of different behaviors has also been suggested as forming the basis for personality differences [54–56]. Therefore, as observed inter-populational variance may provide further support for zebrafish 'emotional'-like behavior, these differences were also assessed here. Specifically addressing this aspect in the present study, Experiment 4 was designed to examine whether sub-populations of subjects selected based on their differing anxiety levels will also display robust differences in habituation. Table 2 shows, however, that despite robust behavioral differences in anxiety-related behaviors observed between high- and low-anxiety groups, this did not result in major differences in the ability of most of zebrafish behaviors to habituate. Therefore, habituation and sensitivity to anxiety in zebrafish novelty paradigms seem to represent distinct behavioral domains, the high-throughput

phenotyping of which may target them differentially, even within a single experimental session. Our *habituome*-based approach may not only allow us to address a wider spectrum of complex neurobiological (e.g., affective and cognitive) phenomena, but can also enhance multi-domain screening for potential therapies, including both anxiolytic and cognitive enhancer agents.

It currently remains unclear how habituation indices cluster according to various other factors, such as pharmacological agents, genetic mutations and environmental enrichment [57,58]. While our approach provided large-scale insight into habituation profiles for selected experimental manipulations (Fig. 3), it may foster further research screening various modulating agents. For example, as habituation is sensitive to genetic differences in rodents [2,59,60] and humans [61–63], the proposed *habituome*-based approach to genetically modified zebrafish can be applied to generate a gene-phenotype map and differentiate their motor, affective and cognitive profiles. This, in turn, may markedly enhance our ability to analyze the complex genetic underpinnings of animal behavior.

This study also highlights the potential differences between manual and automated recording of zebrafish behavior. While the reliability of video-tracking zebrafish behavior has already been established [31–33,44,64], our results further support the higher accuracy and precision of video-tracking tools. For example, fish exposed to alarm pheromone in the present study demonstrated an increasing intra-session habituation for time in and transitions to the top of the novel tank (Fig. 3). While an earlier study using manual observation showed an overt increasing pattern of habituation, it did not reach statistical significance due to the considerable standard error present [24]. Therefore, revealing the sensitivity of habituation analyses to experimenter bias and subjective variation, our data supports the need for continued development of reliable automated neurophenotyping tools to quantify zebrafish behaviors, including their habituation.

Finally, our observation that sensitivity to anxiety in zebrafish does not determine the ability of their behaviors to habituate is important from a theoretical point of view. Most likely representing an adaptive behavioral strategy, this suggests that zebrafish maintain a balance between anxiety behaviors that habituate (e.g., top preference, reflecting exploration) and do not habituate (e.g., erratic movements, providing constant 'vigilance'), both critical for animal survival. With the continued development of new IT-based tools for zebrafish behavioral analyses, greater progress can be made to enhance our understanding of complex trait interconnectivity in this model organism. Given the similarity between habituation in various species [3,4,15,16], it can be expected that similar approaches can be used to construct *habituomes* in other species, including rodents and humans. Thus, in combination with cross-species [65–67] and multi-domain behavioral analyses [68–71], zebrafish *habituome*-based research may serve as an important "bridge" in neurobehavioral phenotyping, revealing novel associations within the systems biology approach.

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