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Individual differences in activity levels in zebrafish (*Danio rerio*)

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Abstract

Individual differences and variation in behavioural responses have been identified in many animal species. These differences may be the result of genetic or environmental factors or the interaction between them. Analysis of individual differences in behaviour may be important for many reasons. The zebrafish is a powerful model organism that is rapidly gaining popularity in behavioural brain research. However, individual differences have rarely been explored in zebrafish although significant variation in their performance has been reported. In the current study we identified individual differences in activity levels of zebrafish using a genetically heterogeneous population. Groups of zebrafish classified as high, medium, or low activity performers demonstrated consistent activity levels over a period of 7 days, and also in a subsequent open field task, suggesting stable individual differences as opposed to stochastic variation among subjects. We also uncovered a sex dependent relationship between behavioural measures. Female zebrafish in the high activity group preferred the top portion of the tank, whereas low activity females preferred the lower portion but males did not show such a relationship. The relationship between these two behaviours in females implies the potential existence of a behavioural syndrome persisting between contexts. Furthermore, females demonstrated a higher level of consistency in their behaviour as compared to males, and the behavioural differences were found to be independent of both body size and weight of the tested subjects. The identification of individual differences in activity levels in zebrafish will allow the investigation of underlying genetic and/or environmental underpinnings.

Keywords

individual differences; locomotory activity; sex differences; zebrafish

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INTRODUCTION

The zebrafish has become a popular animal model for behavioural neuroscience over the past several decades [1 – 3]. Numerous studies of zebrafish behaviour have found a high level of variance in the performance of zebrafish but it remains unclear whether this variability may be due to consistent and stable individual differences or to random stochastic variation. Recently, researchers have started to look at the possibility of individual differences in behaviour and investigated whether such differences may represent personalities or behavioural syndromes [4 – 7]. Boldness and aggressiveness are two of the behavioural personality types commonly explored in zebrafish [5 – 10]. Boldness is usually measured as locomotor activation (e.g. total distance travelled) in response to a novel environment (e.g. open-field) or a predator, or as time swimming near a novel object [9]. Aggressiveness is often quantified as the number of aggressive encounters using the mirror task [4 – 6, 11]. These behavioural measures have been reported to exist on a continuum with certain individuals scoring high on measures of boldness and/or aggressiveness, while others scoring low [7, 12].

Individual variation in behavioural traits may be regarded as fuel for evolution, variation upon which natural selection may act [13 – 15]. In zebrafish, such variation is linked to reproductive success [5], and is thought to be under strong selective pressure [6, 15]. Artificial selection for boldness and high exploratory behaviour (e.g. high locomotor activity) in zebrafish has allowed investigators to develop distinct lines [7, 10] suggesting a high degree of heritability for these traits [6].

In addition to the boldness and aggressiveness axes, three other behavioural axes have been identified in fish: sociability, exploration and activity [16]. Activity as a behavioural axis has received the least attention albeit its common use in the measurement of other behavioural traits is evident [16 – 17]. Activity level as a trait itself has been suggested to be an independent measure of behaviour in some contexts, e.g. when quantified in a familiar environment [16].

Locomotor activity of zebrafish has often been found to be a sensitive measure with which the effects of specific stimuli or of other manipulations including that of the testing environment may be quantified [16]. Experimenters regularly report high within-group variance for locomotor activity in zebrafish. The observed variance may be accounted for by stochastic variation in animal behaviour and/or experimental error variation. An alternative explanation for the observed variation is that it is due to genetic predisposition and/or environmental effects leading to consistent individual differences among the subjects tested. For example, some zebrafish populations and strains have been reported to exhibit a great deal of genetic variation [18]. Previous studies have reported significant variation in different measures of zebrafish behaviours including boldness [5], predator induced behavioural responses [4], predator inspection behaviour [19], and shoaling [9, 20]. However, it has been unclear whether the observed variation was due to consistent individual differences or stochastic error variation [17].

Although several studies have demonstrated behavioural variation in zebrafish, to our knowledge, individual differences in general locomotor activity have not been shown, i.e. the question whether the variation among individuals remains consistent across time and/or across experimental tests, has not been answered. To investigate individual variation in activity levels, we chose a genetically heterogeneous population of zebrafish expected to exhibit greater between-subject variability. We used locomotor activity to measure individual responses and determined whether these responses remained consistent over a period of 7 days and across two contexts, familiar and novel environments.

METHODS

Animals and housing

A total of 61 fully mature short-fin wild-type zebrafish exhibiting the gold pigment phenotype were purchased from Big Al's Aquarium (Mississauga, Ontario). They were housed in our facility (University of Toronto Mississauga Vivarium) for 1 month to acclimatize to the environment before testing. Animals were maintained in 37 L glass tanks (50 × 25 × 30 cm, length × width × height) in groups ($n = 15$ per tank) with biological filtration and kept on a 09:00 – 21:00 light cycle. The water quality was monitored on a daily basis and kept within optimal parameters (pH: 6.5 – 7.5, conductivity: 100 – 200 microsiemens, temperature: 26 – 28 °C), Zebrafish were fed ground flake food (3:1 ratio of TetraMin flake (Melle, Germany) and Spirulina (Jemco Inc. Lambertville, New Jersey)) twice a day. A week prior to testing, individual zebrafish were moved to individual 2.7 L trapezoid tanks (Top length: 28 cm, bottom length: 23 cm, width: 11 cm, height: 15cm) to allow habituation and individual identification where they remained until testing. On testing Day 7, one zebrafish jumped out of the testing tank and died, therefore data for this fish was only available for testing Days 1 – 6.

Behavioural apparatus

Zebrafish were individually tested on Day 1 through 7 in a 37 L tank (50 × 25 × 30 cm). The tank had white corrugated plastic on the bottom, back and its sides to provide a consistent testing background and prevent access to external visual cues. On day 8, animals were tested in an open field (70 × 70 × 30 cm). The open field had white corrugated plastic on all four sides, as well as on the bottom to create a uniform testing area. Two identical experimental setups were run in parallel, with the exception of the open field task. Two JVC video cameras (GZ-MG150) were used to record zebrafish behaviour from the front view in the 37 L tanks (multiple days of testing). A single JVC video camera was used to record activity in the open field tank from the top view. The order in which animals were tested was randomized each day. Locomotor activity in zebrafish has been shown to exhibit a diurnal cycle that is regulated by circadian rhythms [21]. To minimize the effect of circadian rhythms on the experimental outcome, all experiments were carried out between 11:00 and 15:00 hour.

Behavioural testing procedure

On Day 1, individual zebrafish were placed in the middle of the 37 L tank and their activity was recorded for a total duration of 10 minutes. Using percentile rankings, three separate

groups were subsequently created based on locomotor activity scores obtained during this single test (High: $n = 20$, Medium: $n = 22$, Low: $n = 20$). In between testing sessions, zebrafish were placed back in their 2.7 L home tank. Each zebrafish was subsequently tested again on Days 2 – 7 in the same 37 L tank, and their behaviour was recorded. On day 8, individual zebrafish were placed in the center of the open field and their behaviour was recorded for 10 minutes.

Quantification of behaviour

Video files were extracted and analyzed using Ethovision XT 8.0 (Noldus Information Technology, The Netherlands), an automated video tracking software application which utilizes the pixel subtraction method, dynamic subtraction. Total distance travelled was used as a measure of locomotor activity. Distance from bottom was used as a measure of fear, commonly used in previous studies [22 – 23].

Experimental Design and Statistical Analysis

We employed a $3 \times 2 \times 8$ between and within-subject repeated measures design with Activity level as a between-subject factor with 3 levels (High, Medium, Low), Sex as a between subject factor with 2 levels (male or female) and Day as the repeated measure factor with 8 levels (Day 1 to 8). Data were analysed using SPSS 14 written for the PC. Initially, a two-factor repeated measures ANOVA was performed with Sex as the between-subject factor and days as the repeated measures for locomotor activity. Due to significant sex $\times$ day interactions, the data was split and a two factor repeated measures ANOVA was performed with activity levels as the between-subject factor and days as the repeated measures (8 levels) for each sex separately. The analysis of distance from bottom utilized the same repeated measures ANOVAs but with only 7 levels for the repeated measures because distance from bottom was not measured in the open field task. In case of equal or similar sample sizes across treatment groups, as was the case in our study, parametric statistical tests have been shown to be insensitive to the violation of the normality of distribution and variance inhomogeneity criteria. Nevertheless, in one measure (distance from bottom) square root transformation was employed to successfully homogenize variances and the statistical analysis was performed on the scale transformed data.

RESULTS

Locomotor Activity

Analysis of overall locomotor activity in the 37 L tank showed a gradual decrease in total distance travelled over the 7 days of testing, followed by a significant increase in activity when tested in the Open Field Task (Figure 1). A repeated measures ANOVA confirmed this observation and showed a significant interval effect ($F(7, 413) = 91.836$, $p < 0.001$). Although there was no significant sex effect ($F(1, 59) = 0.799$, $p = 0.375$), there was a significant interval $\times$ sex interaction ($F(7, 413) = 3.137$, $p < 0.01$). Thus subsequently, we performed statistical analyses separately for males ($n = 32$) and females ($n = 29$). Females in the high, medium, and low activity groups habituated to the novel environment during testing days 1 through 7, and again showed an increase in activity in the subsequent open field (Figure 2). Notably, all groups maintained consistency relative to their initial group

assignment. A repeated measures ANOVA confirmed these observations and demonstrated a significant interval effect ($F(7, 203) = 25.605, p < 0.001$), as well as an activity effect ($F(2, 29) = 7.390, p < 0.01$), but found no significant interval $\times$ activity interaction ($F(14, 203) = 0.807, p = 0.661$).

Males exhibited less apparent consistency than females, with the high and medium groups showing convergence after a few days. However, the high and low groups still maintained an apparently distinct activity level throughout multiple days of recording, i.e. remained consistently as classified based upon their initial group assignment. It is also notable that when males were tested in the Open Field Task, differences among all three groups were apparent again and were consistent with their original group classification (Figure 3). A repeated measures ANOVA confirmed these observations and found all effects significant (interval, $F(7, 203) = 82.838, p < 0.001$; activity, $F(2, 29) = 10.578, p < 0.001$; interval $\times$ activity interaction, $F(14, 203) = 2.167, p = 0.01$).

Day to day locomotor activity correlated significantly with the previous day ($0.679 > r < 0.872, p < 0.05$) in the 37 L tank. Although weight correlated with locomotor activity on Day 1 ($r = 0.314, p = 0.019$), it did not correlate with any other days ($p > 0.05$). Length was not correlated with locomotor activity on any days ($p > 0.05$) (Table 1), an important finding given that locomotor speed is believed to be dependent upon the linear dimensions of the fish.

Distance from Bottom

Average distance from bottom did not seem to change with days of testing, i.e. animals in the high, medium and low activity groups did not apparently alter their position in the water column over the 7 days of testing. This observation was confirmed by the non-significant interval effect found by ANOVA ($F(6, 330) = 1.561, p = 0.158$). Also importantly, neither the activity effect ($F(2, 55) = 0.418, p = 0.660$) nor its interaction with interval was found significant ($F(12, 330) = 0.642, p = 0.806$). Although no significant sex differences were found ($F(1, 55) = 0.511, p = 0.478$), ANOVA revealed a significant activity $\times$ sex interaction ($F(2, 55) = 3.222, p = 0.047$). Therefore, the data was split into males ($n = 32$) and females ($n = 29$) for further analysis.

Although there were no apparent changes over the 7 days of testing, females in the high activity group appeared to spend more time in top portions of the tank compared to the medium and low groups (Figure 4). A repeated measures ANOVA confirmed this observation and found the effect of day ($F(6, 156) = 1.410, p > 0.20$) and the day $\times$ activity interaction ($F(12, 156) = 0.353, p > 0.95$) to be non-significant, but the main effect of activity was significant ($F(2, 26) = 3.394, p < 0.05$).

There were no obvious observable trends in the male population with regard to daily changes or group differences in the measure distance from bottom (Figure 5). A repeated measures ANOVA found no significant day effect ($F(6, 174) = 0.821, p > 0.55$), activity effect ($F(2, 29) = 0.784, p > 0.45$), or day $\times$ activity interaction: $F(12, 174) = 0.656, p > 0.791$.

Last, total distance travelled correlated significantly with distance from bottom on Day 1 in females ($r = 0.381$, $p = 0.04$), but not in males ($r = -0.241$, $p > 0.05$) (Figure 6).

DISCUSSION

Individual differences in behaviour have been identified in many vertebrate species [24 – 26] including humans [27]. Activity levels of zebrafish have been investigated in the past and have often been found to exhibit a high degree of variance. Nevertheless, the question of whether the observed variance represents random error variation or consistent individual differences among subjects has not been resolved in zebrafish. The current study reconciles this issue and demonstrates that indeed reliable individual differences do exist in zebrafish and they manifest as consistently differing performance across multiple testing days and in two different testing environments, a novel open field and a more familiar tank.

In most behavioural studies, zebrafish are introduced to a novel environment [28]. Similar to rodents, depending on the level of fear zebrafish initially may exhibit novelty-induced freezing, erratic movement (under more aversive conditions), or the opposite: increased activity due to elevated exploratory drive (in case of less aversive conditions) [2, 28 – 30]. Also occasionally observed in zebrafish is the initial preference for the bottom of the tank within the first few minutes of being exposed to a new place, a response that subsequently may dissipate as the fish becomes habituated to the environment [22, 30 – 31]. Notably, however, this response has been argued to be less of a reliable indicator of fear due to its context dependence [2, 23, 36]. We report no changes in depth preference in the 37 L tank over the first 7 testing days but we detected a significant habituation of activity especially robust between days 1 and 2. We propose that the habituation of activity may be the result of reduced exploratory drive and/or diminishing fear due to the increasing familiarity (memory) of the test tank. We also suggest that the small empty test tank was only mildly aversive, even on the first day, which may be due to its dimensions (less open space represents reduced probability to be exposed to aerial predators [23]), or to prior holding conditions of our experimental fish. For example, in the current study, zebrafish were individually housed in 2.7L tanks throughout the testing period and previously zebrafish were found to exhibit attenuated anxiety responses in a novel environment when individually housed compared to group housed fish [32]. Individually housed zebrafish were also found to exhibit lower cortisol levels [32] a sign of reduced stress reactivity [33].

Importantly, individual differences in activity levels were independent of the length or weight of zebrafish. Although female zebrafish are on average larger than males [7], here we found the effect of locomotor activity to be independent of these physical characteristics similarly to what has been described in the bluegill sunfish [34]. The lack of correlation between activity levels and these physical measures of body size suggests that the individual differences in locomotor activity were unlikely to be due to simple performance factors such as strength or size. Therefore, we propose that the differences among the high, medium and low activity performer fish may be the result of differences among the individuals in their brain function.

Interestingly, sex-dependent individual differences were identified in the current study. For example, the differences among the high, medium and low groups in activity levels were found to be more consistent among females than males across the 7 days in the smaller 37 L tank. Sex differences are rarely reported for zebrafish, but in the rodent literature females are often found more variable due to hormonal changes associated with the estrous cycle. Zebrafish females are oviparous and do not exhibit such cycles [35] but the observed consistent individual differences among them are in accordance with higher variability in females compared to males (compare figures 2 and 3). Why consistent individual differences were found more robust in female zebrafish than in males is a matter of speculation at this point. Nevertheless, it is important to note that the sex-dependency of individual differences may be task specific and may not generalize to all contexts. In the open field, it appears the individual differences were more robust in males than in females. There may be numerous reasons why females and males respond differently to certain tasks, which may include exploratory drive, territoriality and aggression, or other behaviours associated with reproduction. The current data do not allow us to distinguish among these possibilities but suggest that perhaps males and females exhibit coping strategies that are context dependent.

What makes up a behavioural strategy will be investigated by analysing a broader spectrum of behavioural measures obtained in a range of environments (contexts). Nevertheless, our current results already show that the high, medium and low activity groups also differed in another behavioural measure, distance from bottom, at least in females. The latter behaviour has been used in the past as a measure of anxiety/fear [36] and boldness [7]. Elevated activity and decreased preference for the bottom of the tank, found in the current study, is often interpreted as signs of decreased fear and increased boldness in zebrafish [7, 36]. A combination of such characteristics may be regarded as a behavioural syndrome, traditionally described as sets of correlated behaviours that persist over time and situations. Behavioural syndromes have been used to describe animal (and human) temperament, coping style, or personality [14 – 17]. Correlations for behavioural syndromes in zebrafish have been reported previously between boldness and activity [4], boldness and exploration [10], and boldness and dominance status [8]. The relationship we found between distance travelled and distance from bottom resembles the activity-boldness spectrum that has been identified, for example, in the context of anti-predatory behaviour [4].

The current study opened several questions, but it also clearly identified the existence of consistent individual differences in activity in zebrafish. These individual differences may be utilized in future experimental work in multiple ways. One could “convert” such differences into genetic differences across lines or strains of fish by selectively breeding extreme performers or by inbreeding zebrafish and thus increasing genetic drift (random fixation of alleles) [7, 12]. One could also conduct systematic and comprehensive gene expression analyses [37] and compare the individuals or classes of individuals as defined in the current study to understand the potential biological mechanisms underlying the observed behavioural differences. It would also be important to investigate the developmental stability of individual differences, that is, the question whether such differences exist earlier, perhaps even during the larval stage of the fish, and whether they change as the fish mature. Finally, as mentioned above, one could also conduct a range of behavioural studies and explore the existence of sex-specific behavioural syndromes or, alternatively, the possibility that

swimming activity is a unique feature that does not correlate with other behavioural measures. Irrespective of what research direction one will take, the current work now opened up these possibilities.

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Research highlights

Low, medium and high activity zebrafish were identified on test day 1

Activity differences remained consistent throughout 7 days

Activity differences remained consistent in a different test environment

The consistency of differences was sex and environment dependent

Figure 1.

Total distance travelled (cm) during each 10 minute session decreased after the first day of testing in the 37 L tank (Days 1 through 7), which was followed by increased activity in the open field task. Mean $\pm$ S.E.M. are shown. Note the slightly higher activity levels in females in the 37 L tank (Days 1 – 7) and lower activity for females in the open field task compared to males.

Figure 2.

Total distance travelled (cm) by female zebrafish in a small (37 L) tank across 7 days and in a subsequent large open field. Mean $\pm$ S.E.M. are shown. Females identified as high, medium and low activity performers on the first day remained consistently different in their activity levels in the 37 L tank for the subsequent days of testing (Days 2 – 7) and also showed consistent activity level differences in the open field task.

Figure 3.

Total distance travelled (cm) by male zebrafish in a small (37 L) tank across 7 days and in a subsequent large open field. Mean $\pm$ S.E.M. are shown. Males identified as high and medium activity performers on the first day started to converge on subsequent days of testing demonstrating less consistent individual differences across testing days. Males identified as low activity performers on the first day, however, continued to show low activity throughout the rest of the testing period (Day 2 – 7). Note the increase in activity levels in the open field task, and also the consistent order of activity levels of the originally defined groups.

Figure 4.

Distance female experimental zebrafish swam from the bottom (cm) across 7 days of testing in the 37 L tank. Mean $\pm$ S.E.M. are shown. Note the lack of significant changes across days in all three groups. Also note that the effect of activity (the difference among groups) was found significant.

Figure 5. Distance (cm) from the bottom of the tank for male zebrafish is shown for each testing session (Day 1 – 7). Mean $\pm$ S.E.M. are shown. Note that despite the apparent trend, no significant activity related differences were found and the effect of day was also non-significant.

Figure 6.

Correlation between total distance traveled (cm) and distance from bottom (cm) calculated based upon the first session in the 37 L tank (1st day). A moderate (but significant) correlation between total distance travelled and average distance from bottom was observed in female zebrafish but not in males. Pearson product moment correlation coefficients and the associated significance values are shown.

Table 1

Correlation among distance travelled during 10 min sessions over a 7 day period (1 session per day), and weight and length of experimental zebrafish. Pearson's product moment correlation coefficients are shown. Daily locomotor activity significantly correlated with the previous day activity level of experimental zebrafish. Body weight and length of the subjects also significantly correlated with each other. Although weight significantly correlated with locomotor activity on Day 1, no significant correlations were observed between this variable (or between length) and activity levels on other days, suggesting that locomotor activity of zebrafish was independent of size and weight.

	Weight	Length	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Open Field
Weight	1.000									
Length	0.697**	1.000								
Day 1	0.314*	0.173	1.000							
Day 2	0.105	0.121	0.679**	1.000						
Day 3	0.000	-0.193	0.520**	0.683**	1.000					
Day 4	-0.047	-0.117	0.483**	0.676**	0.783**	1.000				
Day 5	-0.057	-0.115	0.482**	0.727**	0.742**	0.772**	1.000			
Day 6	-0.033	-0.07	0.476**	0.699**	0.761**	0.819**	0.774**	1.000		
Day 7	-0.153	-0.166	0.470**	0.752**	0.766**	0.779**	0.816**	0.872**	1.000	
Open Field	-0.005	0.133	0.413**	0.592**	0.508**	0.537**	0.497**	0.624**	0.604**	1.000

Asterisks denote level of significance

Correlation coefficients without asterisks are non-significant.

* p < 0.05

** p < 0.01.