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Research Report

Understanding spatio-temporal strategies of adult zebrafish exploration in the open field test

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ABSTRACT

Zebrafish (*Danio rerio*) are emerging as a useful model organism for neuroscience research. Mounting evidence suggests that various traditional rodent paradigms may be adapted for testing zebrafish behavior. The open field test is a popular rodent test of novelty exploration, recently applied to zebrafish research. To better understand fish novelty behavior, we exposed adult zebrafish to two different open field arenas for 30 min, assessing the amount and temporal patterning of their exploration. While (similar to rodents) zebrafish scale their locomotory activity depending on the size of the tank, the temporal patterning of their activity was independent of arena size. These observations strikingly parallel similar rodent behaviors, suggesting that spatio-temporal strategies of animal exploration may be evolutionarily conserved across vertebrate species. In addition, we found interesting oscillations in zebrafish exploration, with the per-minute distribution of their horizontal activity demonstrating sinusoidal-like patterns. While such patterning is not reported for rodents and other higher vertebrates, a nonlinear regression analysis confirmed the oscillation patterning of all assessed zebrafish behavioral endpoints in both open field arenas, revealing a potentially important aspect of novelty exploration in lower vertebrates.

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1. Introduction

The open field test (OFT) is commonly used to study animal exploration and emotionality in various species, including rodents (Carola et al., 2002; Choleris et al., 2001; Karl et al., 2003; Koplik et al., 1995; Walsh and Cummins, 1976), primates (Ferguson and Bowman, 1990a; Ferguson and Bowman, 1990b; Ferguson et al., 1996; Mothes et al., 1996), birds (Frederick, 1976; Gallup and Suarez, 1980; Kembro et al., 2008; Rodenburg et al., 2003; Spetch and Edwards, 1986) and fish (Budaev et al., 1999; Gerlai and Csányi, 1988; Gerlai et al.,

1990; Miklosi et al., 1992; Warren and Callaghan, 2006). However, despite its extensive application in biopsychology, the exact nature of animal OFT behaviors is not fully understood (Calatayud et al., 2004; Kalueff et al., 2006; Kalueff et al., 2007).

The OFT has recently been applied to zebrafish to assess their swimming behavior and locomotion (Blaser and Gerlai, 2006; Echevarria et al., 2008), also revealing some similarities in strategies of novelty exploration between rodents and zebrafish (Bencan et al., 2009; Egan et al., 2009; Levin et al., 2007). For example, both adult zebrafish and rodents exhibit robust thigmotaxis (preferring staying close to the walls) (Blaser et

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al., 2010; Champagne et al., 2010; Lamprea et al., 2008; Simon et al., 1994) and scototaxis (preferring dark over light environment (Hascoet et al., 2001; Maldonado and Navarro, 2000; Maximino et al., 2010b; Maximino et al., 2010a)). Like rodents, zebrafish demonstrate habituation responses, changing their exploration behavior as they explore the novel tanks (Bolivar, 2009; Bolivar et al., 2000; Wong et al., 2010). Finally, both rodents and zebrafish actively establish homebases — safe preferred loci serving as strategic reference points in novel arenas (Eilam and Golani, 1989; Eilam and Golani, 1990; Rosemberg et al., 2011; Stewart et al., 2010).

Since zebrafish display robust behavioral responses to novelty (Bencan and Levin, 2008; Bencan et al., 2009; Egan et al., 2009; Levin et al., 2007), we used OFT in adult zebrafish to assess in-depth their novelty-evoked behavior. One recently recognized striking feature of rodent exploration is its ability to withstand changes in novelty characteristics (Eilam et al., 2003; Fonio et al., 2009; Kalueff et al., 2006). For example, exposed to different novel arenas, rodents scale their horizontal activity to the size of the arena (Eilam et al., 2003), but display stable spatiotemporal distribution of activity despite alterations in arena size, color and shape (Kalueff et al., 2006). The relation between novelty and exploration is a key issue in neurobehavioral research, as it underlies the animal's innate behavioral organization in new environments. The traditional characteristics of animal activity in novelty-based paradigms include distance traveled, average velocity, the number of stops, average inter-stop distance (Table 1) and their spatial distribution (Edut and Eilam, 2003; Kalueff et al.,

2006; Kalueff et al., 2007). In contrast, temporal patterning of animal behavior assesses the relative timing and duration of these activities, and focuses on the temporal dynamics of these behaviors (Eilam, 2003; Eilam et al., 2003; Kalueff et al., 2006; Rosemberg et al., 2011).

While rodent exploration was once viewed as a stochastic process, it is currently recognized as a well-organized activity with complex spatio-temporal patterning of behaviors (Eilam, 2003; Eilam and Golani, 1989; Eilam and Golani, 1990; Kalueff et al., 2006; Tchernichovski and Golani, 1995). Thus, both spatial and temporal analyses become necessary to fully characterize animal OFT behavior. Although the spatio-temporal characteristics of rodent behavior in novel environments (Eilam, 2003; Eilam et al., 2003; Kalueff et al., 2006) and zebrafish spatial strategies (Blaser and Gerlai, 2006; Cachat et al., 2011; Champagne et al., 2010) are relatively well-studied, temporal organization of zebrafish OFT activity has not been examined in detail (Rosemberg et al., 2011). Here we analyze zebrafish OFT activity and its temporal patterning (assessed as a per-minute distribution of activity), and investigate whether these characteristics can be modulated by different novel environments (Fig. 1).

2. Results

As shown in Figs. 2–4, zebrafish generally displayed different amounts of horizontal locomotor activity across the smaller, square OFT1 and the larger, rectangular OFT2. While distance

Table 1 – Summary of mathematical analyses of the “wave-like” nature of adult zebrafish open field test (OFT) behavior. Curve fitting via nonlinear regression closely describes the per-min distribution of zebrafish horizontal activity as a function of a 10th degree polynomial. The best-fit curves and corresponding functions, generated using MATLAB (see Statistical analysis for details) further confirmed the oscillating pattern of zebrafish behavior exhibited in Figs. 2–3.

Endpoint	OFT type	Oscillation function	Oscillation pattern
Distance traveled (m)	OFT1	$y = -1.6e-010 \cdot x^{10} + 3.1e-008 \cdot x^9 - 2.5e-006 \cdot x^8 + 0.00011 \cdot x^7 - 0.003 \cdot x^6 + 0.05 \cdot x^5 - 0.52 \cdot x^4 + 3.3 \cdot x^3 - 11 \cdot x^2 + 17 \cdot x - 4.3$	Present
	OFT2	$y = -5.4e-010 \cdot x^{10} + 8.4e-008 \cdot x^9 - 5.6e-006 \cdot x^8 + 0.00021 \cdot x^7 - 0.0046 \cdot x^6 + 0.066 \cdot x^5 - 0.59 \cdot x^4 + 3.2 \cdot x^3 - 9.5 \cdot x^2 + 14 \cdot x - 4.3$	Present
Velocity (m/s)	OFT1	$y = 5.6e-011 \cdot x^{10} - 6.7e-009 \cdot x^9 + 3.1e-007 \cdot x^8 - 6.3e-006 \cdot x^7 + 1.7e-005 \cdot x^6 + 0.0016 \cdot x^5 - 0.032 \cdot x^4 + 0.25 \cdot x^3 - 0.88 \cdot x^2 + 0.77 \cdot x + 4.5$	Present
	OFT2	$y = -3.1e-010 \cdot x^{10} + 4.7e-008 \cdot x^9 - 3.1e-006 \cdot x^8 + 0.00011 \cdot x^7 - 0.0026 \cdot x^6 + 0.037 \cdot x^5 - 0.33 \cdot x^4 + 1.8 \cdot x^3 - 5.4 \cdot x^2 + 8.2 \cdot x - 0.87$	Present
Immobility duration (s)	OFT1	$y = -8.5e-010 \cdot x^{10} + 1.4e-007 \cdot x^9 - 9.7e-006 \cdot x^8 + 0.00038 \cdot x^7 - 0.0091 \cdot x^6 + 0.14 \cdot x^5 - 1.3 \cdot x^4 + 7.5 \cdot x^3 - 23 \cdot x^2 + 35 \cdot x - 14$	Present
	OFT2	$y = 8.1e-010 \cdot x^{10} - 1.2e-007 \cdot x^9 + 7.9e-006 \cdot x^8 - 0.00028 \cdot x^7 + 0.006 \cdot x^6 - 0.079 \cdot x^5 + 0.61 \cdot x^4 - 2.4 \cdot x^3 + 2.9 \cdot x^2 + 6.7 \cdot x - 4.9$	Present
Immobility frequency	OFT1	$y = -1.1e-010 \cdot x^{10} + 2.3e-008 \cdot x^9 - 1.9e-006 \cdot x^8 + 8.4e-005 \cdot x^7 - 0.0022 \cdot x^6 + 0.037 \cdot x^5 - 0.36 \cdot x^4 + 2 \cdot x^3 - 5.3 \cdot x^2 + 4.7 \cdot x + 4.6$	Present
	OFT2	$y = 8.8e-010 \cdot x^{10} - 1.3e-007 \cdot x^9 + 8.7e-006 \cdot x^8 - 0.00031 \cdot x^7 + 0.0068 \cdot x^6 - 0.091 \cdot x^5 + 0.72 \cdot x^4 - 3.1 \cdot x^3 + 4.9 \cdot x^2 + 3.8 \cdot x - 3.8$	Present
Inter-stop distance (m)	OFT1	$y = -1.1e-009 \cdot x^{10} + 1.7e-007 \cdot x^9 - 1.2e-005 \cdot x^8 + 0.00046 \cdot x^7 - 0.011 \cdot x^6 + 0.16 \cdot x^5 - 1.4 \cdot x^4 + 7.8 \cdot x^3 - 23 \cdot x^2 + 30 \cdot x - 5.6$	Present
	OFT2	$y = -7.2e-010 \cdot x^{10} + 1.1e-007 \cdot x^9 - 7.8e-006 \cdot x^8 + 0.0003 \cdot x^7 - 0.007 \cdot x^6 + 0.1 \cdot x^5 - 0.95 \cdot x^4 + 5.4 \cdot x^3 - 17 \cdot x^2 + 27 \cdot x - 11$	Present
Average immobility bout duration (s)	OFT1	$y = -5.1e-010 \cdot x^{10} + 8.4e-008 \cdot x^9 - 5.9e-006 \cdot x^8 + 0.00023 \cdot x^7 - 0.0053 \cdot x^6 + 0.075 \cdot x^5 - 0.64 \cdot x^4 + 3 \cdot x^3 - 6.6 \cdot x^2 + 4 \cdot x + 4.9$	Present
	OFT2	$y = -4.9e-011 \cdot x^{10} + 1.1e-008 \cdot x^9 - 9.5e-007 \cdot x^8 + 4.6e-005 \cdot x^7 - 0.0013 \cdot x^6 + 0.024 \cdot x^5 - 0.28 \cdot x^4 + 1.9 \cdot x^3 - 7.4 \cdot x^2 + 13 \cdot x - 4$	Present

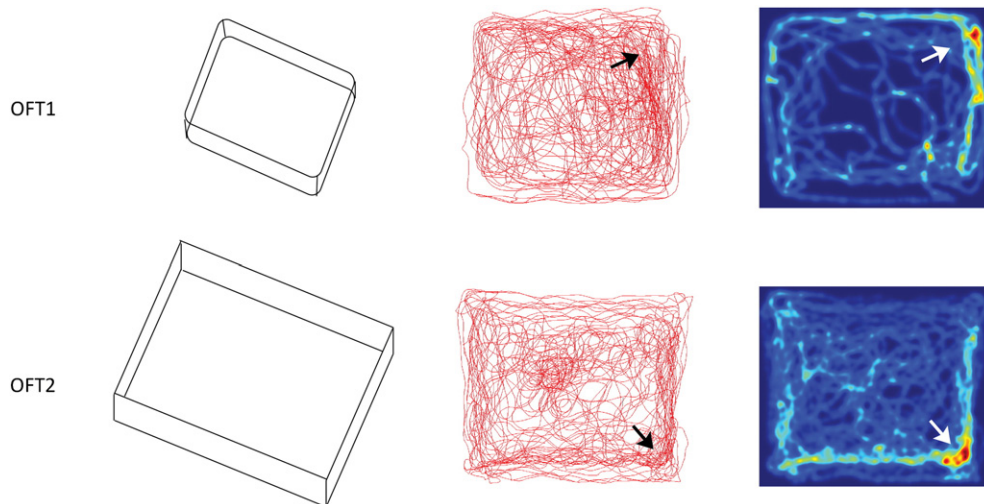


Fig. 1 – The open field test (OFT) arenas used in this study, with swim traces and ‘density maps’ constructed for two representative zebrafish by Noldus Ethovision XT7. OFT arena size ranged from small (OFT1) to large (OFT2; see the [Experimental procedures](#) section for details). Density maps were also used to visually reconfirm the homebase using the time spent in each zone (as described previously ([Stewart et al., 2010](#))), in which a color gradient ranging from yellow to red reflected time spent in location (in this diagram, arrows indicate the location of homebases).

traveled differed significantly between arenas, the fish velocity, immobility duration and immobility frequency remained consistent throughout the differing arenas (NS, not significant). Inter-stop distance and average immobility bout duration were also similar between the two OFT arenas (NS).

Examining a per-minute distribution of zebrafish horizontal OFT1 and OFT2 activity ([Figs. 2–3](#)), we found that temporal patterning in a given minute of the trial remained stable regardless of the OFT arena used. Two-way ANOVA revealed no significant difference between two OFT arenas in terms of velocity, distance traveled, immobility duration, and immobility frequency ($F_{(1,39)}=0.001\text{--}0.014$, NS). Inter-stop distance and the average immobility bout duration ($F_{(1,39)}=0.001\text{--}2.26$, NS) also remained stable across different OFT arenas ([Fig. 3](#)).

Furthermore, the per-minute distribution of zebrafish horizontal activity also demonstrated an overt “wave-like” nature, with fish behavior appearing to oscillate every 5–7 min across the duration of the 30 min trials ([Fig. 2–3](#)). Nonlinear regression analysis confirmed the oscillation patterning of behavior for all endpoints in both OFT arenas, with similar “waves” for each endpoint ([Fig. 4](#)), representing a 10th degree polynomial function ([Table 1](#)).

Zebrafish vertical behavior in a novel environment, assessed here in OFT3, was organized similarly to that reported in the novel tank test ([Stewart et al., 2011; Wong et al., 2010](#)). The fish demonstrated an initial bottom dwelling, followed by increased vertical exploration as they became acclimated to the novel environment. Overall, the fish tracks revealed an extensive amount of distance traveled near the bottom of the tank, with the rest of the locomotory path being gradually allocated to the very top of the tank (data not shown). While active swimming did occur in the middle region of the tank, it appeared to be mainly transitional — as part of the animals’ path between top and bottom. Overall, the trial was characterized by an initial geotaxis (bottom

preference) during the first few minutes, followed by a consistent growth of top dwelling for the remainder of the trial. No overt oscillations of vertical activity measures were observed in zebrafish in this experiment (data not shown).

3. Discussion

Overall, there were several important observations in this study. First, we showed that fish adjust the amount of their locomotion to the size of the novel arena, but do not change the temporal patterning of activity in different arenas ([Figs. 2–3](#)). Thus, the present study is the first report in adult zebrafish focusing on an important aspect of animal novelty exploration — its spatio-temporal “stability” ([Avni et al., 2008; Eilam and Golani, 1989; Eilam and Golani, 1990](#)). Reflecting the inherent behavioral organization in a new environment, this aspect has not been studied in zebrafish, despite the growing utility of the OFT paradigms in various fish models ([Budaev et al., 1999; Gerlai and Csányi, 1988; Gerlai et al., 1990; Miklosi et al., 1992; Warren and Callaghan, 2006](#)). Recent novel tank ([Wong et al., 2010](#)) and OFT ([Echevarria et al., 2008](#)) studies in adult zebrafish suggest that their exploration may be similar to that in rodents, likely reflecting strategies of exploration that are evolutionarily conserved in different species.

Exploration is used by animals to investigate novel environments, and plays an important part in their natural behavior ([Hughes, 1968; Montgomery and Monkman, 1955; Thompson, 1953](#)). Rodent exploration has been extensively studied in the past decades ([Baron, 1964; Blanchard et al., 1974; File and Wardill, 1975; Glanzer, 1961; Montgomery, 1953; Montgomery, 1955](#)), and is well represented in the recent literature ([Dvorkin et al., 2008; Eilam, 2003; Horev et al., 2007; Kalueff et al., 2006; Streng, 1971](#)). The temporal organization of mouse OFT exploration appears to be stable and independent of extrinsic

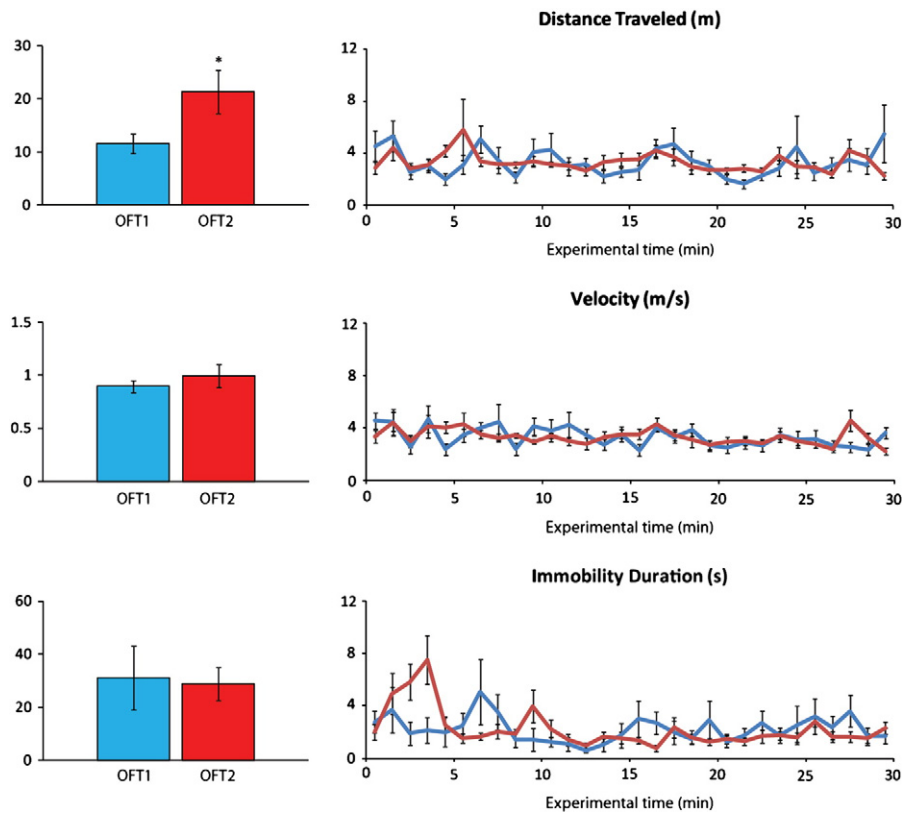


Fig. 2 – Behavior of adult zebrafish (n=20 in each group) exposed for 30 min to two different open field tests (OFT 1 and OFT2, see Fig. 1 for details). The bar diagrams represent the total endpoint values (Y-axis) for the entire 30-min duration of the trial (* $P < 0.05$; U-test, OFT1 vs. OFT2). The line diagrams represent the temporal patterning of zebrafish behavioral activity, with the data normalized and presented as % of total group's activity (Y-axis; taken as 100%) scored during the entire 30-min trial. Note the oscillation-like patterns for these endpoints throughout the duration of the trial (see Table 1 and Fig. 4 for details).

properties of novelty (e.g., size, color, and shape) (Eilam, 2003; Kalueff et al., 2006), suggesting that rodents use conservative temporal strategies for their exploration. The fact that zebrafish in our study displayed a similar phenomenon (Figs. 2–3) expands the notion of conservative behavioral organization of novelty exploration to lower vertebrate species, such as fish.

Notably, adult zebrafish locomotor activity in our study (Fig. 2) varied depending on the size of the tank, similar to findings in rodents exposed to different OFT arenas (Eilam, 2003; Eilam et al., 2003; Kalueff et al., 2006). Since the larger rectangular OFT2 tank was of similar size to that used in the rodent OFT studies, a smaller arena (OFT1) was also utilized in our study, to allow the results to be translatable between the two model organisms, relative to their sizes. However, the fact that zebrafish exploration patterning withstands marked changes in OFT size (like rodents (Kalueff et al., 2006)), suggests an even higher similarity of animal exploration strategies in novelty-based paradigms between these species.

Interestingly, zebrafish freezing bouts remained relatively constant across arena sizes, also paralleling previous rodent studies (where the number of immobility episodes/stops did not change with increasing arena size, representing an invariant feature of their exploratory behavior (Drai et al., 2001; Eilam, 2003; Kalueff et al., 2006)). We also found that the inter-stop distance did vary across the OFT arenas (Fig. 3), thereby supporting rodent data, in which inter-stop distance

grew with increasing OFT size (Eilam, 2003). Collectively, these results demonstrate stability in temporal organization of exploration in adult zebrafish, similar to that previously reported in rodents (Kalueff et al., 2006).

The potential role of animal stopping behavior has already been discussed in the literature, reflecting an important aspect of exploration (Eilam and Golani, 1989; Golani et al., 1993; Kalueff et al., 2006). For example, in addition to reflecting anxiety-evoked freezing, stopping may be part of information-gathering, decision-making and/or risk assessment behavior, which animals tend to maintain throughout their exploration (Blanchard et al., 1990; Blanchard et al., 2005; Kindermann et al., 2009). Our data on the temporal stability of zebrafish stopping in the OFT experiments (Figs. 2–3) indirectly supports this notion, raising the possibility that a similar role may exist for zebrafish immobility (which may be a more complex behavior than initially recognized). The oscillating patterning of immobility frequency, duration and inter-stop distance (Fig. 2–3) further implicates stopping behavior in zebrafish exploration.

Although not in the scope of this study, we also noted that 85% of the fish established prominent homebase behaviors (in which one area of the tank is chosen as a preferred point of reference during the test, where the fish frequently return and spend a longer duration (see Fig. 1 and (Stewart et al., 2010) for details). Similar to previous studies (Stewart et al., 2010), analyses of the distance traveled, frequency of visits, and time spent within

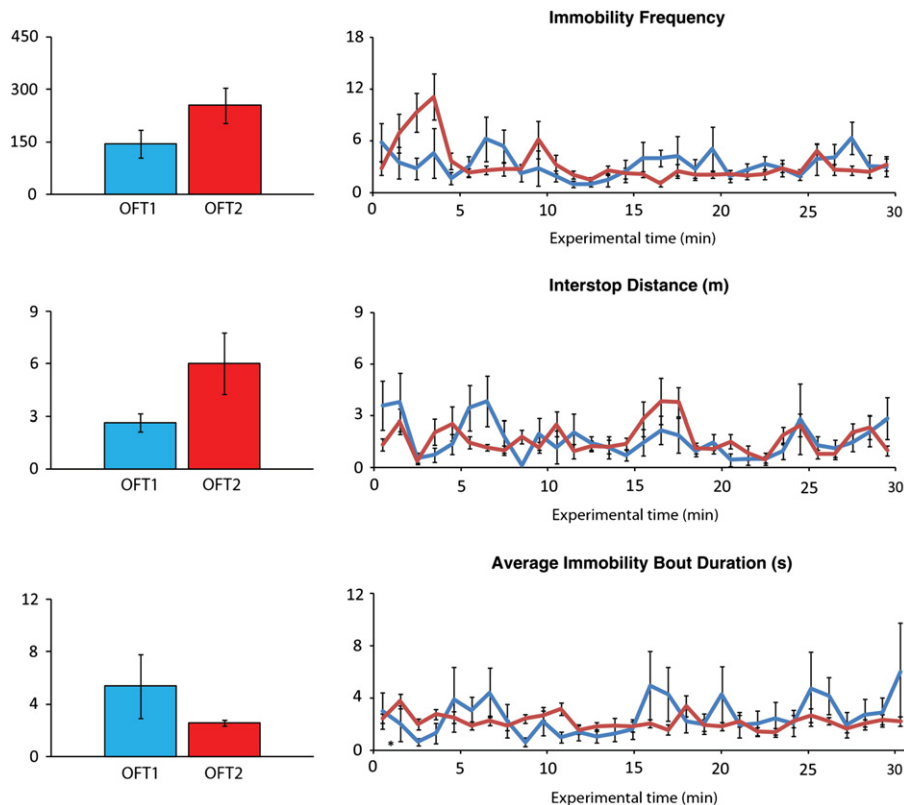


Fig. 3 – Behavior of adult zebrafish (n=20 in each group) exposed to two different open field arenas (OFT 1 and OFT 2) for 30 min (legend as in Fig. 2). Inter-stop distance was calculated by dividing the distance traveled by the number of stops (immobility frequency) of the zebrafish (n=20 in each group). Average immobility bout duration was calculated by dividing the number of immobility bouts by immobility frequency of the zebrafish. Data are represented as the average of total behavior (Y-axis) for each OFT type. Note oscillation-like patterns for these endpoints throughout the duration of the trial (see Table 1 and Fig. 4 for details).

the homebase zones revealed similar temporal dynamics of homebase behavior across different OFT arenas. Relatively constant levels of the homebase behaviors were maintained across the different OFT arenas, with zebrafish generally spending 10–40 s/min per homebase, visiting there 1–6 times/min, and traveling 0.5–1 m there each minute (data not shown). The presence of homebase behavior in this study and similarity of its spatio-temporal patterning to previous reports in zebrafish (Rosemberg et al., 2011; Stewart et al., 2010) confirms the validity of our model, showing the typical characteristics of zebrafish exploration in novel environments.

Finally, we also observed a sinusoidal oscillation-like temporal patterning in zebrafish horizontal activity in both OFT arenas (Fig. 2–4), with the per-minute behavioral distribution of OFT1 and 2 displaying “waves” of exploration with the frequency of 5–7 min across the 30-min trials (Fig. 2–3; see Table 1 for a summary of results). This suggests that zebrafish alternate between phases of lower and higher exploratory activity when they explore novel environments. Whether other animal species use this strategy of exploration, and what can be the exact biological role of this behavior, is currently unclear, and merits further scrutiny.

While animal exploration depends on spatial mapping of the arena, they also use navigation in novel environments. For example, rats utilize dead reckoning, which involves processing

self-movement cues to map a trajectory from the location where the movements were initiated (Wallace et al., 2006). It is also possible that, like many species (including humans), fish use path integration to calculate a route using landmarks. Path integration provides the animal continuous information about its location in its internal representation of space (Benhamou et al., 1990; Etienne and Jeffery, 2004), and such potentially egocentric sensory coding in zebrafish may be possible, given high accuracy in some of their locomotion.

Another limitation of the present study is its focus on two-dimensional patterning of zebrafish locomotion in a horizontal plane. To address this question, we performed a second experiment, in which zebrafish temporal stability was analyzed in vertical coordinates. In contrast to the horizontal OFT1 and OFT2 data, vertical behavior in adult zebrafish remained relatively constant in the OFT3. The tendency for the fish to demonstrate an initial geotaxis followed by gradual exploration of the upper region of the tank was apparent here, and is consistent with previous data in the novel tank tests (Grossman et al., 2010; Stewart et al., 2011; Wong et al., 2010). However, this approach offered only a partial methodological solution to this problem, and further analyses may be needed. For example, quantifying zebrafish exploratory behavior in three dimensions (Cachat et al., 2010a; Cachat et al., 2010b) may assess zebrafish behavior more fully, representing an important avenue to examine in future studies.

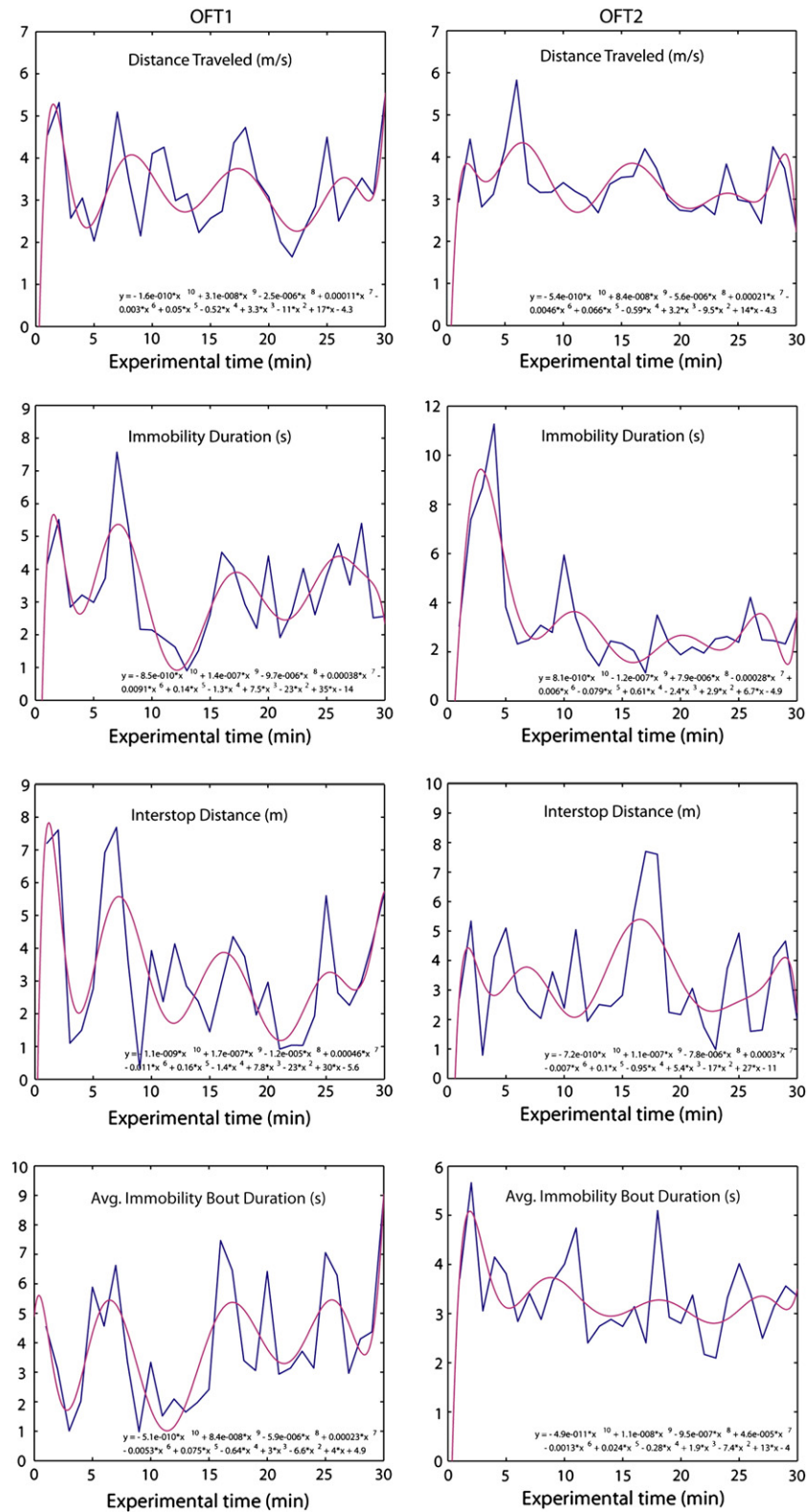


Fig. 4 – The wave-like (oscillation) nature of behavioral activity of adult zebrafish (n=20 per group) exposed to two different open field tests (OFT 1 and OFT2) for 30 min. Plots used in Figs. 2 and 3 were analyzed in MATLAB, with curves fitted to reflect behavior as a function of a 10th degree polynomial (bottom of each panel). Note that all endpoints in both OFT arenas (Figs. 2–3) exhibit robust oscillations (approximately every 5–7 min) in behavioral patterning across 30-min OFT trials (see Table 1 for details).

Likewise, the observed oscillations in horizontal activity across time may represent another conservative temporal strategy of zebrafish behavior. However, while such sinusoidal-like behavioral patterning is not common in rodent OFT exploration, it may be an innate species-specific behavior, and may represent a valuable exploration strategy in fish species. In line with this, zebrafish homebase activity parallels the oscillations of high/low swimming activity observed here, further revealing intrinsic complexity of zebrafish novelty exploration.

In summary, our data suggests that the spatiotemporal patterning of zebrafish OFT behavior is conserved and stable, whereas the amount of their exploratory locomotion is adjusted, depending on properties of the novelty, such as arena size. These phenotypes in zebrafish strikingly parallel previous rodent OFT observations, and may contribute to our fundamental understanding of how various animals explore novelty. Oscillation of exploration-related behaviors (Fig. 4) was another aspect of zebrafish responses to novelty, likely to reflect a useful behavioral strategy of novelty exploration in fish.

4. Experimental procedures

4.1. Animals and housing

A total of 40 adult (4–6 month-old; ~50:50 male:female ratio) ‘wild type’ short-fin zebrafish were used in this study, approved by the Institutional Animal Care and Use Committee (IACUC) at Tulane University. The animals ($n=20$ in each group) were obtained from a local commercial distributor (50 Fathoms, Metairie, LA) and given at least 20 days to acclimate to the animal facility. The fish were housed in groups of approximately 20–30 fish per 40-L tank. All tanks were filled with facility water, with room and water temperatures maintained at $\approx 25^\circ\text{C}$ and water pH at 7.0–8.0. Illumination ($1170\pm 67\text{ lx}$) was provided by ceiling-mounted fluorescent light tubes on a 12-h cycle (on 6:00 h; off 18:00 h), consistent with the zebrafish standard of care (Westerfield, 2007). All animals used in this study were experimentally naïve and fed Tetraamin Tropical Flakes (Tetra U.S., Blacksburg, VA) twice a day.

4.2. Apparatus and behavioral testing

Behavioral testing to assess horizontal locomotory patterns was performed using two different novel OFT arenas filled with aquarium water to the level of 12 cm (Fig. 1). OFT1 was a small white square tank (14 cm height \times 29 cm width \times 37 cm length) while OFT2 was a larger, rectangular white tank (12 cm height \times 39 cm width \times 47 cm length). These apparatuses, differing in size, were selected based on their potential to reveal differences in zebrafish exploration and homebase activity that may be potentially associated with distinct OFT environments.

To assess vertical behavior, a clear third tank was utilized, OFT3 (25 cm height \times 20 cm width \times 41 cm length). All apparatuses rested on level ground, maintained the same distance (114 cm) from the camera. Top-view recording was used for OFT1 and 2, while side-view recording was used for OFT3. Behavioral testing took place between 12:00 and 16:00 h, with illumination ($1170\pm 67\text{ lx}$) consistent with animal’s housing. At the beginning of the trials each fish was gently placed in the center

of the tank and video-recorded for 30 min, alternating between the OFT arenas every 3 trials, while OFT3 experiments were conducted separately. Each fish was only subjected to one 30 min trial, and to only one of the two OFT apparatuses. The experimenters were not present in the room during the recording, to prevent disturbances to the fish.

The trials were recorded via camera (Sony Handycam DCR-SR47, New York, NY) and analyzed off-line using Ethovision XT7 (Noldus IT, Wageningen, Netherlands). Event rules were set to precisely and consistently register behavioral profiles within the arena. Subsequently, additional endpoints, such as the duration in a particular area (s), mobility and immobility (s), distance traveled (m), and velocity (m/s), could be gathered for statistical comparison for OFT1 and 2. Inter-stop distance (average distance between successive stops) was also calculated by dividing the distance traveled by the number of stops (immobility frequency) of the zebrafish. Average immobility bout duration was calculated by dividing the number of immobility bouts by immobility frequency of the zebrafish. Fish traces were also generated in this study by video-tracking systems, to visualize zebrafish locomotory behaviors. Both traces (Fig. 1) represented the tracking of locomotor activity, with the density maps also accounting for location and duration. Vertical exploration was assessed using OFT3, in which fish tracks were generated with Ethovision XT7 and compared for similarity, specifically focusing on data on mobility in the top vs. bottom of the tank.

4.3. Statistical analysis

Total endpoint values were calculated by taking the sum of the activity that occurred during 1–30 min for each individual fish, then averaging across the entire 20-fish cohort for each OFT. Data for the total endpoint values was further analyzed using the Mann–Whitney *U*-test, comparing OFT1 and 2 scores. Data for per-minute distribution was normalized and expressed as % of total 30-min score and was analyzed using SPSS 18.0.0 (SPSS, Inc., Chicago, IL) by two-way ANOVA (factors: OFT type; test time/minutes) followed by a post-hoc Tukey test for significant ANOVA data. Data is presented as mean \pm SEM; significance was set at $P<0.05$. To further characterize the temporal patterning of zebrafish activity, we applied the regression analysis to all endpoints recorded in this study. Briefly, the per-minute distribution data was analyzed using nonlinear regression through MATLAB (MathWorks, Inc., Natick, MA) to approximate the mathematical expression of zebrafish activity, and by a method of successive approximations to find the best-fitting parameters. Using the least squares approach to minimize the sum of squared residuals, a series of curves were optimally fitted across the data points to yield mathematical expressions closely representing the patterning of horizontal activity across time (Table 1, Fig. 1).

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