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Behavioral phenotyping in zebrafish: Comparison of three behavioral quantification methods

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The zebrafish has been popular in developmental biology and genetics, but its brain function has rarely been studied. High-throughput screening of mutation or drug-induced changes in brain function requires simple and automatable behavioral tests. This article compares three behavioral quantification methods in four simple behavioral paradigms that test a range of characteristics of adult zebrafish, including novelty-induced responses, social behavior, aggression, and predator-model–induced responses. Two quantification methods, manual recording and computerized videotracking of location and activity, yielded very similar results, suggesting that automated videotracking reliably measures activity parameters and will allow high-throughput screening. However, observation-based event recording of posture patterns was found generally not to correlate with videotracking measures, suggesting that further refinement of automated behavior quantification may be considered.

The zebrafish is a diploid vertebrate with a good balance of complexity and simplicity. It is small (4 cm long) and easy to keep and breed (Westerfield, 1993). Its aquarium environment is isolated from the experimenter's, which facilitates elimination of disturbing external stimuli. The zebrafish is prolific: A single female produces 200 eggs per spawning and can spawn several times a week. The fry grow quickly (reaching free-swimming stage within 5 days) and become sexually mature within 2 months (Detrich, Westerfield, & Zon, 1999). Large numbers of zebrafish can be kept in a small space, making the ease of housing comparable to that of invertebrate model organisms, such as *C. elegans* and *Drosophila*, and superior to that of other vertebrate species, such as the mouse or the rat. Coupled with these excellent features, its complex brain structure, similar in basic layout to other vertebrate brains, including our own (see Tropepe & Sive, 2003), and its sophisticated (albeit not yet well-utilized) behavior make the zebrafish an ideal model organism for neuroscience.

Several genes discovered in the zebrafish are evolutionarily conserved and have homologues in mammals, including our own species (e.g., Cerda, Conrad, Markl, Brand, & Herrmann, 1998). Syntenic relationships between regions of zebrafish and mammalian chromosomes are also known (Barbazuk et al., 2000; Woods et al., 2000). A large number of genetic tools allowing random or targeted introduction of mutations and the identification of the mutant genes are available. For example, genetic markers, linkage maps, and oligonucleotide microarrays aid localization and identification of randomly induced mutations (Donovan et al., 2000; Geisler et al., 1999; Guo et al., 2000; Hukriede et al., 1999; Knapik et al., 1998; Stickney et al., 2002; Zhang, Talbot, & Schier, 1998). Reverse genetic methods (McCallum, Comai, Griene, & Henikoff, 2000; Nasevicius & Ekker, 2000; Wienholds, Schultz-Merker, Walderich, & Plasterk, 2002) and the sequencing of the genome of zebrafish at the Sanger Center also favor this species, and most genetic tools and sequence information are in the public domain (e.g., Genbank [the Sanger Center Web site] and ZFIN; Sprague, Doerry, Douglas, & Westerfield, 2001). In sum, the genetics of the zebrafish place this species on par with the mouse or the fruit fly (Eisen, 1996; Granato & Nusslein-Volhard, 1996; Grunwald, 1996).

By now, hundreds of mutant zebrafish have been generated (see, e.g., Currie, 1996; Eisen, 1996; Grunwald, 1996; Haffter & Nusslein-Volhard, 1996; Holder & McMahon, 1996), but the majority of studies have focused on developmental questions (e.g., Canger et al., 1998; Concha & Adams, 1998; Detrich et al., 1999; Eisen, 1991, 1996; Fetcho & O'Malley, 1995; Schier, 1997). Only a very few have attempted to investigate the genetics of behavior or brain function, and most of these studies have focused on the perceptual systems: the visual system (Baier et al., 1996; Brockerhoff et al., 1996; Neuhauss, 2003; Neuhauss et al., 1999), the olfactory system (e.g., Kratz,

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Dugas, & Ngai, 2002), the auditory system, and inner ear functions (Bang, Yelick, Malicki, & Sewell, 2002; Granato et al., 1996; Malicki et al., 1996). The motor function of the zebrafish has been studied in the embryo (e.g., Fetcho & Liu, 1998; Liu & Fetcho, 1999; Lorent, Liu, Fetcho, & Granato, 2001), as well as in the adult (Gerlai et al., 2000). Motor responses in the context of circadian activity patterns have been investigated in small fry (Zhdanova, Wang, Leclair, & Danilova, 2001). Furthermore, analyses of conditioned place preference (Darland & Dowling, 2001) and alcohol-dependent strain differences in social behavior (Dlugos & Rabin, 2003) suggest that the zebrafish has a sophisticated behavioral repertoire and that functional changes of the brain, induced by drugs of abuse, can be detected at the behavioral level. Strain differences in the development of the zebrafish, due to early ethanol exposure, have also been demonstrated recently (Loucks & Carvan, 2004), and behavioral effects of such exposure have been shown (Carvan, Loucks, Weber, & Williams, 2004).

The above demonstrates that behavioral studies using the zebrafish have started and that this species has promise in forward genetics and in behavioral neuroscience in general. This optimism was also reflected in a recent symposium "Zebrafish, a New Behavioral Model System" organized by one of us (see www.noldus.webaxxs.net/mb2005/program/index.html) at the Measuring Behavior 2005 conference (Wageningen, The Netherlands). The speakers at the symposium agreed that using this species as the subject of forward genetics or as a model system for drug screening and toxicology will have great utility in behavioral neuroscience. Before one can fully utilize the zebrafish, however, appropriate phenotypical test methods must be worked out. Particularly important is the development of a methodology that allows fast and reliable detection of functional changes in the brain in a manner that may be scaled up for high-throughput applications.

One fruitful way to detect functional alterations of the brain is to conduct a behavioral analysis (see, e.g., Gerlai & Clayton, 1999). Successful examples demonstrating the utility of behavior-analysis–based mutation screening abound in other species (see, e.g., Byers, Davis, & Kiger, 1981; Levin et al., 1992). The goal of the present article is to conduct a proof-of-concept analysis and show that behavioral paradigms and behavioral quantification methods useful for high-throughput screening are also feasible in the zebrafish.

Four simple paradigms developed previously (Gerlai, Lahav, Guo, & Rosenthal, 2000) are employed: a novel open tank, a social preference task, an aggression test, and a predator model test. These paradigms represent a variety of test conditions under which we previously detected idiosyncratic behavioral responses induced by alcohol (Gerlai et al., 2000). Here, the different test conditions will allow us to ask how three recording methods are capable of quantifying test-specific behavior—that is, environmentally induced changes in behavior. The three behavioral quantification methods compared are (1) manual recording of swim location and locomotor activity, a method employed before with the zebrafish; (2) videotracking-based analysis of swim paths that allows automation of recording and, thus, scaling up, a necessary requirement for high-throughput testing; and (3) computer-aided analysis (event recording) of motor and posture patterns, a method often utilized by ethologists.

METHOD

Animals and Housing

One hundred eighteen adult, 3- to 6-month-old male and female zebrafish (*Danio rerio*) were used. All the fish were purchased from a local vendor (Pet Pacifica, Honolulu) and were of a genetically heterogeneous (randomly bred) stock whose exact origin is not known. Most of the fish exhibited the *long fin* phenotype to a varying degree. The disadvantage of undefined genetic heterogeneity is that such a stock is difficult to use for forward genetic (e.g., random chemical-mutagenesis–based) studies (large genetic variability), but the advantage of this stock is hybrid vigor (e.g., ease of maintenance) and similarity to natural wild populations in terms of phenotypical features. Furthermore, genetic variability and, thus, increased phenotypical variance may allow one to better investigate phenotypical correlations, one of the goals of the present study.

The fish were kept in groups of 20 for 2 weeks in 40-l quarantine tanks (50 × 30 × 26 cm, length × depth × width) and then moved to large 160-l (90 × 60 × 30 cm) home tanks, where they were kept in groups of 80 until the experiments started. Thermostat-controlled heaters maintained the water temperature at 26º in all the tanks, and the water was filtered by Fluval 204 (small tanks) or Fluval 404 (large tanks) canister filters that contained filter foam (mechanical filtration), activated carbon (removal of organic waste and small particles), and BioMax rings (biological filtration). The fish tanks were illuminated using fluorescent light tubes (20 W/tank) switched on at 7:00 h and off at 19:00 h. The tanks also received natural light (sunrise around 6:00 h and sunset around 19:30 h). The fish were fed twice daily with a 50/50 mix of ground TetraMin flakes (Melle, Germany) and freeze-dried krill (Aquatic Ecosystems, Apopka, FL).

General Experimental Procedures

The behavior of the fish was recorded between 10:00 and 17:00 h in four test paradigms similar to those described previously (Gerlai et al., 2000). The fish were tested first in the novel open tank and then in the group preference paradigm, followed by an aggression (mirror) test and, finally, a predator model task. This constant order of the tests was established previously in order to minimize interference among tests and to minimize environmental error variation (Gerlai et al., 2000). In each test, the fish were placed individually into the experimental tank (20 × 25 × 12 cm, length × depth × width) and were monitored for 10 min. The intertest interval was 2 min. Upon conclusion of all four tests, the fish were returned to their home tank and were kept there for future experimentation. A CCD camera (Panasonic WV-CP470) fed the live image (frontal view) into the computer (Dell Dimension 8300, Pentium IV), and this image was processed using the EthoVision 3.0 video-tracking software (Noldus Information Technology, Wageningen, The Netherlands). A second camera (Sony DCR TRV 70) was used to record the frontal view of the experimental tank onto MiniDV tapes. The videorecordings on these tapes were later downloaded onto the computer and were analyzed using manual activity and location quantification, and also with The Noldus Observer Color Pro software.

Behavioral Tests

Novel open tank. Exposure to a novel test chamber, as well as handling by the experimenter, is an inherent part of most laboratory animal behavioral tests. The novel open tank task is intended to analyze behavior in response to these factors. In this task, zebrafish may exhibit elevated activity that habituates with time (Gerlai, 2003),

and they may also show fear-related behaviors (Gerlai et al., 2000). These behavioral responses have previously been investigated with crude manual recording of locomotory activity and swim location. In addition to this method, both computerized event recording of motor and posture patterns and detailed videotracking-based analysis of swim path patterns are performed on all subjects.

The experimental tank was filled with mature fresh water that was aged and biologically filtered and had previously been exposed to zebrafish. The tank was illuminated from above by two 13-W fluorescent lightbulbs, and the test room was kept dark to obscure the external environment. Three sides of the tank were covered by a gray cardboard paper. The experimental fish was placed singly in the small experimental tank (21 × 12 × 24 cm), and after a 20-sec period, its behavior was recorded for 10 min. Upon completion of the recording, the fish was gently removed and placed in a small holding chamber.

Group preference. The zebrafish is a highly social species. It forms schools, a group of individuals that swim close to one another. Individual zebrafish are expected to be motivated to join a school. This preference for the group, also termed *group cohesion*, formed the basis of a behavioral test in which the effect of alcohol was investigated (Gerlai et al., 2000). The present test is a modification of this previously employed paradigm. After the novel open tank test, the experimental fish was removed from the test tank, was held in a small container for 1 min, and was placed back into the test tank. The partitions on the right and left sides of the experimental tank were removed to allow unobstructed view of two adjacent stimulus tanks. One of these tanks contained 10 stimulus fish, conspecific zebrafish of the same size and age as those of the test subject, and the other tank contained only fresh water but no stimulus fish. The positioning of the stimulus fish—that is, whether they were presented on the left or the right side of the experimental tank—was randomly balanced across experimental fish. Behavior was recorded for 10 min. At the end of the test, the experimental fish was gently removed and again placed into a small holding container until the next recording session (the aggression test) started.

Aggression test (the inclined mirror task). Solitary zebrafish encountering another individual often exhibit agonistic behavior, a response different in form and alcohol dose response characteristics from social behavior (Gerlai et al., 2000). Agonistic behaviors were tested following the group preference task. The partitions were returned to the sides of the experimental tank, and a mirror was placed behind the tank at a 22.5º angle to the back of the aquarium in such a way that the mirror image on the left side appeared closer and that on the right side appeared farther away. Since solitary fish of the same gender encountering each other often exhibit agonistic behaviors, rather than group cohesion, the "approaching" mirror image would be expected to elicit aggression. The behavior of the zebrafish was monitored in this test again for 10 min. The rationale for the positioning of the mirror was that it provided a lateral view of the "opponent," a sight that best elicits aggression (Gerlai et al., 2000). Furthermore, it allowed the experimental fish to view its "opponent" from closer or farther away while swimming along the longitudinal axis of the tank, which we expected would allow the experimenter to better quantify aggressive tendencies. Following the test, the experimental fish was again placed in a small container until the predator model test started.

Predator model test. The antipredatory behavior of the zebrafish is believed to be adaptive and, thus, likely to be under the influence of genetic factors (Csányi, 1986; Gerlai, 1993). Furthermore, predator-model–elicited behavioral responses have been shown to be dependent on level of exposure to alcohol (Gerlai et al., 2000). These features suggest that predator-elicited responses are phenotypical characteristics that will allow the detection of mutational or pharmaceutical-agent–induced functional changes in the brain.

In the present study, a predator model similar in size and shape to that used before by Gerlai et al. (2000) was employed. The model was made of a 50-ml falcon tube that was filled with charcoal and water (and thus appeared black). The model had two plastic "eyes" (diameter, 8 mm; white "iris" and black "pupil") glued to the conical end of the tube. The right and left partitions were once again removed from the experimental tank, and the predator model was placed into the stimulus tank adjacent to the experimental tank and was moved using a transparent plastic rod attached to its back during the 1st and 10th minutes of the 10-min-long recording session. The positioning of the stimulus presentation was identical to the one used in the group preference task. That is, if the experimental fish was allowed to view a group of conspecifics on the right side of its experimental tank in the group preference test, for example, the predator model was also presented on that side.

Quantification of Behavior

Manual quantification. The method was similar to those published previously (Gerlai et al., 2000). The zebrafish were analyzed using manual quantification of the location and locomotor activity of the fish. Videotapes were replayed on a Sony (DVCAM, DSR-11) MiniDV digital cassette player connected to a 14-in. JVC TV monitor. A transparency with a grid pattern was placed on the monitor. Using The Observer software, the experimenter recorded the duration of time the fish spent in the upper or the lower half of the tank and on the left and the right sides of the tank. The time that the fish spent on the side opposite to the stimulus tank that contained the stimulus fish or the predator model and the time that the fish spent in the upper half of the tank were statistically analyzed. In addition, the total number of times that the fish entered the left, the right, the upper, and the lower halves of the tank was also analyzed, which served as a measure of general locomotor activity.

Videotracking. Videotracking was accomplished using the EthoVision Color Pro (Version 3.0) software (Noldus), an approach that was expected to allow quantification of swim path patterns, including the location and locomotor characteristics of the fish, more precisely than a manual recording method could, and without the need for the experimenter to view videotapes. The EthoVision software was configured to accept live input from a video camera fed directly into the computer. The signal from the camera was fed through a piccolo video card and was read by EthoVision. Before each test, a background image was recorded of the empty experimental tank. After the subject had been placed in the tank, the program compared each incoming image sample with the original background. Image samples were taken at a rate of 10/sec. The software was configured to use a subtraction method of stimulus detection: The pixel values of each new sample image were subtracted from those of the background image, and discrepancies were detected. Detection threshold levels, the minimum difference between the values of 2 pixels accepted by the computer, were also set to minimize environmental noise (from water droplets, reflections, bubbles, etc.). Surface area was also recorded, defined as the number of adjacent pixels with differences above noise threshold. The pixel cluster with the largest surface area was interpreted as an "object," corresponding to the experimental subject, and the *x*-, *y*-coordinates of the center of that object were recorded. If no object of at least 25 pixels was located, the program recorded the coordinates of the last known location of the object (fewer than 5% of the samples). Tracks were recorded for the full 10 min of the test period. After recording was complete, the tracks were visually inspected for artifacts, and these were removed manually. This correction procedure was particularly important in the analysis of behavior in the aggression test, where the mirror image of the test fish was occasionally confused by the software with the actual subject (for further details and implications, see the Results and Discussion sections).

The following parameters were quantified.

1. *Mean distance from bottom*. The distance of the experimental fish from the bottom of the tank was measured every 0.10 sec, and the average distance was calculated for the 10-min recording

session in each task. This measure was chosen because previous observations (e.g., Gerlai et al., 2000) had suggested that proximity of zebrafish to the bottom of the tank may represent a good measure of fear versus habituated state.

2. *Mean distance from stimulus*. The distance of the experimental fish from the glass wall of its test tank adjacent to the stimulus tank was recorded every 0.10 sec, and the mean of these distance values was calculated for the entire session length. Note that the stimulus (the group of conspecifics or the predator model) was presented at the same side for a given experimental fish but that the side changed randomly among experimental fish. Also note that in the novel open tank, no stimulus was presented on either side of the tank and that the side from which distance was quantified was chosen so as to be the same as the one in which the group of stimulus fish or the predator model would be presented. This distance measure was chosen because it allowed us to analyze social cohesion, aggression, and the effect of the predator model.

3. *Total distance moved*. To quantify locomotor activity, the total distance moved by the experimental fish was recorded. Quantification of all the distance measures was conducted after calibration of EthoVision by inputting the actual dimensions of the test tank. The distance measures are expressed in centimeters.

In addition to these measures, we also quantified the *mean heading direction* and the *mean turning angle*. Mean heading, a measure of direction of movement, is defined as the angle of movement, relative to the vertical line. The subject's location was measured every 0.10 sec, and a vector was calculated between that location (n) and the most recent point ($n-1$). The average angle of this vector, relative to the vertical reference line, was taken for the entire session. Thus, 0º means movement straight upward, 180º means straight downward, 270º is horizontally toward the stimulus, and 90º means movement in the opposite direction (i.e., away from the stimulus). Turn angle is a measure of a tendency to change direction of movement, most prevalent in erratic (zig-zagging) or thrashing behaviors. Turn angle is calculated as the difference between two consecutive heading calculations, taken every 0.10 sec and averaged across the session.

Event recording. The behavioral measures described above for our videotracking analysis may not quantify motor and posture patterns. Usually, observation-based event recording is conducted for such purpose. The latter is based on one of the fundamental tenets of ethology, which postulates that an apparently continuous stream of behavior can be broken down into mutually exclusive distinct successive motor patterns that represent species-specific units of behavior (Huntingford, 1984). Indeed, we have successfully employed this approach in different species, including fish (e.g., Gerlai, Crusio, & Csányi, 1990) and rodents (Gerlai et al., 1993). The Ethogram—that is, a complete list of species-specific motor and posture patterns—is not yet established for the zebrafish. Here, we recorded and quantified only six basic simple motor patterns that could be easily recognized and distinguished using The Observer event-recording software (Noldus). We acknowledge that, potentially, there are a large number of motor and posture patterns of the zebrafish that one may be able to define, differentiate, and quantify, but we also argue that the six behavioral units we recorded here are sufficient for our proof-of-concept analysis.

The following behavioral units (motor and posture patterns) were recorded: *swimming* (continuous locomotion with the use of the pectoral and caudal fins), *thrashing* (forceful back-and-forth swimming against the glass wall of the fish tank), *floating* (fish is stationary or is moving very slowly without using its caudal fin; pectoral, dorsal, and anal fins may open and close, or beat, with a low and stable frequency [no more than 1 beat/sec]), *freezing* (a motionless state during which only the gills and, occasionally, the eyes may move, which occurs mostly while the fish is on the bottom, in a corner, or right below the water surface), *erratic movement* (fast [more than 3 cm/sec swim speed] and seemingly aimless zig-zagging with frequent changes of the direction of swimming, which, often, occurs in the bottom of the tank but can be seen in midwater as well), and *creeping* (slow [less than 1 cm/sec speed] movement during which the caudal dorsal and anal fins are motionless and only the pectoral fins beat, most often observed after freezing and/or erratic movement). The duration, relative to session or interval length (%), was calculated for all the behavioral units.

Statistical Analysis

The analysis of the data was conducted using SPSS (Version 12.0.1 for the PC). Behavior of fish across multiple test situations was analyzed using a repeated measures ANOVA. In case of significant results, differences across test situations were further analyzed using the post hoc Tukey honestly significant difference (HSD) test. To investigate potential correlations among swim path parameters and motor and posture patterns, bivariate Pearson correlation coefficients were calculated, and the correlation matrices were subjected to a principal component analysis (PCA). The component matrices were subjected to Varimax rotation with Kaiser normalization. Retention of components was set at the minimum eigenvalue of 1.

RESULTS

The zebrafish exhibited different behavioral responses to the four test situations. These differences were detected similarly by the manual recording of the location and activity of the fish and by computerized videotracking. The results from the manual quantification of the location of the fish are shown in Figure 1A. According to these results, the fish spent about 50% of their time in the upper half of the tank in the novel open tank and in the aggression task, whereas the fish spent more than 60% of their time in the upper half of the tank in the group preference and the predator model tasks, a significant difference

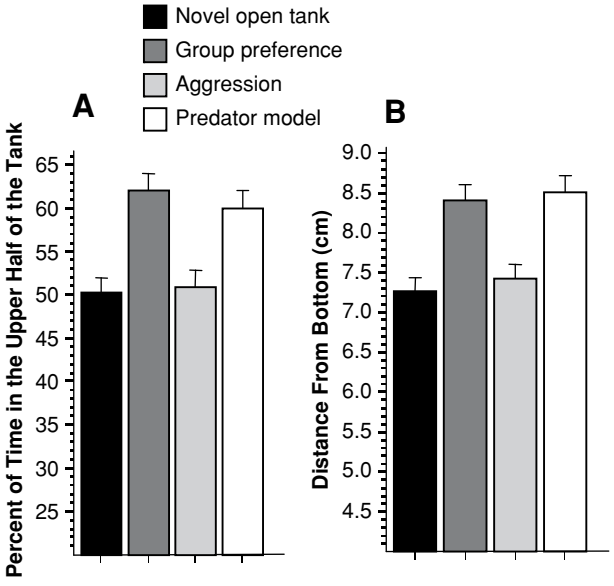

Figure 1. The zebrafish spent differing amounts of time near the surface of the water, depending on the test situation. (A) Percentage of time the fish spent in the upper half of the tank (manual recording). (B) Distance from the bottom (videotracking-recorded data). Means + *SE* are shown. Sample sizes are indicated in the text. Note the highly similar pattern of results obtained with the two recording methods.

among tests [test situation, $F(3,351) = 17.89$, $p < .001$]. A post hoc Tukey HSD multiple comparison confirmed the results and showed that the fish spent significantly ($p < .01$) less time in the upper half of the tank in the novel open tank and in the aggression task than they did in the other test situations. The pattern of results obtained with this method is highly comparable to that obtained with the use of videotracking. Figure 1B shows the results for the behavioral measure *distance from bottom*, quantified using the videotracking software. This measure is obtained by recording the distance of the fish from the bottom of the tank (in centimeters) every 0.10 sec and obtaining the average of the distance values for the entire session (mean distance from bottom). The results show again that in the novel tank and in the aggression task, the fish stayed closer to the bottom (smaller values), whereas they stayed closer to the surface in the other two tasks. An ANOVA supported these observations [$F(3,351) = 23.07$, $p < .001$], and a Tukey HSD test also showed that in the open tank and aggression task, the fish were closer to the bottom than they were in the other two tasks ($p < .01$). The primary goal of the present study was to compare different behavioral quantification methods and to determine whether these methods could detect environmentally induced test-specific behavioral differences similarly. The differences in behavioral responses to the four test environments have been discussed before (Gerlai et al., 2000). Thus, here, we will only briefly state that the time spent near the surface and the differences in this measure are likely species-specific characteristics of zebrafish and are not due to biased stimulus positioning. For example, the increased time spent near the surface in the group preference task was not the result of the fact that the stimulus fish were near the surface. These fish were presented in a small stimulus tank, and their distribution in the tank was fairly homogeneous. Similarly, the predator model was also presented in the middle layers of the water.

Figure 2 shows the results that reflect the distance of the experimental fish from the side of its tank adjacent to the stimulus presented—that is, the side where the group of conspecifics, the predator model, or the closest view of the mirror image in the aggression test was. In the case of manual recording (Figure 2A), the experimenter quantified the amount of time the fish spent in the half of the tank opposite to the stimulus presentation side. An ANOVA showed significant differences across test situations [$F(3,351) = 199.92$, $p < .001$]. Not surprisingly, the fish in the novel open tank (no specific stimulus presented on either side) spent 50% of their time in each half of the tank. Presentation of a group of conspecifics dramatically reduced the time spent in the opposite half of the tank; that is, the experimental fish moved closer to the group of stimulus fish (Tukey HSD, $p < .01$). The analysis of response to the mirror image (aggression task) showed that the experimental fish had no side preference (Tukey HSD, $p > .05$, in comparison with performance in the novel open tank). This was somewhat surprising, since previously (Gerlai et al., 2000), we had observed a robust preference of the test fish to stay close to its mirror image, a discrepancy with respect to the present result that may have been due to differences in our present experimental setup (a smaller tank, leading to a smaller distance change

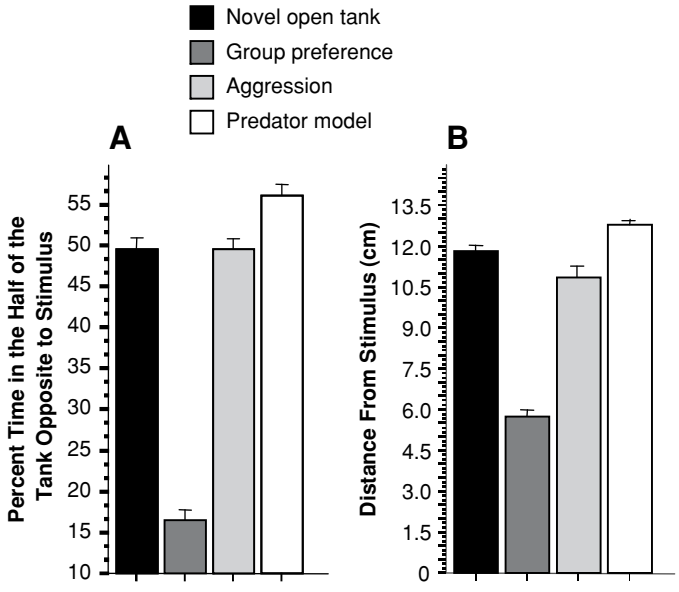

Figure 2. The zebrafish spent differing amounts of time near the target stimulus. (A) Percentage of time the fish spent in the half of the tank away from the stimulus (manual recording). (B) Distance from stimulus (videotracking-recorded data). Means + *SE* are shown. Sample sizes are indicated in the text. Note the similar pattern of results obtained with the two recording methods.

between the test subject and its mirror image from one side of the tank to the other). Last, it is notable that the fish in the predator model task spent significantly more time in the opposite side of the tank—that is, away from the predator model (Tukey HSD, $p < .05$, in comparison with all the other test situations). Importantly, the pattern of results above is closely replicated by the computerized videotracking analysis. Here, the actual distance from the stimulus was quantified precisely. An ANOVA again showed a significant test situation effect [$F(3,351) = 568.94$, $p < .001$], and a post hoc Tukey HSD test confirmed that the distance of the experimental fish from the stimulus was smallest in the group preference task ($p < .01$, in comparison with all the other tests) and largest in the predator model test ($p < .01$, in comparison with all the other tests).

Figure 3 shows the results reflecting the locomotor activity of the zebrafish in the four test paradigms, quantified using two methods: the manual recording (Figure 3A) and the videotracking technique (Figure 3B). The two methods employed were manual recording of shuttling activity among the four quadrants of the tank and videotracking, which measured total distance moved. These measures were chosen for the purpose of comparison, because shuttling activity is often used as a crude measure of locomotor activity in numerous species, including zebrafish (see, e.g., Gerlai et al., 2000), but the most precise way to quantify amount of locomotion is to measure the actual distance moved, using videotracking. Thus, our question was whether the labor-intensive and approximate manually recorded measure would correlate with the precise computerized quantification parameter. Locomotor activity, measured as the total number of transitions among the four quadrants (upper left, upper right, lower left, and lower right) of the test tank (shuttling activity), showed significant differences from test situation to test situation [$F(3,351) = 42.72$, $p < .001$], with activity being highest in the novel open tank (Tukey HSD, $p < .05$, in comparison with all the other test situations) and lowest in the group preference task (Tukey HSD, $p < .01$, in comparison with all the other test situations). The pattern of results obtained with videotracking was fairly similar to this, with one exception: The actual total distance moved, measured in centimeters, was much higher in the group preference task than would have been predicted on the basis of the results obtained with the manual recording method. Although an ANOVA detected significant differences among test situations [$F(3,351) = 22.23$, $p < .001$], as did the manual-recording findings, a post hoc Tukey HSD test did not show the activity level of the fish in the group preference task to be different from that of all the other groups; in fact, activity in this task was significantly

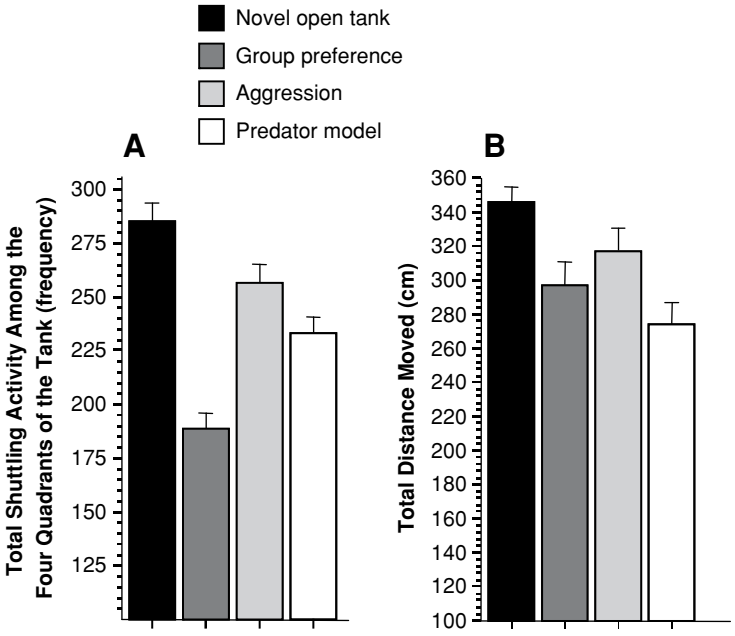

Figure 3. Locomotor activity of the zebrafish as measured by (A) total shuttling activity (frequency of entry to each of the four quadrants of the tank: upper left, upper right, lower left, and lower right) recorded manually and (B) total distance moved recorded by the videotracking software. Means + *SE* are shown. Sample sizes are indicated in the text. Note again that the pattern of results (the test-specific differences) obtained with the two recording methods are comparable. Also note that the total distance moved was erroneously quantified for the aggression task by videotracking, due to technical problems (see the text), and that the detected swim paths had to be manually corrected before analysis.

($p < .05$) different (smaller) only when compared with the novel open field activity level. This discrepant finding was due to the fact that the fish in the group preference task spent most of their time swimming in the half of the tank closer to the stimulus fish and, thus, performed less shuttling activity between the left and the right sides of the tank. This resulted in a reduced value's being recorded by the manual method. But given that these fish were actively moving, trying to join their schoolmates in the other tank (see below), the videotracking system did not detect, and correctly so, such a dramatic reduction of locomotor activity as that found with the manual-recording method.

Figure 4 shows the results for mean heading direction, quantified using the videotracking software. No significant differences were found among test situations [$F(3,351) = 0.91, p > .40$]; that is, all values were around 180º, demonstrating that, on average, the fish swam in all directions in each task. Figures 4B and 4C show heading direction during the first and last (10th) minutes of the session in each task. The results suggest that heading direction will be a useful measure for more refined data analysis aimed at shorter time intervals preceding or following the presentation of particular stimuli. For example, in our analysis, an ANOVA revealed a significant test dif-

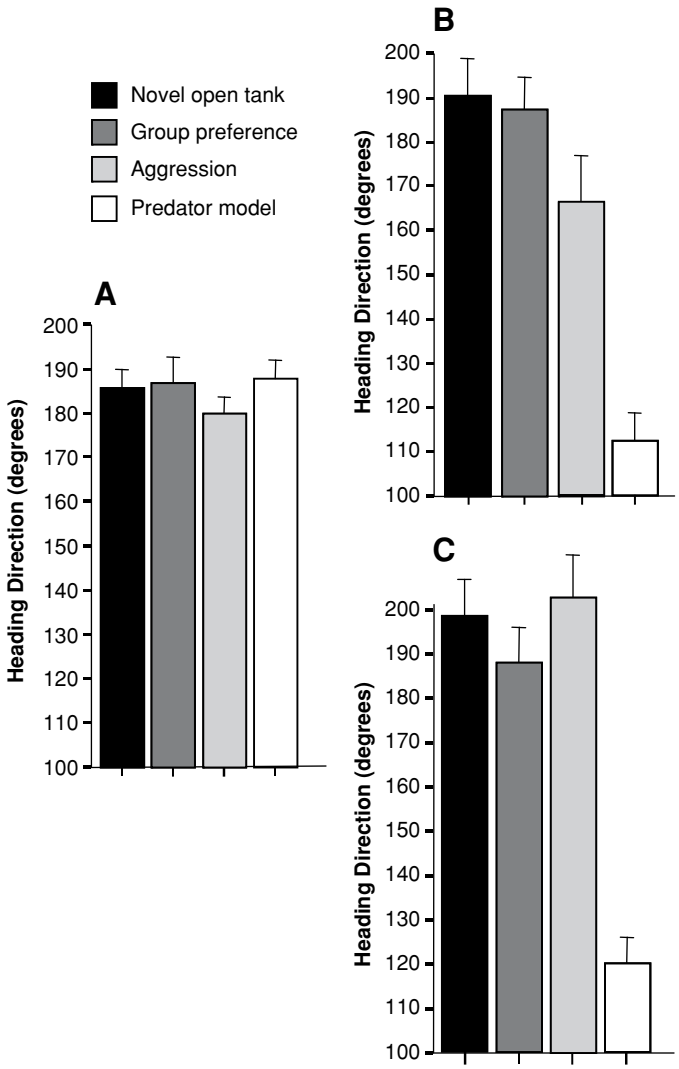

Figure 4. Heading direction quantified by videotracking, in the four test situations. Means + *SE* are shown. Sample sizes are given in the text. (A) The overall (average) heading direction throughout the entire session. Panels B and C show heading direction recorded for the 1st and the 10th (last) minutes of the session, respectively. Note the lack of significant difference in overall heading direction. Also note the significantly reduced values during the first and last minutes of recording in the predator model test.

ference for both the first [$F(3,351) = 21.27$, $p < .001$] and the last [$F(3,351) = 23.13$, $p < .001$] minutes of the session, a finding that was due to the significant reduction of heading direction values in the predator test, in comparison with the other situations, during the first and the last minutes of the test (Tukey HSD, $p < .01$), demonstrating that during the presentation of the predator model (1st and 10th min), the zebrafish tended to head away from the stimulus presentation side.

The mean turning angle (Figure 5), reflecting the angular change in swimming direction, was largest in the two paradigms associated with social interaction—that is, the group preference and the aggression tasks. The differences were significant [ANOVA test effect, $F(3,351) = 111.61$, $p < .001$; Tukey HSD test: group preference and aggression test values differed from those for the other two tasks at $p < .01$, and no other differences were significant at $p = .05$]. According to our personal observations, the increased turning angle may reflect the intense thrashing (swimming against the glass—i.e., toward the group of conspecifics in the group preference task; see Figure 7C) and the aggressive display dance (not quantified) often observable in the aggression task.

In addition to the manual and videotracking-based analysis of the location and activity parameters of zebrafish behavior, we were also interested in the quantification of motor and posture patterns. These patterns are characteristic of the movements of zebrafish and may reflect unique features not captured by the traditional activity parameters. The Ethogram—that is, the list of characteristic species-specific motor and posture patterns—of zebrafish is not established yet. Here, we used only six basic motor and posture patterns for the sake of addressing the principal question: Are motor/posture patterns (recorded by event recording) and the activity parameters (recorded by videotracking) redundant measures of behavior?

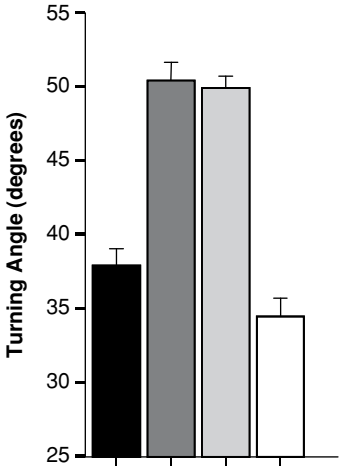

Figure 5. Turning angle quantified by videotracking in the four test situations. Means + *SE* are shown. Sample sizes are given in the text. Note the robust turn angle increase (smaller turning radius and, thus, increased angular change in movement) in the group preference and aggression tasks.

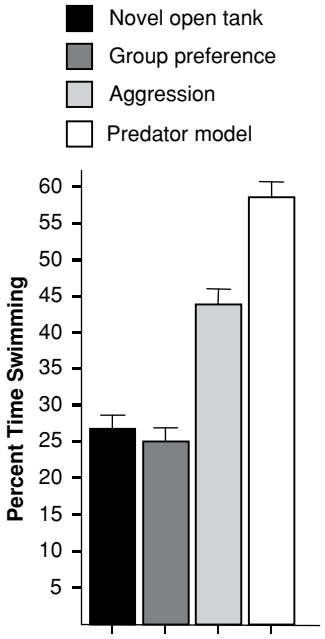

Figure 6. Percentage of time the zebrafish swam in the four test situations. Means + *SE* are shown. Sample sizes are given in the text. Note that this behavioral measure was quantified using observation-based event recording. Also note that the test-specific differences show a pattern dissimilar to the activity parameters recorded by videotracking.

Figure 6 shows the results of quantification of *swimming*. This active locomotory response is differentiated from another active swim pattern termed *thrashing* (see Figure 7). Swimming differs in form and, perhaps, even in what behavioral state it represents, from thrashing. Swimming is a more relaxed locomotion, whereas thrashing is more forceful, and the latter is always directed toward the glass wall (i.e., the fish swims back and forth, with its head pushed against the glass). The analysis of swimming revealed a significant effect of test situation [$F(3,351) = 137.27$, $p < .001$]. A post hoc Tukey HSD test showed that the value of swimming was highest in the predator model test, which differed from the second highest value obtained in the aggression test ($p < .01$), and the latter also differed significantly ($p < .01$) from the group preference and novel open tank values.

The fish performed significantly different amounts of thrashing in the four test paradigms [Figure 7A; $F(3,351) = 120.92$, $p < .001$], and the pattern of differences for thrashing was highly different from that for swimming. Thrashing (Figure 7A) was highest in the group preference paradigm and lowest in the predator model test (Tukey HSD, $p < .05$; all the groups differed from each other). When we define thrashing, we refer to the form—that is, the appearance—of the behavior. However, we acknowledge that this motor pattern may represent different aspects of zebrafish behavior, depending on the direction of thrashing—that is, whether it

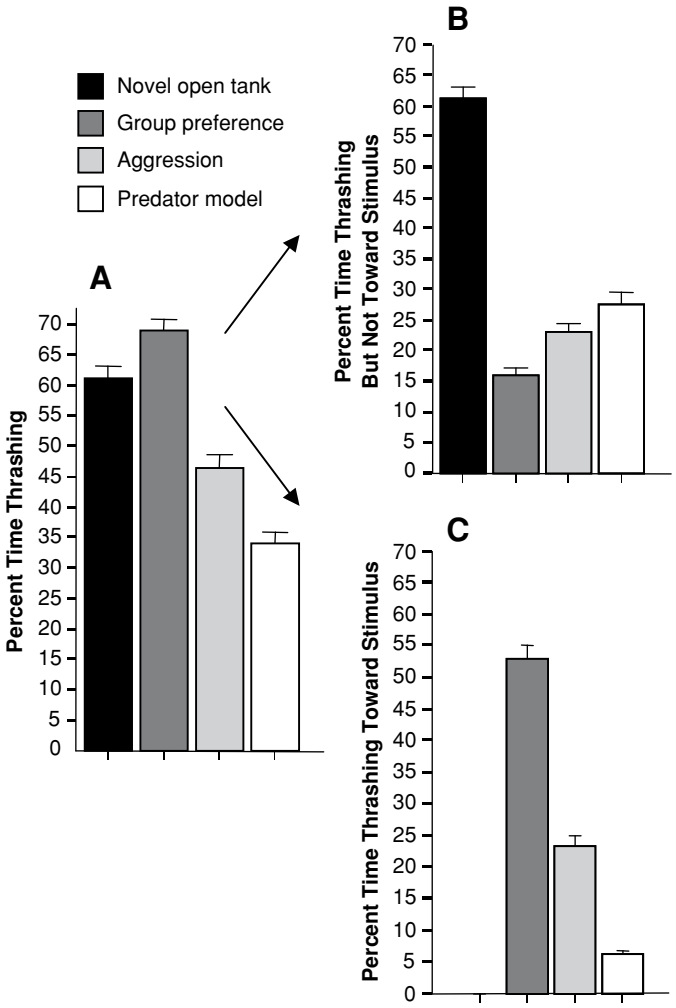

Figure 7. Percentage of time the zebrafish performed thrashing in the four test situations. Means + *SE* are shown. Sample sizes are given in the text. Panel A shows thrashing irrespective of where it occurred. Panel B shows thrashing occurring near all the glass walls but the one adjacent to the stimulus tank. Panel C shows thrashing occurring near the glass wall adjacent to the stimulus tank. Again note that the pattern of differences among thrashing values recorded in the four test situations do not appear to correspond to patterns of activity parameters recorded by videotracking.

is performed toward or away from the stimulus presented. These two possibilities were distinguished in the analysis and are presented in Figures 7B and 7C. An analysis of thrashing on all the glass walls but the one adjacent to the stimulus showed significant test-situation–dependent differences [$F(3,351) = 209.84$, $p < .001$]. However, here, the value was smallest in the group preference task (Tukey HSD, $p < .05$, in comparison with all the other test situations). Similarly, the thrashing shown in Figure 7B is smaller, and not larger (as is shown in Figure 7A), in the aggression task, in comparison with the value obtained in the predator model task (Tukey HSD, $p < .05$). Clearly, the differences between the values shown in panels A and B of Figure 7 were due to the fact that the fish performed thrashing toward or away from the stimulus in a task-dependent manner (Figure 7C). The analysis of thrashing toward the stimulus (Figure 3C) showed a significant [$F(3,351) = 220.25$, $p < .001$] test situation effect, and a Tukey HSD test confirmed that the fish performed significantly differently in each test situation, with the largest values in the group preference task and the second largest in the aggression test. That is, a large proportion of the thrashing in the group preference task, and also in the aggression task, was directed toward the stimulus, since the experimental fish were attempting to get closer to their school mates or their opponents. In the novel open tank, no specific external stimulus was presented, and thus no thrashing toward the stimulus was recorded. Last, in the

predator model task, thrashing was rarely performed by the test fish toward the stimulus side, since the experimental fish tended to avoid this side. However, given that the predator model was presented only for the first and the last minutes of the 10-min session, some thrashing toward the stimulus side did occur.

Figure 8 depicts motor and posture patterns that occurred rarely or for only short periods of time. These include erratic movement (Figure 8A), floating (Figure 8B), creeping (Figure 8C), and freezing (Figure 8D). Despite the low occurrence and, thus, the relatively higher variability, in comparison with the mean, these behaviors also showed test-dependent significant differences [erratic movement, $F(3,351) = 30.24$, $p < .001$; creeping, $F(3,351) = 7.67$, $p < .001$; floating, $F(3,351) = 3.45$, $p < .05$; freezing, $F(3,351) = 3.41$, $p < .05$]. Tukey HSD post hoc analyses showed a significantly ($p < .05$) higher erratic movement in the novel open tank than in all the other tests, a significantly elevated ($p < .05$) amount of creeping in the predator model task, in comparison with all the other tests, and significant ($p < .05$) differences in floating and freezing between the novel open tank and the predator model test.

The means obtained with videotracking and event recording show an apparently different pattern across tests. That is, unlike in the case of videotracking and manual recording, generally no correlation is evident. Nevertheless, it is possible that behavioral measures obtained with videotracking and event recording covary at the interindividual level. To address this question, we analyzed bivariate Pearson correlation coefficients. Given the number of variables and test situations, the correlation matrix obtained was large (it contained $[n \times (n-1)]/2 = 946$ bivariate correlation coefficients, where $n = [5$ video-tracking measures +

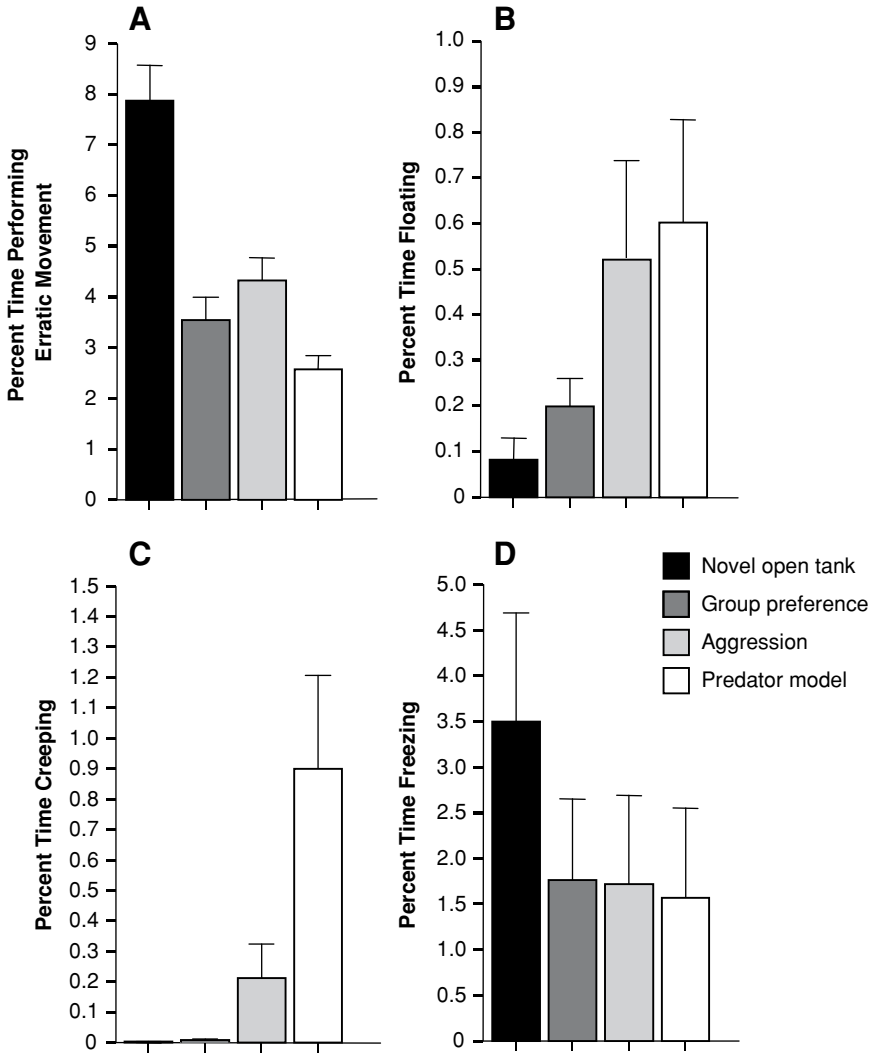

Figure 8. Motor and posture patterns. (A) Erratic movement, (B) floating, (C) creeping, and (D) freezing are shown (means + *SE*). Note that these behaviors occurred less frequently and for shorter durations of time than did those shown in Figures 5 and 6 but that they also reveal significant test-specific differences.

6 event-recording measures] ∗ 4 test situations). To reduce this complexity, we subjected the correlation matrix to a multivariate statistical procedure, the PCA. Briefly, this procedure allows one to group behavioral measures that correlate with each other. A correlation group of behaviors is represented by a principal component, and behaviors belonging to such a group are listed under a component (also often called a *factor*) with large (.30 or larger) loadings, the correlation coefficient between the behavioral measure and the component. In addition, the PCA also addresses the question of how the same behavioral measures recorded in different tests correlate. For example, it is not obvious that swimming in the group preference task necessarily represents the same behavior as swimming, say, in the predator model test.

Table 1 shows the principal component loading structure for all behavioral measures recorded by event recording and videotracking in all four test situations. Thirteen principal components were obtained with greater than 1 eigenvalues, and these components are thus retained. These components explained more than 75% of the total variance, a reasonably large value. It must also be noted

Table 1
Rotated Principal Component Matrix

	Component												
Measure	1	2	3	4	5	6	7	8	9	10	11	12	13
Mean distance from bottom, open tank				.768									
Mean distance from stimulus, open tank												.614	
Total distance moved, open tank			.809										
Mean heading direction, open tank													.618
Mean turn angle, open tank						.705							
Mean distance from bottom, group preference				.810									
Mean distance from stimulus, group preference					−.833								
Total distance moved, group preference			.882										
Mean heading direction, group preference													.744
Mean turn angle, group preference					.723	.540							
Mean distance from bottom, aggression				.758									
Mean distance from stimulus, aggression			.706										
Total distance moved, aggression			.795										
Mean heading direction, aggression					.480								.346
Mean turn angle, aggression						.670							
Mean distance from bottom, predator				.715									
Mean distance from stimulus, predator			.315									.582	
Total distance moved, predator			.828										
Mean heading direction, predator										.360		.432	
Mean turn angle, predator						.780							
Swimming duration, open tank	−.348									.767			
Thrashing duration, open tank		−.362								−.738			
Erratic movement duration, open tank									.653				
Floating duration, open tank								.599					
Freezing duration, open tank		.873											
Creeping duration, open tank								.881					
Swimming duration, group preference	−.503				−.683					.307			
Thrashing duration, group preference	.481				.685								
Erratic movement duration, group preference							.303		.576			.491	
Floating duration, group preference							.539					.425	
Freezing duration, group preference		.954											
Creeping duration, group preference								.920					
Swimming duration, aggression	−.811												
Thrashing duration, aggression	.834												
Erratic movement duration, aggression									.762				
Floating duration, aggression							.862						
Freezing duration, aggression		.862											
Creeping duration, aggression											.877		
Swimming duration, predator	−.855												
Thrashing duration, predator	.839												
Erratic movement duration, predator	.408								.566				
Floating duration, predator							.806						
Freezing duration, predator		.943											
Creeping duration, predator											.860		

Note—Principal components were extracted from a bivariate Pearson correlation matrix. The extraction method was principal component analysis with Varimax rotation and Kaiser normalization. Rotation solution converged in 10 iterations. Note that as a result of the Varimax method, the principal components are orthogonal; that is, they do not correlate with each other. The table shows major *loadings* of behavior—that is, loadings larger than .30. Loadings represent the bivariate correlation coefficient between the corresponding behavior and the principal component. Note that most of the principal components characterize either event-recorded motor and posture patterns or videotracking-recorded behavioral measures, but not both.

that given the rotation procedure (Varimax), the extracted principal components are orthogonal; that is, the correlation between them is zero. Principal Component 1 contains large loadings of motor/posture patterns recorded with event recording exclusively. It represents swimming and thrashing, with opposite signs recorded in all four test situations. Videotracking quantified behavioral measures are not represented in this component. Principal Component 2 is also exclusively of motor and posture patterns, and no videotracking measures have large loadings on this factor. It represents freezing in all four tests. Principal Component 3 has videotracking measures exclusively, and it mainly represents total distance moved in all four test situations and mean distance from the stimulus in two situations. Principal Component 4 again is of videotracking measures exclusively, and it is made up of large loadings of the measure *distance from bottom*. Principal Component 5 is the first mixed factor in which both videotracking and motor/posture patterns are present with large loadings. This component reflects the strength of social cohesion. Principal Component 6 has videotracking measures exclusively: turn angle across all situations. Conversely, Principal Component 7 has motor/posture patterns exclusively, mainly floating across all four situations. Principal Component 8 has only three large loadings, the two largest being for creeping in the novel open tank and in the group preference test and a smaller one being for floating in the novel open tank. Principal Component 9 is made up of erratic movement in all four tests. Principal Component 10 is a mixed factor, but mainly characterizes swimming versus thrashing in the novel open tank. Principal Component 11 is made up of creeping in two tests. Principal Component 12 is a mixed factor whose composition is difficult to interpret. Finally, Principal Component 13 represents heading in three test situations.

In summary, the pattern of loadings above (Table 1) shows that a given principal component usually represents either videotracking *or* event-recording parameters, but rarely both; that is, behavioral measures quantified by videotracking or by event recording generally do not correlate with each other, a finding that is in line with the results shown in Figures 1–8. It is also interesting to note that some principal components have large loadings of the same behavioral measure recorded in multiple test situations, which implies that some common features or factors affect behavior similarly among multiple test paradigms.

DISCUSSION

The behavioral paradigms used in this article were previously developed with simplicity and automation in mind (Gerlai et al., 2000), but quantification of behavior was conducted using only manual recording of the location and activity of the fish. This time-consuming and labor-intensive method is inappropriate for high-throughput mutation screening, the ultimate goal behind the development of the paradigms. Here, a computerized videotracking-based quantification of swim paths, as well as a computer-aided observation-based analysis of motor patterns, event recording, was conducted, along with the manual recording of activity and swim location.

A comparison of the results of manual activity recording and videotracking suggests that videotracking could appropriately quantify the activity, as well as the location, of zebrafish. Thus, we conclude that manual recording can be replaced with this automated computerized method. Furthermore, unlike manual recording, videotracking could measure the precise location of the fish and could record numerous characteristics of their swim path, including speed, turning angle, heading direction, and so forth, which could not be quantified previously, using the manual method. With its better precision and the larger number of swim path characteristics it could quantify, videotracking is expected to better detect differences between experimental groups.

A drawback we noted with regard to videotracking concerns the experimental setup. For example, the human observer could easily recognize the experimental fish and tell it apart from its mirror image in the aggression task, whereas the videotracking system had a hard time differentiating the two. Thus, albeit sophisticated, videotracking is not foolproof. The test setup must be chosen carefully to avoid tracking errors. Numerous recommendations may be made in this regard. First, reflections must be avoided. For example, we now place the mirror in the aggression task on the side of the tank, and we use matte plastic sheets covering the otherwise reflective glass bottom and back side of the test tank. Second, lighting conditions must be optimal; for example, homogeneous illumination of the fish and the background must be achieved. Third, removal of visual disturbances—for example, floating debris or bubbles—is important. To achieve this, the water must be filtered, and nonpressurized water that has set for at least 24 h must be used (the latter prohibits bubbles from forming as a result of compressed gases coming out of solution in the water). Last, careful attention must be paid to the settings of EthoVision—for example, specification of the minimum and maximum sizes of the target subject and adjustment of contrast levels.

Another result that emerged from the comparison of behavioral quantification methods employed in the present study is that the videotracking measures did not correlate with the event-recording measures. The latter motor patterns showed paradigm-dependent changes different from those measures obtained with videotracking. This observation was confirmed by a multivariate method, PCA. This result may seem surprising at first. However, one must note that motor patterns, as recorded here, represent a qualitative description of behavior and do not reflect intensity of behavior as much as videotracking measures do. For example, a fish can swim faster or slower, and thus, the recorded duration of time spent swimming (event recording) may not properly reflect the actual amount of locomotion (the length of swim path recorded by videotracking). Clearly, videotracking was superior to event recording in this regard. However, the videotracking measures employed here did not differentiate finer motor

patterns as well as the human observer could with event recording. Thus, the two methods were complementary to each other. Briefly, this implies that the "standard" videotracking measures will miss some aspects of behavior and may not capture potential mutation-induced changes, the ultimate goal of the present study. It is expected that programming changes to be implemented for videotracking in the future will enable us to record numerous motor patterns without having to use the labor-intensive and slow observation-based event-recording method and that this will further increase the sophistication of automated behavior quantification.

Some motor and posture patterns may be easy to record using videotracking. For example, thrashing is characterized by a stereotypical pattern of swimming back and forth on or near the glass wall, and thus, this behavior may be quantified by EthoVision if one defines the area within which the behavior occurs (within 2 cm from the glass) and the swim pattern (e.g., no more than a 10-cm-long swim in one direction and/or more than 90º angular swim change within less then 2 sec). Similarly, several other motor and posture patterns could be recorded by EthoVision, including erratic movement (defined by the high speed of swimming and the frequent swim direction changes), leaping (defined by a single fast bout of swimming), freezing (no movement), and so forth.

Other motor or posture patterns may be more difficult to quantify. Nevertheless, we are planning to conduct a systematic analysis of swim path and posture patterns in order to identify characteristic swim trajectories corresponding to particular motor or posture patterns, and after the identification of such trajectories, we will program the videotracking system. The first step in this analysis will be the establishment of a detailed Ethogram of the zebrafish. Once a large number of motor and posture patterns have been descriptively defined, the exact time periods within which a particular motor or posture pattern occurs during the recording will be identified, and the swim path patterns corresponding to all these periods will be analyzed. The library of characteristic swim path patterns common to the identified periods corresponding to a particular motor or posture pattern will allow us to properly program EthoVision to automatically detect and quantify these patterns. A conceptually similar idea has been proposed for the analysis of force-transducer–recorded activity parameters in mice (Fitch, Adams, Chaney, & Gerlai, 2002), and similar approaches using videotracking and computer-vision–based software learning algorithms are also being developed in the private sector (reviewed in Gerlai, 2002). Currently, the key problem in this analysis is the synchronization of time for the manually recorded motor patterns and the videotracking-system–recorded swim paths. This seemingly simple problem requires serious attention because the softwares employed in this study, although compatible with each other, do not record time similarly. Whereas the EthoVision videotracking system measures the precise time, Observer suffers from a problem, as follows. The human experimenter is expected to press a key corresponding to a behavior when the behavior starts. However, to achieve high precision, the experimenter needs to know in advance what behavior is going to start. This, of course, is not possible, so the result is a significant lag, a period during which the experimenter recognizes what has started and then presses the appropriate key. As a result of this lag, and because the length of this lag depends on reaction time and many other subjective, experimenter-dependent factors, precise synchronization of time at the resolution of seconds or better is very cumbersome, at least as far as the Noldus Observer and EthoVision programs are concerned.

In summary, our present results demonstrate that automated behavior quantification using videotracking is feasible with adult zebrafish, since this method properly quantified the differences observed in different behavioral paradigms. Importantly, this means that scaling up the tests—and thus, higher throughput—is feasible. The simplicity of these behavioral tests and their previously shown ability to detect acute alcohol-treatment–induced behavioral alterations (Gerlai et al., 2000), combined with the present findings demonstrating the feasibility of automated behavior quantification, suggest that the zebrafish may be utilized in forward genetic or pharmacological analysis of alcohol effects. Despite its good potential, however, one must also acknowledge that, although promising, zebrafish behavior as an emerging line of research is still in its infancy. A lot of fundamental behavioral characterization studies are needed before the zebrafish will be regarded as a "mainstream" model organism of behavioral neuroscience and behavioral genetics.

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