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

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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, 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

sources (Hara, 1994a; Kotrschal, 2000). Too few species have been characterized to generalize a role for SCCs. SCCs may convey information about potential predators (Peters *et al.*, 1991; Kotrschal, 1996, 2000; Kotrschal *et al.*, 1996, 1997), though in at least one perhaps specialized case SCCs clearly convey information about food (Silver and Finger, 1984; Finger, 1997).

All three chemosensory modalities are established anatomically by the end of the first week a.f. and continue to develop into adult life. Olfactory neurons are reputedly the first to appear. A pair of olfactory placodes form at the anterior tip of the head ~17 h after fertilization (a.f.), pioneer neurons grow inward beginning ~24 h a.f., and sensory axons begin forming synaptic connections in the olfactory lobe soon after (Hansen and Zeiske, 1993, 1998; Whitlock and Westerfield, 1998). Odor receptor mRNAs are detectable in olfactory neurons ~30–35 h a.f. (Barth *et al.*, 1996; Byrd *et al.*, 1996; Vogt *et al.*, 1997; Argo *et al.*, 2003). The olfactory organ exists as an open pit beginning ~35 h a.f., and begins folding into the adult rosette beginning ~2 weeks a.f. (Hansen and Zeiske, 1993). Thus, embryos hatch during day 3 a.f. with an olfactory system that appears anatomically and biochemically complete. Taste buds have been first observed 4–5 days a.f. on the lips and in the oropharyngeal cavity (Hansen *et al.*, 2002), and on day 5 a.f. on the outer body surface coincident with the first observations of exogenous feeding (Kotrschal *et al.*, 1997). SCCs have been first observed on day 3 a.f. (Kotrschal *et al.*, 1997). The numbers of olfactory neurons, taste buds and SCCs increase with animal growth (Barth *et al.*, 1996; Byrd *et al.*, 1996; Kotrschal *et al.*, 1997).

Zebrafish embryos are provisioned with yolk stores that are rapidly depleted during the first week a.f., and consequently larvae require food shortly after hatching (Fuiman and Webb, 1988; Westerfield, 1993). A behaviorally functional chemosensory system may thus be critical to the survival of these early larvae. We therefore characterized behavioral responses to waterborne chemosensory stimulants (e.g. amino acids) during the first days after hatching, and have established that at least one chemosensory system is operating at a behavioral level by day 4 a.f., ~1 day after hatching. The assay we employed established an early developmental time point of chemosensory functionality; such an assay might be useful in future studies for characterizing odorant sensitivity or developmental plasticity or screening for chemosensory mutations.

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 the 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

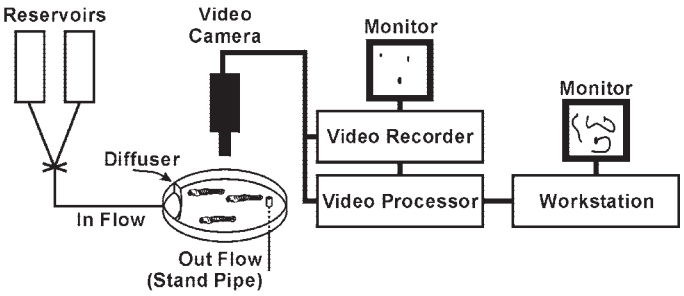

Figure 1 Diagram of the experimental set up. Either aquarium water alone or aquarium water plus amino acids flowed through the experimental dish. Dish diameter was 90 cm, fish lengths were ~3 mm. Zebrafish (five per trial) were videotaped, and their swimming paths were analyzed using computer assisted 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 $\times$ 2 treatments = 16 dishes (80 fish); 4 days a.f., $n = 12$ dishes $\times$ 2 treatments = 24 dishes (120 fish); and 5 days a.f., $n = 12$ dishes $\times$ 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 1 Activity of zebrafish in still water (Still, phase 1 of the behavioral assay) and following introduction of flowing aquarium water (Flow Control, phase 2 of the assay) on days 3, 4, and 5 after fertilization

Activity variable	Day 3 a.f.		Day 4 a.f.		Day 5 a.f.	
	Still	Flow Ctrl	Still	Flow Ctrl	Still	Flow Ctrl
Number fish swimming	0.27 (0.60)	0.54 (0.65)	2.04 (1.64)	2.54 (1.75)	4.90 (0.44)	5.00 (0.32)
Mean swimming speed (mm/s)	0.38 (0.16)	0.37 (0.16)	1.24 (1.23)	1.56 (1.77)	2.64 (1.21)	2.91 (1.22)
Mean RCDI (degrees/s)	467.41 (134.66)	412.67 (134.66)	449.35 (119.21)	431.89 (121.79)	318.08 (108.99)	313.35 (65.43)
Mean NGDR	0.07 (0.09)	0.05 (0.05)	0.22 (0.24)	0.21 (0.22)	0.52 (0.19)	0.48 (0.12)

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 circuity.

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).

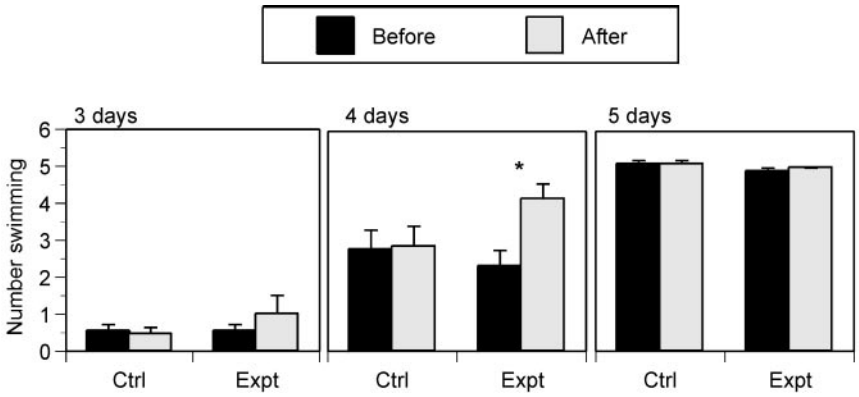

Figure 2 Number of fish swimming before and after addition of test stimulus at 3, 4 and 5 days after fertilization (mean per dish +SE). Test stimulus was either aquarium water (Ctrl) or amino acid mixture (Expt). *Significant difference between the before and after means (paired t-test, $P < 0.01$).

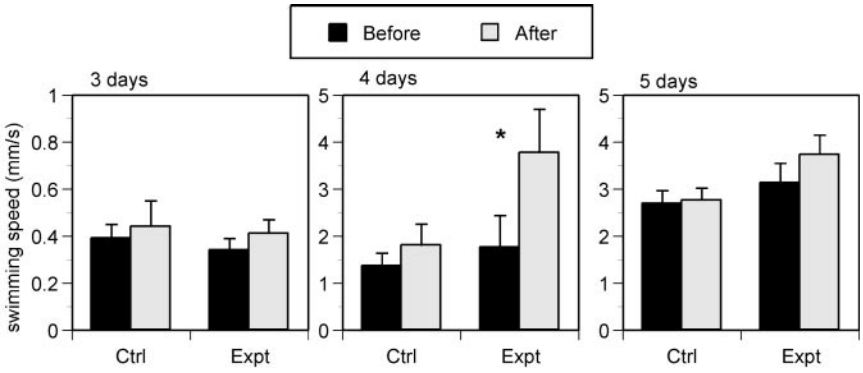

Figure 3 Swimming speeds of zebrafish on days 3, 4 and 5 after fertilization before and after the addition of test stimulus (weighted average per dish, means +SE). Stimulus was either aquarium water (Ctrl) or amino acid mixture (Expt). *Significant difference between the before and after means (paired t-test, $P < 0.10$). Note differences in Y-axis scales, indicating transition from negative to positive buoyancy between days 3 and 4 a.f.

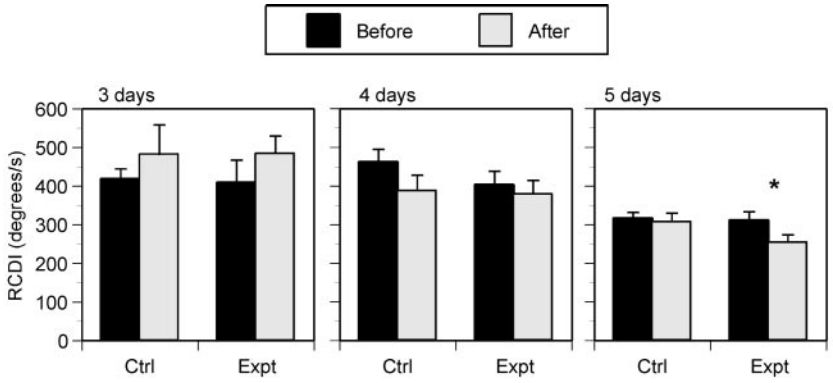

Figure 4 Rate of change of direction index (RCDI) of fish before and after exposure to test stimulus (weighted average per dish, means +SE). Stimulus was aquarium water (Ctrl) or amino acid mixture (Expt). *Significant difference between the before and after means (paired *t*-test, $P < 0.05$).

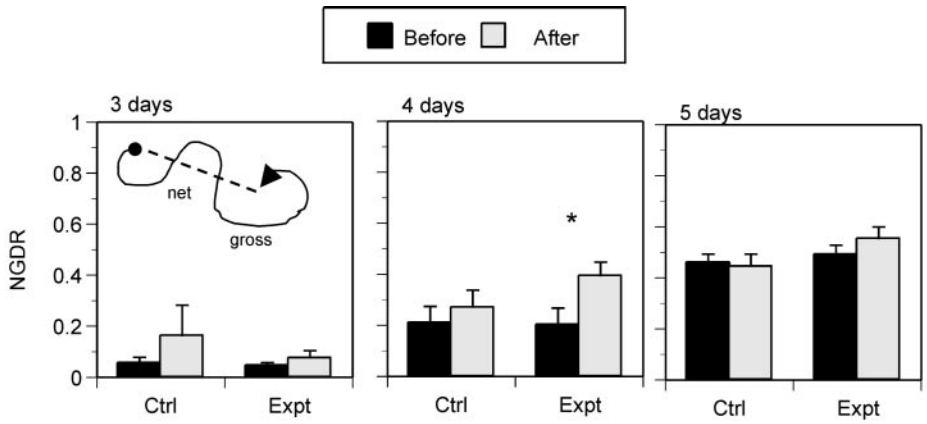

Figure 5 Net to gross displacement ratio (NGDR) for zebrafish swimming paths before and after addition of either aquarium water (Ctrl) or amino acid mixture (Expt) (weighted average per dish, means +SE). NGDR is the ratio of net displacement (dashed line in inset) to total path length (solid line with dot indicating start of path and arrow head indicating end of path). Increasing NGDR values indicate fish are moving further away from their starting positions. *Significant difference between the before and after means (paired *t*-test, $P < 0.05$).

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 circuity, 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 electrophysiologial 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 $\alpha = 0.05$ with a power of 0.97, and even significant at $\alpha = 0.005$ 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.

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References

Argo, S., Weth, F. and Korsching, S. (2003) *Analysis of penetrance and expressivity during ontogenesis supports a stochastic choice of zebrafish odorant receptors from predetermined groups of receptor genes*. Eur. J. Neurosci., 17, 833–843.

Banks, C.J. (1957) *The behavior of individual coccinellid larvae on plants*. Br. J. Anim. Behav., 5, 12–24.

Barth, A.L., Justice, N.J. and **Ngai, J.** (1996) *Asynchronous onset of odorant receptor expression in the developing zebrafish olfactory system*. Neuron, 16, 23–34.

Byrd, C.A., Jones, J.T., Quattro, J.M., Rogers, M.E., Brunjes, P.C. and **Vogt, R.G.** (1996) *Ontogeny of odorant receptor gene expression in zebrafish,* Danio rerio*.* J. Neurobiol., 29, 445–458.

Caprio, J. (1982) *High sensitivity and specificity of olfactory and gustatory receptors of catfish to amino acids.* In Hara, T.J. (ed.), Chemoreception in Fishes. Elsevier Scientific Publishing Co., Amsterdam, pp. 109–134.

Dempsey, C.H. (1978) *Chemical stimuli as a factor in feeding and intraspecific behaviour of herring larvae*. J. Mar. Biol. Assoc. UK, 58, 739–747.

Dixon, A.F.G. (1959) *An experimental study of the searching behavior of the predatory coccinellid beetle* Adalia decempunctata *(L.)*. J. Anim. Ecol., 28, 259–281.

Døving, K.B., Maristol, M., Andersen, J.R. and **Knutsen, J.A.** (1994) *Experimental evidence of chemokinesis in newly hatched cod larvae (*Gadus morhua *L.)*. Mar. Biol., 120, 351–358.

Eaton, R.C. and **Nissanov, J.** (1985) *A review of Mauthner-initiated escape behavior and its possible role in hatching in the immature zebrafish,* Brachydanio rerio. Environ. Biol. Fishes, 12, 265–279.

Ellingsen, O.F. and **Døving, K.B.** (1986) *Chemical fractionation of shrimp extracts inducing bottom food searching behavior in cod* Gadus morhua. J. Chem. Biol., 12, 155–168.

Essler, H. and **Kotrschal, K.** (1994) *High resolution analysis of swim path patterns of intact and olfaction-deprived minnows (*Phoxinus phoxinus*) stimulated with food and potential predator odour*. J. Fish Biol., 45, 555–567.

Finger, T.E. (1997) *Evolution of taste and solitary chemoreceptor cell systems.* Brain Behav. Evol., 50, 234–243.

Friedrich, R.W. and **Korsching, S.I.** (1997) *Combinatorial and chemotopic odorant coding in the zebrafish olfactory bulb visualized by optical imaging*. Neuron, 18, 737–752.

Fuiman, L.A. and **Webb, P.W.** (1988) *Ontogeny of routine swimming activity and performance in zebra danios (Teleostei: Cyprinidae)*. Anim. Behav., 36, 250–261

Fuss, S.H. and **Korsching, S.I.** (2001) *Odorant feature detection: activity mapping of structure response relationships in the zebrafish olfactory bulb*. J. Neurosci., 21, 8396–8407.

Hansen, A. and **Zeiske, E.** (1993) *Development of the olfactory organ in the zebrafish* Brachydanio rerio. J. Comp. Neurol., 333, 289–300.

Hansen, A. and **Zeiske, E.** (1998) *The peripheral olfactory organ of the zebrafish,* Danio rerio*: an ultrastructural study*. Chem. Senses, 23, 39–48.

Hansen, A., Reutter, K. and **Zeiske, E.** (2002) *Taste bud development in the zebrafish,* Danio rerio. Dev. Dyn., 223, 483–496.

Hara, T.J. (1982) *Structure–activity relationships of amino acids as olfactory stimuli.* In Hara, T.J. (ed.), Chemoreception in Fishes. Elsevier Scientific Publishing Co., Amsterdam, pp. 135–157.

Hara, T.J. (1994a) *Olfaction and gustation in fish: an overview.* Acta Physiol. Scand., 152, 207–217

Hara, T.J. (1994b) *The diversity of chemical stimulation in fish olfaction and gustation*. Rev. Fish. Biol., 4, 1–35.

Haynes, L.J., Stangster, D.M., Steven, A.W. and **Thomas, S.** (1967) *Chemical factors inducing exploratory feeding behaviour in fish—e.f.b.-inducing properties of marine invertebrates*. Comp. Biochem. Physiol., 20, 755–765.

Jayaram, K.C. (1981) The Freshwater Fishes of India, Pakistan, Bangladesh, Burma, and Sri Lanka—A Handbook. Calcutta: Zoological Survey of India.

Kasumyan, A.O. and **Ponomarev, V.Y.** (1986) *Study on the behavior of zebrafish,* Brachydanio rerio*, in response to natural chemical food stimuli*. Vopr. Ikhtiol., 26, 665–673

Kasumyan, A.O. and **Ponomarev, V.Y.** (1990) *The ontogeny of feeding behavior in relation to natural chemical signals in cyprinid fishes*. Vopr. Ikhtiol., 30, 447–456.

Kimmel, C.B., Ballard W.W., Kimmel S.R., Ullmann, B. and **Schilling T.F.** (1995) *Stages of embryonic development of the zebrafish.* Dev. Dyn., 203, 253–310.

Kotrschal, K. (1996) *Solitary chemosensory cells: why do primary aquatic vertebrates need another taste system?* Trends Ecol. Evol., 11, 110–114.

Kotrschal, K. (2000) *Taste(s) and olfaction(s) in fish: a review of specialized sub-systems and central integration*. Pflugers Arch., 439 (3 Suppl), R178–R180.

Kotrschal, K., Krautgartner, W.-D. and **Hansen, A.** (1997) *Ontogeny of solitary chemosensory cells in the zebrafish,* Danio rerio. Chem. Senses, 22, 111–118.

Kotrschal, K., Peters R. and **Døving, K.B.** (1996) *Chemosensory and tactile nerve response from the anterior dorsal fin of a rockling,* Gaidropsarus vulgaris *(Gadidae, Telostei)*. Prim. Sens. Neuron, 1, 297–308.

Lipschitz, D.L. and **Michel, W.C.** (2002) *Amino acid odorants stimulate microvillar sensory neurons.* Chem. Senses, 27, 277–286.

Lipschitz, D.L. and **Michel, W.C.** (1999) *Physiological evidence for the discrimination of L-arginine from structural analogues by the zebrafish olfactory system*. J. Neurophysiol., 82, 3160–3167.

Lytle, C.R. and **Perdue, E.M.** (1981) *Free, proteinaceous, and humic-bound amino acids in river water containing high concentrations of aquatic humus*. Environ. Sci. Technol., 15, 224–228.

Michel, W.C. and **Derbidge, D.S.** (1997) *Evidence of distinct amino acid and bile salt receptors in the olfactory system of the zebrafish,* Danio rerio. Brain Res., 764, 179–187.

Michel, W.C. and **Lubomudrov, L.M.** (1995) *Specificity and sensitivity of the olfactory organ of the zebrafish* Danio rerio. J. Comp. Physiol. A, 177, 191–199.

Michel, W.C., Steullet, P., Cate, H.S., Burns, C.J., Zhainazarov, A.B. and **Derby, C.D.** (1999) *High-resolution functional labeling of vertebrate and invertebrate olfactory receptor neurons using agmatine, a channel-permeant cation.* J. Neurosci. Methods, 90, 143–156.

Mikheev, V.N., Pavlov, D.S. and **Pakulska, D.** (1992) *Swimming response of goldfish,* Carassius auratus*, and the tetra,* Hemigrammus caudovittatus*, larvae to individual food items and patches*. Environ. Biol. Fishes, 35, 351–360.

Ogawa, K. and **Caprio, J.** (2000) *Glossopharyngeal taste responses of the channel catfish to binary mixtures of amino acids*. Chem. Senses, 25, 501–506.

Peters, R., Kotrschal, K. and **Krautgartner, W.-D.** (1991) *Solitary chemoreceptor cells of* Ciliata mustela *(Gadidae, Telostei) are tuned to mucoid stimuli*. Chem. Senses, 16, 31–42.

Silver, W.L. and **Finger, T.E.** (1984) *Electrophysiological examination of a non-olfactory, non-gustatory chemosense in the searobin,* Prionotus carolinus. J. Comp. Physiol. A, 154, 167–174.

Smith, J.N.M. (1974) *The food searching behaviour of two European thrushes. II. The adaptiveness of the search patterns*. Behaviour, 19, 695–706

Steele, C.W., Owens, D.W. and **Scarfe, A.D.** (1990) *Attraction of zebrafish,* Brachydanio rerio*, to alanine and its suppression by copper*. J. Fish Biol., 36, 341–352.

Steele, C.W., Scarfe, A.D. and **Owens, D.W.** (1991) *Effects of group size on the responsiveness of zebrafish,* Brachydanio rerio *(Hamilton Buchanan), to alanine, a chemical attractant*. J. Fish Biol., 38, 553–564.

Sterba, G.S. (1963) Freshwater Fishes of the World. The Viking Press, New York.

Valentincic, T. and **Caprio, J.** (1997) *Visual and chemical release of feeding behavior in adult rainbow trout*. Chem. Senses, 22, 375–383.

Valentincic, T., Lamp, D.F. and **Caprio, J.** (1999) *Expression of a reflex biting/snapping response to amino acids prior to first exogenous feeding in salmonid alevins*. Physiol. Behav., 67, 567–572.

Vogt, R.G., Lindsay, S.M., Byrd, C.A. and **Sun, M.** (1997) *Spatial patterns of olfactory neurons expressing specific odor receptor genes in 48-hour-old embryos of zebrafish* Danio rerio. J. Exp. Biol., 200, 433–443.

Westerfield, M. (1993) The Zebrafish Book: A Guide for the Laboratory use of Zebrafish (Brachydanio rerio). University of Oregon Press, Eugene, OR.

Whitlock, K.E. and **Westerfield, M.** (1998) *A transient population of neurons pioneers the olfactory pathway in the zebrafish*. J. Neurosci., 18, 8919–8927.

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