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THE BEHAVIORAL DETECTION OF BINARY MIXTURES OF AMINO ACIDS AND THEIR INDIVIDUAL COMPONENTS BY CATFISH

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Summary

The question of whether a binary mixture of amino acids is detected by fish as a unique odor or whether the qualities of the individual components are retained within the mixture was investigated in channel (*Ictalurus punctatus*) and brown bullhead (*Ameiurus nebulosus*) catfish, species that are highly similar in their olfactory receptor and behavioral responses to amino acid odorants. Catfish respond with greater appetitive food-searching (swimming) behavior to amino-acid-conditioned olfactory stimuli than to non-conditioned amino acids. In the present study, appetitive food-searching behavior was measured by counting the number of turns of the fish greater than 90 ° within 90 s of stimulus onset and, in some tests, by video tracking. The two methods yielded highly correlated results. Channel catfish conditioned to a binary mixture composed of equimolar amino acids responded with searching behavior to the amino acid that produced the larger-amplitude electro-olfactogram (EOG) response as they did to the conditioned stimulus. In further studies, bullhead catfish were conditioned either to a binary mixture or to a single amino acid and tested to determine whether a binary mixture was detected as the component evoking the larger EOG response. In all initial tests (trials 1–3), the more stimulatory component of a binary mixture was not discriminated from the binary mixture; however, the less stimulatory component and all other amino acids tested were discriminated from the mixture. By increasing the concentration of the originally less potent component in a binary mixture, making it the more stimulatory compound, it was now detected as not significantly different from the binary mixture; however, the original more potent component (i.e. now the less potent stimulus) was detected as significantly different from the mixture. However, with 5–10 additional discrimination training trials, the less stimulatory component in a binary mixture influenced the perception of the binary mixture because the binary mixture was no longer detected only as its more stimulatory component. The data suggest that a two-step learning process occurs within the olfactory bulb and possibly higher-order telencephalic nuclei.

Key words: olfaction, learning, olfactory discrimination, binary mixture, behaviour, amino acid, catfish, *Ictalurus punctatus*, *Ameiurus nebulosus*.

Introduction

In nature, animals typically encounter odor mixtures rather than single odorants. Thus, mixture identification and blend recognition are problems faced by all animals that possess the sense of smell (Smith, 1996). Depending upon olfactory neural processing, mixtures are detected either as sensations of the components of the mixture, which remain identifiable, or as a unique quality not detected in the components (Rescorla et al., 1985; Westbrook and Charnock, 1985). Irrespective of the differences in olfactory transduction of water-soluble and volatile chemicals, similar coding principles are expected to apply to mixture perception for both aquatic and terrestrial animals. The evidence suggests that arthropods [e.g. honeybees (*Apis mellifera*) (Getz and Smith, 1990; Smith and Cobey, 1994) and the spiny lobster (*Panulirus argus*) (Steullet and Derby, 1997; Daniel et al., 1996)] have the ability to detect separate components in a binary mixture. Generalization (i.e. the response to a compound that is slightly different from the conditioned stimulus; Smith, 1996) between binary mixtures and their components can, however, also occur and has been reported for rats (Linster and Smith, 1999). In humans, only a small difference in intensity of two odorants in a binary mixture is necessary for the quality of the higher-intensity odorant to predominate or for only that odorant to be perceived (Laing and Willcox, 1983). For humans, mixtures of a small number of chemically pure odorants rarely result in qualities not apparent in the components (Rabin and Cain, 1989); however, only a maximum of 3–4 components within a mixture can be detected as separate (Livermore and Laing, 1996).

The present report investigates how aquatic vertebrates (catfish), organisms that have fewer different putative olfactory receptor genes by an order of magnitude (Ngai et al., 1993b)

than mammals (Ressler et al., 1994; Buck, 1996), detect simple odorant mixtures consisting of two amino acids. It is most likely that fish process olfactory information in at least the initial portions of the olfactory pathway within the central nervous system in a manner similar to that of mammals. In both groups of animals, there is a convergence of the axons of olfactory receptor neurons onto specific glomerular regions of the olfactory bulb, where there is evidence of a combinatorial coding of odor information (Friedrich and Korsching, 1997; Johnson et al., 1998; Hara and Zhang, 1998; Rubin and Katz, 1999).

Olfactory discrimination of single amino acids has been investigated in catfish using conditioning techniques, and these studies established that olfaction rather than taste facilitates the conditioned discrimination of amino acid stimuli (Valentinčič and Caprio, 1994; Valentinčič et al., 1994, 2000). Conditioned amino acid stimuli excite the catfish to search vigorously for food for longer periods than following stimulation with non-conditioned stimuli (Valentinčič and Caprio, 1994; Valentinčič et al., 2000). Data on olfactory discrimination of single amino acid stimuli are currently available for two related catfish species, channel (*Ictalurus punctatus*; Valentinčič and Caprio, 1994; Valentinčič et al., 1994) and brown bullhead (*Ameiurus nebulosus*; Valentinčič et al., 2000) catfish. No major differences in the relative stimulatory effectiveness of amino acids determined from electrophysiological recordings (Caprio, 1980) or from olfactory discrimination of amino acids have been reported for these two closely related species of freshwater catfish.

In the present report, we investigated binary mixture discrimination using the same conditioning paradigm as that used previously for testing olfactory discrimination of single amino acids. The major question addressed is whether catfish detect binary mixtures of amino acids as a unique quality or whether the qualities of the individual components continue to be detected. All amino acids tested individually have been shown previously to be detected as different odorants (Valentinčič et al., 2000). The first series of tests determined whether a binary mixture composed of equimolar components is detected as the more potent component within the mixture (option 1) or as a novel sensation unique to the mixture (option 2) by channel catfish. The results confirmed option 1. A second series of tests performed in brown bullhead catfish determined whether, by altering the relative effectiveness between the component stimuli in a binary mixture, the more potent stimulus [i.e. the component that resulted in a larger-amplitude electro-olfactogram (EOG) when tested individually] would be detected initially as the binary mixture. The results confirmed this prediction. Lastly, it was tested and confirmed that additional discrimination training facilitated the detection of the presence of the less stimulatory component of a binary mixture.

Materials and methods

Experimental animals

Brown bullhead catfish (*Ameiurus nebulosus* Lesueur, 1819) (1–3 years old), obtained from ponds in the vicinity of Ljubljana, Slovenia, and 1-year-old channel catfish (*Ictalurus punctatus* Rafinesque, 1818), obtained from Louisiana State University ponds, Baton Rouge, LA, USA, were maintained initially in large 500 l storage containers. The fish were fed daily and were used for electrophysiological and behavioral experiments for 2–24 months subsequent to their transfer from natural ponds to captivity. For behavioral experiments, catfish were placed individually into separate 80 l test aquaria and maintained at 21–24 °C for brown bullhead catfish and at 29–30 °C for channel catfish. The higher maintenance temperature for the channel catfish was above the tolerance limits of the bacterium *Edwardsiella ictaluri*, which chronically infects this species in Louisiana and is deleterious to their olfactory epithelium (Morrison and Plumb, 1993).

Stimulus compounds and delivery: electrophysiological experiments

All amino acid solutions and their mixtures were prepared from the finest grade chemicals obtained from Fluka (Fluka Chemie AG, Switzerland) and Sigma (St Louis, MO, USA) and were tested within 1–2 h of their preparation. Stimulus solutions were delivered to the olfactory organ through silicone tubing from a laboratory-designed manual sample-injection valve. A constant flow (5 ml min^{-1}) of dechlorinated tapwater bathed the olfactory organ. To present a stimulus, a manual valve was switched to the sample tube containing 0.5 ml of the odorant, which then flowed for 10 s to the olfactory organ. Immediately thereafter, the flow was manually switched back to dechlorinated tap water. The initial odorant concentration delivered to the olfactory organ was approximately 83 % of that injected. A 2 min interstimulus interval allowed for stimulus clearance from the previous odorant presentation.

Electrophysiology: the electro-olfactogram

Catfish were anesthetized with Tricaine (ethyl-3-aminobenzoate methanesulfonate) (1:8000 dilution) present in the gill irrigation water and immobilized with an intramuscular injection of Flaxedil (0.16 mg 100 g^{-1} body mass for brown bullhead catfish and 0.1 mg 100 g^{-1} body mass for channel catfish). EOG recordings, as previously described (Caprio, 1995), were performed in brown bullhead (14–22 cm body length) and channel (12–18 cm body length) catfish, which were of the same approximate size and age as the catfish tested in the behavioral experiments. Briefly, the EOG was recorded with calomel electrodes *via* capillary pipettes filled with Ringer–agar (0.5 % agar solution), amplified by a direct-coupled amplifier and displayed on a pen recorder. The capillary pipette connected to the active electrode was placed in the water immediately above (<1 mm) the midline raphe of the olfactory organ on one side. The Ringer–agar bridge of the reference electrode was placed against the skin dorsomedial to the olfactory organ. The magnitude of the EOG was determined as the height (in mm) of the negative phasic displacement from baseline. Individual EOG responses were standardized to the response to 10^{-4} mol l^{-1} L-alanine. The

relative effectiveness index was calculated as the maximum amplitude of the response to an odor stimulus divided by the maximum amplitude of the response to 10^{-4} mol l^{-1} L-alanine. Responses are presented as medians and interquartile ranges of the relative effectiveness index for the different fish tested.

Stimulus delivery: behavioral experiments

Chemical stimuli were delivered to the test aquaria from removable Pasteur pipettes, which were positioned approximately 1 cm above the turbulent water surface caused by the aquarium aeration systems. Stimulus delivery was controlled 3 m from the aquaria by the injection of air into Tygon tubing connected to 5 ml syringes pre-loaded with stimulus or control solutions. Chemical stimuli usually reached the catfish 12–25 s after introduction into the aquarium. During the experiments, the test mixture in the center of the stimulus eddies (i.e. odor plumes) was diluted 300- to 3000-fold prior to reaching the fish, as determined by micro-electrochemical measurements (Moore et al., 1989; Valentinčič and Caprio, 1994). The actual test concentrations of each chemical are therefore 300–3000 times lower than reported in the text. The test odorants did not change the pH of the aquarium water measured with a pH electrode 10 cm away from the stimulus injection point.

Conditioning procedures, behavioral responses and evaluation

Details of the conditioning procedures for the channel (Valentinčič and Caprio, 1994; Valentinčič et al., 1994) and bullhead (Valentinčič et al., 2000) catfish have been presented previously. Briefly, channel and brown bullhead catfishes in aquaria (50 cm×40 cm×40 cm) without shelters were conditioned either to a binary mixture of amino acids or to one of the components of the mixture followed by a food reward delivered 90 s after the introduction of the conditioning stimulus. A maximum of four conditioning and four test sessions were performed daily. After the conditioned response had stablilized (after approximately 50 trials for both bullhead and channel catfish), both conditioned and unconditioned amino acids were tested. In the initial experiments, two methods of evaluation, video-tracking and direct visual counts of the behavioral events, were employed. The behavioral response was quantified using an image motion computer (Vidmex-V, Columbus Instruments, OH, USA) and by visual counts of turns of greater than 90° within 90 s of stimulus addition. The number of turns was recounted during video-replay. For video-tracking, the black gravel aquarium substratum provided the background that activated the dark pigmentation in the fish. A high contrast between the fish and the aquarium background (the white walls of the room) was necessary to enable the image motion computer continuously to track the centroid of the pigmented animals. Video-tracking (distance in cm) and visual counts of the number of turns greater than 90° made by the fish yielded highly correlated results (Fig. 1: N=881, square-root-transformed data, r=0.93). Since the two methods yielded similar results, counts of turns alone were used in later experiments. A non-parametric Wilcoxon sum of ranks test compared the responses of the fish to water controls with those to the conditioning and test stimuli.

Preparation of binary mixtures

Only those amino acids that had previously been shown to be discriminated by channel and bullhead catfish (Valentinčič and Caprio, 1994; Valentinčič et al., 1994, 2000) were tested in the present study. For channel catfish, equimolar concentrations of amino acids were used in the preparation of binary mixtures. The relative potency of the components was determined from EOG recordings. For bullhead catfish, concentrations of component amino acids in a specific binary mixture were adjusted relative to their equipotent concentrations (i.e. the concentration of each of the components that results in similar EOG response magnitudes). To determine the equipotent concentrations for the components of a specific binary mixture, EOG dose–response relationships were first determined for each component amino acid. The specific equipotent concentrations were then determined by drawing a line parallel to the abscissa intersecting the dose–response plots of the two component amino acids between 10^{-4} mol l^{-1} and 10^{-7} mol l^{-1} (i.e. the range of odorant concentrations expected to be available to the fish during appetitive and consummatory feeding behaviors) (Fig. 2). Stimulus concentrations relative to this equipotent concentration for each binary mixture were then determined depending upon the stimulus paradigm used.

Experimental paradigms

Paradigm 1 tested whether binary mixtures composed of equimolar concentrations of L-alanine and L-arginine (where

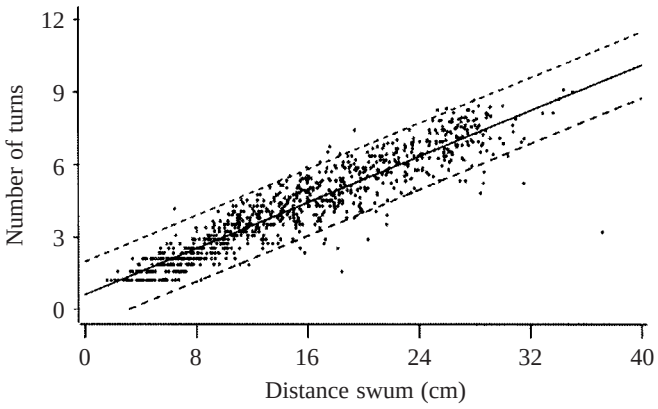

Fig. 1. Swimming (food-searching) activity of catfish was determined using an image motion computer (video-tracking, distance swum in cm) and visual counting of the events (turns greater than 90° during the initial 90 s following addition of odorant to the aquarium) during the replay of video tapes of the experiments. The two quantification methods yielded highly correlated results (r=0.93). Since the results were binomially distributed, the data were square-root-transformed; dashed lines indicate 95% confidence limits; only 34 of 881 response values were located outside the confidence limits.

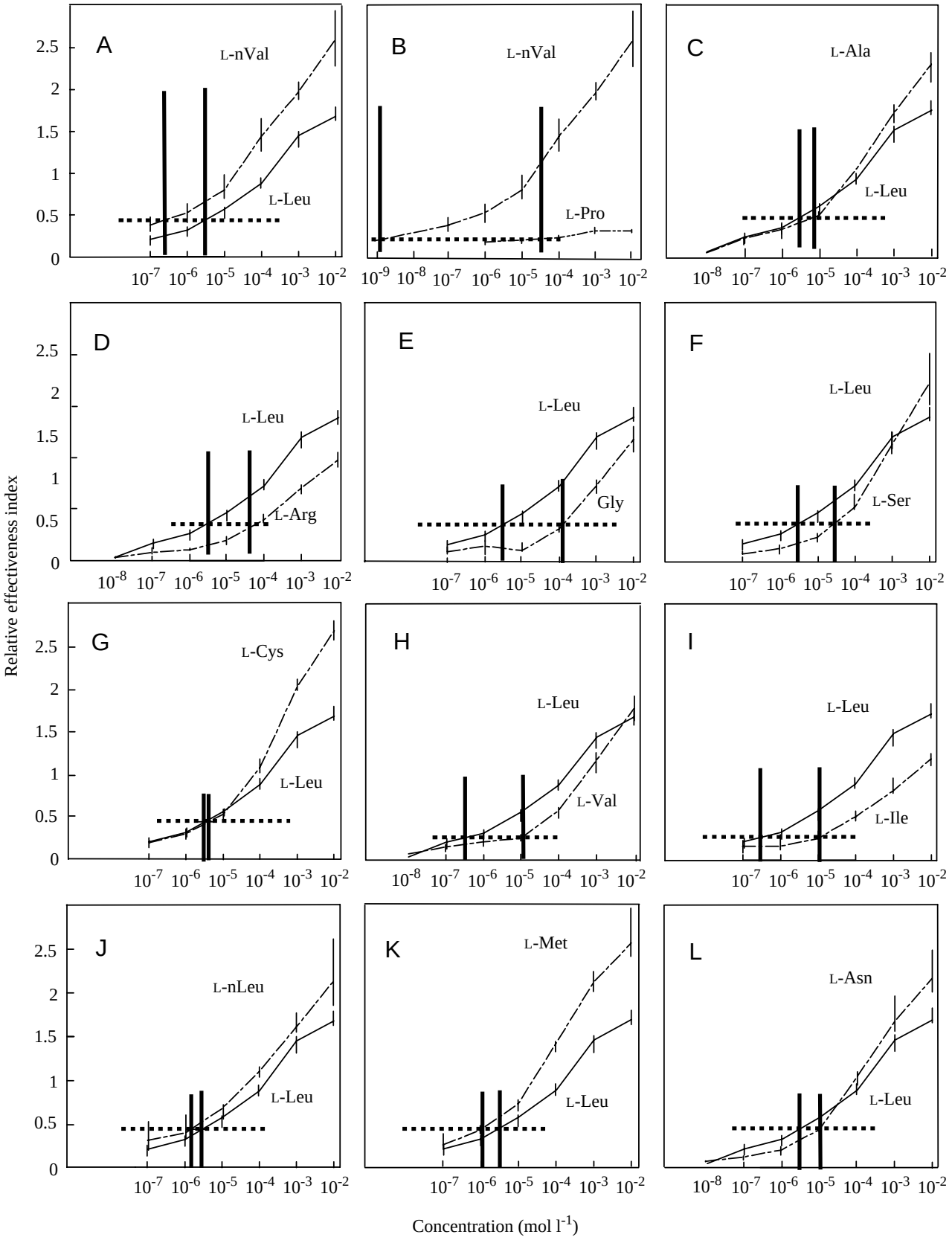
A
L-nVal
L-Leu
B
L-nVal
L-Pro
C
L-Ala
L-Leu
D
L-Leu
L-Arg
E
L-Leu
Gly
F
L-Leu
L-Ser
G
L-Cys
L-Leu
H
L-Leu
L-Val
I
L-Leu
L-Ile
J
L-nLeu
L-Leu
K
L-Met
L-Leu
L
L-Asn
L-Leu
Relative effectiveness index
Concentration (mol l-1)

Fig. 2

Fig. 2. Dose–response curves for 13 (A–L, *N*=7 for each chemical) amino acids, which were components of binary mixtures, determined in brown bullhead catfish. Component concentrations that would be equally stimulatory were determined by intersecting the dose–response plots for each component with a line parallel to the abscissa (thick dotted lines) such that the resulting concentrations indicated by the thick vertical lines fell within the range 10^{-4} to 10^{-7} mol l^{-1} (A, C–L). For B, the equal stimulatory concentrations were also determined behaviorally. The relative effectiveness index is the response to the tested amino acid divided by the response to the standard, 10^{-4} mol l^{-1} L-alanine. Thin vertical lines indicate the median and interquartile ranges for the electrophysiological (electro-olfactogram, EOG) responses.

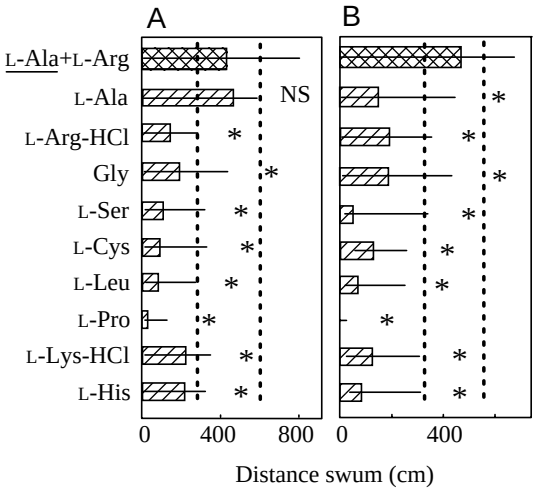

Fig. 3. Two repeated tests (A,B) of swimming responses to the binary mixture L-alanine+L-arginine and to nine test amino acids in channel catfish conditioned to a mixture of L-alanine+L-arginine at concentrations in which L-alanine (underlined) was the more stimulatory component. The binary mixture was composed of equimolar components, and the tested amino acids were injected at 2×10^{-2} mol l^{-1}. In this and succeeding figures (Figs 4–6), the amino acids were estimated (see Materials and methods) to be diluted 300- to 3000-fold before contact with the receptors. To illustrate that no discrimination learning occurred during the tests, the initial 2–3 repeats of the same experimental paradigm are presented throughout this report (Figs 3–8). The abscissa reports distance swum in centimeters (median and interquartile range), the vertical dotted lines indicate the range of median responses to the conditioned mixture, and an asterisk indicates a significant difference at $P<0.01$; NS, not significantly different (Wilcoxon test; N=11 fish).

alanine is 2.5 times more concentrated than its equipotent concentration with arginine-HCl) (see Fig. 3) and L-norvaline plus L-leucine (where norvaline is 10 times more concentrated than its equipotent concentration with leucine) (see Fig. 4) were tested in channel catfish (in Baton Rouge, LA, USA). On the basis of the results of paradigm 1, paradigms 2 and 3 for bullhead catfish were constructed. The series of experiments using bullhead catfish was performed in Ljubljana, Slovenia, where channel catfish are not readily available.

Paradigm 2 tested (i) whether brown bullhead catfish (*Ameiurus nebulosus*, previously *Ictalurus nebulosus*) detect binary mixtures as their more stimulatory component. Brown bullhead catfish were conditioned to binary mixtures of L-norvaline plus L-leucine (where norvaline is 10 times the equipotent concentration and leucine is 10 times less than the equipotent concentration; see Fig. 5) and the same binary mixture with the reverse ratio (see Fig. 6). Responses to single amino acids were compared with responses to the conditioned mixtures.

Paradigm 3 also tested whether brown bullhead catfish detect binary mixtures as their more stimulatory components, but here the catfish were conditioned to a component amino acid, rather than to the binary mixture (paradigm 2). Responses to different binary mixtures containing either the conditioned amino acid or another amino acid as the more stimulatory component of the mixture were compared with responses to the conditioned amino acid stimulus (see Fig. 7). Using this paradigm, the equal response magnitude for the pair L-proline +L-norvaline was also established and tested to determine how a binary mixture composed of equipotent components is detected (see Fig. 8).

Paradigm 4 tested the ability of brown bullhead catfish with additional discrimination testing to be affected by the less stimulatory component in a binary mixture (see Fig. 9). In this paradigm, the binary mixture is the conditioned stimulus, and the conditioned mixture and the more stimulatory component alone are presented 5–12 times successively.

Results

Channel catfish: conditioned to binary mixtures

In both the first and second discrimination tests, channel catfish conditioned to the binary mixture of L-alanine+L-arginine-HCl, where L-alanine was the more potent electrophysiological component, discriminated L-arginine-HCl and all other amino acids tested, except L-alanine, from the mixture (Fig. 3). On the basis of the dose–response functions, the L-arginine-HCl concentration in this previous example would need to be increased by 2.5-fold to be equally stimulatory as L-alanine. Quantification of the behavioral results, either by counting the number of turns greater than 90 ° within 90 s of stimulus delivery or by video-tracking the distance traveled (Fig. 3A,B), gave similar results. Channel catfish conditioned to the binary mixture of L-norvaline+L-leucine, where L-norvaline was the more potent electrophysiological component, almost always discriminated L-leucine and all other test amino acids from the mixture, which was detected as being similar to (i.e. not significantly different from) L-norvaline (Fig. 4). On the basis of the dose–response functions, the L-leucine concentration in the previous example would need to be increased tenfold to be equally stimulatory as L-norvaline.

Brown bullhead catfish: conditioned to binary mixtures

Brown bullhead catfish conditioned to a mixture of L-norvaline+L-leucine, where L-norvaline was the more potent

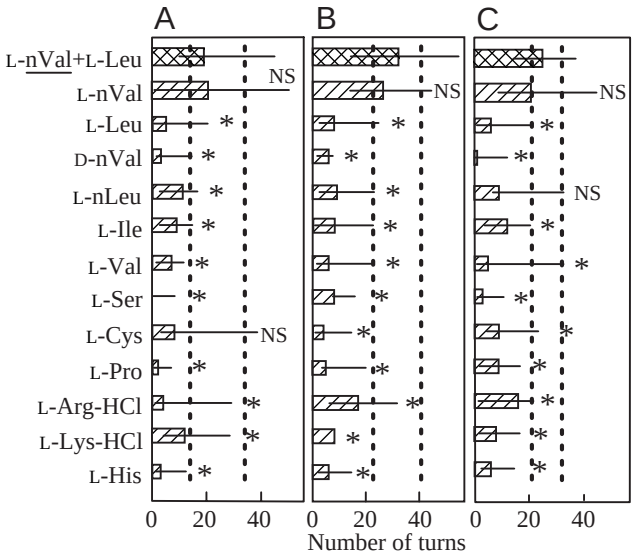

Fig. 4. Three repeated tests (A–C) of swimming (food-searching) responses of channel catfish conditioned to a mixture of equimolar L-norvaline and L-leucine, in which L-norvaline (underlined) was the more stimulatory component (concentrations as in Fig. 3). The abscissa reports the number of turns (median and interquartile range), the vertical dotted lines indicate the range of median responses to the conditioned mixture, and an asterisk indicates a significant difference at $P<0.01$; NS, not significantly different (Wilcoxon test; $N=11$ fish).

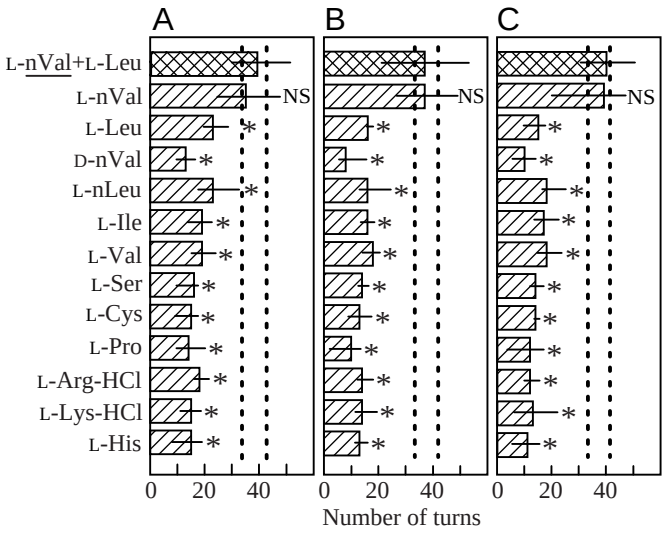

Fig. 5. Three repeated tests (A–C) of brown bullhead catfish conditioned to L-norvaline+L-leucine at concentrations where L-norvaline (underlined) was the more stimulatory component (i.e. 2×10^{-2} mol l^{-1} L-norvaline and 3×10^{-3} mol l^{-1} L-leucine). The abscissa reports the number of turns (median and interquartile range), the vertical dotted lines indicate the range of median responses to the conditioned mixture, and an asterisk indicates a significant difference at $P<0.01$; NS, not significantly different (Wilcoxon test; $N=9$ fish).

component (100 times more concentrated than the equipotent concentration with L-leucine), discriminated L-leucine and other amino acids from the binary mixture. L-Norvaline, the more stimulatory component, was detected as not significantly different from the mixture (Fig. 5A–C). In reciprocal experiments, another group of nine catfish was conditioned to a mixture of L-leucine+L-norvaline, where L-leucine was the more stimulatory component (100 times more concentrated than the equipotent concentration with L-norvaline). The results indicated that bullhead catfish discriminated L-norvaline (in two out of three series) and other amino acids from the binary mixture, which was detected as being similar to L-leucine (Fig. 6A–C).

Brown bullhead catfish: conditioned to single amino acids

In all tests in L-leucine-conditioned brown bullhead catfish, binary mixtures of L-leucine plus one of 11 different amino acids, where the non-leucine amino acid was more potent (30 times more concentrated than the equipotent concentration with L-leucine), were detected as significantly different from L-leucine (Fig. 7A,C). In reciprocal tests in which L-leucine (30 times more concentrated than the equipotent concentration with the other amino acid) was the more potent stimulus, the binary mixtures were almost always detected as being similar to L-leucine (Fig. 7B,D).

All the previous results clearly demonstrated that channel and bullhead catfish initially detect a binary mixture as the component that is more stimulatory. The next experimental

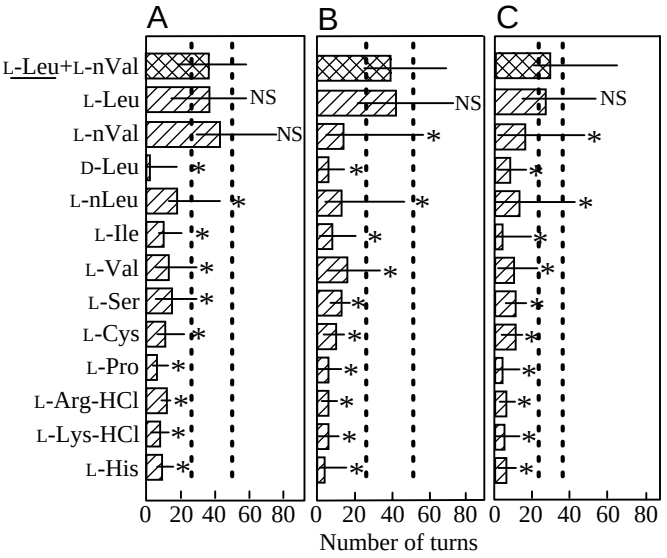

Fig. 6. Three repeated tests (A–C) of brown bullhead catfish conditioned to a mixture of L-norvaline+L-leucine at concentrations where L-leucine (underlined) was the more stimulatory component (i.e. 3×10^{-2} mol l^{-1} L-leucine and 2×10^{-3} mol l^{-1} L-norvaline). The abscissa reports the number of turns (median and interquartile range), the vertical dotted lines indicate the range of median responses to the conditioned mixture, and an asterisk indicates a significant difference at $P<0.01$; NS, not significantly different (Wilcoxon test; $N=13$ fish).

paradigm tested binary mixtures of L-proline+L-norvaline in which the components were adjusted in concentration such that

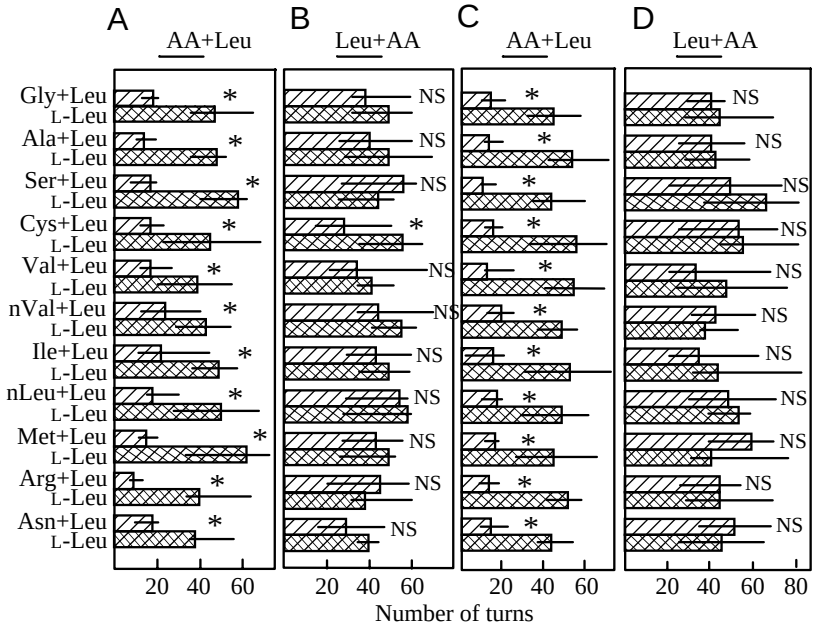

Fig. 7. Bullhead catfish conditioned to L-leucine were tested with different binary mixtures containing L-leucine plus another amino acid (AA) at two different component ratios. (A,C) Mixtures contain the non-leucine (underlined) amino acid as the more stimulatory component; (B,D) mixtures contain L-leucine (underlined) as the more stimulatory component (concentrations tested were as follows: the conditioning amino acid, 3×10^{-2} mol l^{-1} L-Leu; the binary mixtures in A and C, Gly/Leu, 1 mol l^{-1}/3×10^{-4} mol l^{-1}; Ala/Leu, 7×10^{-2} mol l^{-1}/3×10^{-4} mol l^{-1}; Ser/Leu, 3×10^{-1} mol l^{-1}/3×10^{-4} mol l^{-1}; Cys/Leu, 4×10^{-2} mol l^{-1}/3×10^{-4} mol l^{-1}; Val/Leu, 10^{-1} mol l^{-1}/3×10^{-5} mol l^{-1}; nVal/Leu, 2×10^{-3} mol l^{-1}/3×10^{-4} mol l^{-1}; Ile/Leu, 10^{-1} mol l^{-1}/3×10^{-5} mol l^{-1}; nLeu/Leu, 2×10^{-2} mol l^{-1}/3×10^{-4} mol l^{-1}; Met/Leu, 10^{-2} mol l^{-1}/3×10^{-4} mol l^{-1}; Arg/Leu, 4×10^{-1} mol l^{-1}/3×10^{-4} mol l^{-1}; Asn/Leu, 10^{-1} mol l^{-1}/3×10^{-4} mol l^{-1}; the binary mixtures in B and D, Gly/Leu, 10^{-2} mol l^{-1}/3×10^{-2} mol l^{-1}; Ala/Leu, 7×10^{-4} mol l^{-1}/3×10^{-2} mol l^{-1}; Ser/Leu, 3×10^{-3} mol l^{-1}/3×10^{-2} mol l^{-1}; Cys/Leu, 4×10^{-4} mol l^{-1}/3×10^{-2} mol l^{-1}; Val/Leu, 10^{-3} mol l^{-1}/3×10^{-3} mol l^{-1}; nVal/Leu, 2×10^{-5} mol l^{-1}/3×10^{-2} mol l^{-1}; Ile/Leu, 10^{-3} mol l^{-1}/3×10^{-3} mol l^{-1}; nLeu/Leu, 2×10^{-4} mol l^{-1}/3×10^{-2} mol l^{-1}; Met/Leu, 10^{-4} mol l^{-1}/3×10^{-2} mol l^{-1}; Arg/Leu, 4×10^{-3} mol l^{-1}/3×10^{-2} mol l^{-1}; Asn/Leu, 10^{-3} mol l^{-1}/3×10^{-2} mol l^{-1}). The abscissa reports the number of turns (median and interquartile range), and an asterisk indicates a significant difference at $P<0.01$; NS, not significantly different (Wilcoxon test). $N=15$ fish tested.

the components were equipotent, L-norvaline was more potent or L-proline was more potent. For the first of these, an L-proline:L-norvaline concentration ratio of 30 000:1, where the two components were similarly potent, the mixture was discriminated seven times and not discriminated five times from the conditioned stimulus, L-proline (Fig. 8A). For the second and third tests, the results were as predicted from all the previous experiments. In a binary mixture in which L-norvaline was more potent (i.e. an L-proline:L-norvaline ratio of <30 000:1), the mixture was detected as being different from L-proline (Fig. 8B). In a binary mixture in which L-proline was more potent (i.e. an L-proline:L-norvaline ratio of 300 000:1), the mixture was detected in 12 out of 14 trials as L-proline (Fig. 8C). With further discrimination training (repeated tests using the same binary mixture; results not shown), however, there was an increase in the probability of detecting the slight difference in the mixture provoked by the less stimulatory component in the mixture.

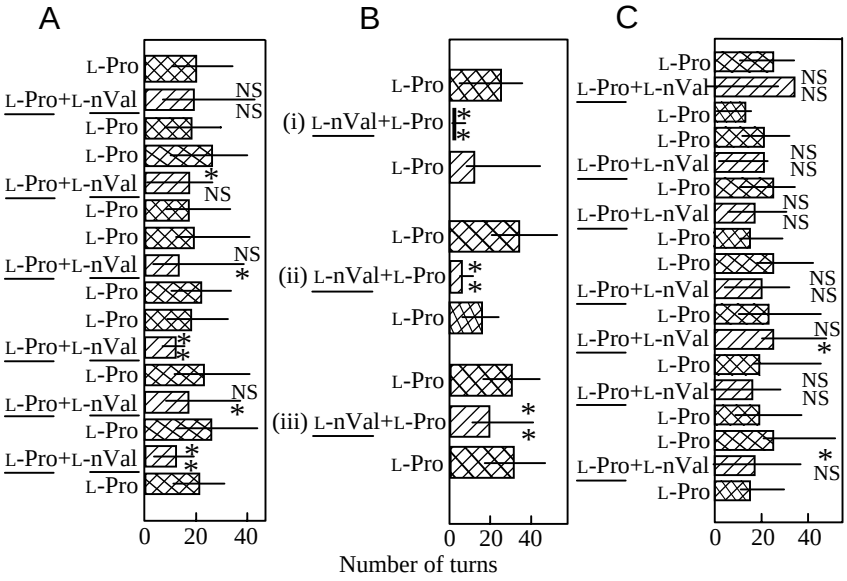

Fig. 8. Bullhead catfish were conditioned to L-proline (3×10^{-2} mol l^{-1}), and responses to different binary mixtures of L-proline+L-norvaline were studied. Responses to mixtures in which (A) L-proline and L-norvaline are equipotent stimuli (i.e. L-proline is 30 000 times more concentrated than L-norvaline; L-proline, 3×10^{-2} mol l^{-1}; and L-norvaline, 10^{-6} mol l^{-1}, (B) in which L-norvaline (underlined) is the more stimulatory component [L-proline is (i) three times (L-proline, 3×10^{-2} mol l^{-1}; L-norvaline, 10^{-3} mol l^{-1}), (ii) 30 times (L-proline, 3×10^{-2}; L-norvaline, 10^{-4} mol l^{-1}) and (iii) 3000 times (L-proline, 3×10^{-2} mol l^{-1}; L-norvaline, 10^{-6} mol l^{-1}) more concentrated than L-norvaline], and (C) in which L-proline (underlined) is the more stimulatory component (i.e. L-proline is 300 000 times more concentrated than L-norvaline; L-proline, 3×10^{-1} mol l^{-1}; L-norvaline, 10^{-6} mol l^{-1}). The abscissa reports the number of turns (median and interquartile range), and an asterisk indicates a significant difference at $P<0.01$; NS, not significantly different (Wilcoxon test). $N=15$ fish tested.

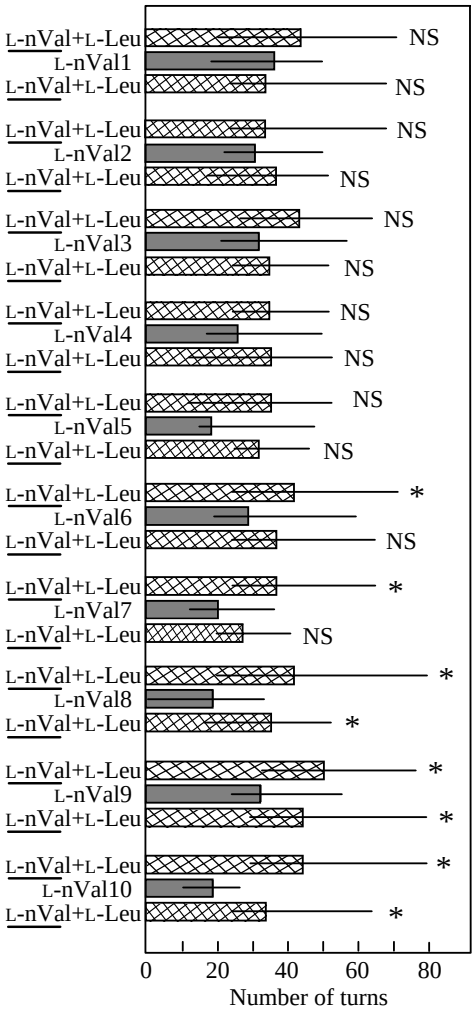

Fig. 9. Effects of the less stimulatory component on the perception of a binary mixture of amino acids studied using successive presentations of the conditioned binary mixture of amino acids and the nonconditioned more stimulatory amino acid alone. Bullhead catfish were first conditioned to the mixture of L-norvaline plus L-leucine, with L-norvaline (underlined) as the more stimulatory component. Concentrations are the same as in Fig. 5. Numbers adjacent to L-norvaline indicate the sequence of the test trials. The abscissa reports the number of turns (median and interquartile range), and an asterisk indicates a significant difference at $P<0.01$; NS, not significantly different (Wilcoxon test). $N=6$ fish tested.

Discrimination training

Experience can improve the detection of the less stimulatory component in a binary mixture. Brown bullhead catfish conditioned to the binary mixture L-norvaline+L-leucine in which L-norvaline was the more stimulatory component (at 100 times the equipotent concentration), L-norvaline was not discriminated from the mixture in the first five series of comparisons, but was discriminated from the mixture in the majority of subsequent tests (Fig. 9). The same results were obtained in a series of replicate tests in bullhead catfish conditioned to L-leucine+L-norvaline, in which L-leucine was the more stimulatory component (at 100 times the equipotent concentration). In this latter case, L-leucine was not discriminated from the mixture in the first three test series, but was discriminated from the mixture in three of four subsequent tests.

Discussion

Odorant mixtures made up of two components (i.e. binary mixtures) are detected by humans as their more stimulatory component (Laing and Willcox, 1983). However, human subjects can identify more than one component in an odorant mixture composed of multiple components, and the recognition of the less stimulatory component improves with learning (Rabin and Cain, 1984). In spite of this, perfumers, who are professionally trained to discriminate odors, are unable to detect more than three or four components in a complex mixture (Laing and Francis, 1989; Laing and Glemarec, 1992; Livermore and Laing, 1996). Since humans can identify at least a few individual components in a six-component odorant mixture, it is assumed that simple mixtures are coded analytically (i.e. as neural codes of their individual components) in the nervous system. Large complex mixtures, however, are detected primarily as unique new qualities, since discrimination and identification of individual components in such a complex array is extremely difficult (Laing and Livermore, 1992).

The perception of odorant mixtures has also been studied in detail in two arthropod species, the honeybee and the spiny lobster. The experimental paradigm in behavioral studies in the honeybee included the proboscis extension reflex, which is normally released by glucose, the unconditioned taste stimulus, and can be conditioned to occur after presentation of olfactory stimuli alone (Menzel et al., 1974). The honeybee data indicate a strong relationship between the perception of the binary mixture and its individual components, thus indicating that the mixture is not detected as being intermediate between the odors of the components and that single substances can characterize odorant mixtures (Getz and Smith, 1990; Laska et al., 1999). Information concerning individual components of simple mixtures is also preserved in the nervous system of the southern spiny lobster, in which the conditioning of an aversive response was used to study odorant discrimination between single compounds and mixtures (Derby et al., 1989). Generalization among complex stimuli was not common, and the ‘across-nerve patterns’ of activity typical for single compounds were preserved at least at the level of the olfactory receptor cell in the lobster (Derby et al., 1996).

A major finding of the present experiments was that the relative EOG amplitudes in response to the components of a binary mixture were an effective predictor of how channel and brown bullhead catfish would initially detect behaviorally an experimental binary mixture. We hypothesized that the neural code for the component of a binary mixture that caused the larger-magnitude EOG response would be dominant in the olfactory neural circuitry that results in the initial perception.

The odorant-induced direct current change (EOG) in the water above the olfactory organ reflects the averaged result of all the depolarizations and hyperpolarizations of the responding population of olfactory receptor neurons. Since the binding affinities of most amino acids to their respective receptors in teleosts are probably similar (Brown and Hara, 1981; Bruch and Rulli, 1988), the magnitude of the EOG response is a relative measure of the number of receptor neurons activated by an odorant. Because of the random distribution of the molecular olfactory receptors across the sensory epithelia of the olfactory organ of ictalurid catfish (Ngai et al., 1993a), the relative effectiveness of odorant stimuli is independent of the relative rostro-caudal positioning of the salt bridge of the active EOG electrode in the water over the olfactory organ (Chang and Caprio, 1996). Thus, the experimental position of the active EOG electrode in the present experiments did not affect the relative potency of the compounds tested.

In 98 of 100 mixture discrimination experiments performed in this study with brown bullhead catfish, the responses to the binary mixtures neither habituated nor generalized, because the binary mixtures were detected as their more stimulatory component over long periods (up to 3 months of testing) and over numerous experiments (over 200 conditioning and test trials with different chemicals). The only two exceptions to the hypothesis that occurred are currently unexplainable. These two anomalies included the cases (i) where L-norvaline in the first test series was not discriminated from the conditioned stimulus, the binary mixture of L-norvaline+L-leucine, with L-leucine as the more stimulatory component. (Fig. 6A), and (ii) where the mixture of cysteine+L-leucine, with L-leucine as the more stimulatory component, was unexpectedly discriminated from the conditioned stimulus, L-leucine (Fig. 7B). The one exception to the hypothesis observed during the preliminary 12 discrimination tests with channel catfish probably occurred as a result of the discrimination testing of equimolar L-alanine and L-arginine solutions in which their physiological potencies were too similar to be effectively discriminated by the fish without additional successive discrimination training tests (Fig. 3B).

The smallest difference in the stimulatory effectiveness of components of a binary mixture required in the present experiments for it to be reliably detected as the more stimulatory component was three times the equipotent concentration; however, to ensure that one component was indeed more stimulatory in this experimental paradigm, the stimulus concentration of the more potent component in the majority of tests was adjusted to be 10–100 times the equipotent concentration (Fig. 2A,C–L). Such an increase in concentration of an amino acid stimulus results in a relatively small incremental increase in EOG response magnitude since EOG dose–response functions to amino acids in catfish are best described by power functions with an exponent of only approximately 0.2 (Byrd and Caprio, 1982). At the other end of the spectrum, the concentration of L-proline had to be elevated 300 000 times relative to L-norvaline before catfish detected the binary mixture of L-norvaline+L-proline as L-proline, because the EOG response to L-proline was only 9 % of the response to L-norvaline when both were tested individually at 10 mmol l^{-1} (Fig. 2B). Such a high L-proline concentration probably saturated the available L-proline receptor sites and maximized the output of the neurons containing these sites, such that the response of the olfactory organ to L-proline exceeded that of the neurons responding to the low concentration of L-norvaline. Evidence to support the hypothesis that amino acid odorants that are more potent excite more receptor neurons than those that are less stimulatory was provided in a recent optical imaging study of presynaptic activity in glomerular modules of the zebra fish olfactory bulb (Friedrich and Korsching, 1997). Amino acid odorants with long neutral side-chains, such as L-norvaline, excited large areas of presynaptic terminals compared with those excited by L-proline.

To show further that a difference in relative stimulatory effectiveness between the two components in a binary mixture was a critical cue as to how catfish initially detected these mixtures, additional experiments with equipotent stimuli were tested. At electrophysiologically (EOG) equipotent concentrations, the detection (possibly perception) of a binary mixture vacillated between the components (i.e. either component during different tests was detected as the binary mixture). Even after 70 conditioning trials, the responses of channel and bullhead catfish to either of the equipotent components of a conditioned binary mixture were variable: a binary mixture of equipotent amino acids was detected as one component in some trials and as the other component in other trials. These results in catfish are remarkably consistent with how components of binary odorant mixtures are perceived by humans (Laing and Willcox, 1983; Livermore and Laing, 1998) and rats (Linster and Smith, 1999). In both cases, simple mixtures were not perceived as independent of their components. Further, as found in the present study for catfish, the relative intensity of the odorants in a binary mixture was important to which individual odors were initially identified by humans (Laing and Willcox, 1983).

Although catfish initially never discriminated during the first three discrimination tests of all 17 binary mixtures tested the more stimulatory component from the respective binary mixture, adjustments in the discrimination training emphasized the differences between the conditioned mixture and the more stimulatory component alone. These adjustments included discrimination training consisting of 5–15 repeated presentations of both the conditioned (rewarded) binary mixture and the unconditioned (unrewarded) more stimulatory component. With this revised paradigm, the less stimulatory component in the mixture influenced the perception of the binary mixture (i.e. behavioral responses to a binary mixture became significantly different from responses to the more stimulatory component of each mixture, which became smaller). This phenomenon of discrimination learning (i.e. learning to tell the difference between two stimuli) is well known in visual discrimination (McFarland, 1993; Sutherland and Mackintosh, 1964, 1971). Given the long duration of the perception of a binary mixture as its more stimulatory

component in the present experiments, the saliency of the dominant component is probably not lost after the additional discrimination learning, but the catfish became aware and attentive to the modification of the sensory input pattern provoked by the less stimulatory component of the binary mixture. A similar phenomenon has also recently been shown to occur in honeybees, which showed a gradual improvement in their olfactory ability for discrimination between odorants with repeated trials (Laska et al., 1999).

On the basis of the results of the present experiments, it is most likely that a two-step learning process occurred during the detection of a binary mixture by catfish; there was an initial learning of the more stimulatory component in the binary mixture, followed by increased awareness (i.e. learning) of the presence of the less stimulatory component. The present data suggest that the more salient stimulus within a binary mixture was learned first, and the catfish failed to pay attention to the minor difference between the more stimulatory component alone and the entire mixture. In discrimination studies using single amino acid stimuli, brown bullhead catfish were in most cases able to learn the difference (i.e. discriminate) between two similar amino acids, one being the conditioned stimulus (e.g. the discrimination between L-valine and L-leucine), but in a few cases failed at this task (e.g. the discrimination between L-valine and L-isoleucine and, in L-alanine-conditioned catfish, the discrimination between L-alanine, glycine and L-serine; Valentinčič et al., 2000). During testing, once the catfish had learned to discriminate between the conditioned amino acid and another amino acid with a similar structure, they continued to be able to discriminate the compounds in subsequent tests; i.e. the lack of discrimination typical of generalization was never again observed.

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