

## Shoaling develops with age in Zebrafish (*Danio rerio*)<sup>☆</sup>

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### ABSTRACT

The biological mechanisms of human social behavior are complex. Animal models may facilitate the understanding of these mechanisms and may help one to develop treatment strategies for abnormal human social behavior, a core symptom in numerous clinical conditions. The zebrafish is perhaps the most social vertebrate among commonly used laboratory species. Given its practical features and the numerous genetic tools developed for it, it should be a promising tool. Zebrafish shoal, i.e. from a tight multimember groups, but the ontogenesis of this behavior has not been described. Analyzing the development of shoaling is a step towards discovering the mechanisms of this behavior. Here we study age-dependent changes of shoaling in zebrafish from day 7 post fertilization to over 5 months of age by measuring the distance between all pairs of fish in freely swimming groups of ten subjects. Our longitudinal (repeated measure within subject) and cross sectional (non-repeated measure between subject) analyses both demonstrated a significant increase of shoaling with age (decreased distance between shoal members). Given the sophisticated genetic and developmental biology methods already available for zebrafish, we argue that our behavioral results open a new avenue towards the understanding of the development of vertebrate social behavior and of its mechanisms and abnormalities.

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### 1. Introduction

The zebrafish has been enjoying much popularity in embryology for the past three decades (for examples see Schweitzer and Driever, 2009; Holder and Xu, 2008) thanks to this species' transparent embryo and its numerous other practical features that make it an ideal laboratory model organism (e.g. Lin et al., 2009). Indeed, the zebrafish has been employed as a model for a variety of human diseases including cancer (Stoletov and Klemke, 2008), movement (Flinn et al., 2008) and sleep disorders (Zimmerman et al., 2008) among other conditions (for a review see Lieschke and Currie, 2007).

As increasing number of genetic tools has become available for zebrafish (Patton and Zon, 2001; Keller and Murtha, 2004) the popularity of this species has grown in a variety of disciplines

including behavioral neuroscience (Sison et al., 2006). However, unlike the body of knowledge available on embryonic development and genetics of zebrafish, the behavior of this species is still poorly characterized (Kato et al., 2004). This is a crucial drawback because behavioral analysis has the potential to reveal a variety of functional changes in the brain and has been argued to be an important screening method in forward genetics as well as pharmacology (Gerlai, 2002). Nevertheless, most recently numerous studies have appeared that demonstrated the utility of zebrafish behavioral analysis in the investigation of vertebrate brain function (Gerlai, 2010; Egan et al., 2009). Some of these recent papers utilized the analysis of zebrafish's social behavior (Gerlai et al., 2009; Miller and Gerlai, 2007, 2008; Saverino and Gerlai, 2008; Speedie and Gerlai, 2008).

Social behavior is a complex phenomenon whose biological mechanisms and development are not well understood in vertebrates. Abnormal social behavior is a defining characteristic of a variety of human psychiatric and neurodevelopmental conditions including depression (Bell-Dolan et al., 1993), anxiety disorders (Liebowitz et al., 1985), and the autism spectrum disorders (Reichow and Volkmar, 2010). The underlying mechanisms of the abnormal social behavior associated with these diseases are not well understood (Bartz and Hollander, 2006). Given the similarities between zebrafish and other vertebrates, including humans, in the layout of the brain (Tropepe and Sive, 2003), in the neurochemical properties of the brain (Zhdanova, 2006), and in characteristics of many other levels of biological organization (Egan et al., 2009), including nucleotide

Abbreviation: Dpf, days post fertilization.

<sup>☆</sup> Ethical statement: the research reported in our manuscript has been conducted in accordance with the local University, Provincial and Federal (Canadian Council for Animal Care) guidelines and has been reviewed and approved by the Local Animal Care Committee.

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sequence of genes, the zebrafish is thought to be suitable as a model for investigating the biology and genetics of vertebrate brain functions (Gerlai, 2003). Briefly, findings from studies in zebrafish are expected to generalize well to humans and may shed insights on complex human conditions including the autism spectrum disorders (Tropepe and Sive, 2003).

The zebrafish is a shoaling fish, it aggregates, i.e. forms multi-member groups in nature and in the laboratory. Shoaling is thought to provide the individual fish with multiple benefits, including access to mates, efficient foraging, and defense against predators (Griffiths et al., 2004; Ledesma and McRobert, 2008; Morrell and James, 2008). Although the adaptive nature of shoaling is well documented, the biological mechanisms and the development of this behavior are far from understood. In the current paper, we focus on the latter: we investigate, for the first time, whether shoaling develops, i.e. whether it changes with age, in zebrafish. Our prior personal observations suggested that newly hatched zebrafish disperse while adult zebrafish have been documented to exhibit robust shoaling, a strong preference for staying close to conspecifics (Al-Imari and Gerlai, 2008; Miller and Gerlai, 2007, 2008; Saverino and Gerlai, 2008). A previous study investigating the effects of kin exposure on preference for conspecifics in zebrafish revealed an imprinting-like effect of olfactory cues at an early age of the fish (6 days post fertilization, dpf) and suggested that some preference for conspecifics already exists at this stage of development (Gerlach et al., 2007). Preference for conspecifics was also demonstrated to be based solely upon visual cues in another study (Engeszer et al., 2007), which found measurable preference for conspecifics at postflexion stage (about 12 dpf) of zebrafish. However, it is not known whether the preference responses quantified in the above studies represented shoaling or other type of responses (e.g. agonistic) because the subjects were tested singly and could not interact with the stimulus fish. Furthermore, the question whether shoaling changes with age, i.e. the developmental trajectory of shoaling itself, has not been investigated up till now.

To address the above questions, we analyzed age-dependent changes of shoaling behavior in freely moving groups of zebrafish. The main rationale for our study is as follows. If significant age-dependent (i.e. developmental) changes in shoaling behavior are identified, this discovery could open new research avenues for numerous investigations. For example, one could study the ecological/adaptive importance of developmental alteration of social behavior. Another important goal would be to investigate the mechanisms of the age-dependent changes in social behavior, a classical developmental biology question. Here we report behavioral findings showing a significant developmental change in shoaling in zebrafish and based on this we argue that zebrafish will be an excellent tool with which the mechanisms of vertebrate social behavior may be investigated.

## 2. Methods

### 2.1. Animals and housing

In total, one thousand one hundred and ninety zebrafish (*Danio rerio*) of the AB strain were utilized for the three experiments outlined below. The fish were bred in-house and originated from progenitors obtained from the Zebrafish International Research Center (ZIRC) (Eugene, Oregon). All experiments described below were approved by the University of Toronto Animal Care Committee. All fish used in this study were bred, raised and housed in the same environment. Gender could not visually be determined when testing commenced (at 7 days post fertilization). After completion of the experiments and after the subjects had reached maturity, the gender distribution within the shoals tested was determined to be 50% male 50% female.

Upon hatching, the animals were housed in groups of ten in 1 l plastic aquaria. After 5 weeks post fertilization the animals were transferred to 2.8 l Plexiglas aquaria that were part of a recirculating

filtration aquaculture rack system which had a mechanical, biological, and activated carbon (chemical) filter as well as a UV sterilizing unit (Aquanearing Inc. (San Diego, Ca, USA). Water was maintained at 27 °C. The system water used on the rack as well as during the development and testing of the fish was reverse osmosis purified and was supplemented with 60 mg/l Instant Ocean Sea Salt to achieve water chemistry appropriate for zebrafish.

Zebrafish were kept at a 12 h light/12 h dark cycle with lights on at 7 am and off at 7 pm. All fish were fed twice daily with Larval Artificial Plankton 100 (particle size below 100 µm, ZeiglerBros, Inc., Gardners, PA, USA) until 2 weeks post spawning, after which animals were fed twice daily with nauplii of brine shrimp (*Artemia salina*) until they were 4 weeks old. Older and adult fish were fed a 1:1 mixture of flake food (Tetramin Tropical fish flake food, Tetra Co, Melle, Germany) and powered spirulina (Jehmco Inc., Lambertville, NJ, USA).

### 2.2. Open field task

All zebrafish that were housed together were tested together, forming a given shoal. Each shoal was identified by an ID number and remained constant (same shoal members) throughout the experiments (the unit of statistical analysis here is the shoal, and the sample sizes (n) shown below represent the number of shoals tested). Each group (shoal) consisted of ten fish. The home tank was placed next to the testing arena for transfer. Fish were netted as a group (in most cases all 10 fish could be captured with one net, due to the size of the net and the holding tank) and immediately released in the center of the arena. Transfer (air time) from the holding tank to the arena was not more than 3 s. The fish were released simultaneously in the center of a square plexi-glass tank, the open field, and were allowed to explore the field freely. Each trial lasted 6 min and the behavior of fish during the trial was recorded with an overhead video camera (JVC Everio Hard Drive GZ-MG750BU). After the open field trial, the group was returned to its home tank. In experiments 1 and 2, the arena size was kept proportional to the body length of the growing fish (and thus constant for a particular age group), a practice recommended by others (Gallego and Heath, 1994; Masuda et al., 2003; Vogel, 2008). But in experiment 3, two separate age groups of fish (30 and 60 day old) were tested in six different arena sizes each, and the order of use of different arena sizes was randomized. Behavioral testing was always conducted between 0900 and 1600 h.

### 2.3. Experiment 1: longitudinal developmental analysis of shoaling

The purpose of this experiment was to investigate the trajectory of potential age-dependent changes of shoaling behavior in zebrafish. Nineteen groups, each consisting of ten fish, were utilized in this experiment. Each was tested at 7, 18, 26, 42, 49, 59, 66, 70, and 76 dpf. That is, the same groups of fish were followed throughout their development, a repeated measure design.

### 2.4. Apparatus

Distance traveled has been argued to be the function of the linear dimension of the fish, e.g. their body length, therefore arena sizes or movement parameters such as speed or total distance swum are usually normalized to the length of fish, i.e. expressed in body lengths (Hale, 1999). In zebrafish, speed has also been found to be positively and linearly correlated with body length (Kimmel et al., 1974) and thus we decided to keep the linear dimensions of our open field experimental tanks proportional to the body length of our developing fish. We employed open fields whose linear dimensions were 28x the average body length of the zebrafish tested in them. This ratio gave us sufficiently large tanks in which the fish were not physically forced to stay close to each other and thus any shoaling observed would be the result of social cohesion and not of the physical constraints of the

environment. On the other hand, this tank to fish size ratio was sufficiently small so as to allow high quality video recording and analysis (large enough subjects on the video screen). For the youngest age group, the 3.2 mm long 5 dpf old fish, the youngest free swimming age, we constructed a 90×90×30 mm (width×length×depth) tank. All open field tanks employed for the older age groups reported here were proportional, i.e. scaled up versions of this tank. That is for the 7, 18, 26, 42, 49, 59, 66, 70 and 76 dpf fish we used open fields whose linear dimensions were 1.7, 2.4, 3.3, 4.5, 5.0, 5.5, 6.5, 6.9, and 7.5 times those of the smallest open field, respectively. The level of water in these open fields was kept at 90% of the depth of the tanks.

### 2.5. Experiment 2: cross sectional analysis of age differences in shoaling

In the above described longitudinal developmental study shoaling responses could be influenced by repeated handling and exposure to the open field task. Depending on the salience of the stimulus or the context in which it is delivered, repeated exposure to stimuli may lead to habituation or sensitization even in simple invertebrates (Carew et al., 1971; Pinsker et al., 1970). In mammals repeated postnatal handling has been shown to lead to habituation (reduction) of stress induced anxiety later in life (e.g. Meerlo et al., 1999). Similarly, repeated exposure to the same object (e.g. Dere et al., 2007 and references therein) or environment (e.g. Gerlai and Roder, 1993 and references therein) has been shown to lead to habituation in mice. Unfortunately, whether handling and repeated exposure to testing would lead to habituation or sensitization in zebrafish, and under what circumstances, has not been systematically investigated, but our personal observations suggested that inappropriate handling could easily lead to sensitization (increased fear responses) in zebrafish. Similar observations have been published with other fish species suggesting fear or stress inducing effects of repeated handling (Pauker et al., 2005). Briefly, repeated handling induced elevation in fear could in principle lead to enhanced shoaling, i.e. tighter shoal cohesion. To address this potential confound, we conducted a non-repeated measure cross sectional experiment. We analyzed shoaling behavior of 7 different age groups of fish with “AGE” as the between subject factor, i.e. fish were tested in the open field only once. The order of testing fish of different ages was randomized. All other parameters (housing conditions, recording methods, size of open field, etc.) were identical to those of the longitudinal developmental study. The age groups corresponded closely to those tested in the longitudinal analysis but we also decided to include two older groups, a 121 dpf and a 173 dpf group. The open field sizes were as explained in the first experiment but for the two oldest groups we used larger tanks (to keep the linear dimensions of these tanks consistently 28 times the body length of the subjects), so the dimensions of the tanks were 8.4 and 10.6 times of the smallest open field (described above).

### 2.6. Experiment 3: randomization of open field size

In both the longitudinal developmental and the cross sectional age effect analyses the size of the open field was proportional to body size of the tested fish. However, one could argue that perhaps the absolute size of the open field itself could drive the observed behavioral changes. For example, a larger open area may induce elevated fear as fish in this environment may be more vulnerable to aerial predation and the increased fear may lead to tighter shoals, a typical antipredatory response in zebrafish (Miller and Gerlai, 2007). The purpose of the third experiment was to address this question, i.e. to investigate whether exposure to different sizes of open fields itself alters shoaling within the same age-group of fish. Naive groups of fish were exposed to different tank sizes as described in experiment 1, but instead of increasing tank sizes over time, exposure to the different arena sizes was randomized. As in experiment 1, a repeated measure design was used to expose the same groups of fish to different tank

sizes. Two age groups of fish were tested, 30 dpf (juvenile) and 60 dpf (adult).

### 2.7. Apparatus

For the fish that were 30 dpf at the time of testing, we used one arena that was identical in size to what was employed in experiment 1 (M: 150×150×50 mm, length×width×depth), referred to as the Medium sized tank. We also tested two smaller (XS=100×100×33 mm, and S=120×120×40 mm) and two larger (L=200×200×66 mm, and XL=250×250×83 mm) arenas. For the groups tested at their age of 60 dpf again we used an arena whose size corresponded to what was used in experiment 1 (M=450×450×83 mm) and we also tested two smaller (XS=300×300×100 mm, and S=400×400×133 mm) and two larger arena sizes (L=500×500×167 mm, and XL=600×600×200 mm). The linear dimensions of the medium sized arenas (M) for both age groups were 28 times the body length of the corresponding age group fish as employed before, however the extra small (XS) was 18 times, the small (S) 22 times, the large (L) was 36 times and the extra large (XL) was 45 times the body length of the corresponding age group fish.

### 2.8. Procedure

For each age cohort we tested ten groups of zebrafish with each group (shoal) containing ten individuals. Each of these shoals was housed separately, i.e. the holding density was ten fish per 3-liter tank. Once every 2 days each group was subjected to the open field task, a repeated measure design, as outlined below. The group of ten zebrafish was released from the center of the arena and the fish were allowed to freely explore the arena for 6 min. After the trial, the fish were returned to their home tank.

### 2.9. Behavioral recording and quantification

All sessions were recorded with a JVC HDD (JVC Everio Hard Drive GZ-MG750BU) overhead camera. Recorded digital files were converted to AVI format using Cyberlink Powerdirector. Still images were obtained for every 5 sec of the complete duration of the 6 min recorded trials. From each of the still images, the distances between a given fish from all other fish were measured and averaged for that given fish. Thus for a ten member shoal we obtained ten averages (one for each fish) and calculated the mean of the averages (the inter-individual distance) and its standard error, values that characterized the shoal itself. The quantification was performed using a custom software application developed in-house described in detail elsewhere (Miller and Gerlai, 2007). The software loaded the video file recorded, and a human observer inputted the sampling rate (which was 0.2 Hz, i.e. one image every 5 sec as mentioned above). The software then provided the observer with a still image at the requested starting time point and once the recording of the location of fish was completed from that given still image it advanced to the next image by the preset amount of time (i.e. by 5 sec). Recording of the location of each fish on the image was achieved manually, i.e. the human observer located the fish on the image and clicked on it with the mouse. Although labor intensive, this method is very precise given that the human observer is unlikely to be confused as to what represents the fish on the image and thus recording errors were minimal. Prior to locating the fish, the observer calibrated the area of the testing arena by overlaying a square outline on the boundaries of the tank. By providing the software with the measurements of the testing arena, it could then calculate the distances between the fish from the coordinate points extracted from each location. These distances quantified were subsequently exported to a text file for statistical analysis as described above.

### 3. Results

#### 3.1. Experiment 1: longitudinal developmental analysis of shoaling

The longitudinal analysis of the development of shoaling in freely moving zebrafish suggested a robust change in social behavior. At the youngest age tested zebrafish appeared rather dispersed: at age 7 dpf they exhibited an average distance from each other (Fig. 1, first data point) that equaled 13.22 body lengths ( $SD = \pm 1.009$ ). Previously, the average distance among shoal members of zebrafish in 10 member shoals was estimated to be approximately 4 body lengths in adult fish (Miller and Gerlai, 2008). The value we obtained for the 7 dpf zebrafish was significantly above this value ( $t = 25.85$ ,  $df = 7$ ,  $p < 0.001$ ). Does this large average distance observed in 7 dpf fish represent random distribution, increased repulsion (inter-individual distances above random chance), or some cohesion (shoaling, i.e. inter-individual distances below random chance)?

Unfortunately, determining what inter-individual distance value (the average distance between all possible pairs of fish within a shoal) would correspond to random chance has been a controversial and a complicated problem (Clark and Evans, 1954). Thus to address this question we have run a Monte Carlo simulation with parameters identical to our set up (10 fish in an arena measuring  $28 \times 28$  body length). We ran the simulation 10,000 times and plotted the results, which gave us a Gaussian (normal) distribution of average distance values with a mean equaling 14.6 body lengths and standard deviation of 1.7 (inset, Fig. 1). We compared our empirical results to the results obtained from this simulation (random chance) using the independent samples  $t$ -test (assuming unequal sample sizes and variance) and found that the empirical value we obtained for 7 day old zebrafish was significantly smaller than random chance ( $t = -5.784$ ,  $df = 18.19$ ,  $p < 0.001$ ). This suggests that although 7 dpf zebrafish do not form as tight shoals as adults do, these young fish are already attracted, albeit modestly, to each other. As a cautionary note, however, we also need to point out that our Monte Carlo simulation had no assumptions about any “rules” zebrafish may follow in their

environment. Fish must respond to numerous environmental stimuli other than those of their shoal mates. These stimuli may also influence the way fish distribute themselves. Systematic analysis of what environmental stimuli and how may influence the distribution of zebrafish in their environment (laboratory tanks or natural habitat) has not been conducted. Therefore, although the test tanks we employed presented a fairly homogeneous environment, it is possible that the distribution of fish in these tanks, even without any shoaling, could deviate from random chance.

Fig. 1 shows that the distance among shoal members decreases with age and reaches 5.81 body lengths at 76 dpf, a value very close to the one that was previously found optimal in adults in a study using high resolution temporal analysis of shoal cohesion (Miller and Gerlai, 2007). Repeated measure ANOVA confirmed this observation and revealed a significant increase of shoal cohesion with age (decrease of average distance among shoal members,  $F(8, 128) = 32.95$ ,  $p < 0.0001$ ). Post hoc multiple comparison tests including Tukey HSD are not appropriate for repeated measure designs. Furthermore, the periods between different age groups were not always consistent due to practical limitations. To avoid type one error and to make the age group comparisons more consistent, we conducted the following analysis. We identified four pairs of age groups for which the age difference between the groups was similar (7 vs. 26 dpf; 26 vs 42 dpf; 42 vs 59 dpf; and 59 vs. 76 dpf) and conducted four separate repeated measure ANOVAs (each with two levels) with a Bonferroni correction for multiple comparisons. The results showed a significant age effect for all ( $F_{7vs26}(1, 17) = 10.31$ ,  $p < 0.05$ ;  $F_{26vs42}(1, 17) = 15.92$ ,  $p < 0.01$ ;  $F_{42vs59}(1, 17) = 72.81$ ,  $p < 0.001$ ) but the comparison of 42 vs 59 dpf groups ( $F(1, 17) = 0.003$ ,  $p > 0.05$ ).

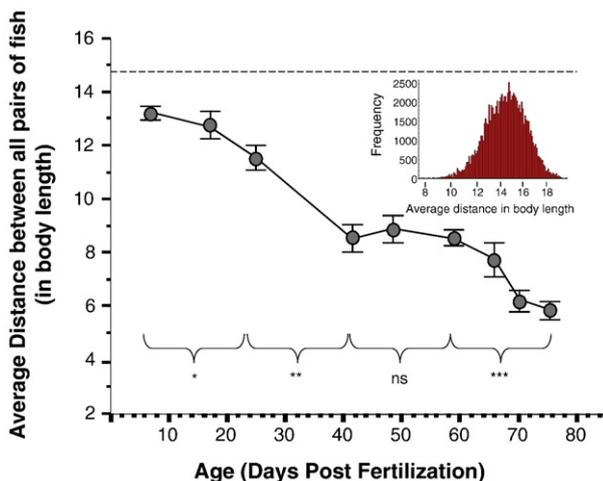
#### 3.2. Experiment 2: cross sectional analysis of age differences in shoaling

The first experiment showed a significant increase of shoal density over the course of the development of zebrafish. It is possible however, that repeated handling of the subjects (longitudinal study) affected shoal cohesion, for example, by inducing increased anti-predatory responses leading to tighter shoal cohesion as explained above. To exclude this temporal confound, we performed a cross sectional analysis in which each group of fish was tested (and handled) only once but multiple age groups were analyzed the same time in a randomized order. The developmental time points (age groups) corresponded approximately to those tested in the longitudinal analysis but we added two older groups to extend the age range.

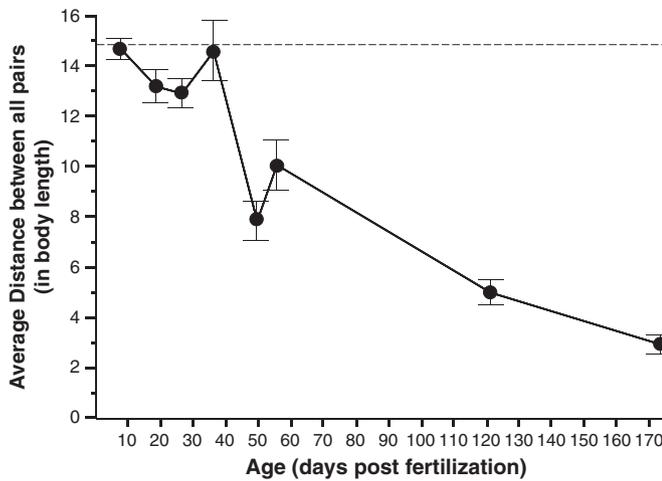
In general, the cross sectional analysis confirmed what we found before: a significantly increased shoal density (reduction of distance among shoal members) with age (Fig. 2) ANOVA showed this age-dependent change to be significant ( $F(1,7) = 23.121$ ,  $p < 0.001$ ) and post hoc Tukey Honestly Significant Difference (HSD) test revealed a significant difference in shoal density between various age groups. Fish tested at 7 dpf, 18 dpf, 26 dpf, and 36 dpf were not significantly different from each other (and they were also not different from random chance,  $|t| < 1.021$ ,  $df > 3$ ,  $p > 0.05$ ) but these age groups were significantly different from age groups tested at 49 dpf, 121 dpf, and 173 dpf ( $p < 0.05$ ) and these latter age groups were also significantly below random chance ( $|t| > 2.51$ ,  $df > 5$ ,  $p < 0.05$ ). Age groups in the upper age range (121 dpf, and 173 dpf) were found not to differ significantly from each other ( $p > 0.05$ ).

#### 3.3. Experiment 3: randomization of open field size

Although the second experiment confirmed that shoal cohesion increases with the age of zebrafish, this increase could still be due to two separate factors: one, increased social cohesion as the fish develop, or two, increased tank size. Throughout our experiments the tank size was kept proportional to the body length of the tested subjects and thus increased with the age of the fish. The rationale for



**Fig. 1.** The average distance between all pairs of fish within the shoal significantly decreases with the age of the fish, a longitudinal analysis. Mean  $\pm$  SEM is shown.  $n = 19$  shoals (each shoal consisting of 10 fish) were tested from 7 dpf to 76 dpf of age, i.e. on nine occasions. The arena size was kept proportional to the average body length of the fish ( $28 \times$  the body length) and the average distance between all different pairs of fish is also expressed in body lengths. The inset shows the results of a Monte Carlo simulation and shows the distribution of average distance between all pairs of fish in 10-fish shoals in case of random positioning of shoal members within the shoal. The mean of this distribution, i.e. random chance, is indicated on the main graph by the dashed line. Comparison between particular age points is indicated above the X-axis (ns = not significant, \* $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ). For further details see [Methods and Results](#).



**Fig. 2.** The average distance between all pairs of fish within the shoal significantly decreases with the age of the fish, a cross sectional analysis. Mean  $\pm$  SEM is shown.  $n = 8$  shoals with each shoal consisting of 10 fish. Each shoal was tested once. The arena size was kept proportional to the average body length of the fish (28x the body length) and the average distance between all different pairs of fish is also expressed in body lengths. For further details, including the results of post hoc Tukey HSD test, see [Methods and Results](#).

this was that the ability to traverse a set distance is believed to be proportional to the length of the fish and thus numerous investigators standardized according to body length of the subject (Gallego and Heath, 1994; Masuda et al., 2003; Vogel, 2008; Hale, 1999). However, as explained above, it is possible that altering the tank size itself may have driven the observed behavioral changes and that zebrafish respond to absolute rather than the relative tank size. To address the question whether increasing tank size increases shoaling (decreases the distance among fish), we conducted experiment 3 in which we tested two age groups of fish, a 30 dpf (juvenile) and a 60 dpf (adult) group.

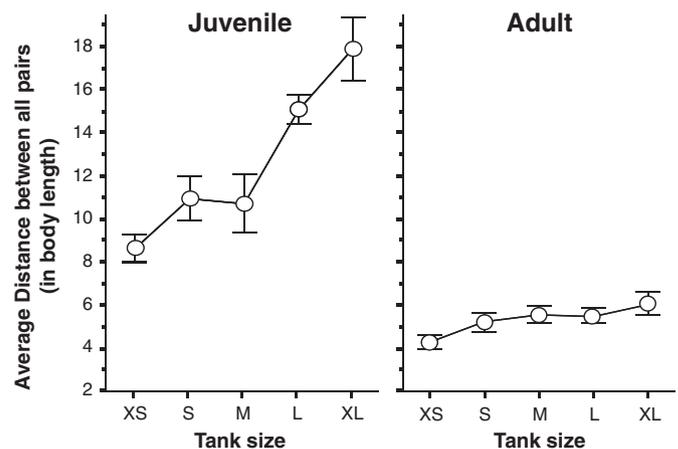
Neither age group analyzed showed significantly decreased average distances as the tank size increased (Fig. 3). In subjects aged 60 dpf the distances among subjects was not significantly different among the various tank sizes employed ( $F(1,4) = 2.486$ ,  $p > 0.05$ ), and the average distance was 5.28 body lengths, a value that corresponds well to what we have found both in the longitudinal (Fig. 1) and cross sectional (Fig. 2) analyses. The fish tested at 30 dpf exhibited a significant tank size dependent increase in average distance among shoal members (ANOVA,  $F(1,4) = 11.058$ ,  $p < 0.05$ , also see Fig. 3). Tukey HSD test showed that fish exposed to the largest tank ( $25 \times 25$  cm), compared to those in the smallest tank ( $10 \times 10$  cm) significantly ( $p < 0.05$ ) differed from each other. It is notable, however, that this change is opposite in direction to what we have observed in the longitudinal and cross sectional studies. Briefly, increasing tank sizes did not lead to decreasing average distance among shoal members in either age group studied. Why increasing tank sizes led to decreased shoal cohesion in the younger group of fish but not in the older is an interesting question from several viewpoints including a potential ecological aspect of this finding (perhaps younger fish need to disperse in larger volume of water to forage more efficiently for more homogeneously distributed food), and a potential fish husbandry viewpoint (what would be the ideal fish density and tank size one needs to use to achieve optimal foraging and growth rate in the laboratory?). Such questions will be addressed in future experimental analyses.

#### 4. Discussion

Developmental changes in shoaling have not been demonstrated in zebrafish. Zebrafish have previously been found to exhibit strong

preference for conspecifics (Saverino and Gerlai, 2008; Gerlach et al., 2007). For example, the sight of conspecifics has been shown to support associative learning and thus this stimulus was considered rewarding (Al-Imari and Gerlai, 2008). Social behavior (preference for particular conspecific color variant) was also shown to be influenced by early exposure to the given color variant (Engeszer et al., 2007). Here we describe, for the first time, maturation of social behavior, i.e. changes in shoaling, in developing zebrafish. Our results suggest that newly hatched zebrafish form only loose aggregates, groups in which the positioning of the individual fish is close to random chance. However, as the fish develop, shoal cohesion significantly increases. Importantly, our data also demonstrate that the increase of shoal cohesion is independent of repeated exposure to the test environment, i.e. could be observed both in a longitudinal as well as in a cross sectional study. Last, the age-dependent increase of shoal cohesion cannot be explained by differences in the size of the test chambers employed for the different age groups. In the younger (30 dpf) fish we found a negative correlation between shoal cohesion and tank size, and in the older age group tested (60 dpf) we found no correlation. Thus the increasing tank sizes we employed as the fish matured could not explain the age-dependent increase we observed in shoal cohesion. Therefore, we conclude that shoal cohesion significantly increases in zebrafish from the first few days of free swimming stage to adulthood.

What may be the ecological and evolutionary relevance of the age-dependent change in shoal cohesion of zebrafish is not known. It is possible that differential selection pressures may operate and differential ecological niches open as the fish mature. For example, larger piscivores may attack single prey when the prey reaches a particular size but may attempt to scoop up a group of prey when the prey is small Anderson, 2010; Barnes et al., 2010; Holmes and McCormick, 2009; Ioannou et al., 2009). Thus being in a tight shoal may only have advantages for larger prey fish, such as adult zebrafish, but not for smaller individuals. Similarly, being able to be close to potential mates has an obvious fitness advantage but only for adult, reproductively mature fish (Pitcher and Parrish, 1993). Furthermore, it is also likely that small microorganisms are more evenly distributed



**Fig. 3.** Increasing tank size does not decrease the average distance between all pairs of fish within the shoal. Mean  $\pm$  SEM is shown. Two age groups, 30 dpf juveniles (J) and 60 dpf adults (A) were exposed to different tank sizes in a randomized manner.  $n = 10$  shoals (with 10 fish in each shoal) were tested for each age group. Each shoal was tested in five differently sized tanks (XS, S, M, L, and XL). The medium (M) sized tank was identical to what was used in previous experiments, i.e. it was 28x the average body length of the fish tested. The arena sizes for juveniles were as follows: XS =  $10 \times 10$  cm, S =  $12 \times 12$  cm, M =  $15 \times 15$  cm, L =  $20 \times 20$  cm, XL =  $25 \times 25$  cm. The arena sizes for adults were as follows: XS =  $30 \times 30$  cm, S =  $40 \times 40$  cm, M =  $45 \times 45$  cm, L =  $50 \times 50$  cm, and XL =  $60 \times 60$  cm. Note that the increasing tank sizes led to significantly increased (but not decreased) average distance between all pairs of fish in the juveniles and increasing tank sizes had no significant effect on adults. For additional details see [Methods and Results](#).

than larger insects or swarms of plankton and thus being dispersed may be more optimal from a foraging perspective for younger smaller fish and less so for older larger fish (Anderson, 2010). Which of these, or perhaps what other, selection forces may drive the age-dependent increase of shoaling in zebrafish is an experimental question that empirical studies will address in the future.

Another important question concerns the mechanisms underlying the developmental change in shoal cohesion. These mechanisms are not known at this point. Developmental analyses, including anatomical and molecular characterization of changes usually focus on embryonic stages of zebrafish (Schweitzer and Driever, 2009; Holder and Xu, 2008), i.e. the period of development up to 5 dpf, the free swimming stage. Our behavioral results, however, suggest that potential changes beyond this stage of development may also be important to investigate. To address this question one may need to conduct a thorough neuroanatomy analysis looking for structural changes, or analysis of changes in gene expression using microarrays, or perhaps more targeted methods including RT-PCR and/or immunostaining for particular proteins such as neurotransmitter receptors. Analysis of neurochemicals (levels of neurotransmitters and their metabolites) may also be conducted. Clearly, identification of the mechanisms underlying the changes in shoaling behavior will not be easy and may require a number of multidisciplinary analyses. It may also be noted that higher temporal resolution sampling of the different age groups may allow one to detect particular periods during which development of shoaling is accelerated or decelerated. Focus on such periods, if exist, may also aid mechanistic analyses.

The last question we wish to discuss concerns forward genetics and drug screening. The zebrafish has been utilized particularly successfully in high-throughput mutation screens (forward genetics) (Patton and Zon, 2001) but more recently also in drug screens (Chakraborty et al., 2009). This is partly due to the prolific nature and easy and cost effective of maintenance of this species in the laboratory. The problem for behavioral brain research related investigations, however, has been the paucity of behavioral tasks (Sison et al., 2006), i.e. the limited availability of appropriate screening tools. The current work suggests that analysis of shoaling may be an important way one can test complex functional changes in the brain of zebrafish. Alteration of shoaling has been achieved using different environmental stimuli including the presence or absence of food, the presentation of a model of an aerial predator (Miller and Gerlai, 2007) as well as the delivery of the natural alarm substance of zebrafish (Speedie and Gerlai, 2008) suggesting that this behavioral response is modifiable and may be an appropriate readout for drug screens and perhaps mutation screens as well. Although the currently employed method to quantify shoaling is labor intensive, development of automated quantification of shoaling is underway. These new methods will enable the investigator to characterize the behavior of the shoal as a whole and also its individual members in a sophisticated manner. Once commercially available, these methods will allow medium to high throughput mutation and drug screens and thus will greatly facilitate the analysis of social behavior and its mechanisms.

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## References

Al-Imari L, Gerlai R. Sight of conspecifics as reward in associative learning in zebrafish (*Danio rerio*). Behav Brain Res 2008;189:216–9.  
Anderson JJ. Ratio- and predator-dependent functional forms for predators optimally foraging in patches. Am Nat 2010;175:240–9.

Barnes C, Maxwell D, Reuman DC, Jennings S. Global patterns in predator-prey size relationships reveal size dependency of trophic transfer efficiency. Ecology 2010;91:222–32.  
Bartz JA, Hollander E. The neuroscience of affiliation: forging links between basic and clinical research on neuropeptides and social behavior. Horm Behav 2006;50:518–28.  
Bell-Dolan DJ, Reaven NM, Peterson L. Depression and social functioning: a multidimensional study of the linkages. J Clin Child Adol Psych 1993;22:306–15.  
Carew TJ, Castellucci VF, Kandel ER. An analysis of dishabituation and sensitization of the gill-withdrawal reflex in *Aplysia*. Int J Neurosci 1971;2(2):79–98 Aug.  
Chakraborty C, Hsu CH, Wen ZH, Lin CS, Agoramorthy G. Zebrafish: a complete animal model for in vivo drug discovery and development. Curr Drug Metab 2009;10:116–24.  
Clark PJ, Evans FC. Distance to nearest neighbor as a measure of spatial relationships in populations. Ecology 1954;35:445–53.  
Dere E, Huston JP, De Souza Silva MA. The pharmacology, neuroanatomy and neurogenetics of one-trial object recognition in rodents. Neurosci Biobehav Rev 2007;31:673–704.  
Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, et al. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav Brain Res 2009;205:38–44.  
Engeszer RE, Alberici Da Barbiano L, Ryan MJ, Parichy DM. Timing and plasticity of shoaling behavior in the zebrafish, *Danio rerio*. Anim Behav 2007;74:1269–75.  
Flinn L, Bretaud S, Lo C, Ingham PW, Bandmann O. Zebrafish as a new animal model for movement disorders. J Neurochem 2008;106:1991–7.  
Gallego A, Heath MR. The development of schooling behavior in Atlantic herring *Clupea harengus*. J Fish Biol 1994;45:569–88.  
Gerlach G, Hodgins-Davis A, MacDonald B, Hannah RC. Benefits of kin association: related and familiar zebrafish larvae (*Danio rerio*) show improved growth. Behav Ecol Sociobiol 2007;61:1765–70.  
Gerlai R. High-throughput Behavioral Screens: the First Step towards Finding Genes Involved in Vertebrate Brain Function Using Zebrafish. Molecules 2010;15:2609–22.  
Gerlai R. Phenomics: fiction or the future? Trends Neurosci 2002;25:506–9.  
Gerlai R. Zebra Fish: an uncharted behavior genetic model. Behav Genet 2003;33:461–8.  
Gerlai R, Roder J. Female specific hyperactivity in S100 beta transgenic mice does not habituate in open-field. Behav Brain Res 1993;59:119–24.  
Gerlai R, Fernandes Y, Pereira T. Zebrafish (*Danio rerio*) responds to the animated image of a predator: towards the development of an automated aversive task. Behav Brain Res 2009;201:318–24.  
Griffiths S, Brockmark S, Höjesjö J, Johnsson J. Coping with divided attention: the advantage of familiarity. Proc R Soc Lond B 2004;271:695–9.  
Hale ME. Locomotor mechanics during early life history: effects of size and ontogeny on fast-start performance of salmonid fishes. J Exp Biol 1999;202:1465–79.  
Holder N, Xu Q. The zebrafish: an overview of its early development. Methods Mol Biol 2008;461:483–91.  
Holmes TH, McCormick MI. Influence of prey body characteristics and performance on predator selection. Oecologia 2009;159:401–13.  
Ioannou CC, Morrell LJ, Ruxton GD, Krause J. The effect of prey density on predators: conspicuousness and attack success are sensitive to spatial scale. Am Nat 2009;173:499–506.  
Kato S, et al. A computer image processing system for quantification of zebrafish behavior. J Neurosci Meth 2004;134:1–7.  
Keller ET, Murtha JM. The use of mature zebrafish (*Danio rerio*) as a model for human aging and disease. Comp Biochem Physiol C Toxicol Pharmacol 2004;138:335–41.  
Kimmel CB, Patterson J, Kimmel RO. The development and behavioral characteristics of the startle response in the zebrafish. Dev Psychobiol 1974;7:47–60.  
Ledesma JM, McRobert SP. Innate and learned shoaling preferences based on body coloration in juvenile mollies, *Poecilia latipinna*. Ethology 2008;114:1044–8.  
Liebowitz Michael R, Gorman Jack M, Fyer Abby J, Klein Donald F. Social phobia. review of a neglected anxiety disorder. Arch Gen Psychiatry 1985;42:729–36.  
Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. Nat Rev Genet 2007;8:353–67.  
Lin Y, Chen Y, Yang X, Xu D, Liang S. Proteome analysis of a single zebrafish embryo using three different digestion strategies coupled with liquid chromatography-tandem mass spectrometry. Anal Biochem 2009;394:177–85.  
Masuda R, Shoji J, Nakayama S, Tanaka M. Development of schooling behavior in Spanish mackerel *Scomberomorus niphonius* during early ontogeny. Fish Sci 2003;69:772–6.  
Meerlo P, Horvath KM, Nagy GM, Bohus B, Koolhaas JM. The influence of postnatal handling on adult neuroendocrine and behavioral stress reactivity. J Neuroendocrinol 1999;11:925–33.  
Miller N, Gerlai R. Quantification of shoaling behavior in zebrafish (*Danio rerio*). Behav Brain Res 2007;184:157–66.  
Miller NY, Gerlai R. Oscillations in shoal cohesion in zebrafish (*Danio rerio*). Behav Brain Res 2008;193:148–51.  
Morrell LJ, James R. Mechanisms for aggregation in animals: rule success depends on ecological variables. Behav Ecol 2008;19:193–201.  
Patton EE, Zon LI. The art and design of genetic screens: zebrafish. Nat Rev Genet 2001;2:956–66.  
Pauker CP, Ward DL, Sponholtz PJ, Hilwig KD. Effects of repeated hoopnetting and handling on bonytail chub. J Freshwater Ecol 2005;20:649–54.  
Pinsker H, Kupfermann I, Castellucci V, Kandel E. Habituation and dishabituation of the gill-withdrawal reflex in *Aplysia*. Science 1970;167:1740–2.

- Pitcher TJ, Parrish JK. Functions of shoaling behavior in teleosts. In: Pitcher TJ, editor. *Behavior of Teleost Fishes*. London: Chapman and Hall; 1993. p. 363–439.
- Reichow B, Volkmar FR. Social skills interventions for individuals with autism: evaluation for evidence-based practices within a best evidence synthesis framework. *J Autism Dev Disord* 2010;40:149–66.
- Saverino C, Gerlai R. The social zebrafish: behavioral responses to conspecific, heterospecific, and computer animated fish. *Behav Brain Res* 2008;191:77–87.
- Schweitzer J, Driever W. Development of the dopamine systems in zebrafish. *Adv Exp Med Biol* 2009;651:1–14.
- Sison M, Cawker J, Buske C, Gerlai R. Fishing for genes influencing vertebrate behavior: zebrafish making headway. *Lab Anim* 2006;35:33–9.
- Speedie N, Gerlai R. Alarm substance induced behavioral responses in zebrafish (*Danio rerio*) *Behav. Brain Res* 2008;188:168–77.
- Stoletov K, Klemke R. Catch of the day: zebrafish as a human cancer model. *Oncogene* 2008;27:4509–20.
- Tropepe V, Sive HL. Can zebrafish be used as a model to study the neurodevelopmental causes of autism? *Genes Brain Behav* 2003;2:268–81.
- Vogel S. Modes and scaling in aquatic locomotion. *Integr Comp Biol* 2008;48:702–12.
- Zhdanova IV. Sleep in Zebrafish. *Zebrafish* 2006;3:215–26.
- Zimmerman JE, Naidoo N, Raizen DM, Pack AI. Conservation of sleep: insights from non-mammalian model systems. *Trends Neurosci* 2008;31:371–6.