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Olfactory discrimination of complex mixtures of amino acids by the black bullhead *Ameiurus melas*

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On the basis of previous findings of behavioural discrimination of amino acids and on the knowledge of electrophysiology of the catfish (genera *Ictalurus* and *Ameiurus*) olfactory organs, behavioural experiments that investigated olfactory discrimination of amino acid mixtures were carried out on the black bullhead *Ameiurus melas*. Repeated presentations of food-rewarded mixtures released increased swimming activity measured by counting the number of turns $>90^{\circ}$ within 90 s of stimulus addition. Non-rewarded amino acids and their mixtures released little swimming activity, indicating that *A. melas* discriminated between the conditioned and the non-conditioned stimuli. Two questions of mixture discrimination were addressed: (1) Are *A. melas* able to detect components within simple and complex amino acid mixtures? (2) What are the smallest differences between two complex mixtures that *A. melas* can detect? Three and 13 component mixtures tested were composed primarily of equipotent amino acids [determined by equal electroolfactogram (EOG) amplitude] that contained L-Cys at $\times 100$ the equipotent concentration. *Ameiurus melas* initially perceived the ternary amino acid mixture as its more stimulatory component alone [*i.e.* cysteine (Cys)], whereas the conditioned 13 component mixture containing the more stimulatory L-Cys was perceived immediately as different from L-Cys alone. The results indicate that components of ternary mixtures are detectable by *A. melas* but not those of more complex mixtures. To test for the smallest detectable differences in composition between similar multimixtures, all mixture components were equipotent. Initially, *A. melas* were unable to discriminate the mixtures of six amino acids from the conditioned mixtures of seven amino acids, whereas they discriminated immediately the mixtures of four and five amino acids from the conditioned mixture. Experience with dissimilar mixtures enabled the *A. melas* to start discriminating the seven-component conditioned mixture from its six-component counterparts. After fewer than five training trials, *A. melas* discriminated the mixtures of nine and 10 amino acids from a conditioned mixture of 12 equipotent amino acids; however, irrespective of the number of training trials, *A. melas* were unable to discriminate the 12 component mixture from its 11 component counterparts.

Key words: conditioning; fish; mixture; olfaction; perception.

INTRODUCTION

Ethological observations of feeding behaviour in anosmic black bullhead *Ameiurus melas* (Rafinesque 1820) formerly thought to be brown bullhead *Ameiurus nebulosus*

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(LeSueur 1819) (Valentincic *et al*., 2000*a*) and channel catfish *Ictalurus punctatus* (Rafinesque 1818) (Valentincic & Caprio, 1994*a*) showed that the taste system releases innate behavioural responses: reflexive biting and snapping, and swimming and turning behaviours (Valentincic, 2004). In contrast, conditioning experiments showed that the olfactory system enables learning of odour stimuli, where conditioned olfactory stimuli release an intense and long lasting food search activity (Valentincic *et al*., 1994, 2000*a*, *b*), which is always larger than similar innate responses.

This series of experiments investigated whether *A. melas*, an organism that has $\times 10$ fewer olfactory receptor genes (Ngai *et al*., 1993) than mammals (Ressler *et al*., 1994; Buck, 1996), could discriminate simple (composed of three amino acids) and more complex (up to 13 amino acids) conditioned mixtures from similar mixtures and from their components. In vertebrates, olfactory discrimination is based on differences between inner representations of odours in the glomerular region of the olfactory bulb (OB). The first processing centre of olfactory information within the central nervous system, the olfactory bulb, is where olfactory receptor neurons (ORN) expressing the same molecular olfactory receptors converge onto dendrites of mitral cells (MC) within glomeruli (Buck, 1996). Here, chemical properties of odorants and their mixtures release activity of specific glomeruli and thus represent the odorants in the combinatorial olfactory code (Friedrich & Korsching, 1997, 1998; Laurent, 1999; Malnic *et al*., 1999; Bozza & Mombaerts, 2001; Friedrich & Laurent, 2001, 2004; Kauer & White, 2001; Nikonov & Caprio, 2001; Wachowiak & Cohen, 2002; Wachowiak *et al*., 2002; Friedrich, 2006). This chemotopic code is subsequently modified within the MC layer by lateral inhibition (Yokoi *et al*., 1995), centre surround inhibition (Aungst *et al*., 2003) and central influences (Folgueira *et al*., 2004*a*, *b*).

Glomerular activity patterns to closely related odorants are similar, whereas those to different odorants are more distinct (Friedrich & Korsching, 1997; Sachse *et al*., 1999; Galizia & Menzel, 2000, 2001; Korsching, 2001). Therefore, odorants that animals have difficulty in discriminating show considerable overlap in their neural representations (Daly *et al*., 2001) as the ability to discriminate odours depends on differences between their respective glomerular activity patterns of the component stimuli, which are dependent upon both odorant quality and quantity. In rats *Rattus norvegicus* (Rubin & Katz, 1999), mice *Mus musculus* (Fried *et al*., 2002; Wachowiak & Cohen, 2002) and zebrafish *Danio rerio* (Hamilton 1822) (Friedrich & Korsching, 1997), high concentrations of odorants activate greater areas of the bulb glomeruli than lower odorant concentrations. Large odorant concentrations also elicit large areas of activity in the antennal lobes (*i.e.* the arthropod equivalent of the OB) of the fruit fly *Drosophila melanogaster* (Wang *et al*., 2003), honeybee *Apis mellifera* (Sachse & Galizia, 2003), African cotton leafworm *Spodoptera littoralis* (Carlsson *et al*., 2002), sphinx moth (Hansson *et al*., 2003) and tobacco budworm *Heliothis virescens* (Skiri *et al*., 2004). The broadening and modifications of the glomerular activity patterns with increasing odorant concentration (Carlsson & Hansson, 2003) potentially modify the ability to distinguish between odorants (Wright *et al*., 2005). One of the aims of the present behavioural experiments was to determine minor differences between similar odours that animals can discriminate behaviourally.

As a result of both the relative simplicity of the fish olfactory system (Ngai *et al*., 1993) and its capability for distinguishing amino acids (Valentincic *et al*., 1994), fishes are suitable model organisms to investigate olfactory discrimination. When

testing amino acids and their binary mixtures, fishes always perceived large differences between different odorants initially, subsequent discrimination training enabled *I. punctatus* to perceive minor differences between similar odours (Valentincic *et al*., 2000*a*; Valentincic, 2005). *Ictalurus punctatus* always perceived first the more stimulatory component of a binary mixture (Valentincic *et al*., 2000*b*). Similarly, in the OB of *D. rerio*, one of the component responses frequently dominated the responses to a binary mixture of amino acids (Tabor *et al*., 2004). For comparison with *A. melas*, humans are similarly able to detect and identify components of binary and ternary odorant mixtures, but not components of large mixtures containing more than four components (Laing & Willcox, 1983; Rabin & Cain, 1989; Livermore & Laing, 1996, 1998), which humans perceive as unique novel odours (Rescorla *et al*., 1985; Rabin & Cain, 1989). Interactions that originate from simultaneous stimulation with multiple components provoke perceptual qualities in the olfactory system that are different from those of the single components alone (Laing & Francis, 1989; Chandra & Smith, 1998; Wiltrout *et al*., 2003); the so-called configural interactions (Smith, 1998) incapacitate component recognition. Configural interactions, overshadowing and synthetic coding do not imply component detection, whereas analytic coding does not imply imprecision in component detection. Initially *A. melas* detect binary mixtures as their more stimulatory components alone, whereas experience enables the detection of modifications of the more stimulatory component sensation by the less stimulatory component (Valentincic *et al*., 2000*b*). For the analytic coding in *A. melas*, a question is asked, whether the more stimulatory component in the mixture is detected initially in ternary mixtures and whether the more stimulatory component can be detected in large multimixtures. Hypothetically, synthetic coding should preclude such detection. In the present behavioural investigation, the ability of *A. melas* to detect the more stimulatory component of ternary and large complex mixtures was tested along with the smallest detectable differences that *A. melas* could detect between complex mixtures.

To provide physiologists with fundamental data on maximum capabilities of the olfactory system at the level of olfactory discrimination two questions were addressed: (1) are mixtures of three amino acids perceived initially as their more stimulatory component? (2) Are mixtures of 13 amino acids also perceived initially as their more stimulatory component? In contrast to the results with ternary mixtures, *A. melas* did not detect the more stimulatory component in a 13 component mixture. Since *A. melas* perceived multi-component mixtures as blends, the smallest detectable differences between similar blends were then determined, testing if the differences between seven and six-component, and between 12 and 11 component mixtures were detectable by *A. melas*. At the same time, the present study provided an indication of the minimal differences between two mixtures that facilitate olfactory discrimination and should be further investigated in olfactory bulb studies.

MATERIALS AND METHODS

AMEIURUS MELAS AND DESCRIPTION OF EXPERIMENTS

One hundred *A. melas* were caught in Pernica carp ponds near Maribor, Slovenia, and 2 months before the experiments were placed in individual 80 l aquaria (50 cm × 40 cm

$\times$ 40 cm) and allowed to acclimate to the aquarium for a period of 2 months. During this long acclimation period, *A. melas* started to feed regularly and the probability of occurrence of fear and escape behaviour diminished substantially. *Ameiurus melas* were treated for *Ichthyophthirius multifiliis* and bacterial diseases using malachite green, the antibiotic Flubactin (Krka Pharmaceuticals; http://www.krka.biz/en/), and 3‰ sodium chloride. All healthy animals were included in the testing.

In the behavioural experiments, 2 ml of either the conditioning or test solutions of amino acids was added to the water surface, which was continuously mixed by the aquarium aeration system. Large mechanical agitations provided by air bubbles prevented the detection of small mechanical disturbance during the stimulus additions into the aquarium. As determined by injecting diluted milk into the aquaria, eddies containing high concentrations of the test solutions reached the heads of *A. melas* in the most remote corners of the aquaria in $<$30 s, intensity of mixing with air bubbles was set to clear milk clouds in $<$90 s. Ten seconds after the stimulus delivery into the aquarium, its concentration within the high concentration centres of eddies was 300–3000 times lower than the injected amino acid concentration (Valentincic & Caprio, 1994*a*; Valentincic, 2004). Concentrations of amino acids differed in the various mixtures and the expected contact concentration to the fish of the individual amino acids in the mixture was between 3×10^{-5} and 10^{-8} M. Ninety seconds after the introduction of the conditioning stimulus, *A. melas* received a food reward consisting of a portion of Atlantic cod *Gadus morhua* L. 1758 muscle. *Ameiurus melas* was only fed during these conditioning sessions. Due to mixing, the added solution became homogeneous with the rest of the aquarium water in *c*. 2 min. The calculated final dilution factor for the added amino acid solution was $\times$40 000 (valid for each amino acid in the mixture). This concentration was only slightly higher than the background concentration of most amino acids in aquarium water. Food-searching activity usually ceased after $<$2 min and *A. melas* did not respond further to the faded stimulation. After a maximum of four sequential experiments, there was a minimum of a 4 h interval before the next experiment. At 25° C, even without additional filtration, the $>$4 h intervals between successive experiments were sufficient for partial breakdown of the remaining amino acids to below detectable levels. Experiments were performed in artificial illumination (16L:8D). The time of day did not influence responsiveness by *A. melas* to the chemical stimuli.

All experiments were video-recorded, and swimming behaviour was quantified by counting the number of $>90^\circ$ turns. The *A. melas* turned abruptly, which facilitated counts of the number of turns within the 90 s experiment. During preliminary experiments, the distance travelled was measured from two-dimensional video-recordings using video tracking of the centroid of the *A. melas* (Vidmex V; www.colinst.com), which served as an independent verification of the behavioural measure of activity. The distance travelled correlated significantly (Pearson correlation coefficient $= 0{\cdot}738$, $n = 223$; $P < 0{\cdot}01$) with the number of turns determined for the same experiments (Valentincic *et al*., 2000*b*). The test and conditioned experiments were conducted sequentially on the same day, and the results compared using the Wilcoxon rank-sum test. It was extremely important that non-rewarded stimuli, which were highly similar to the conditioned stimulus, were presented only once in a sequence test-conditioned stimulus. The similar conditioned and non-rewarded stimuli were presented either in the alternating sequence, test–conditioned–test–conditioned stimuli, or a single test stimulus was presented between two conditioned stimuli, thus resetting the conditioned responses to their conditioned intensities. Conditioned stimuli and the test stimuli that followed each other were statistically compared; thus, the tests were independent of each other.

Different groups of 15 *A. melas* were conditioned to a single conditioning stimulus: the conditioning mixtures consisted of three, seven, 12 and 13 amino acids. After 30–50 conditioning sessions, *A. melas* substantially increased their food-searching activity. The non-conditioned stimuli were used after *c*. 40 conditioning sessions and a maximum of three conditioning and three test stimuli were tested daily. Later, during five to 15 comparisons of sequential test and conditioned responses, discrimination training improved discrimination capability of *A. melas*, which started discriminating the similar stimuli (Valentincic *et al*., 2000*b*). To validate improvements to discrimination, six to eight sequential test and conditioned responses were compared for each pair of stimuli.

THE CONDITIONED AMINO-ACID MIXTURES

It has previously been demonstrated that *I. punctatus* (Valentincic *et al*., 1994) and *A. melas* (Valentincic *et al*., 2000*a*) discriminate all amino acids tested, which include: glycine (Gly), L-alanine (L-Ala), L-valine (L-Val), L-norvaline (L-nVal), L-leucine (L-Leu), L-norleucine (L-nLeu), L-methionine (L-Met), L-cysteine hydrochloride (L-CysHCl), L-arginine hydrochloride (L-ArgHCl), L-lysine hydrochloride (L-LysHCl), L-aspartate (L-Asp), L-asparagine (L-Asn) and L-histidine (L-His). In the present study, amino-acid solutions were prepared in dechlorinated tap water in polystyrene beakers (VWR Scientific Inc.; www.vwrsp.com) <30 min before experimentation. The amino acids were of the highest commercially available grade obtained from Sigma-Aldrich (www.sigmaaldrich.com) or Merck (www.merck.com). Hydrochlorides of L-CysHCl and basic amino acid L-ArgHCl and L-LysHCl were tested, which being highly water-soluble did not alter the pH. Neutral amino acids did not alter the pH of the well-buffered aquarium water. Hydrochlorides of basic amino acids L-ArgHCl and L-LysHCl were tested, which are highly water-soluble but did not alter the pH.

THE EQUIPOTENT AMINO-ACID MIXTURE DESIGN AND THE MORE STIMULATORY COMPONENT

To design amino-acid ternary and multimixtures from equipotent amino acids, the same procedure was applied and data source as previously reported for the binary mixture experiments (Valentincic *et al*., 2000*b*). Receptor potentials of activated olfactory receptor neurons sum to provide electro-olfactogram (EOG) amplitude, which reflects stimulus strength in activating the populations of olfactory receptor neurons (Dolensek & Valentincic, 2010). The determinations of equipotent amino-acid concentrations are shown for mixtures of seven [Fig. 1(a)] and 12 [Fig. 1(b)] amino acids, the horizontal equipotent concentration line crosses the dose–response curves of the amino acids.

SYNTHETIC *v*. ANALYTIC MIXTURE PERCEPTION

To test if *A. melas* perceive mixtures synthetically (as a unique novel sensation or analytically) as components of the mixture, a single more stimulatory component was added to the mixture (see Table I). The three and 13 component mixtures were prepared with amino acids at their equal EOG concentrations, but with *L-CysHCl* at ×100 greater (italicized) than the equipotent concentration. The ternary mixture consisted of 3×10^{-2} *M L-CysHCl* (the more stimulatory amino acid italicized), 10^{-3} M L-Leu, and 5×10^{-5} M L-nVal. The 13 component conditioned mixture was composed of 3×10^{-2} M *L-CysHCl*, 3×10^{-2} M L-Gly, 3×10^{-4} M L-Ala, 10^{-2} M L-Serine (L-Ser), 10^{-2} M L-Val, 3×10^{-5} M L-nVal, 10^{-2} M L-isoleucive (L-Ile), 10^{-3} M L-Leu, 3×10^{-4} M L-nLeu, 10^{-4} M L-Met, 3×10^{-3} M L-Asn, 10^{-2} M L-ArgHCl and 10^{-2} M L-LysHCl.

THE TERNARY MIXTURE EXPERIMENT

The responses to the conditioned ternary mixture were initially compared with responses to the individual test stimuli at the following injected concentrations: 10^{-2} M L-Ser, 10^{-2} M L-nVal, 10^{-2} M L-Ile, 10^{-5} M L-Leu and 10^{-3} M L-Asn. Next, binary mixtures of amino acids were tested consisting of the same components at the same concentrations as in the conditioned ternary mixture: *L-CysHCl* + L-Leu, L-*CysHCl* + L-nVal and L-Leu + L-nVal. The tested ternary mixtures were: (1) 10^{-2} M *L-Leu*, 3×10^{-4} M L-CysHCl and 5×10^{-5} M L-nVal, (2) 5×10^{-3} M *L-nVal*, 3×10^{-4} M L-CysHCl and 10^{-3} M L-Leu and (3) 3×10^{-4} M L-CysHCl, 5×10^{-5} M L-nVal and 10^{-3} M L-Leu with all components equally stimulatory. Finally, responses of the ternary mixture-conditioned *A. melas* were compared to the responses to the 13 component mixture with all amino acids equally stimulatory or with L-Cys at a ×100 times more stimulatory than the other equally stimulatory amino acids.

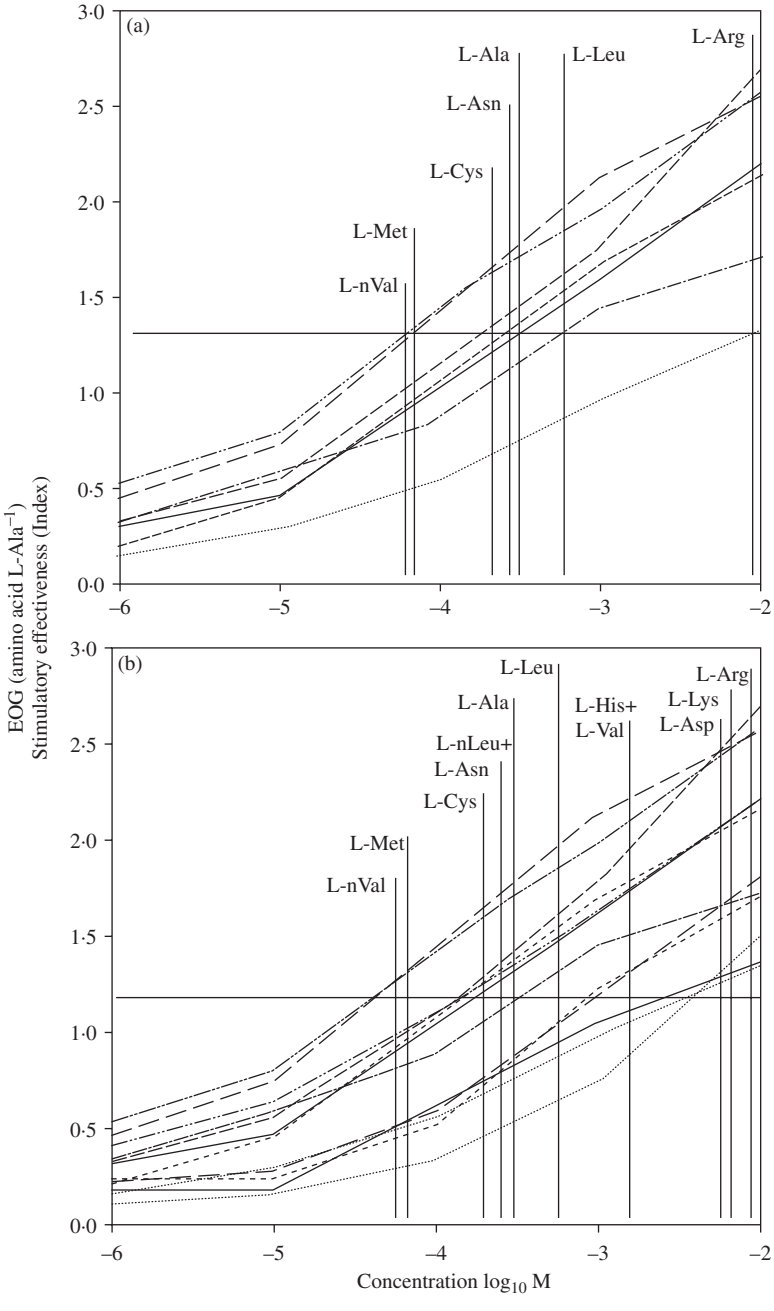

FIG. 1. Concentrations of amino acids that result in equal relative electro-olfactogram (EOG) magnitudes [EOG of amino acid EOG^{-1} of L-alanine (L-Ala)] for components of complex (a) seven and (b) 12 amino-acid mixtures (Valentincic *et al*., 2000*b*). Data points for increasing $\log_{10}$ concentrations are connected by straight lines; horizontal lines indicate concentrations of different amino acids that result in equal relative EOG response magnitudes. L-nVal, L-norvaline; L-Met, L-methionine; L-Cys, L-cysteine; L-Asn, L-asparagine; L-Leu, L-leucine; L-Arg, L-arginine; L-nLeu, L-norleucine; L-His, L-histidine; L-Val, L-valine; L-Lys, L-lysine; L-Asp, L-asparagine.

TABLE I. Olfactory detection of the more stimulatory component (italicized) in the mixture and detection of the smallest differences between different multi-component mixtures in *Ameiurus melas*

Conditioned stimuli	More stimulatory component detection	Differences between $n-1$ component mixtures
Ternary mixture containing the more stimulatory *Cys*	Mixture detected as more stimulatory component	Initially not detected but detected after experience
Large (13 component) mix containing the more stimulatory *Cys*	Complex mix detected more stimulatory component not detected	Except for the more stimulatory component containing mix in most cases not detected
Mixture of seven equally stimulatory amino acids	Does not apply	Initially not detected but detected after experience
Mixture of 12 equally stimulatory amino acids	Does not apply	Initially not detected but not detected after experience

Cys, cysteine.

EXPERIMENTS WITH THE CONDITIONED MIXTURE CONTAINING 13 AMINO ACIDS WITH L-CYS AS THE MORE STIMULATORY COMPONENT

The responses to the conditioned mixture of 13 amino acids were compared with responses to the single amino acids, including L-CysHCl at 10^{-2} M injected and 10^{-5} M probable contact amino-acid concentration. Responses to the conditioned mixture of 13 amino acids were also compared to responses to the mixtures of 12 amino acids. Lastly, responses to the conditioned mixture were compared with responses to the mixture of 13 equipotent amino acids, with the responses to the mixture of seven, the more stimulatory L-CysHCl containing amino-acid mixture, and seven and six equipotent amino-acids mixtures.

SMALLEST DETECTABLE DIFFERENCES BETWEEN SIMILAR MULTIMIXTURES

To study the smallest differences in mixture composition that *A. melas* can discriminate, amino acids at their equal EOG magnitude concentrations were tested [Fig. 1(a), (b)] (Valentincic *et al*., 2000*b*). The seven equipotent amino acid conditioned mixture contained 6×10^{-5} M L-nVal, 7×10^{-5} M L-Met, 2×10^{-4} M L-CysHCl, $2{\cdot}4 \times 10^{-4}$ M L-Asn, 4×10^{-4} M L-Ala, 6×10^{-4} M L-Leu and 9×10^{-3} M L-ArgHCl. The additional equipotent amino acids comprising the 12 component mixture were $2{\cdot}4 \times 10^{-5}$ M L-nLeu, $1{\cdot}5 \times 10^{-3}$ M L-His, $1{\cdot}5 \times 10^{-3}$ M L-Val, 6×10^{-3} M L-Asp and 7×10^{-3} M L-LysHCl. Responses to the conditioned mixtures comprising the seven and 12 equipotent amino acids were compared with responses to the mixtures containing one to three fewer components.

RESULTS

TERNARY MIXTURES

Detection of the more stimulatory component in the mixture

After a few pre-tests with L-arginine (L-Arg) [Fig. 2(a)], *A. melas* conditioned to the ternary mixture composed of *L-Cys* (the more stimulatory component), L-nVal

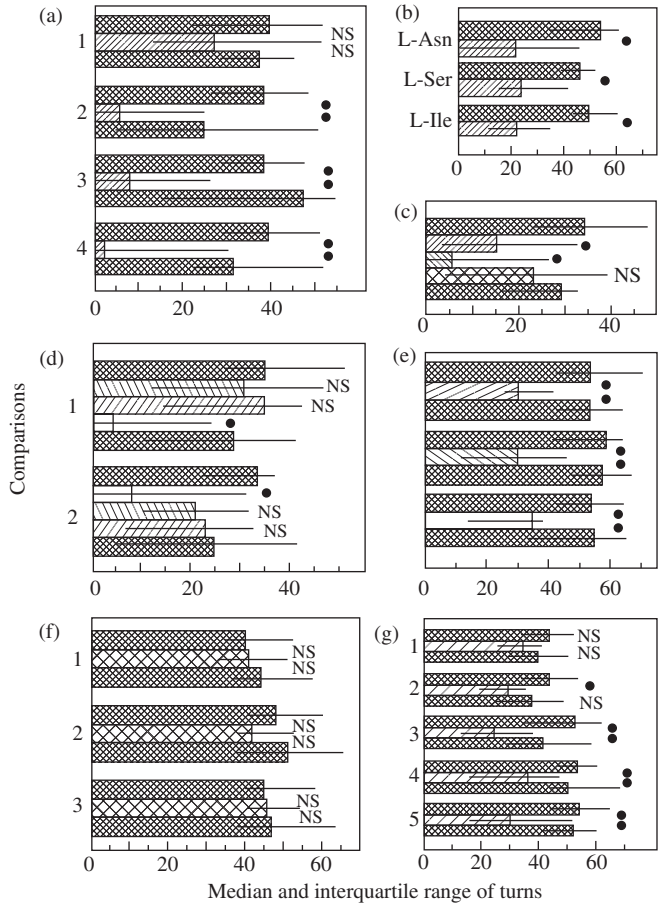

FIG. 2. All the panels (▩) represent the conditioned ternary mixture containing L-cysteine (*L-CysHCl*) as the more stimulatory component which is italicized. (a) The initial discrimination of the conditioned ternary mixture of *L-CysHCl*, L-norvaline (L-nVal) and L-leucine (L-Leu) with *L-CysHCl* as the more stimulatory component from L-arginine (L-Arg) (▨). (b) Discrimination of L-asparagine (L-Asn), L-serine (L-Ser) and L-leucine (L-Leu) (▨) from the conditioned ternary mixture. (c) Discrimination of components of the conditioned mixture from the conditioned ternary mixture, L-CysHCl (⊠), L-nVal (▨), and L-Leu (▨). (d) Discrimination of binary mixtures composed of ternary mixture components (*L-CysHCl*+L-nVal (▨), *L-CysHCl*+L-Leu (▨), L-nVal+L-Leu (□) from the conditioned ternary mixture. (e) Discrimination of ternary mixtures composed of the same components at different concentrations *L-Leu*+L-CysHCl+L-nVal (▨), *L-nVal*+L-CysHCl+L-Leu (▨), L-nVal+L-Leu+L-CysHCl (□) from the conditioned ternary mixture. (f) Discrimination of the conditioned ternary mixture from a mixture of 13 amino acids with the same more stimulatory *L-CysHCl* (⊠). (g) Discrimination of the conditioned ternary mixture from a mixture of 13 equipotent amino acids (▨). Sequences of tests are reported alphabetically figure labels. Numbers along the vertical axis indicate sequence of experiments. Bars = median of turns, lines = interquartile range of turns, Wilcoxon sum of ranks test, $n = 15$, dots indicate significant difference between results of test and adjoining conditioned stimulus test, $P < 0.05$, NS = non-significant. The significant dots located between the compared bars indicate a comparison of sequential conditioned and non-conditioned tests and dots located on the top of the bars are for comparison of the conditioned stimulus experiments with experiments conducted one experiments before or after the conditioned experiment.

and L-Leu immediately discriminated the conditioned mixture from the less stimulatory amino-acid components [Fig. 2(c)]. They were, however, unable initially to discriminate the conditioned mixture from the more stimulatory component alone [Fig. 2(c)]. Behavioural responses to several other single amino acids were significantly different (Wilcoxon rank-sum test, $n = 15$; $P < 0{\cdot}05$) and <50% of the response to the conditioned mixture [Fig. 2(a), (b)]. In two repeated tests, *A. melas* discriminated the conditioned ternary mixture from the binary mixture containing the two less stimulatory components [Fig. 2(d)]; however, in most tests they were unable to discriminate the conditioned mixture from the two binary mixtures containing the more stimulatory L-Cys [Fig. 2(d)]. Furthermore, *A. melas* were generally able to discriminate ternary mixtures containing a different more stimulatory component than L-Cys and the ternary mixture composed of the same equally stimulatory amino acids from the conditioned ternary mixture [Fig. 2(e)]. Interestingly, *A. melas* were unable to discriminate the conditioned ternary mixture from the 13 amino-acid mixture that also contained the more stimulatory L-Cys [Fig. 2(f)]. With little additional experience *A. melas* were able to discriminate the conditioned ternary mixture from the mixture composed of 13 equally stimulatory amino acids [Fig. 2(g)].

MULTICOMPONENT MIXTURES

Discrimination of large multimixtures containing equally stimulatory amino acids and a single more stimulatory component

During conditioning to a 13 component amino-acid mixture, *A. melas* increased their activity to near the maximum level by the 20th conditioning session [Fig. 3(a)]. Conditioned older *A. melas* [4 to 5 years old; Fig. 3(a)] had lower searching activity (made fewer turns) than the young *A. melas* [2 to 3 years old; Fig. 4(a)]. In two repeated series, *A. melas* discriminated single amino acids, including L-Cys, from the conditioned 13 component amino-acid mixture [Fig. 3(b)] containing L-Cys as the more stimulatory component. The initial response to single amino acids, including L-Cys, was generally <40% of that observed to the conditioned 13 component mixture. Unlike for binary and ternary mixtures, *A. melas* failed to detect L-Cys as equal to the conditioned 13 component amino-acid mixture [Fig. 3(b)]. Usually, mixtures of 12 amino acids not containing one of the conditioned mixture components, but containing the more stimulatory L-Cys, were not discriminated from the conditioned 13 component mixture [Fig. 3(d)]. When L-Cys was omitted from the 13 component mixture, *A. melas* did discriminate the conditioned and non-conditioned mixtures in five of 13 comparisons [Fig. 3(c)]. The mixture of 13 amino acids with L-Cys as the more stimulatory component was easily discriminated from the mixture of the same 13 equipotent amino acids, containing L-Cys at a concentration that was equipotent to the other components [Fig. 3(e)]. Mixtures of six to seven amino acids that either contained or lacked the more stimulatory L-Cys were also discriminated from the conditioned 13 component mixture [Fig. 3(e)].

Determination of the smallest difference in number of components between multimixtures that A. melas *can discriminate*

Prior tests suggested that a 13 component amino-acid mixture with L-Cys as the more stimulatory component was not reliably discriminated from the same mixture minus L-Cys [Fig. 3(c)]. Using mixtures of equipotent amino acids, the ability of *A.*

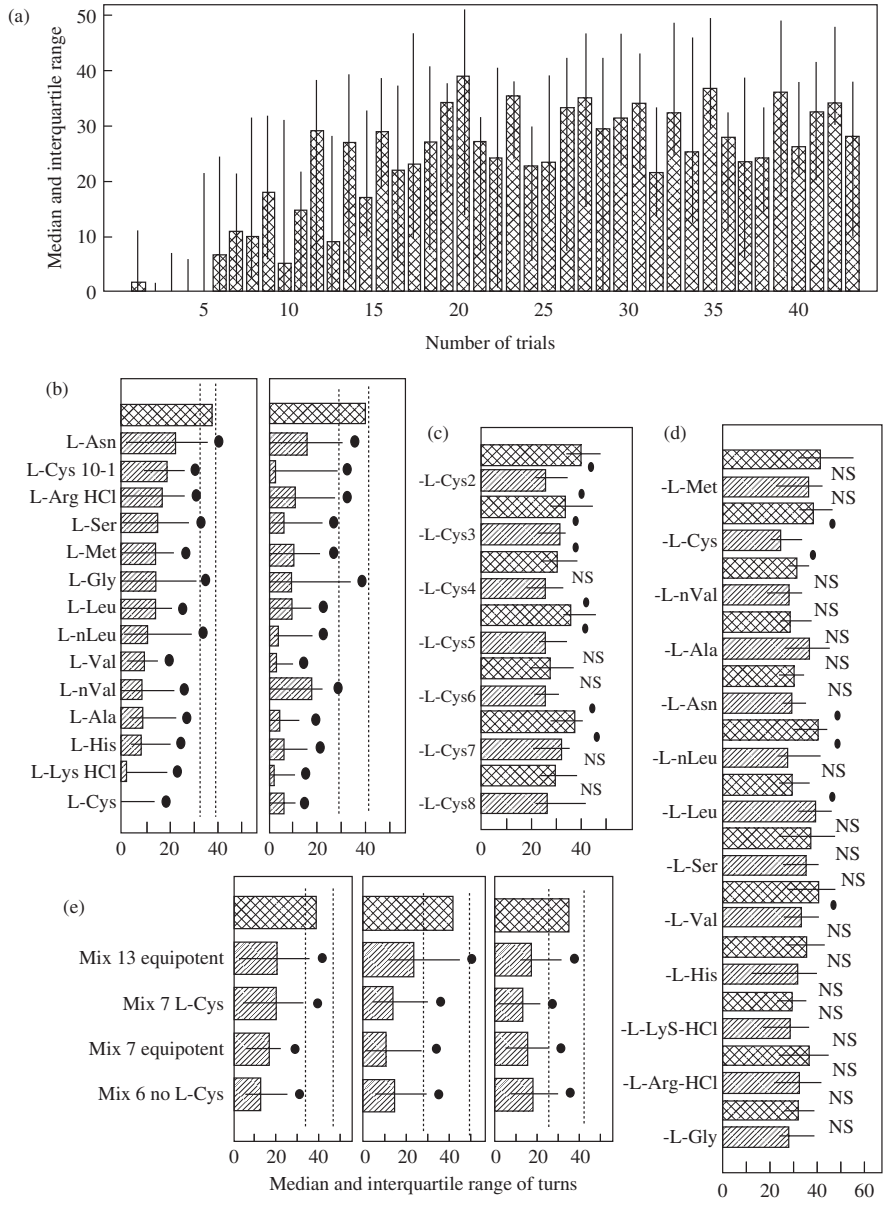

FIG. 3. (a) Conditioning of *Ameiurus melas* to a mixture of 13 amino acids (⊠) composed of 12 equipotent amino acids as determined by electro-olfactogram (EOG) recordings and L-cysteine hydrochloride (*L-CysHCl*) at ×100 its equipotent concentration. (b) Two series of responses to mixture components (▨) and the response to the conditioned mixture of 13 amino acids (⊠). (c) Discrimination of the mixture of 12 amino acids without L-CysHCl (▨) from the conditioned mixture of 13 amino acids containing the more stimulatory *L-CysHCl* (⊠). (d) Comparison of responses to the conditioned 13 component amino-acid mixture (⊠) with responses to its 12 component counterparts (▨). (e) Comparison of responses to the conditioned 13 amino-acid mixture (⊠) with responses to 13 equipotent amino-acid mixture and mixtures of six and seven amino acids (▨). Statistical tests and marks (●) are the same as in Fig. 2 ($n = 15$), interrupted vertical lines on (b) and (e) indicate interquartile range for all the conditioned stimulus tests in the reported series. In (c) and (d), responses to the test stimuli are compared statistically with responses to the conditioned stimuli before and after series of tests.

melas to discriminate large mixtures that are different by one to three fewer components than the conditioned mixture were re-examined. Two different groups of *A. melas* were conditioned to either seven [Fig. 4(a)] or 12 equipotent amino acids. Initially, the *A. melas* were unable to discriminate reliably a six-component amino-acid

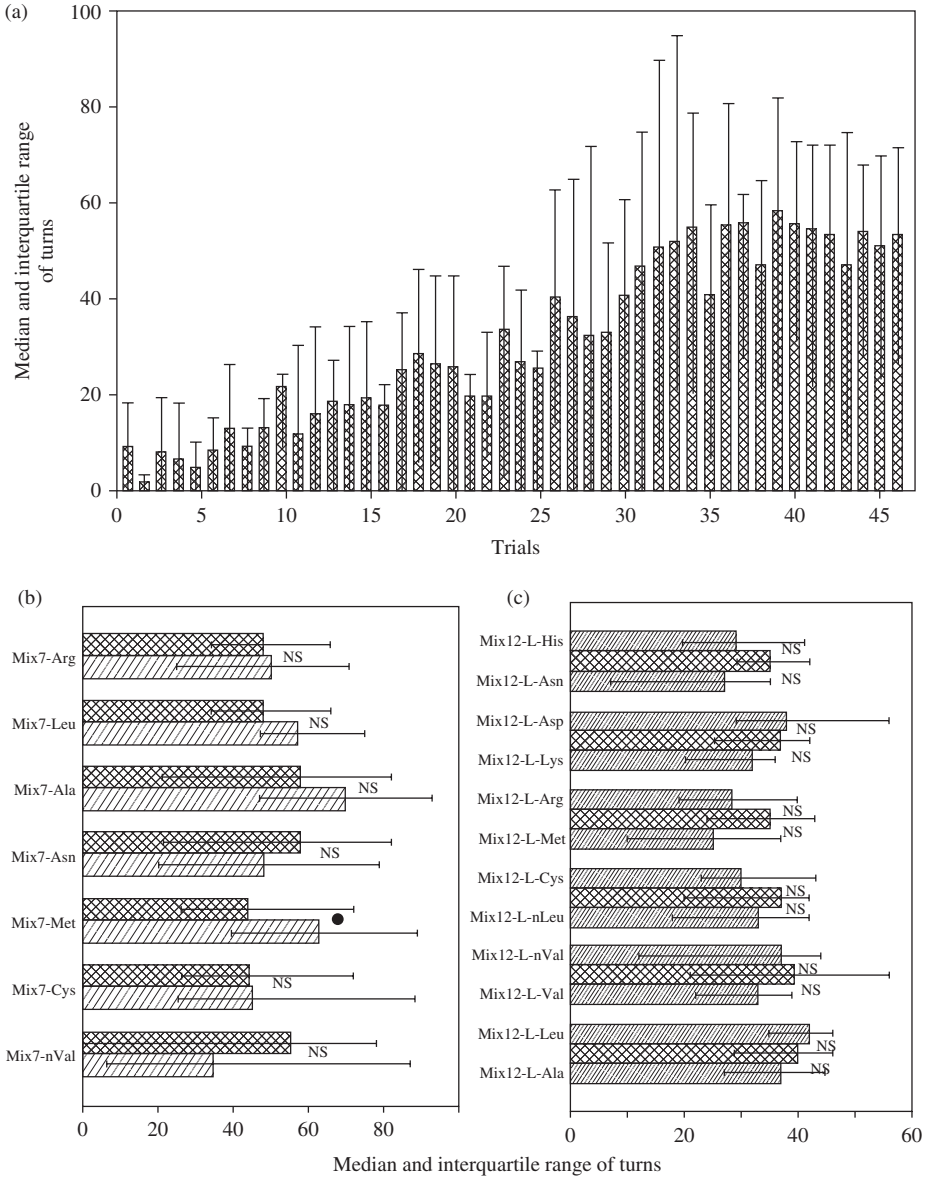

FIG. 4. Initial olfactory discrimination of mixtures composed of equally stimulatory amino acids. (a) Conditioning of *Ameiurus melas* to a mixture of seven amino acids (▨). (b) Comparison of responses to a seven-component conditioned mixture (▨) with the first response to six amino-acid mixture (▨) and (c) comparison of conditioned responses to a 12 component amino-acid mixture (▨) with responses to different 11 component mixtures (▨). Statistical tests and marks (●) are the same as in Fig. 2 ($n = 15$).

mixture from the seven-component conditioned mixture [Fig. 4(b)] or an 11 component amino-acid mixture from a 12 component conditioned mixture [Fig. 4(c)].

With additional testing of these mixtures [Fig. 5(a)–(c)], *A. melas* began to discriminate the six-component mixture from the conditioned seven-component mixture [Fig. 5(d), (e)]. *Ameiurus melas* immediately discriminated a four component amino-acid mixture from a seven-component conditioned mixture [Fig. 5(a)]; the number of $>90^{\circ}$ turns made by the experimental fish in response to the four-component mixture was <50% of the conditioned response. Discrimination between the seven-component conditioned mixture and its five-component counterparts, L-Met and L-nVal [Fig. 5(b)], L-Ala and L-Arg [Fig. 5(c)] or L-Met and L-Ala, were missing in this mixture, typically occurred immediately, the median activity after stimulation with the five-component mixtures was *c*. 70% of the activity released by the conditioned stimulus. After the experience with the four and five amino-acid mixtures, *A. melas* discriminated the six-component mixtures that did not contain a particular single amino acid, either L-nVal [Fig. 5(d)] or L-Arg [Fig. 5(e)], from the conditioned mixture of seven amino acids. When the difference between the conditioned and the non-conditioned mixture was very small, the large number of comparisons between the conditioned mixture responses *v*. the non-conditioned mixture responses made the differences between the conditioned and the non-conditioned responses more or less equal.

In spite of the initial inability to discriminate mixtures of 11 amino acids from the conditioned 12 component mixture [Fig. 4(c)], *A. melas* immediately discriminated a nine amino-acid mixture from the conditioned mixture of 12 equipotent amino acids [Fig. 6(a)], in most comparisons the non-conditioned responses were *c*. 60% of the conditioned response. *Ameiurus melas* also discriminated 10 component mixtures, mixtures missing either-Ala and L-Arg [Fig. 6(b)] or L-Ala and L-nVal [Fig. 6(c)] from the conditioned 12 component mixture. Irrespective of experience, *A. melas* were unable to discriminate the 11 component amino-acid mixtures that did not contain either L-Arg [Fig. 6(d)] or L-nVal [Fig. 6(e)] from the conditioned mixture of 12 amino acids. In nine of 17 comparisons, *A. melas* did not discriminate the 11 component mixture that did not contain L-Ala [Fig. 6(f)] from the conditioned 12 component mixture. The small differences and the variability of the behavioural responses to the conditioned and the non-conditioned stimuli illustrated the limits of olfactory discrimination for two similar amino-acid mixtures.

DISCUSSION

STUDIES ON OLFACTORY DISCRIMINATION IN *AMEIURUS MELAS*

In fishes, taste and olfactory systems detect the same chemicals, such as amino acids. It is relatively easy to remove the nose surgically and study responsiveness to taste stimuli in anosmic *I. punctatus* (Valentincic & Caprio, 1994*a*). In anosmic *I. punctatus*, the frequency of innate behavioural responses to taste stimuli was proportional to the number of supra-threshold stimuli at the taste receptors and to the physiological effectiveness of the stimulus (Valentincic & Caprio, 1994*b*). On the other hand, it is impossible to remove the entire taste systems of *I. punctatus* and

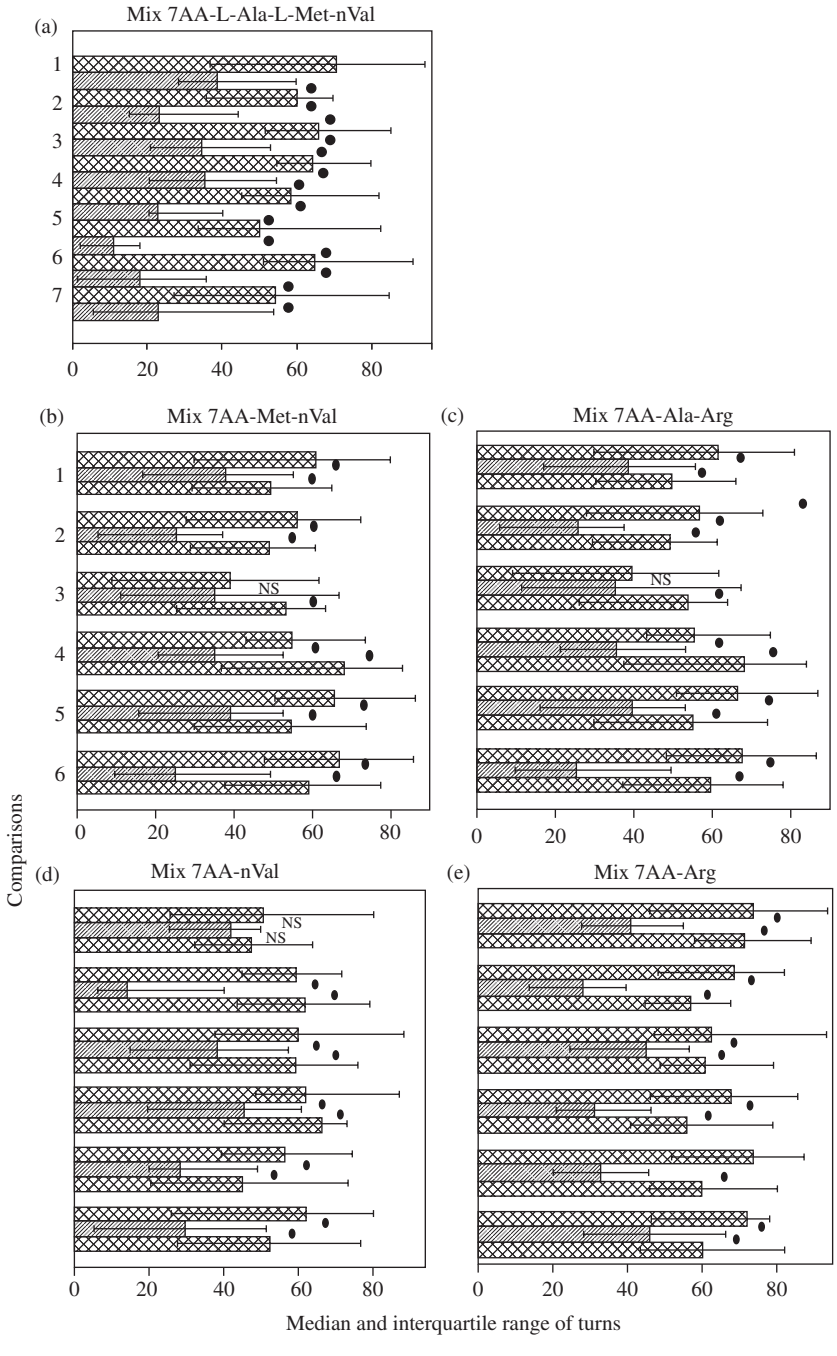

FIG. 5. (a) Discrimination of the conditioned mixture of seven equipotent amino acids (▨) from a mixture of four equipotent amino acids (▨). (b), (c) Comparison of the responses to the conditioned mixture (▨) with those to two different five-component mixtures (▨). (d), (e) Comparison of the responses of the conditioned mixture (▨) with those of two different six-component mixtures (▨). Statistical tests and marks (•) are the same as in Fig. 2 ($n = 15$). Numbers along the vertical axis indicate sequence of experiments valid for all the panels.

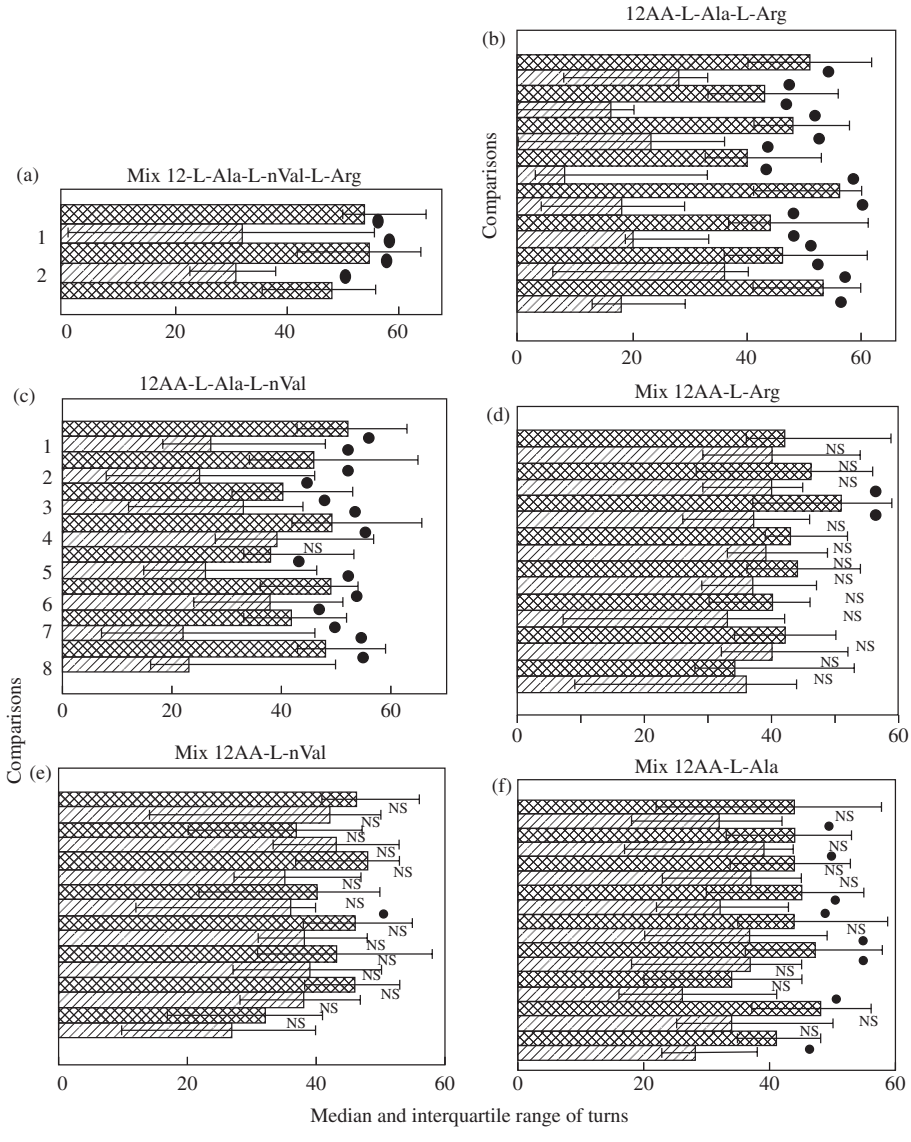

FIG. 6. (a) Discrimination of the conditioned mixture of 12 equipotent amino acids (▨) from mixtures of nine equipotent amino acids (▨). (b), (c) Comparison of the responses to the conditioned mixture (▨) with those to two different 10 component mixtures (▨). (d)–(f) Comparison of the response to the conditioned mixture (▨) to those of three different 11 component mixtures (▨). Statistical tests and marks are the same as in Figs 2 and 3 ($n = 15$). Numbers along the y-axis indicate sequence of experiments valid for all the panels.

A. melas; their taste buds are distributed within the mouth, on the barbells and over the entire body surface (Caprio, 1978). Behavioural responsiveness to olfactory stimuli can be studied based on differential properties of the two systems. Taste systems of different fish species are narrowly tuned and only detect a limited number of

amino acids. Olfactory systems, however, are broadly tuned and fishes can detect all the amino acids. The narrowly tuned *A. melas* taste system detects L-Pro, L-Ala and L-Cys best, the feeding responses to the other amino acids are controlled by olfactory stimuli only (Valentincic *et al*., 2000*a*). It was impossible to change the intensity and duration of the feeding response to the taste stimuli by learning in anosmic animals (Valentincic & Caprio, 1994*a*, *b*); any changes in the strength and duration of the feeding responses to taste stimuli of the same intensity depend on motivation and inhibition by other behaviours. The food-searching responsiveness to olfactory stimuli is controlled by learning (Valentincic & Caprio, 1994*a*; Valentincic *et al*., 1994, 2000*a*; Valentincic, 2005). Naive *I. puctatus* (Valentincic & Caprio, 1994*a*) and *A. melas* (Valentincic *et al*., 2000*a*) respond to olfactory stimuli with short and slow search swims; however, after conditioning, the frequently rewarded amino acid stimulates long lasting and rapid search swimming (Valentincic *et al*., 1994, 2000*a*). On the basis of large responses to the conditioning stimuli, it is possible to investigate properties of the olfactory system. In addition, reflex responses to most taste stimuli do not occur at concentrations that are below 10^{-5} M amino acids. In this study, at least four phenomena confirmed that the behavioural responses to amino-acid stimuli were controlled by the olfactory system: the large responses to the conditioned stimuli, behavioural responses to all amino acids including those that are not detected by the taste system, the absence of learning chemical stimuli in anosmic *A. melas* (unpubl. data) and lack of behavioural responses to taste stimuli at concentrations of amino acids below 10^{-5} M.

LEARNED OLFACTORY DISCRIMINATION IN FISHES

During experiments with humans, language is used to communicate mixture perception and its description to the experimenters whereas, in animals, evidence of olfactory discrimination can be provided only by conditioning animals to one stimulus and testing the behavioural responses to other non-conditioned stimuli (Cleland *et al*., 2002). In fishes, learning chemical stimuli during olfactory conditioning is a stepwise process where large differences between odorants are learned first followed by fine differences between similar stimuli during subsequent discrimination training trials (Valentincic *et al*., 2000*b*; Valentincic, 2004). After 30–50 conditioning trials (Valentincic & Caprio, 1994*a*), a single experience with any non-rewarded odorant caused the fish to respond less to all odorants dissimilar to the conditioned stimulus, *i.e.* a 'dissimilarity generalization' [Fig. 2(a)–(c)]. In this study, *A. melas* were highly motivated (very hungry) throughout the experiments, they always responded more to the stimulus that predicted food reward than to non-conditioned stimuli. For similar stimuli, a differential reinforcement of the conditioned odour (Linster *et al*., 2002), additional olfactory discrimination training (Valentincic *et al*., 2000*b*) or ample experience with similar odorant mixtures (Figs 5 and 6 and Table I) improved olfactory discrimination to the limits of the olfactory system capabilities.

DETECTION OF THE MORE STIMULATORY COMPONENT IN TERNARY MIXTURES

Ameiurus melas initially perceived binary (Valentincic *et al*., 2000*b*) and ternary mixtures [Fig. 2(c) and Table 1] of amino acids as their more stimulatory component. In *D. rerio*, the more stimulatory component in a binary mixture dominated

glomerular excitation (Tabor *et al*., 2004). Therefore, the initial binary mixture perception (sensation) would not be, as in A. *melas*, discriminated from the perception of the more stimulatory component alone (Table I). *Ameiurus melas*, however, failed to detect the more stimulatory amino acid in a 13 component mixture even when tested at $\times 100$ the equipotent amino-acid concentration [Fig. 3(b) and Table I]. This argues strongly against the ability of the fish to detect individual components within large mixtures. Humans also recognize components of binary and ternary mixtures, but do not recognize components within mixtures composed of more than four-components (Laing & Livermore, 1992). *Ameiurus melas* failed to discriminate a conditioned ternary mixture with L-Cys as the more stimulatory component from a 13 amino-acid mixture containing the same more stimulatory component; based on current theories, there is no explanation for this phenomenon.

On the other hand, the *A. melas* conditioned to a mixture of 13 amino acids containing the more stimulatory L-Cys discriminated it immediately from a seven-component amino-acid mixture with the same more stimulatory component. These results suggest that fishes perceive complex mixtures as blends, *i.e.* as novel qualities (Rabin & Cain, 1984; Rescorla *et al*., 1985), whereas for simple binary and ternary mixtures fishes initially perceive the mixture as equal to its more stimulatory component alone. In multimixtures, 'configurational' interactions (Laing & Francis, 1989; Chandra & Smith, 1998; Linster & Smith, 1999; Kay *et al*., 2003; Wiltrout *et al*., 2003) yield the mixture qualitatively different from undetectable components. The overlapping chemotopic activity patterns of different components probably mask the contribution of a single more stimulatory component. The 13 component mixture was initially not equal to its more stimulatory component alone, which supports synthetic (Chandra & Smith, 1998) rather than analytic multimixture perception.

THE SMALLEST DIFFERENCES BETWEEN SIMILAR MIXTURES THAT ALLOW DISCRIMINATION

Because differences between two chemotopic patterns either enable or prevent odorant discrimination, it is essential to establish the finest difference between two odorants that *A. melas* can detect. Equal or very similar chemotopic activation patterns hypothetically occur when testing mixtures composed of the same amino acids that are different by only one component. Complex mixtures composed of the same amino acids at their equal EOG magnitudes (Silver *et al*., 1976; Valentincic *et al*., 2000*b*) that are different by one to three amino acids enabled a precise stepwise manipulation of the odorant similarity (Figs 5 and 6 and Table I).

Initially *A. melas* were incapable of discriminating a seven-component conditioned mixture from its six-component counterpart [Fig. 4(b) and Table I]. Most animals required additional time and repetition to learn to discriminate among similar odorants (Uchida & Mainen, 2003; Abraham *et al*., 2004). Ample practice with stimuli dissimilar to the conditioned stimulus [Fig. 5(a)–(d)] also provided the *A. melas* with an opportunity to learn small differences between the similar amino-acid mixtures. After practice with several dissimilar mixtures, *A. melas* could perceive fine differences between mixtures of six and seven equipotent amino acids [Fig. 5(e), (f) and Table I]. *Ameiurus melas* could almost immediately discriminate a conditioned mixture composed of 12 equipotent amino acids from the nine and 10 component mixtures composed of the same [-2(-3) amino acids] equipotent amino

acids [Fig. 6(a)–(d)]. Irrespective of practice, however, *A. melas* were unable to discriminate a 12 equipotent amino-acid conditioned mixture from its 11 component counterparts [Fig. 6(e)–(g) and Table I]. Hypothetically, with an increasing number of components in the mixture, the differences in the stimulated areas of the OB between n and $n - 1$ odorant mixtures become smaller. In addition, due to potentially overlapping chemotopic activation patterns for neutral amino acids, the differences in the activated areas of the OB for 11 and 12 component mixtures were even less than for mixtures with non-overlapping chemotopic activation patterns that were different by one component. Due to unequal number of neutral and basic amino acids in multimixtures, it was not possible to detect differences in perception originating from neutral only or basic only amino acids.

At the presynaptic level in the vertebrate OB, the observed activation of converging ORN axons represents an unmodified chemotopic representation of amino-acid stimuli at the tested concentrations (Friedrich & Korsching, 1997). Chemotopy as observed for MCs (Friedrich & Laurent, 2001; Korsching, 2001) is a mirror image of the presynaptic sensory input, modified by dendro-dendritic interactions between mitral and granule cells (Yokoi *et al*., 1995). Previous reports suggest that temporal modifications of the input representation that occur at the MC layer (Friedrich & Laurent, 2001; Spors & Grinvald, 2002; Abraham *et al*., 2004; Friedrich *et al*., 2010) improve odorant discrimination with time (Laurent *et al*., 2001). In behavioural experiments, the temporal improvements of odorant discrimination were described as latency to odorant recognition (Wise & Cain, 2000), whereas other investigators state that the time to odour recognition is constant irrespective of odorant similarity (Ditzen *et al*., 2003). In this study, learning experiments indicate that odorant similarity influences the number of comparisons needed for learning the fine differences between similar stimuli. After *A. melas* learned the fine odorant discrimination, additional time for discrimination, irrespective of the degree of the similarity between stimuli, was unnecessary.

STABILITY OF LEARNED RESPONSES TO SINGLE AMINO ACIDS AND THEIR MIXTURES

To avoid confusion, conditioned and non-conditioned stimuli were presented sequentially to *A. melas*. Sequential presentations of the non-rewarded stimuli that *A. melas* could not discriminate from the conditioned stimulus diminished the intensity of the conditioned response nearly immediately, after two to four trials. Therefore, the rewarded conditioned stimulus was always tested immediately before and after the test of any stimulus equal or similar to the conditioned stimulus [Figs 3(c), (d), 4(c), 5(a)–(f) and 6(a)–(g)] (Valentincic *et al*., 1994, 2000*a*, *b*). Supposedly, the final capacity for odorant discrimination depends exclusively on the brain's ability to perceive fine differences in glomerular activity patterns within the OB. Up to 5 month long intervals between successive tests did not interrupt odorant discrimination (Valentincic *et al*., 2000*a*), although a few tests were needed to re-establish the previous level of discrimination between conditioned and non-conditioned stimuli. Experience seemingly allowed for refinement, most probably a greater refinement in perception. Whenever refinement did not occur, such as in the cases of L-Ile *v*. L-Val (Valentincic *et al*., 2000*a*) and mixtures of 11 *v*. 12 equipotent amino

acids [Fig. 6(e)–(g) and Table I], discrimination was not possible irrespective of the discrimination training tasks applied.

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