Chapter 7

A Simple and Effective Method to Condition Olfactory Behaviors in Groups of Zebrafish

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Abstract

We describe a simple assay for studying and conditioning olfactory behaviors of adult zebrafish. The apparatus consists of a circular flow-through tank into which odorants can be administered in a controlled fashion. Odorants (conditioned stimuli; CS) are repeatedly paired with food flakes (unconditioned stimuli; UCS) that are provided inside a tethered floating feeding ring. In response to conditioning, zebrafish develop an odorant-dependent place preference and restrict appetitive swimming behavior to the vicinity of the feeding ring. This robust assay can also be conducted with groups of zebrafish and thus provides a potentially important tool for large behavioral screens.

Key words: Conditioned olfactory behavior, circular flow-through tank, conditioned stimulus, unconditioned stimulus, place preference, appetitive swimming.

1. Introduction

Zebrafish are a favorable model for neurobiological investigations of olfaction. Their olfactory system is representative of that in higher vertebrates, but is reduced in size and complexity. This system is also accessible for physiological study and is easily manipulated by standard genetic approaches. In combination with tractable olfactory behaviors, zebrafish thus constitute a powerful tool for studying the cellular mechanisms that underlie chemosensory behavior and learning. We have recently established an assay for conditioning appetitive olfactory behaviors of adult zebrafish (1). In this chapter we detail how this assay is

A.V. Kalueff, J.M. Cachat (eds.), *Zebrafish Neurobehavioral Protocols*, Neuromethods 51,
DOI 10.1007/978-1-60761-953-6_7, © Springer Science+Business Media, LLC 2011

conducted and demonstrate that it can also be used to condition olfactory behaviors through group training.

Upon encountering certain odorants (e.g., an amino acid emanating from a food source), fish initiate appetitive swimming behaviors. These behaviors vary significantly across species (2), but most fish that are used in laboratory settings initiate chemotactic swimming: when fish encounter a decrease in odorant concentration, they will turn to orient themselves towards the direction of increased odorant concentration. This behavior ultimately leads the animal to the source of the odorant (3). We have shown that naïve zebrafish respond to the amino acids L-alanine and L-valine in a similar fashion (1). They increased their swimming behavior and executed more turns (>90°) when compared to normal swimming.

Appetitive chemotactic behaviors can be intensified via positive reinforcement conditioning (4–6). This was first demonstrated in sedentary catfish after repeatedly exposing them to amino acid mixtures paired with food rewards. The catfish ultimately learned to associate the conditioned amino acids with imminent feeding and responded with increased appetitive swimming (3). We have shown that zebrafish also display increased appetitive swimming after olfactory conditioning to both the natural amino acids L-alanine and L-valine, and the neutral odorant phenylethyl alcohol (1). However, appetitive swimming behavior and its modifications through conditioning can be difficult to identify in zebrafish. Zebrafish are naturally active, swimming quickly and displaying frequent directional turns (>90° turns). This activity is often increased during behavioral experiments (due to stress and/or anticipation of reward) and can obscure the detection of appetitive swimming behaviors, which are also characterized by a high frequency of >90° turns. Thus, while appetitive swimming is a useful behavioral measure for work in sedentary species with low levels of normal swimming (i.e., catfish), it may not always be useful for work with active fish species.

To overcome this limitation, we designed an olfactory conditioning method that involves a place preference paradigm. A place preference ensues with repeated positive reinforcement of a set of environmental cues, so that these cues ultimately acquire the motivational properties of the reward (7). We rewarded zebrafish after odorant administrations, and restricted the reward retrieval to the inside of a floating feeding ring. We demonstrated that zebrafish quickly learned to associate this ring with feeding, and that this occurs in an odorant-dependent manner (1). This localized feeding behavior is robust and easily identified, even in highly active fish.

Here we demonstrate that our assay can also be used to condition zebrafish through group training. Our assay is easily conducted, leads to robust olfactory dependent place

conditioning and can be used to train large numbers of fish. These are important criteria for any behavioral assay used for large-scale behavioral screens that are becoming increasingly important in neurobiological investigations seeking to understand genetic and cellular underpinnings of zebrafish behavior.

2. Method

Animals: Our assay can be conducted with zebrafish aged between 2 and 6 months, weighing 0.3–1.0 g. We tested both outbred wild-type zebrafish obtained from a local pet store (AquaCreations, Halifax, NS, Canada) and animals from an established laboratory line (AB strain, University of Oregon). No differences in performance were observed between zebrafish of the different ages or populations listed above.

2.1. Equipment Setup

2.1.1. Tank

The tank is a circular white polypropylene bucket (diameter = 28.5 cm; height = 40 cm) containing a flow-through water system (**Fig. 7.1a**), which provides a rapid, uniform inflow and drainage of the 8 cm-deep water column. The main water inflow (WI in **Fig. 7.1)** is fastened to the vertical wall of the bucket and terminates in a horizontal circular hose, fixed to the bottom of the tank. Regularly spaced (10 cm intervals) holes (I.D. ~ 1 cm) along the underside of this circular hose ensure that the water enters the bucket uniformly. It is important to cover each inflow hole with a mesh (1 mm spacing), because zebrafish will swim into and get trapped inside the inflow tube. As outflow, a polyvinyl chloride standpipe (I.D. ~ 4.5 cm; height = 8 cm) is installed in the middle of the bucket. To ensure that water is drawn off equally from the entire height of the water column, the standpipe needs to be covered with a wider sleeve (I.D. ~ 8 cm; height = 12 cm), in which equally spaced horizontal slits (kerf = 1 mm) are cut at 1 cm intervals. We found it equally important to cover the top of the sleeve, as fish will sometimes jump and may be lost through an uncovered drain.

Odorants are injected via a plastic tube (I.D. = 0.5 cm; see odorant injection tube in **Fig. 7.1a**) that is connected to the main water inflow via a Y-connector. The odorant injection tube is gated by a 3-way Luer valve, to which syringes can be connected (**Fig. 7.1a** inset). The valve needs to be closed when no injections are taking place as the water inflow will draw air into the system

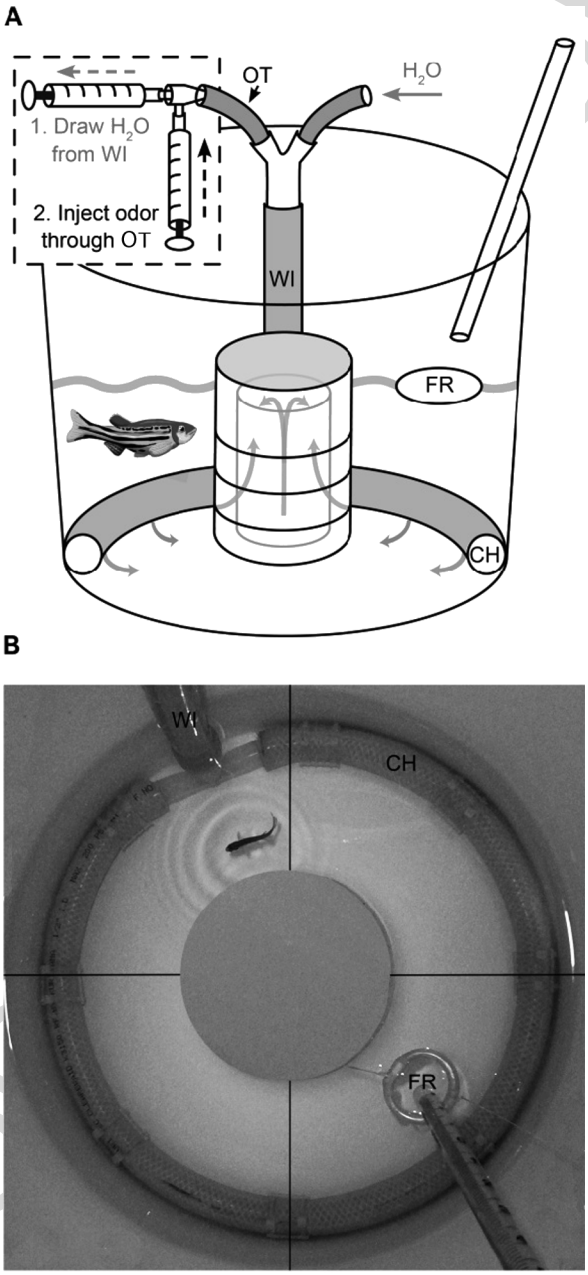

Fig. 7.1. Schematic diagram of the conditioning apparatus (**a**) and still video image (**b**) as recorded from above. Odorants are injected remotely into the main water inflow (WI) and perfuse the bucket through inflow holes spaced along the underside of a circular hose (CH). Following odorant injections, the fish are rewarded inside the feeding ring (FR). The process of injecting odorants is illustrated in the *inset*. To prevent injection of air bubbles into the system, the odorant injection tube (OT) is initially filled by drawing water with a large syringe from the main water inflow (Step 1). Odorants are then injected with a separate odorant syringe (Step 2). The injection tube is rinsed after each trial by drawing water back into the tube (Step 1).

and create large bubbles. To feed the fish, a hollow, plastic feeding tube (I.D. ~1 cm) is mounted to the side of the bucket (above the water level) and aimed at a tethered, floating feeding ring (I.D. = 4 cm; *see* FR in **Fig. 7.1**). We feed the fish with floating food flakes (Nutrafin Staple Fish Food, Hagen Inc., Montreal, QC, Canada), which remain in the lumen of the feeding ring. Both the odorant injection tube(s) and the feeding tube must be sufficiently long for the experimenter to apply both stimuli without being seen by the fish. We also recommend placing the apparatus on high shelves that stand on rubber or styrofoam padding. Zebrafish are very sensitive to vibrations and may respond to the presence of the experimenter rather than odorant injections.

Zebrafish behavior can be monitored and recorded with a standard video camera (30 frames per second) that is placed above the tank. We use a commercially available surveillance video system (Novex Inc., Toronto, ON, Canada) to acquire and view the video clips on our computer. We found it advantageous to have real-time monitoring of the performances of the individual fish and also of the experimenter (e.g., hastened odorant injections may create bubbles that are sensed by the fish). In this way, it is possible to identify potential problems during pilot experiments and prior to conducting lengthy data analyses. Finally, laboratory lighting may be enhanced with fluorescent lights, which should be mounted above the setup. The light is diffused by covering the tanks with white translucent plastic film, leaving only a small hole through which the camera objective can be fit.

2.1.2. Water Flow

Care must be taken to ensure that odorants are administered in a controlled fashion, with predictable onset and clearance. To determine how injected stimuli behave in our apparatus (**Fig. 7.2**), we injected food dye (same volume as odorant injections) into the water inflow and repeatedly drew water samples from the bucket for several minutes. We analyzed the optical density of each dye sample with a spectrophotometer and used these values to create stimulus profiles for each bucket. Using this method, we have determined that injected stimuli are diluted 10^4-fold within 4 min of administration, provided that the volume of the bucket is replaced with fresh water approximately once every minute. We tested a variety of differently sized buckets (0.4–4 l) and the same clearance is achieved in all of these if enough flow is supplied to replace their volume approximately once every minute.

2.1.3. Odorants

The most commonly used appetitive odorants for teleost fish are commercially available L-type amino acids. The amino acid L-alanine (BioChemika > 99.0% purity; Sigma Chemical Co.) is very useful for behavioral work in zebrafish, because it elicits robust appetitive swimming that can also be modified through

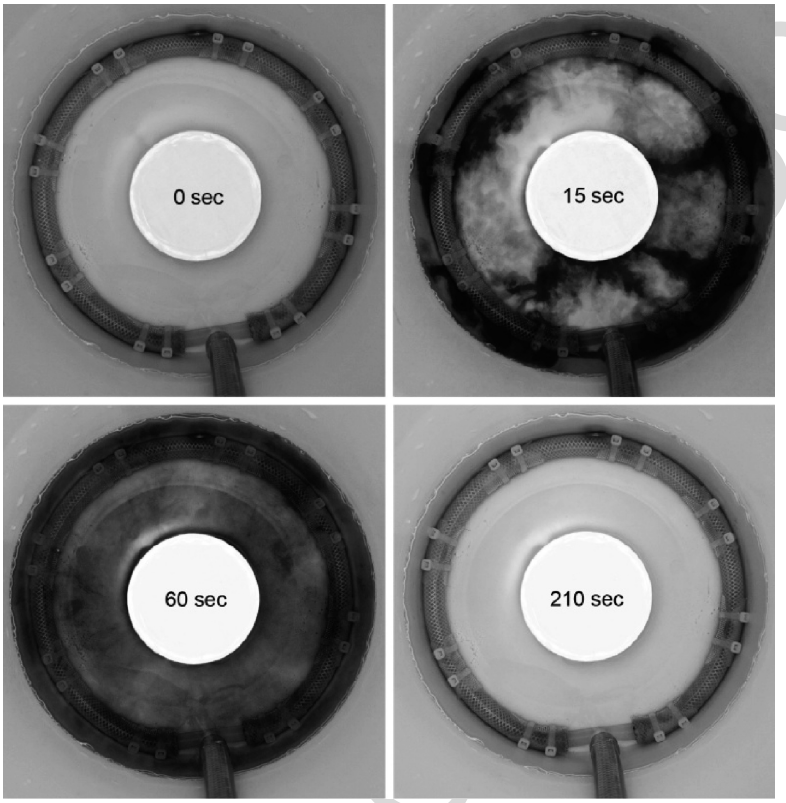

Fig. 7.2. Image series acquired from the conditioning apparatus following injection of 10 mL dye (*black*). The dye quickly spreads through apparatus (15 s) and is evenly distributed within 1 min. A 10^4-fold stimulus clearance is achieved in less than 4 min.

conditioning. As a behaviorally neutral odorant (e.g., conditioned stimulus in classical conditioning), we have used the synthetic fragrance phenylethyl alcohol (PEA; International Flavors and Fragrances Inc.). This odorant does not evoke behaviors in naïve zebrafish, but can be conditioned to elicit appetitive behaviors. Odorants should always be prepared freshly before use and can be injected into the perfusion system as concentrated aliquots. The final stimulus concentration that zebrafish can detect varies widely among odorants, but most amino acids are apparently detected at a final concentration of 10 μM (1,8–10). In all experiments described in this chapter, we used PEA at a final concentration of 1×10^{-4} M as conditioning stimulus.

2.2. Conditioning Procedure

1. Place groups of zebrafish in the buckets. To date, we have trained and tested groups of four individuals of the same sex. Ensure that all fish in a conditioning group are of the same size (*see* **Section 3**).

2. Once the fish are placed in the tanks, adjust the camera and cover the apparatus. Let the fish acclimatize for 24–48 h and do not feed them during this time.
3. We suggest that training be started in the morning, shortly after the light period begins. This will allow sufficient time for conducting all training sessions and ensure that inter-session intervals can be made sufficiently long (*see* 7.).
4. Rinse and prepare the odorant injection tube by drawing water from the main inflow with a large (~ 30 mL) syringe. Close the Luer valve and discard this water. Fill and connect the odorant syringe to the Luer valve (**Fig. 7.1a** inset). Once these steps are completed, record a short video segment (1 min) of the behaving fish. This "baseline" behavior can later be used for comparisons with odorant-evoked behaviors.

   ! Important: Ensure that the odorant injection tube is filled with water prior to injecting odorants. The fish will react to air bubbles that are injected along with the odorant (i.e., through an empty tube).
5. Start olfactory conditioning trials by injecting the odorant (conditioned stimulus) into the water inflow and restarting the video recording. After 45 s [15 s for odorant infusion (**Fig. 7.2**) plus 30 s for behavioral observation], administer food flakes (unconditioned stimulus; a single ~ 2 mg flake for each fish in each trial) through the feeding tube. Watch the fish on the monitor and determine if they retrieve the food rewards and then terminate the recording. In our experiments these are the only feedings that the fish receive. We conduct 12 trials daily and believe that this provides ample food during conditioning.
6. Rinse the injection tube after each trial by drawing water from the main inflow (**Fig. 7.1a** inset). We usually draw enough water to fill a 30 mL syringe and discard this. This ensures that the injection tube is rinsed and prepared for the next trial.
7. Repeat the trial four times during training sessions in the morning, midday, and evening (12 trials per day). Wait at least 15 min between individual trials and 2 h between training sessions. We find that closer spacing of trials and sessions often results in development of odorant-independent place conditioning, where the fish simply remain near the feeding ring in anticipation of feeding.
8. After 4–5 days (48–60 trials), the fish are trained. To determine if each fish within a group has been successfully

conditioned, it is necessary to individually test their performance in the conditioned task. Divide the group and place each fish by itself in a separate conditioning tank. Let the fish acclimatize for 24–48 h and feed them daily, but not through the feeding ring.

9. Prior to testing, we conduct 1 "refresher" trial with individual fish. These trials are conducted in the same manner as training trials and may be necessary for the fish to acclimatize fully to being isolated in the apparatus. A single training trial does not induce an odorant-dependent place preference in individually trained zebrafish, and we therefore believe that the "refresher trial" does not produce conditioned behaviors observed in tests. Conduct this trial at least 1 h before testing the fish.
10. The final test consists of four trials conducted with individual fish to determine if they respond to the odorants with conditioned behaviors. Each trial is performed in an identical manner to the training trials described above, but no food is given to the fish following odorant injections. Perform each probe trial individually, separated by approximately 2 h intervals to minimize habituation to the (now unrewarded) odorants.
11. Before placing new fish into the apparatus, inject household bleach (5.25% sodium hypochlorite) into the system via the odorant injection tube and then turn off the water for 30 min. Rinse well (overnight). This will clean the apparatus and any odors or debris left from the previous training session.

2.3. Control Experiments

To test if olfactory place conditioning is dependent on the specific pairing of odorants and food, these stimuli can be administered independently of each other. For this chapter, we exposed the fish to the odorant (PEA) 12 times daily, on the same schedule as conditioning would normally occur, but we did not feed the fish during these trials. Instead we fed the fish (through the feeding ring) at various times during inter-session intervals. In previous experiments we also assessed the possible involvement of mechanosensation (i.e., the sensing of volume displacement from odorant injections during trials) and gustation in producing learned behaviors (1).

3. Trouble shooting

1. In our apparatus a circular inflow hose is installed in the behavioral arena (**Fig. 7.1a**). It is not uncommon for a fish to be initially hidden beneath this inflow tube. Typically, odorant infusions are enough to lure the fish out of hiding (i.e., they respond with swimming activity), but if this is not the case, we suggest insertion of a mesh barrier into the behavioral arena. We have built such barriers with 1 mm Nitex mesh and these effectively prevented the fish from accessing the tubing (not used in the experiments described here). It is best to make the barriers removable, because the mesh traps debris and requires cleaning after experiments.
2. One or several fish in a group may become stressed in the apparatus and this can affect performance during conditioning. Stress may manifest itself in several ways. The fish may swim very quickly and repeatedly around the circumference of the apparatus (circling). If fish do this continuously for a day after acclimation, they will continue to circle the apparatus and will not respond to training. Alternatively, stressed fish may hide under the inflow hose (if there is no mesh to restrict access) and remain there for the duration of the experiment (without visibly responding to odorants). As with the circling behavior, fish that remain under the tube for a day after acclimation will generally not be useful for conditioning. It is thus important to check for these and other behaviors after the acclimation period. If necessary, replace the stressed fish and let the group acclimate for another day before starting the conditioning experiment. In our experience, fish that are not "stressed" after acclimation will not become stressed during conditioning; nevertheless, we recommend continuous monitoring for any signs of stress.
3. Some fish fail to retrieve the food reward at the end of a training session. This is not uncommon, especially in a group where competition for food exists (*see* also below). If a fish does not retrieve the food reward or approach the feeding ring for a whole day of training (due to stress or competition), it may not be conditioned adequately. It is important to be aware of such individuals during data analysis. If the fish are individually identifiable, it may be helpful to remove the fish in question and continue training the remainder of the group.

4. During training, when the fish are conditioned multiple times in quick succession, it is common that they develop a nonspecific place preference and the feeding ring regardless of the presence of an odorant (1). This place preference becomes more robust as successive rewards are administered more rapidly. To ensure that fish develop an *odorant-dependent* place preference, it is therefore very important that inter-trial intervals are sufficiently long (minimally 15 min). This permits the fish to return to baseline behavior after each trial and impairs the development of a nonspecific place preference. In preliminary experiments we have found that longer spacing of training trials (one trial every ~ 45 min) prevents the development of a nonspecific place preference, but not the odorant-dependent place preference (unpublished observations).
5. Finally, in group-training experiments it is important that all fish in a group are similarly sized. We have repeatedly observed larger fish in a group apparently displaying territorial dominance near the feeding ring. This prevented smaller fish from obtaining the food reward and likely affected their acquisition of conditioned behaviors. Similarly, we noticed that groups of fish obtained from the same holding tanks (provided that they were the same size) readily shoal with one another, while groups of fish from different holding tanks (i.e., different families and ages) were more aggressive towards one another. Even after meticulously selecting animals for our group training experiments, we found considerable variability in the way that fish behaved as a group. We therefore suggest careful observation of the animals during training and to be aware that some individuals may not learn the task due to dominance of other fish.

4. Analysis

To determine if individual zebrafish develop a place preference following group training, we test each fish individually and measure the time that it spends in the area of the bucket containing the feeding ring. We divide the total area of the bucket into four quadrants (**Fig. 7.1b**) by placing a grid drawn onto acetate sheets onto the computer screen. The time that fish spend in each quadrant can then be recorded with a stopwatch or appropriate video analysis software (11). Fish that are distributed at chance will spend 25% of the observation period in each of the four quadrants. A place preference to any quadrant then manifests

itself as an increase in the time that a fish spends in a single quadrant (*see* below). Conditioning can also lead to changes in appetitive swimming behaviors (i.e., frequency of >90° turns) and changes in swimming speed. We have scored such changes manually (1), but suggest that future experiments take advantage of more sophisticated and practical computerized behavioral analysis (11).

Data derived from this experiment will consist of repeated measures of the performance of individual fish during training and testing. An appropriate analysis will thus employ a repeated measures analysis of variance to identify changes that occur within and between treatment groups. Within group effects (or a regression analysis) can be used to identify temporal effects of conditioning [i.e., acquisition curves; *see* (11) for acquisition data for group training], while between group effects will reveal if there are any differences in performance between experimental groups. Finally, we found that performance of individual fish is prone to substantial variability between trials, and we therefore use the mean performance of fish in training sessions (mean of four trials) as data to analyze the effects of conditioning.

5. Results and Conclusion

Group-trained zebrafish show a place preference to the quadrant containing the feeding ring (**Fig. 7.3**). In response to the conditioned odorant PEA, individually tested fish ($n = 12$) spent 45.5 ± 1.7% of the test duration (30 s) in the reward quadrant containing the feeding ring. This was significantly increased from the time spent in the reward quadrant prior to odorant administration (27.5 ± 2.5%; repeated measures ANOVA: $p < 0.001$). The conditioned zebrafish also spent significantly more time in the reward quadrant than fish in the control group that were repeatedly exposed to PEA without subsequent food rewards (*see* Control Fish in **Fig. 7.3a**; between subjects effect repeated measures ANOVA: $p < 0.05$). These data thus indicate that group-trained zebrafish respond to the odorant by localizing to the reward quadrant and that this behavior develops as a result of pairing PEA with rewards administered in the feeding ring.

To summarize, our behavioral assay relies upon an inexpensive apparatus, is easily conducted, and is adaptable for use with large numbers of animals. It therefore meets the requirements of many laboratories and could emerge as a popular tool for behavioral research of the olfactory system. In closing, we suggest that any

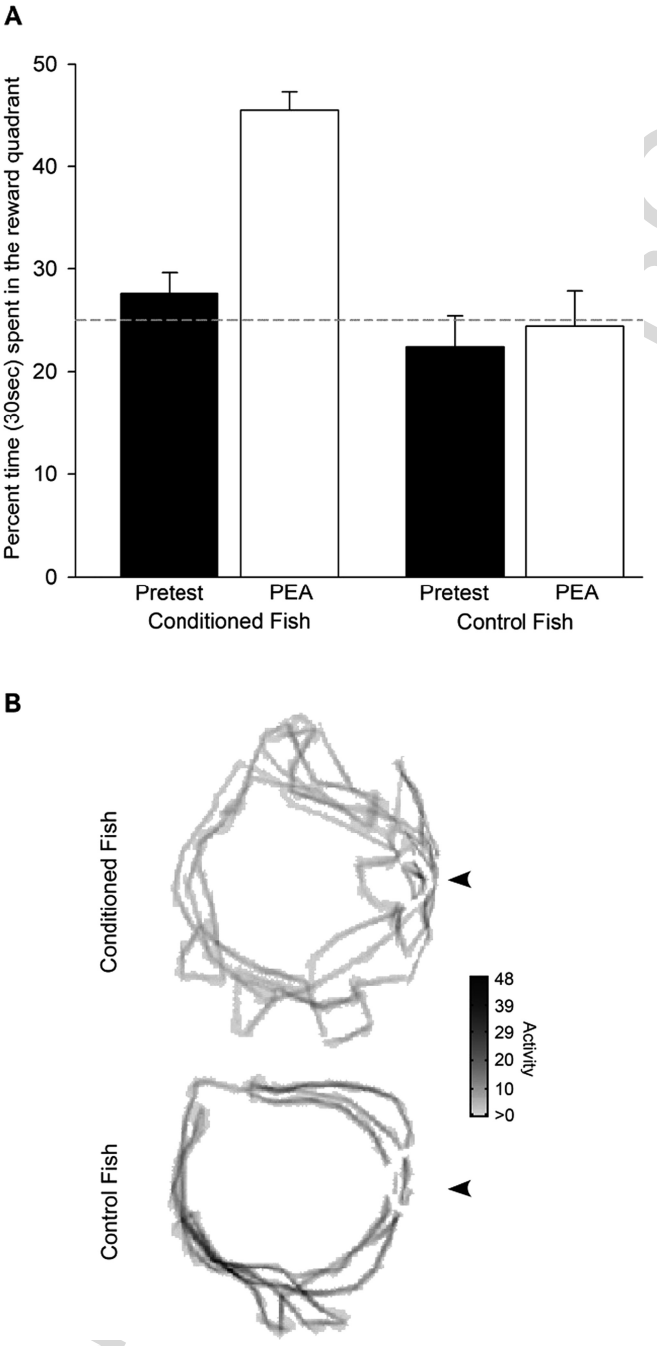

Fig. 7.3. (**a**) Group-trained zebrafish localize to the reward quadrant when exposed to the conditioned odor PEA during individual testing (conditioned fish). Prior to each test, the fish are distributed at chance throughout all areas of the apparatus, indicating that the place preference for the reward quadrant is odorant-dependent. Control fish that received PEA only and were fed at other times during training did not develop a place preference. All data shown in (**a**) are the mean scores from 12 fish tested over four trials and their standard errors. The dashed grey line (25%) indicates chance distribution. (**b**) The distribution of two individual fish during testing is shown in videograms.Conditioned fish moved faster and returned to the ring more often when exposed to PEA. Control fish moved slowly and showed no place conditioning. The videograms were mapped onto a common coordinate system with the same feeding ring location (*arrowhead*). Activity scale: activity frequency over 30 s, sampled at 30 frames s–1 (for more details, *see* (11)).

other small teleost (e.g., medaka, goldfish) could be tested equally well for basic odorant responses (1) and cognitive capabilities through our method.

Acknowledgments

We thank Erika Harding for help with the group training experiments, and Evan McCarvill for the zebrafish illustration in **Fig. 7.1a**. This work was supported by research grants from the Nova Scotia Health Research Foundation (to O.B), the Malacological Society of London (R.W), the Canadian Institutes of Health Research (to A.F) and the Natural Sciences and Engineering Research Council of Canada (to R.P.C).

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