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Chemotopy of Amino Acids on the Olfactory Bulb Predicts Olfactory Discrimination Capabilities of Zebrafish *Danio rerio*

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Abstract

Amino acids reliably evoke strong responses in fish olfactory system. The molecular olfactory receptors (ORs) are located in the membrane of cilia and microvilli of the olfactory receptor neurons (ORNs). Axons of ORNs converge on specific olfactory bulb (OB) glomeruli and the neural responses of ORNs expressing single Ors activate glomerular activity patterns typical for each amino acid. Chemically similar amino acids activate more similar glomerular activity patterns then chemically different amino acids. Differential glomerular activity patterns are the structural basis for amino acid perception and discrimination. We studied olfactory discrimination in zebrafish *Danio rerio* (Hamilton 1822) by conditioning them to respond to each of the following amino acids: L-Ala, L-Val, L-Leu, L-Arg, and L-Phe. Subsequently, zebrafish were tested for food searching activities with 18 nonconditioned amino acids. The food searching activity during 90 s of the test period was significantly greater after stimulation with the conditioned stimulus than with the nonconditioned amino acid. Zebrafish were able to discriminate all the tested amino acids except L-Ile from L-Val and L-Phe from L-Tyr. We conclude that zebrafish have difficulties discriminating amino acid odorants that evoke highly similar chemotopic patterns of activity in the OB.

Key words: amino acid, behavior, discrimination, fish, olfaction

Introduction

Olfactory detection of amino acids was thoroughly studied with molecular, morphological, physiological, and behavioral techniques in channel catfish, *Ictalurus punctatus* (Caprio and Derby 2008); zebrafish, *Danio rerio* (Hansen and Zeiske 1998; Korsching 2001; Niessing and Friedrich 2010); and rainbow trout, *Onchorhyncus mykiss* (Laberge and Hara 2001). For these and other fish species, electrophysiological investigations revealed high olfactory sensitivity to amino acid stimuli (Caprio and Byrd 1984; Michel and Lubomudrov 1995; Nikonov and Caprio 2001). Sensitivities of single olfactory receptor neurons (ORNs) to amino acids were reported for channel catfish (Kang and Caprio 1995; Hansen et al. 2003; Nikonov and Caprio 2004) and zebrafish (Friedrich and Laurent 2004). In the black bullhead catfish (*Ameiurus melas*), it is most likely that ORNs that code for amino acids are those that are excited rather than inhibited by amino acids (Dolensek and Valentincic 2010).

Odorant-activated ORN neural activity is transmitted to the olfactory bulb (OB), where axons of ORNs expressing the same receptor converge onto distinct glomeruli exciting mitral cells, the output neurons of the OB. Different glomeruli receive odorant excitation from specialized ORNs resulting in specific glomerular activation patterns (i.e., chemotopic patterns). Physiological activities of the mitral cells within the same glomerulus provide the structural and functional basis for olfactory perception and discrimination (Johnson and Leon 2007). It is hypothesized that the ability to discriminate between amino acids is predictable from the nonoverlap of combinatorial odor representations in the OB (Friedrich and Korsching 1997, 1998; Friedrich and Laurent 2001, 2004). The first studied OB chemotopic patterns for amino acids were reported in zebrafish where the patterns for acidic, basic, and neutral amino acids were quite distinct, whereas the glomerular activation patterns for similar amino acids, such as neutral amino acids (Friedrich and Korsching 1997), were highly correlated. Behavioral studies of olfactory discrimination of amino acids showed that channel catfish (Valentincic and Caprio 1994a; Valentincic, Wegert, et al.

1994), black bullhead catfish (*A. melas*) (Valentincic, Metelko, et al. 2000; Valentincic 2005), and zebrafish (Lindsay and Vogt 2004; Valentincic and Miklavc 2005; Braubach et al. 2009) discriminate between structurally different amino acids but that discrimination was difficult or even impossible for some structurally similar amino acids.

As for odor elicited neural activations in the OB of rats, which are robust to variability in concentration (Cleland et al. 2007), behavioral studies of olfactory discrimination in channel catfish (Valentincic and Caprio, 1994a, 1994b) and black bullhead catfish (Valentincic et al. 2000a) indicate a concentration invariant perception of odorants. In zebrafish, the initial chemotopic activity that appeared at low L-Ala concentration changed little with concentration increase (Friedrich and Korsching 1997); that is, chemotopic activity patterns remained highly correlated within 2–3 log units of concentration increase (Niessing and Friedrich 2010).

After the original description of chemotopic projections of amino acids (Friedrich and Korsching 1997; Friedrich and Laurent 2004; Yaksi et al. 2007) in zebrafish, it was suggested that neuronal circuits in the OB reduce the overlap between representations of similar odors by decorrelating their similar chemotopic representations (Friedrich and Laurent 2001). The decorrelation of similar chemotopic patterns facilitates discrimination of chemically similar amino acids. Even when the differences in the chemotopic representations of amino acids are small, the decorrelation of their representations provide for categorical differences in activities of mitral cell ensembles (Niessing and Friedrich 2010). In the present investigation, the conditioned zebrafish immediately discriminated between 14 of 19 chemically different amino acids; however, their discrimination of L-Ser and Gly from L-Ala, as anticipated from their highly similar chemotopic activation patterns (Friedrich and Korsching 1997, 1998; Friedrich and Laurent 2001; Yaksi et al. 2007), occurred only after an initial experimental experience with these amino acids. The 2 pairs of amino acids L-Ile versus L-Val and L-Phe versus L-Tyr were not discriminated in wild-type zebrafish (Valentincic and Miklavc 2005). The present investigation behaviorally tested the hypothesis that different chemotopic representations of amino acids in the OB are required for their olfactory discrimination.

Materials and methods

Experimental zebrafish

Wild-type zebrafish were purchased at a Ljubljana aquarium shop and cultured in the Department of Biology, University of Ljubljana, for a period of 7 years. Prior to experimentation, the zebrafish were maintained in 80 L aquaria furnished with Java moss (*Taxiphyllum barbieri* [Iwatsuki 1982], previously *Vesicularia dubyana*, Broth, 1908) and fed with artificial flake food and frozen mosquito larvae. One month before the experiments, the zebrafish were transferred into a recirculating system composed of a ~2000 L reservoir, a 500 L limestone gravel filter, and 10 experimental aquaria ($35 \times 20 \times 25$ cm) to house individual zebrafish. Water temperature was between 23 and 27 °C and the fish were maintained in a light: dark cycle of 16:8 h. To prevent visual detection between zebrafish in neighboring aquaria, the aquaria were separated with nontransparent screens. In the experiments, the zebrafish, which were between 5 and 12 months old, received all their food during the conditioning sessions. All animal procedures were performed in accordance with the animal care guidelines approved by the Veterinary Administration of the Republic of Slovenia. After the experiments, the zebrafish were donated to fish hobbyists in the Ljubljana area.

Amino acid solutions and stimulus application

The tested amino acids included short-chain neutral amino acids: glycine (Gly), L-alanine (L-Ala), and L-serine (L-Ser); neutral amino acids with long side chains: L-valine (L-Val), L-isoleucine (L-Ile), L-leucine (L-Leu), L-norvaline (L-nVal), L-norleucine (L-nLeu), L-methionine (L-Met), and L-asparagine (Asn); aromatic amino acids: L-phenylalanine (L-Phe), L-tyrosine (L-Tyr), and L-tryptophane (L-Trp); acidic amino acids: L-glutamic acid (L-Glu) and L-aspartic acid (L-Asp); basic amino acids: L-arginine (L-ArgHCl), L-lysine (L-LysHCl) and L-histidine (L-His), and the imino acid, L-proline (L-Pro). The amino acids were purchased from either Sigma-Aldrich (http://www.sigmaaldrich.com/site-map.html) or Merck (http://www.jencons.co.uk/app/Home). The hydrochlorides of the basic amino acids were used due to their higher solubility and small pH changes in solution.

Fifty milliliter of amino acid solutions were prepared with aquarium water in polystyrene jars (VWR International; http://si.vwr.com/app/Home); the stock solution concentrations were 3×10^{-3} M. L-Tyr that is insoluble at the above concentration was prepared at 1.5×10^{-3} M concentration. Stimulus solutions were injected into the aquaria from hydraulically operated pipettes via 3.5 m connecting tubing between the syringe and the Pasteur pipette located above the center of each aquarium. Pasteur pipettes were filled with fresh amino acid solutions immediately before the onset of an experimental series. Two milliliters of stock solution were added into a continuous flow of recirculating water entering water surface in each aquarium so that the zebrafish were unable to detect the mechanical noise of the stimulus addition. The final dilution factor for the amino acids after >2 min was 1:10 000. Prior to the experiments, the spread of stimuli within the experimental aquaria was modeled with aliquots of diluted milk suspension of the same volume as the volume of the injected stimulus solution. Irrespective of the fish position within aquarium at the time of stimulus addition, the stimulating solution reached zebrafish in less than 20 s. At that time, the maximal injected solution concentration was 300×–3000× lower than the stock solution

concentration (Valentincic and Caprio 1994a). Two minutes after the addition, the amino acid concentration in the experimental aquaria was 3×10^{-7} M, which is close to the behavioral detection level of channel catfish (Valentincic and Caprio 1994a). The aquarium water was exchanged at a rate of 25 L/h; additional filtration decreased amino acid concentrations in the aquarium water to nearly background levels (10^{-9} to 3×10^{-7} M for different amino acids).

Before each experiment, the glass pipettes were washed 5 times for 1 min with hot water and equilibrated 5 × 2 min with aquarium water. Each day, at the completion of the experiments, the glassware was washed with CEMEX detergent (Kemika, Zagreb, Croatia).

Conditioning and test procedures

The initial acclimation of the test fish included regular feeding for 10 days. Five different groups of zebrafish were conditioned during a minimum of 30 sessions with one of the following amino acids: L-Ala, L-Val, L-Leu, L-ArgHCl, and L-Phe. During the conditioning experiments, zebrafish were offered a food reward (dried mosquito larvae) 90 s after the introduction of the conditioning stimulus into the aquarium. The fish detected the amino acids at concentrations between 3×10^{-5} and 3×10^{-6} M (Valentincic and Caprio 1994a, 1994b). Detection of the conditioned stimulus triggered a longer and more intense food search (conditioned appetitive behavior) than the detection of any nonconditioned stimulus. All behavior was video recorded with digital cameras DM-MV1E (Canon) and Sony HD8V. The swimming activity of each fish was evaluated by counting the number of turns greater than 90° and by measuring the distance traveled with Neuro-inspector software (FDS Research Computer Vision Group, Slovenia). Based on the contrast between the moving fish and its background, the Neuro-inspector software evaluated the length of movement observed in the video of each fish. The number of turns of the fish and the distance traveled were determined. A maximum of 5 conditioning or test sessions were performed daily with a group of 10 fish.

Conditioning and test stimuli

The conditioned stimuli are indicated in bold and the test stimuli which evoke highly similar glomerular activity patterns to the conditioned stimulus and are therefore potentially perceived equally are bold and underlined:

Conditioned stimulus L-**Ala**, test stimuli: Gly, L-Ser, L-Phe, L-Tyr, L-Trp, L-His, L-Asn, L-Val, L-Ile, L-Leu, L-Met, L-ArgHCl, L-Lys HCl, L-Glu, L-Asp, and L-Pro.

Conditioned stimulus L-**Arg** HCl, test stimuli: Gly, L-Ala, L-Ser, L-Phe, L-Tyr, L-Trp, L-His, L-Asn, L-Val, L-Ile, L-Leu, L-Met, L-Lys HCl, L-Glu, L-Asp, and L-Pro.

Conditioned stimulus L-**Leu**, test stimuli: Gly, L-Ala, L-Ser, L-Phe, L-Tyr, L-Trp, L-His, L-Asn, L-Val, L-Ile, L-Met, L-Arg HCl, L-Lys HCl, L-Glu, L-Asp, and L-Pro.

Conditioned stimulus L-**Val**, test stimuli: Gly, L-Ala, L-Ser, L-Phe, L-Tyr, L-Trp, L-His, L-Asn, **l-Ile**, L-Leu, L-Met, L-Arg HCl, L-Lys HCl, L-Glu, L-Asp, L-Pro, L-nLeu, and L-nVal.

Conditioned stimulus L-**Phe**, test stimuli: Gly, L-Ala, L-Ser, **l-Tyr**, L-Trp, L-His, L-Asn, L-Val, L-Ile, L-Leu, L-Met, L-Arg HCl, L-Lys HCl, L-Glu, L-Asp and L-Pro.

Statistics

Conditioned test and control solutions were introduced into the aquaria in alternating sequences so that no test stimulus was used twice without an intermediate rewarded conditioning stimulus. When a conditioned stimulus was tested before and after a test stimulus, the tests were compared with both neighboring conditioned stimuli using Wilcoxon rank test. The entire test series was repeated 2–3 times. If after 2 test series, zebrafish did not discriminate the conditioned from the nonconditioned amino acids, a discrimination training series was performed by presenting 10–18 successive alternating conditioned/test stimulus trials.

Median and interquartile ranges of counts of >90° turns or distance traveled were determined for each response series for the experimental groups of 10 fish. These 2 activity measures were compared using nonparametric Spearman rank correlation coefficients. The Wilcoxon sum of ranks test was applied to evaluate the statistical difference between the test and conditioned experiments.

Comparison of olfactory discrimination data and physiological data from Friedrich and Korsching (1997)

To directly compare behavioral and physiological data on chemotopy of amino acids in the OB of zebrafish, we used Figure 4 of Friedrich and Korsching (1997). The color pictures of the OB chemotopic activity patterns of different amino acids were opened in Photoshop and turned into grayscale mode. Background noise was removed and the central the most excited areas of glomeruli that did not translate into dark gray were filled with black color and saved. In the next step, the grayscale pictures were opened in Image J image processing tool. Assuming concentration invariance of chemotopic representation of odorants and considering the fact that the original data were not collected at equal electroolfactogram response magnitudes for amino acids gray colors were adjusted into black using the Image J threshold procedure. It is notable that it was possible to adjust the black areas of all the chemotopic images of amino acids at the same setting of the threshold filter (0, 221). The total area of the chemotopic representations for each pair of amino acids was calculated using OR procedure (overlapped area counted once) and the differential area for both amino acids was calculated using XOR procedure. An index of amino acid–specific OB dissimilarity (IOBD) for each pair of amino acids was calculated (IOBD = XORaa/ORaa) and introduced into trellis diagram (Figure 11).

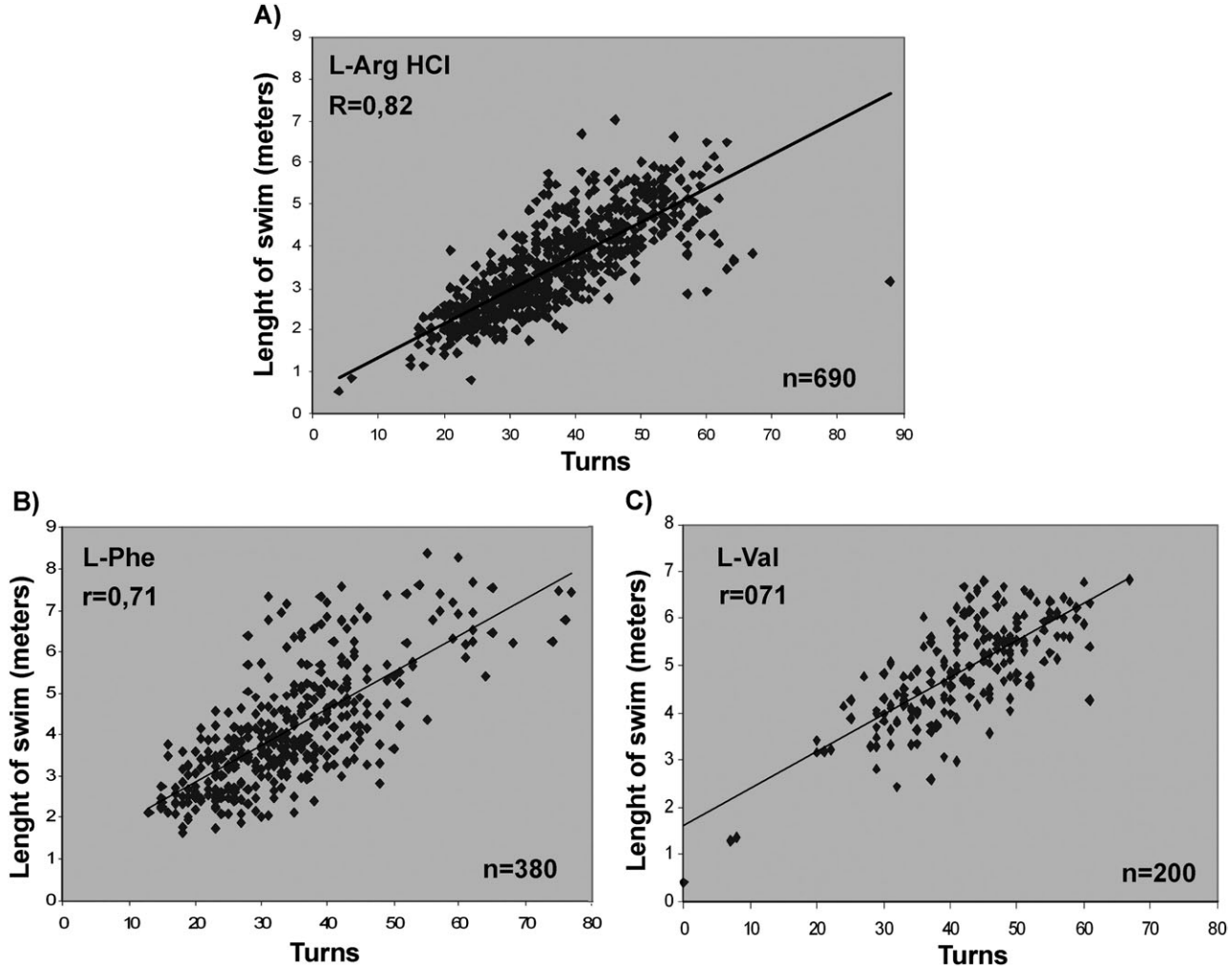

Figure 1 Correlation between the number of turns >90° and the distance traveled in 3 groups of zebrafish. **(A)** Conditioning to L-ArgHCl; **(B)** conditioning to L-Phe; **(C)** conditioning to L-Val.

Results

Activity estimates: the number of turns versus the length of the swim

In daylight, zebrafish are highly active which makes comparisons of behavioral activity in response to a chemical stimulus relatively difficult. Swimming activity after the presentation of a conditioned amino acid usually increased by 50%. To validate the number of turns >90° as an activity measure, the distance of the swimming path as measured from video recordings is correlated with the numbers of >90° turns. In a square shaped aquarium, the number of turns is directly proportional to the distance traveled. The computer software calculates the central point of the fish and measures the movement of a flat projection of the fish swimming path on the CCD sensor of the camera, which is transformed into meters in aquarium. Both methods of evaluation for fish activity have advantages and disadvantages. Large number of turns for slightly different angles during fast zebrafish swimming makes counts of turns imprecise, whereas the length of the swim as measured for the 2D projection of the 3D swimming path makes it distorted relative to the path of the fish. The correlation between the distance traveled and the number of turns was established using nonparametric Spearman rank correlation coefficient. The Spearman correlation coefficients (Figure 1) indicated strong and positive correlations of 0.82 for zebrafish conditioned to L-ArgHCl (n = 690 experiments, Figure 1A), 0.71 for zebrafish conditioned to L-Phe (n = 380 experiments, Figure 1B), and 0.71 for zebrafish conditioned to L-Val (n = 200 experiments, Figure 1C). All 3 correlations are significant ($P < 0.001$). To save time, counts of turns that were collected during the experiments were used in most cases in preference to video replays that enabled the measurements of the swimming distances.

L-Ala–conditioned zebrafish discriminated all amino acids from L-Ala

Zebrafish conditioned to L-Ala responded with greater appetitive swimming to stimulation with L-Ala than to any other

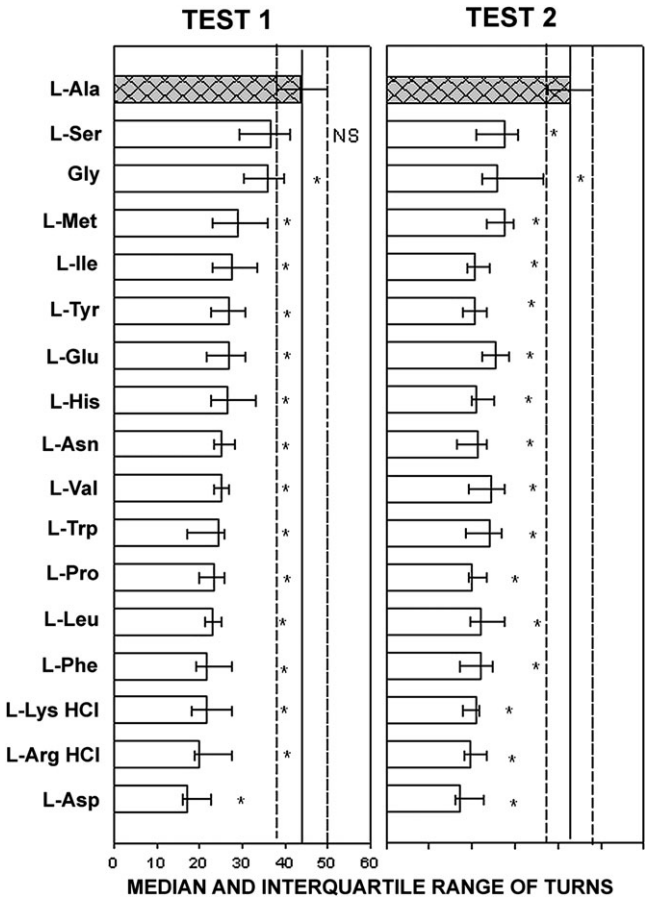

Figure 2 Behavioral responses of zebrafish *Danio rerio* (Hamilton 1822) conditioned to L-Ala (▨) to 16 different test amino acids (□). The conditioned responses are presented as median of medians and minimum and maximum of medians of all conditioning series. The test responses are shown as medians and interquartile range of turns of 10 zebrafish in a series. Statistics: Wilcoxon sum of ranks test: *: significant difference, $P \leq 0.05$ and NS: not significant.

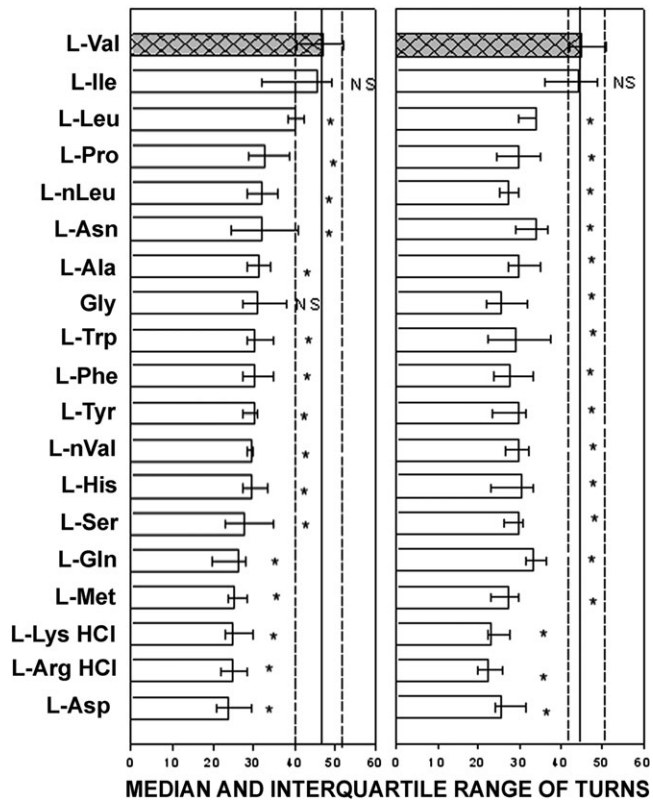

Figure 3 Behavioural responses of zebrafish conditioned to L-Val (▨) after stimulation with test amino acids (□). Statistics are the same as for Fig. 2.

amino acid (Figure 2). In the first test series, all responses to amino acids, except the response to L-Ser, were significantly less than the conditioned response. In addition, in the first test, the response to Gly was also greater than the responses to other nonconditioned amino acids. In the second test series, all the test responses were significantly less than the L-Ala response; that is, responses were 40–60% of swimming activity triggered by the conditioned stimulus (Figure 2). The results of the first test series suggest an initial perceptual similarly among L-Ala, L-Ser, and Gly that is not detectable after the difference between short-chain neutral amino acids is learned.

Responses of zebrafish conditioned to L-Val to other amino acids

L-Val–conditioned zebrafish discriminated all other amino acids except L-Ile from L-Val (Figure 3). In the first test series, in addition to L-Ile, the response to L-Leu was also greater than the other responses to nonconditioned amino acids, whereas in the second test series, only the responses to L-Ile remained similar in magnitude to responses to the conditioned stimulus, L-Val. After 2 test series, the responses to other amino acids were 30–50% of the conditioned activity to L-Val (Figure 3). Additional discrimination training with L-Val versus L-Ile (10 repeated comparisons, Figure 4) did not improve the discrimination of these 2 amino acids. During tests No. 4, 5, 8, and 9 zebrafish responded significantly more to L-Val than to L-Ile (Figure 4), which indicated that some fish discriminated and other fish did not discriminate these 2 amino acids.

To analyze L-Val/L-Ile discrimination further, the distance traveled in meters was analyzed for each individual zebrafish during successive discrimination training tests (Figure 5). Small differences in distance traveled, where the response to the nonconditioned stimuli was generally less than to the conditioned stimulus indicating that some fish in the group ($N = 10$) were capable of discriminating L-Val from L-Ile (e.g., fishes 5 and 7; Figure 5). Other fishes either did not discriminate between these 2 amino acids or their ability to discriminate was slight (e.g., fishes 3, 4, and 9; Figure 5).

Behavioral responses of zebrafish conditioned to L-Leu to other amino acids

Zebrafish that were conditioned to an amino acid possessing a long neutral side chain, L-Leu, responded to olfactory

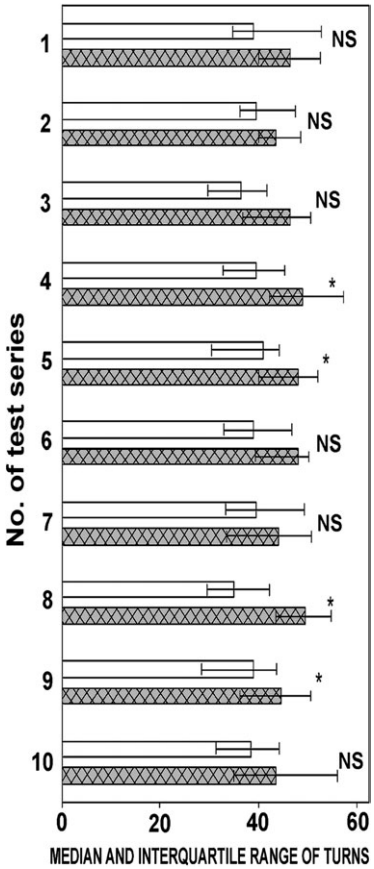

Figure 4 Discrimination training between L-Val (▨) *versus* L-Ile (□) of L-Val conditioned zebrafish. Statistics are the same as for Fig. 2.

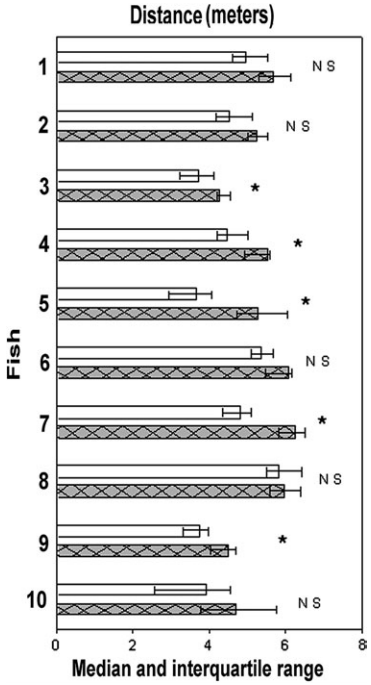

Figure 5 Comparison of distance travelled for individual zebrafish conditioned to L-Val during the discrimination training between L-Val (▨) *versus* L-Ile (□) in the same experiments as on Fig. 4. The bars and range lines are medians and interquartile range of distance travelled in meters. Statistics are the same as for Fig. 2.

stimulation with other amino acids with appetitive search swimming that was 30–50% less intense than the search swim to L-Leu (Figure 6). Except for the greater number of turns to the conditioned stimulus in the second test series, there was no difference between the 2 comparisons; L-Leu was nearly always discriminated from all other amino acids.

Behavioral responses of zebrafish conditioned to L-Arg HCl to other amino acids

Responses of zebrafish to the conditioned stimulus L-Arg HCl were always 30–50% greater than those to other nonconditioned amino acids determined by either distance traveled (Figure 7) or the number of turns made. The difference between the response to L-His and that to the conditioned response was not significant in the first test series but became highly significant ($P < 1\%$) in the second test series. L-LysHCl was always discriminated from L-ArgHCl. Spontaneous activity was slightly less than 50% of the L-Arg–conditioned activity; it was also smaller than the responses to nonconditioned amino acids, which indicated that amino acids per se released an enhanced swimming activity (Figure 7) compared with blanks.

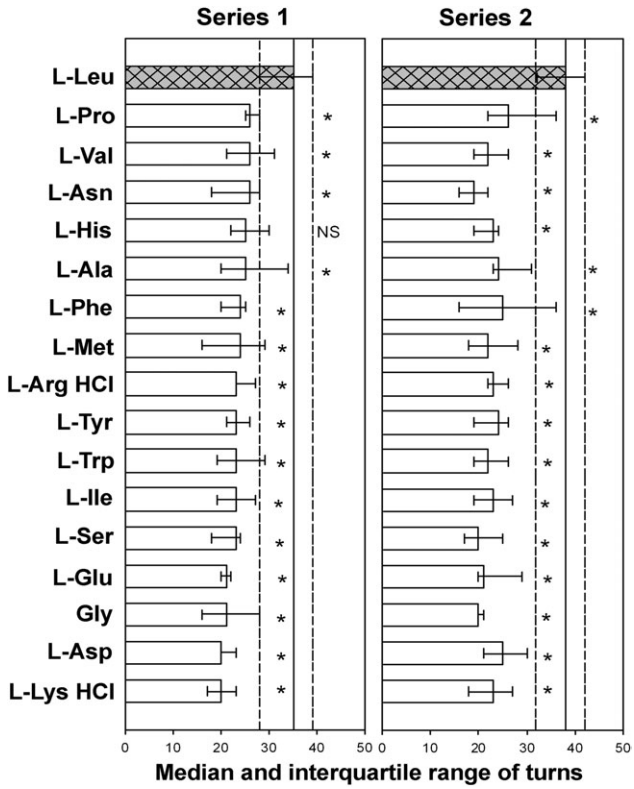

Figure 6 Behavioural response of zebrafish conditioned to L-Leu (▨) to different amino acid solutions (□). Statistics are the same as for Fig 2.

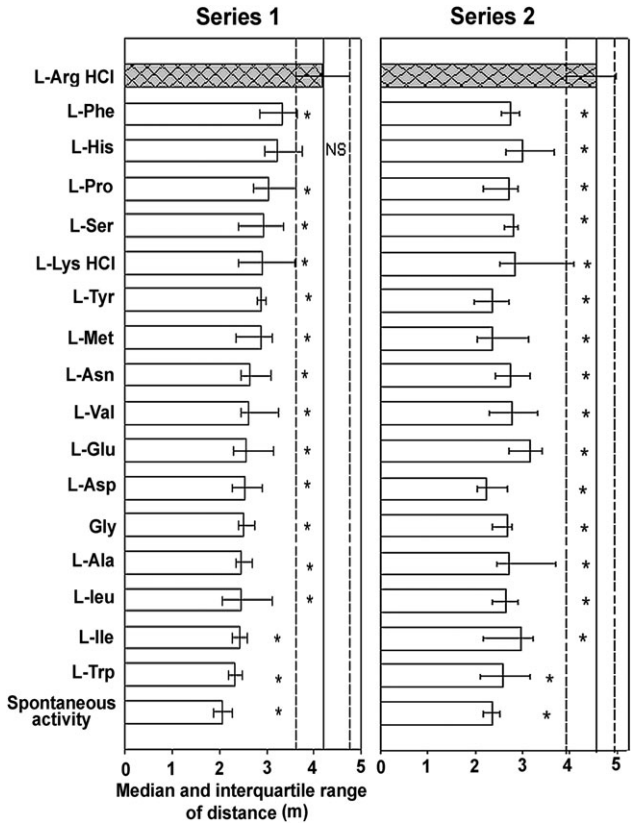

Figure 7 Appetitive responses of zebrafish conditioned to L-Arg HCl (▨) to different amino acids (□) and spontaneous activity with no stimulation. Activity is measured by the length of the swimming path. Statistics are the same as for Figs. 2 and 5.

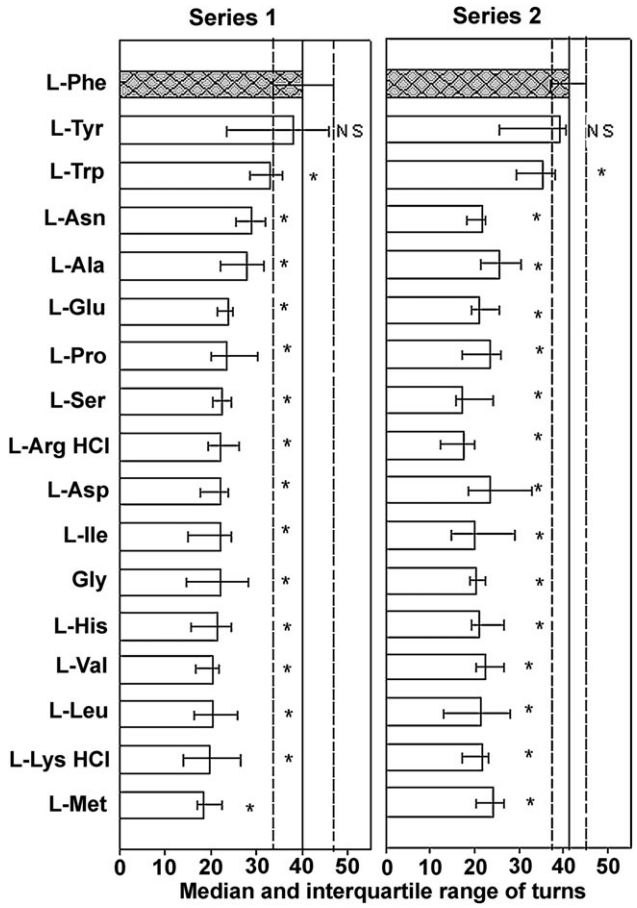

Figure 8 Appetitive response of zebrafish conditioned to L-Phe (▨) to 16 different amino acid solutions (□). Statistics are the same as for Fig 2.

Behavioral response of L-Phe–conditioned zebrafish to other amino acids

L-Phe–conditioned zebrafish responded to all amino acids except L-Tyr with search swims that were significantly less than those to the conditioned stimulus (Figure 8). With the exceptions of L-Tyr and L-Trp, the responses to other amino acids were 45–70% of the median conditioned response to L-Phe. In the first 2 test series, responses to L-Tyr were nearly equal with those to L-Phe, whereas responses to L-Trp, which were significantly less than the responses to L-Phe, were greater than to the other nonconditioned stimuli (Figure 8), indicating an initial perceptual similarity between the aromatic amino acids, L-Phe, L-Tyr, and L-Trp. It is notable that during 18 repeated comparisons of L-Phe versus L-Tyr, discrimination training did not improve the discrimination between L-Phe and L-Tyr (Figure 9). During the discrimination training between L-Phe and L-Tyr, the ability to discriminate similar amino acids was different among individual fish (Figure 10) as some zebrafish discriminated and the other zebrafish did not discriminate L-Phe from L-Tyr; however, the differences in those fish with significantly smaller responses to L-Tyr than L-Phe were extremely small indicating a low discrimination level.

Comparison of olfactory discrimination data and physiological data from Friedrich and Korsching (1997)

Indices of amino acids specific olfactory bulb dissimilarities (IOBD) compare the differences in areas of the inner amino acid representations on the lateral surface of the OB of zebrafish with their total areas (overlapped area counted once, Figure 11). In most cases, the inner representations of amino acids in the OB accurately predict the ability of olfactory discrimination in zebrafish. Three levels of olfactory discrimination are recognized, immediate olfactory discrimination is coupled with IOBD above 0.4, olfactory discrimination after one preexposure to the pair of amino acids is usually coupled with IOBD between 0.3 and 0.4 and inability to discriminate pair of amino acids is in one case coupled with IOBD below 0.3 and in one case between 0.3 and 0.4 (Figure 11). It is notable that there is no discrimination inability at IOBD values above 0.4 and that inability to discriminate amino acids is typically coupled with low values of IOBD. There are few cases of olfactory discrimination of 2 amino acids at low IOBD values; such are the discriminations of L-Phe from L-Ala and L-Phe from L-Ser. Potentially the ease of these discriminations originates from differences of the

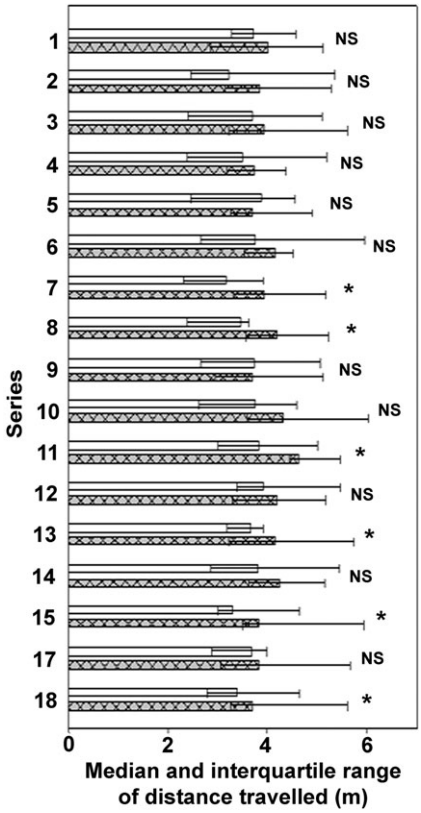

Figure 9 Appetitive responses of zebrafish conditioned to L-Phe during discrimination training between L-Phe ( ) *versus* L-Tyr ( ). Activity was measured by distance traveled. Statistics are the same as for Figures 2 and 5.

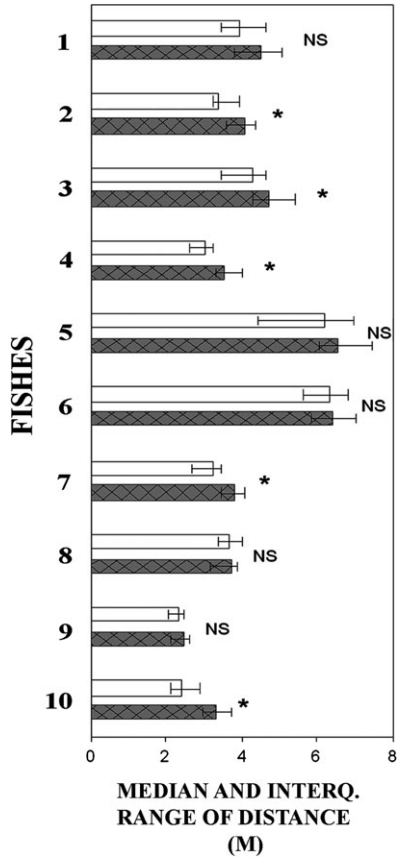

Figure 10 Response of individual zebrafish conditioned to L-Phe during the discrimination training between L-Phe ( ) *versus* L-Tyr ( ) in the same experiments as on Fig. 9. Distances travelled are reported. Statistics are the same as for Figs. 2 and 5.

chemotopic representations that were outside the frame of OB observation (such as in L-Arg and L-Lys, Friedrich and Korsching 1997) or from the fact that the presented physiological data originate from one individual fish.

Discussion

Amino acids are natural food stimuli for omnivorous fish (Caprio 1978; Carr 1988; Valentincic and Caprio 1994a). In Ictaluridae amino acids, through innate taste networks, drive reflexes of biting/snapping, turning and swallowing behaviors (Valentincic and Caprio 1994b). In contrast, olfactory stimuli release intensive feeding behavior only after being associated with a food reward (Valentincic and Caprio 1994a; Valentincic, Wegert et al. 1994; Valentincic 2005; Fields et al. 2007). In mammals, the patterned activations of OB glomeruli form an inner representation of an olfactory stimulus and create an olfactory perception of odor quality (Johnson and Leon 2007). Glomerular activity patterns are described as odotopic (or "chemotopic maps") (Adrian 1950; Johnson and Leon 2007; Yaksi et al. 2007), where specific molecular features of the odorants are associated with precise regions of glomerular activity. Chemotopic maps are distributed hierarchically. The coarse maps of primary odorant features (e.g., neutral, acidic, and basic amino acids) contain within "finer" maps of secondary features (e.g., chain length of neutral amino acids). Functional groups are mapped onto large domains of the chemotopic maps, and the less prominent molecular features, such as chain lengths, are mapped onto domain subregions (Johnson and Leon 2007; Yaksi et al. 2007).

The nature of single amino acid representations in the OB of fish is categorical (Niessing and Friedrich 2010); that is, even chemically similar stimuli can be discriminated because they are represented by distinct activity patterns that provide distinct olfactory sensations. As long as an olfactory stimulus can be detected as different from other stimuli, it can provide a categorical conditioned stimulus for food. The categorical nature of chemical stimuli was revealed by varying concentrations of components in a binary mixture; at a certain concentration ratio of components, the chemotopic patterns switched categorically from one component pattern into another (Niessing and Friedrich 2010).

An additional factor of importance in olfactory discrimination and odorant perception is selective attention, which is required for the conscious detection of small differences between similar odorants. This was shown for binary mixtures

	Ala	Arg	Asp	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Trp	Tyr	Val
Ala		YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	SYES	YES	YES	YES
Arg	0,84		YES	YES	SYES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES
Asp	0,69	0,91		NT	NT	NT	YES	NT	NT	YES	NT	NT	NT	NT	YES
Gly	0,77	0,93	0,75		NT	NT	YES	NT	NT	YES	NT	NT	NT	NT	YES
His	0,44	0,87	0,63	0,73		NT	YES	NT	NT	YES	NT	NT	NT	NT	YES
Ile	0,79	0,9	0,85	0,81	0,77		YES	NT	NT	YES	NT	NT	NT	NT	NO
Leu	0,43	0,69	0,76	0,84	0,64	0,72		YES	YES	YES	YES	YES	YES	YES	YES
Lys	1	0,48	0,9	0,93	0,9	0,93	0,86		NT	YES	NT	NT	NT	NT	YES
Met	0,56	0,69	0,78	0,88	0,69	0,78	0,36	0,77		YES	NT	NT	NT	NT	YES
Phe	0,28	0,83	0,69	0,77	0,44	0,76	0,47	0,9	0,58		YES	YES	SYES	NO	YES
Pro	0,98	0,98	0,99	1	0,98	0,97	0,99	1	0,99	0,98		NT	NT	NT	YES
Ser	0,37	0,88	0,69	0,76	0,49	0,76	0,51	0,92	0,62	0,33	0,98		NT	NT	YES
Trp	0,55	0,86	0,43	0,75	0,56	0,86	0,68	0,91	0,74	0,49	0,97	0,59		NT	YES
Tyr	0,44	0,86	0,65	0,75	0,5	0,78	0,54	0,89	0,67	0,36	0,98	0,49	0,82		YES
Val	0,7	0,89	0,82	0,74	0,73	0,27	0,72	0,92	0,78	0,72	0,97	0,72	0,86	0,89	

Figure 11 Trellis diagram of indices of amino acid–specific olfactory bulbar dissimilarities (IOBD) enabled comparison of olfactory discrimination data (this study) and physiological data (Friedrich and Korsching 1997). IOBD were calculated from the differential area of the inner representations of the 2 amino acids compared (XOR) divided by their total area (overlapped area counted once, OR). Dark shading are indices below 0.3, light shading are indices between 0.3 and 0.4, and white are indices above 0.4, NT is not tested, YES means olfactory discrimination, SYES means not discriminated in the first test and discriminated in all the later tests and NO means no discrimination.

of amino acids where a conditioned food stimulus was perceived initially as a simple categorical stimulus, that is—a single more stimulatory amino acid in the mixture (Valentincic, Kralj, et al. 2000). Later experience or discrimination training facilitated the detection of an additional—minor (the less stimulatory) component in the mixture. Once small differences between similar amino acid mixtures are learned, the presence of minor components of binary and ternary mixtures is detected (Valentincic, Kralj, et al. 2000; Valentincic et al. 2011). Component detection did not occur in mixtures composed of >5 components (Valentincic et al. 2011). At the moment, the categorical nature of multimixture percepts (>4 components) cannot be documented as it likely occurs after the percept is learned to perfection.

Within a certain concentration range of amino acid stimuli, neither their chemotopic representations in the OB nor their perceptions changed considerably (Niessing and Friedrich 2010) as the detection of the same odorant at different concentrations is concentration invariant (Leon and Johnson 2009). This is consistent with our experimental evidence that a conditioned chemical stimulus was, at any concentration, detected as the same stimulus (Valentincic, Wegert et al. 1994; Valentincic, Metelko, et al. 2000). Concentration invariance is, at least in part, an evolutionary consequence of the physiochemical properties of aqueous environments. In water, odorants are contained in eddies (or odor plumes in the air) transported by water currents, which are detected as "bumps" of olfactory stimuli. At the edge of an odor eddy, the concentration of the olfactory stimulus is low, whereas toward the center, the concentration increases to a maximum. The swimming speed determines the detection of the concentration increase and decrease within the eddy. Depending upon the direction of movement, the animal will detect the increasing and the decreasing gradients within a few seconds (Valentincic and Caprio 1994a).

Neural networks of the OB facilitate discrimination of similar neutral amino acids with short side chains potentially by decorrelation (Friedrich and Laurent 2001). Decorrelation is a result of lateral inhibition, mostly through reciprocal synapses between mitral and granule cells (Migliore and Shepherd 2008). If similar bulbar representations cannot be decorrelated (small values of IOBD; Figure 11), the neural networks do not support their discrimination. In wild-type zebrafish, percepts of different pure amino acids are, in most

cases, sufficiently different to be discriminable (IOBD > 0.4); however, the percept of L-Ile versus L-Val (IOBD = 0.27) or the percept of L-Phe versus L-Tyr (IOBD = 0.36) are not different enough for the discrimination to occur (Friedrich and Korsching 1997, 1998, Figure 11). The smallest differences between similar odorants that can be discriminated were determined in a study of mixtures of amino acids in the black bullhead catfish. The relative differences between mixtures comprising 6 and 7 equipotent amino acids enabled their olfactory discrimination, whereas the relative differences between mixtures of 11 and 12 equipotent components were not sufficiently large to be discriminated (Valentincic et al. 2011).

The variability of the chemotopic representations of the same amino acid occurs between individual zebrafish (Friedrich and Korsching 1997) which might affect their discrimination capabilities. A similar effect was noted between individual rats (Strotmann et al. 2000; Schaefer et al. 2001). By analyzing the behavioral responses of single zebrafish to L-Ile versus L-Val, some individuals (20–50%) could discriminate the 2 similar amino acids, whereas 50% of zebrafish failed this discrimination (Figure 5). For L-Phe versus L-Tyr discrimination, the differences in responses were small and unreliable; however, 50% of the individuals could discriminate these 2 amino acids (Figure 10). It remains to be explored if the differences in chemotopic representations and consequently their olfactory discrimination abilities depend upon only the genetic variability of zebrafish or on the differential expression (''variability of expression'') of the same genes in individual zebrafish.

Neurons that process olfactory information in higher forebrain regions ''combine'' information about molecular features from ensembles of active and inactive mitral cells suggesting that pattern processing establishes representations (i.e., perceptions) of odor objects (Yaksi et al. 2009). Are the sparse codes (a small set of active neurons that code information) for neutral amino acids sufficiently different to facilitate within group discrimination of all neutral amino acids possessing short and long side chains? At the behavioral level, there is an initial inability of zebrafish (Figure 2) and black bullhead catfish (Valentincic, Metelko, et al. 2000) to discriminate L-Ala from L-Ser (IOBD 0.37); however, the discrimination became reliable in the second and during all later comparisons. Decorrelation during the initial 600 ms of the olfactory response is hypothesized to result in a reduction of perceptual similarity of Gly, L-Ala, and L-Ser. The tiny difference between these neutral amino acids was likely learned during the first tests to enable their olfactory discrimination later. Similarly, in zebrafish, the discrimination of L-Phe versus L-Trp (IOBD = 0.49) was unreliable in the first test (Figure 8) but became reliable in the second and later tests. In contrast, the discrimination of neutral amino acids with similar long side chains, such as L-Val versus L-Ile (IOBD = 0.27) (Valentincic, Metelko, et al. 2000; Valentincic 2005), and aromatic amino acids such as L-Phe versus L-Tyr (IOBD = 0.36) (Friedrich and Laurent 2001) was not possible initially and did not improve at the population level irrespective of the number of comparisons of the 2 stimuli.

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