Behavioral Responses of Newly Hatched Zebrafish (Danio rerio) to Amino Acid Chemostimulants

Sara M. Lindsay¹,² and Richard G. Vogt¹

¹Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA
²Present address: School of Marine Sciences, University of Maine, Orono, ME 04469, USA

Correspondence to be sent to: Richard G. Vogt, Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA. e-mail: vogt@biol.sc.edu

Abstract

The zebrafish chemosensory systems of olfaction, taste and solitary chemosensory cells (SCCs) are established during the first week after fertilization (a.f.). These systems presumably support the early development of feeding behaviors required as yolk supplies diminish over the same period. Yet there is no previous data reporting early chemosensory responses in zebrafish. We therefore assayed the chemosensory behavior of newly hatched zebrafish on days 3, 4 and 5 a.f. Responses were compared between fish exposed to water alone versus water containing a mixture of 12 amino acids (100 µM each) flowing through a 50 ml test chamber at 4 ml/min; computer-assisted motion analysis was used to quantify responses. Behavioral responses were first observed at day 4 a.f.; the number of fish swimming, their swimming speeds, and their net-to-gross displacement (NGDR) all increased significantly in response to amino acid stimulation. Because taste buds first appear 4–5 days a.f. and the SCCs may not respond to amino acids, these initial chemosensory responses of day 4 fish may be mediated by already established olfactory neurons. The onset of chemosensitivity in day 4 fish corresponded with an easily recognizable developmental phenotype of inactive floating; day 3 fish were inactive and resting on the bottom while day 5 fish were active and moving through the water column. The ease of identifying responsive day 4 fish suggests these animals may be useful for characterizing odorant sensitivity or developmental plasticity or for screening for chemosensory mutations.

Key words: chemoreception, chemosensory behavior, development, fish, odor, olfactory receptor

Introduction

Development of a chemosensory system requires the coordination of a multitude of processes, resulting in a sensory interface between organism and environment that allows the appropriate expression of an assortment of chemosensory based behaviors. The zebrafish, Danio rerio, is a good model for examining mechanisms underlying chemosensory development within the context of behavior. Rapid embryogenesis (<3 days) must be immediately followed by the development of effective feeding behavior as yolk supplies diminish during the first week. The subsequent period of juvenile life and accompanying growth requires dramatic changes in feeding and predator avoidance strategies as mouth size increases and habitat changes. The roles chemosensory systems play in feeding and predator avoidance suggest they too must undergo dynamic change throughout this period.

Zebrafish have three chemosensory systems: olfaction, taste, and solitary chemosensory cells (SCCs). Olfactory neurons are distributed within the olfactory epithelium which is organized into a pair of olfactory rosettes situated above and to either side of the mouth and anterior to the eyes (Hansen and Zeiske, 1993). In adult zebrafish, olfactory neurons are known to respond to amino and bile acids (Michel and Lubomudrov, 1995; Friedrich and Korsching, 1997; Michel and Derbidge, 1997; Lipschitz and Michel, 1999, 2002; Michel et al., 1999; Fuss and Korsching, 2001). Taste neurons are localized within taste buds distributed over the entire outer body surface including the lips and oropharyngeal cavity (Hansen et al., 2002). Taste neurons are known to respond to amino acids and bile acids (Hara, 1994a; Ogawa and Caprio, 2000). SCCs are also typically distributed over the entire outer body surface (Kotrschal et al., 1996; Finger, 1997). In the rockling, SCCs do not respond to amino acids, but rather have a narrow response profile to components in dilute bile or skin washes of other fish (Peters et al., 1991; Kotrschal et al., 1996, 1997). However, in the searobin, the fin rays are used in feeding behavior and SSCs localized on the fin rays do respond to amino acids (Silver and Finger, 1984). In general, olfaction and taste are thought to convey independent information about food.
materials and methods

Animals

Embryos of *Danio rerio* were obtained from an established breeding stock maintained in 75.7 l (20 gallon) aquaria at 28°C on a 16 h:8 h light:dark cycle (lights on 07:00 h); breeding stock were initially obtained from a local pet shop. Aquarium water was filtered, reverse osmosed and deionized house water with sea salts (57mg/l; e.g. ‘Instant Ocean’, Aquarium Systems) and sodium phosphate buffer (315 mg/l ‘pH 7.0 FIXIT’, Aquatronics) added, and pH adjusted to 7.0 (after Westerfield, 1993). A recirculating water supply (~380 l) with common filtration (biofilter) served several adult aquaria and a nursery tank, exposing developing embryos and juveniles to water with a high organic load. Fertilized eggs were deposited into small plastic boxes (~15 × 15 × 2 cm) containing glass marbles plus a plastic aquarium plant and situated on the bottom of the aquarium; for collection, the box was removed by hand and embryos poured into a beaker (modified after Westerfield, 1993; Vogt et al., 1997). A box containing marbles was placed into an aquarium in the evening, and replaced with a new box after lights on. Embryos were collected from this new box after 2 h of accumulation to ensure uniform age ranges of fish in subsequent experiments. Embryos were allowed to develop in screen-bottomed beakers (mesh size 250 µm) submersed in the nursery tank beneath drip irrigation outlets. At 28°C, hatching occurred during the third day a.f. (between 48 and 60 h a.f.). Because newly hatched zebrafish do not require exogenous food until the yolk sac is depleted (approximately day 5 a.f.; Fuiman and Webb, 1988; Westerfield, 1993), larval fish used in these experiments were not fed.

Behavioral assay and data analysis

Freshly made aquarium water was used for all experimental conditions. Fish were assayed for their responses to a mixed solution of 12 amino acids on days 3, 4, and 5 a.f. The test mixture contained L-glutamine, L-methionine, L-alanine, L-cysteine, L-histidine, L-leucine, L-lysine, L-asparagine, glycine, L-serine, aspartic acid and glutamic acid. Concentrated stock solutions of the amino acid mixture were kept frozen (~20°C) in 25 ml aliquots, and diluted with freshly made aquarium water to final concentration of 1 × 10^{-4} M per amino acid (1.2 × 10^{-3} M total).

The behavioral assay is shown in Figure 1; five fish were used in each assay. For each trial, fish were acclimated in

---

Figure 1  Diagram of the experimental set up. Either aquarium water alone or aquarium water plus amino acids flowed through the experimental dish. Dish diameter was 90 cm, fish lengths were ~3 mm. Zebrafish (five per trial) were videotaped, and their swimming paths were analyzed using computer assisted motion analysis; see text for details. Drawings of fish are tracings of 72 h a.f. juveniles from Kimmel et al. (1995).
Petri dishes (90 cm diameter) that had tubes for carrying water in and out (In flow and Out flow). Dishes held 50 ml of water to the top of a stand pipe overflow. Inflowing water passed through a diffuser section, resulting in a broad front of fluid that permeated the Petri dish in ~2 min based on initial observations using dyes. Reservoirs (Ross Toptainer™ enteral feeding units) contained either aquarium water (control) or aquarium water plus amino acids (experimental), and flow speed was maintained by gravity and valves at ~4 ml/min. A three-way valve allowed switching between reservoirs. A video camera was suspended above the dish. An array of four dish–video camera set-ups was constructed for these experiments and located in a temperature controlled room isolated from the experimenter (reservoirs, valves and video recorder were situated and operated from outside the experimental room). Fish were recorded for 15 min in each trial. During the first 5 min (phase 1), no water flowed through the dishes (‘still’ in Table 1). During the second 5 min (phase 2), aquarium water flowed through the dishes. During the last 5 min (phase 3), either aquarium water (controls) or the amino acid mixture (test) flowed through the dishes. Fish were placed in dishes and left undisturbed for one hour before initiating recordings.

Video recordings were analyzed for the number of fish active as well as their average swimming speeds, rate of change of direction (RCDI) and net-to-gross displacement ratio (NGDR). Video recordings were passed through a Motion Analysis VP-110 Video Processor and analyzed on a Sun Workstation (SPARCstation IPC) using Motion Analysis software (Expert Vision) at 10 frames/s. Briefly, this analysis system tracked the X,Y positions of each fish (centroid) in the field of view at a specified frame rate for a specified time period, and then plotted the individual paths. At a frame rate of 10 frames per second these X,Y coordinate positions are separated by 0.1 s intervals. From the lists of X,Y coordinates (paths), the computer then calculated speed (mm/s), RCDI (°/s; otherwise known as turning frequency or angular velocity) and NGDR (the ratio of the shortest linear distance between start and end points of a path, and the total travel distance). Means for swimming speed, RCDI and NGDR were quantified for all paths made by fish during the second or third minute of each 5 min period. We collected paths from the second minute of the assay because the front of inflowing fluid filled the dish in the first minute and all fish had contacted the new fluid by the second minute. However, if only one or no fish were in the field of view of the camera during the second minute, we analyzed path data from the third minute when more fish were visible. If no fish were in the field of view during the second or third minute, then that replicate trial was excluded from the analysis. A ‘weighted average’ was calculated for each parameter to account for variable path durations: the weighted average was the mean value for each path multiplied by the number of frames making up the path. Because multiple fish were active in each dish, the path data were collapsed into a grand mean for each dish, and replicate dish means were analyzed by paired t-tests using PC-SAS version 6.08 (SAS Institute, Cary, NC). Thus, sample sizes indicate the number of dishes tested: 3 days a.f., n = 8 dishes × 2 treatments = 16 dishes (80 fish); 4 days a.f., n = 12 dishes × 2 treatments = 24 dishes (120 fish); and 5 days a.f., n = 12 dishes × treatments = 24 dishes (120 fish). Post-hoc power analyses were made using the PASS 2000 program from NCSS (Kaysville, UT).

Results

Fish hatched during the third day a.f. under the conditions described. Newly hatched fish (day 3 a.f.) were largely inactive and negatively buoyant, lying immobile on the bottom of the nursery beakers. Day 3 a.f. fish do display occasional tail flicks. At some time during day 4 a.f., fish became positively buoyant, floating at the water surface, but still remained largely inactive and immobile similar to the day 3 a.f. fish; positive buoyancy was presumably due to the initiation of swim bladder function. Fish became spontaneously motile during the day 5 a.f., moving throughout the water column with controlled neutral buoyancy. Day 5 fish swimming involved regular but discontinuous beating of the tail; this beating provided the motive force for swimming and

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Still</td>
<td>Flow Ctrl</td>
<td>Still</td>
</tr>
<tr>
<td>Number fish swimming</td>
<td>0.27 (0.60)</td>
<td>0.54 (0.65)</td>
<td>2.04 (1.64)</td>
</tr>
<tr>
<td>Mean swimming speed (mm/s)</td>
<td>0.38 (0.16)</td>
<td>0.37 (0.16)</td>
<td>1.24 (1.23)</td>
</tr>
<tr>
<td>Mean RCDI (degrees/s)</td>
<td>467.41 (134.66)</td>
<td>412.67 (134.66)</td>
<td>449.35 (119.21)</td>
</tr>
<tr>
<td>Mean NGDR</td>
<td>0.07 (0.09)</td>
<td>0.05 (0.05)</td>
<td>0.22 (0.24)</td>
</tr>
</tbody>
</table>

Data shown are means and standard deviations (in parentheses) Paired t-tests comparing still and flow control means on each day were not significant for any variable on any day (all P-values > 0.10) RCDI = average rate of change of direction, or turning frequency; NGDR = net to gross displacement ratio, a measure of path circuitry.
was characteristic of the method of swimming observed in fish as they continued to age and grow. Experimentally induced day 4 movement (see below) involved this same discontinuous tail beating. For the studies described below, day 3 a.f. fish were collected 3 days a.f. from those fish that had hatched and were at the bottom of nursery beakers; 4 day a.f. fish were collected 4 days a.f. from those fish floating at the water surface, and 5 day a.f. fish were collected 5 days a.f. from the swimming population in the beakers (i.e. from the mid-water column).

In the behavioral assay, addition of aquarium water (i.e. introduction of flow) had no significant effect on fish held in still water (comparing phase 1 and phase 2 of the trials for any variable; Table 1). Under phase 3 flow conditions, amino acids elicited little change in the swimming activity of day 3 fish, but prompted a significant increase in the number of fish swimming at 4 days a.f.; the number of fish swimming roughly doubled (Figure 2). No significant difference was observed between stimulated (amino acids) and unstimulated (water) fish at day 5 a.f., possibly due to the high background of normal activity displayed at this age.

In motion analysis of chemotactic organisms, three components of behavior are often analyzed: speed, rate of change of direction (RCDI), and net-to-gross-displacement ratio (NGDR); RCDI and NGDR are both indicators of turning frequency. In this study, swimming speeds of fish at 3 days a.f. were much slower than those at day 4 or 5 a.f. (Figure 3). At day 4 a.f., fish swam twice as fast following addition of amino acids compared to addition of aquarium water alone. This trend for faster swimming in response to amino acids appeared to continue on day 5 a.f., though this difference was not statistically significant (paired t-test, $P = 0.32$; Figure 3).

Rate of change of direction (RCDI) is an indication of how straight the swimming paths are; a lower RCDI might suggest movement that is more directed or less random. On days 3 and 4 a.f., fish showed no significant difference in rates of change of direction in response to either aquarium water or amino acids (paired $t$-tests, $P > 0.10$; Figure 4). On day 5 a.f., however, fish exposed to amino acid mixture turned significantly less frequently during swimming compared to those exposed to aquarium water (Figure 4).

![Figure 2](image2.png)

**Figure 2** Number of fish swimming before and after addition of test stimulus at 3, 4 and 5 days after fertilization (mean per dish +SE). Test stimulus was either aquarium water (Ctrl) or amino acid mixture (Expt). *Significant difference between the before and after means (paired t-test, $P < 0.01$).

![Figure 3](image3.png)

**Figure 3** Swimming speeds of zebrafish on days 3, 4 and 5 after fertilization before and after the addition of test stimulus (weighted average per dish, means +SE). Stimulus was either aquarium water (Ctrl) or amino acid mixture (Expt). *Significant difference between the before and after means (paired t-test, $P < 0.01$). Note differences in Y-axis scales, indicating transition from negative to positive buoyancy between days 3 and 4 a.f.
Net to gross displacement ratio (NGDR) is the ratio of the shortest linear distance between the start and endpoints of a path and the total travel distance (see inset, Figure 5). Increased NGDR also indicates lower turning frequencies or straighter swimming paths. As an indicator of path circuitry, NGDR has a maximum value of 1 when paths are completely straight and a minimum of zero when paths are circular and the start- and endpoints occur at the same spatial coordinates. Thus, a greater NGDR might also suggest movement that is more directed or less random. On day 3 a.f., NGDR values for fish were close to zero, reflecting the fact that fish were largely stationary (Figure 5). NGDR values increased on days 4 and 5 a.f. and on day 4, fish exposed to amino acid mixture showed significantly higher values of NGDR than control fish (i.e. straighter paths) (Figure 5).

**Discussion**

Morphological (e.g. Hansen and Zeiske, 1993, 1998) and molecular biological studies (e.g. Barth et al., 1996; Byrd et al., 1996; Argo et al., 2003) suggest larval zebrafish should be capable of chemoreception soon after hatching. Nevertheless, the earliest chemosensory relevant behavioral data available to date were for zebrafish 25 days old (Kasumyan and Ponomarev, 1990). We have now shown that zebrafish as young as 4 days a.f. are capable of displaying behavioral responses to dissolved amino acids at a stage prior to the onset of active swimming.

Amino acids were chosen as the test compounds in part because they represent a major documented olfactory stimulant of fish (Haynes et al., 1967; Hara, 1982, 1994b; Caprio, 1982; Ellingsen and Døving, 1986; Steele et al., 1990). The amino acid mixture tested was diverse, including ones with uncharged polar and non-polar side chains, as well as those with basic and acidic side-chains. The test concentration of 100 µM per amino acid (1.2 mM total for all 12 amino acids) was possibly physiologically high (we did not test lower concentrations) but well within stimulu–response profiles reported for fish olfactory neurons (e.g. Silver and Finger, 1984). The electrophysiological threshold for adult zebrafish olfactory organs was reported to be on the order of...
0.01–1 µM depending on the odor stimulus (Michel and Lubomudrov, 1995). Activity-dependent labeling of adult zebrafish olfactory neurons stimulated with agmatine and L-glutamine was shown to be greatest at 1 mM and 100 µM respectively (Michel et al., 1999). Adult zebrafish were previously observed to display swimming responses to 100 µM but not 10 µM alanine (Steele et al., 1990). Nevertheless, the ecological relevance of our test concentrations is not so clear. Zebrafish originate in rivers and lakes of India and Bangladesh (Sterba, 1963; Jayaram, 1981) where background levels of total dissolved amino acids may be ~1–10 µM (Lytle and Perdue, 1981); however, these levels would presumably be elevated near actual food sources. The response by larval zebrafish in our study to 1.2 mM (total) concentrations of mixed amino acids is consistent with studies of other larval fish. Non-feeding cod larvae responded to arginine stimuli at 1 mM and 10 µM but not to lower concentrations (Døving et al., 1994). Similarly, eddies of 10–1 mM amino acid elicited biting and snapping responses in non-feeding rainbow and brook trout larvae (Valentincic et al., 1999), responses also observed in adult rainbow trout to 30–0.3 mM amino acid stimuli (Valentincic and Caprio, 1997).

It is likely that the chemosensory response observed in our studies was olfactory based. Olfactory neurons of adult zebrafish respond to both amino acids and bile acids (e.g. Michel and Derbidge, 1997; Lipschitz and Michel, 2002). Solitary Chemosensory Cells (SCCs) may respond to bile acids but not to amino acids (e.g. Peters et al., 1991; Kotrschal et al., 1996), and thus by restricting our study to amino acids we tentatively eliminated the SCCs as a contributing input for the observed behavioral responses. However, the amino acid response of possibly specialized SCCs in the searobin make this a tentative assessment (e.g. Silver and Finger, 1984; Finger, 1997). Taste receptors also respond to both amino and bile acids (Hara, 1994a; Ogawa and Caprio, 2000). However, the taste system appears to develop later than the olfactory system as taste receptor cells are not anatomically evident until 5 days a.f. (Hansen et al., 2002); thus, taste was possibly not functioning at the developmental stages examined in our study.

Our behavioral assay was chiefly designed to test the activity of fish in response to an increase in background odors as opposed to a directionally oriented response to that increase. The simplest measure of such chemosensory-based activity was the number of fish swimming in response to the applied amino acids. Few fish showed any increase in movement activity on day 3 a.f. in response to amino acids (Figure 2), a result to be expected given that zebrafish at this stage are known to remain largely stationary unless disturbed (Eaton and Nissanov, 1985). On day 4 a.f., however, there was a striking and significant increase in the number of fish swimming or moving about in response to amino acids (Figure 2). A similar result has been observed for yolk-sac herring larvae which increased their activity in response to either barnacle naupliar extract or amino acids (Dempsey, 1978). This general increase in the number of day 4 zebrafish swimming was also accompanied by significant increases in swimming speed (Figure 3) and net movement away from starting positions (NGDR, Figure 5), the latter suggesting some degree of directed or oriented swimming. On day 5 a.f., fish also showed an increase in swimming speed together with a significant increase in straight line swimming (decrease in RCDI, Figure 4) relative to controls, indicating that they were clearly responding behaviorally to the applied amino acid mixture. Day 5 fish displayed little difference in the number of fish swimming between control and test conditions; however, spontaneous swimming activity was dramatically and significantly increased for these fish relative to day 4 a.f., presumably obscuring odor stimulated changes in this parameter.

The observed changes in the behavioral parameters measured in our study (Figures 3–5) are consistent with the ontogeny of feeding behavior. Increased swimming speed and straight line swimming (decreased RCDI and/or increased NGDR) have been suggested as components of feeding behavior of adult zebrafish (Kasumyan and Ponomarev, 1986, 1990; Steele et al., 1990, 1991). These parameters have also been suggested to increase in animal search patterns under conditions of low food abundance (Banks, 1957; Smith, 1974). Beetle larvae turning rates were observed to decline as hunger increased (Dixon, 1959). Goldfish and tetra juveniles swam faster in ‘area increased searching’ under low food conditions (Mikheev et al., 1992). Minnows were also observed to respond to food stimulation by searching more of the available area, swimming faster and with a higher maximum but more variable swimming speed (Essler and Kotrschal, 1994).

More importantly, perhaps, this study may present a basis for developing assays useful for studying chemosensory behavioral performance, such as more fully characterizing the ontogeny of chemosensory sensitivity, characterizing chemosensory induced plasticity due to embryonic imprinting of novel chemostimulants, or screening chemosensory mutants. An ideal screen should be simple to perform and score, as well as statistically robust (minimizing type I errors and maximizing power) at ‘reasonable’ sample sizes. Our study suggests that an easily identifiable developmental phenotype, day 4 inactive floating, displays a statistically strong response to applied amino acids. With only 12 replicates of each treatment (total 120 larvae), we detected a significant increase in the number of fish swimming in the dishes after adding amino acid mixture. This result was significant at α = 0.05 with a power of 0.97, and even significant at α = 0.005 with a power of 0.73. The robustness of this measure probably arises from the fact that the larvae we tested were not active swimmers (low noise); by day 5 a.f. all larval fish were swimming all the time.

A behavioral assay for older larvae (e.g. day 5) could be based on measures of swimming such as speed, turning rates
and net-to-gross displacement ratio, but are likely to require larger numbers of individuals in order to obtain high statistical confidence. For example, although changes in average swimming speed and net-to-gross displacement ratio in response to amino acids by day 5 a.f. larvae showed a similar pattern as those on day 4 a.f., they were not statistically different given the sample sizes employed. Turning frequency might be a more useful metric; with 12 replicates of each treatment (total 120 larvae) we detected a significant decrease in turning frequency by larvae in response to amino acids on day 5 a.f. (Figure 4). This result was significant at $\alpha = 0.05$ with a power of 71%; power and sample size analyses indicated that increasing the replication to $n = 15$ would have resulted in a power of 82%, while a sample size of $n = 20$ would have resulted in a power of 92% at the same significance level. Other unexplored behavioral parameters may yield stronger metrics of response, such as increased biting response (after Valentincic et al., 1999) or the redistribution of larvae over time within an asymmetric stimulus field. But zebrafish embryos and larvae can be readily and inexpensively produced and require little maintenance (e.g. no feeding) through the ages tested in this study, making them highly suitable for such investigations.

Sensory systems translate the external world to the organism. We have shown that zebrafish can respond to external chemical cues within 24 h of hatching, only 4 days after fertilization, and perhaps 24 h before the onset of spontaneous swimming. Which chemosensory modality is functioning in this day 4 a.f. response is not definitively clear. Of the three chemosensory modalities, SSCs may not respond to amino acids at all (Peters et al., 1991; Kotrschal et al., 1996, 1997), and taste may not yet be functional on day 4 a.f. (Kotrschal et al., 1997); therefore, the day 4 a.f. response may be an olfactory based chemosensory response. What is clear is that these fish are quick to develop for a chemosensory life outside the egg.

Acknowledgements

The authors thank R. Zimmer and D. Wethey for access to the motion analysis system. R. Zimmer provided helpful advice on chemical stimulants and D. Pentcheff on EV software and data analysis, respectively. I. Hunt von Herbing provided helpful comments on earlier versions of the manuscript. Supported by an NIH-NRSA award (NINDS, fellowship 1F32NS09695-01) to S.M.L. and NIH (NIDCD DC-00588), USDA (CGRP 94-37302-0615) and NSF (IBN-0212510) grants to R.G.V.

References


Accepted November 24, 2003