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Spontaneous behaviour, training and discrimination training in goldfish using chemosensory stimuli

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Abstract. The present behavioural experimental paradigm made use of the responsiveness of goldfish to natural and non-familiar chemosensory stimuli in the context of feeding. With the exception of *Tubifex* food extract, which was spontaneously preferred, goldfish exhibited no spontaneously recordable response to low concentrations of the stimuli tested. Training experiments using non-familiar stimuli (amyl acetate, α-ionone, β-phenylethanol, 10^{-6}, 10^{-7} *M*) required 2–3 months of daily training prior to the animals reaching a 70% positive response level for discrimination. This discrimination was dependent upon a functioning olfactory system as no responses were recorded after bilateral exclusion of olfaction, e.g. dissection of olfactory nerve or olfactory tracts. Amino acids (Ala, Arg, Gln, Gly, Lys), more natural stimuli than those listed above, were preferred when applied at concentrations $< 10^{-5}$ *M*. Goldfish were able to discriminate amino acid odours applied at 10^{-6} or 10^{-7} *M*, but these stimuli elicited no spontaneous response below 10^{-5} *M*. Ten to twenty reinforcements were sufficient to achieve discrimination between amino acids, which again was eliminated after bilateral exclusion of olfactory pathways. In contrast to the 4-week period for long-term memory to non-familiar odours, long-term memory for amino acids lasted at least 3 months.

Key words: Spontaneous reactions – Learning behaviour – Chemosensory stimuli – Goldfish

Introduction

Histological investigations in the 19th century indicated that fish, like other vertebrate species, have similar taste and olfactory receptor cells, respectively (Allison 1953; Andres 1970). The central connections of these peripheral chemoreceptors project to the brain by different pathways and to various nuclei. A difference in the thresholds to gustatory and olfactory stimuli has also been reported in aquatic and terrestrial animals. Since in aquatic animals gustatory and olfactory stimuli are presented in aqueous solutions, it is difficult to determine whether the chemical stimuli are perceived by smell, taste or both systems. A separation of olfactory and taste perceptions can be investigated after surgical bilateral lesion in the olfactory system (Zippel 1970; Zippel et al. 1981, 1988) following differentiation training. Nonfamilar olfactory stimuli have been investigated intensively in the literature (e.g. Herter 1953; Kleerekoper 1982), and training to single stimuli and differentiation training using these odours have been reported (Zippel 1970; Zippel and Voigt 1982).

In the present report, the processes of acclimating goldfish to the experimental setup, investigating the spontaneous behaviour, training or discrimination training to different odour stimuli, and testing behavioural thresholds regularly lasted several months. The experiments described in this paper extended over years and had several aims: (1) to determine the differences in spontaneous behaviour in responses to non-familiar and natural chemical stimuli presented under identical experimental conditions; (2) to determine whether complex natural stimuli (*Tubifex* food extracts, mixtures of amino acids) have different effects on the spontaneous reactions than non-familiar odours and single amino acids; (3) to determine whether the ability to learn and remember is different for non-familiar and more natural chemosensory stimuli; and (4) to describe the effect of bilateral severing of olfactory pathways on the gustatory and olfactory thresholds necessary for behavioural investigations made in functional regeneration experiments.

Materials and methods

Training and testing apparatus. Each training and testing tank (Fig. 1) of dimensions 130 × 20 × 30 cm was filled to a depth of

Abbreviations: FB, funnel biting; FO, funnel orientation

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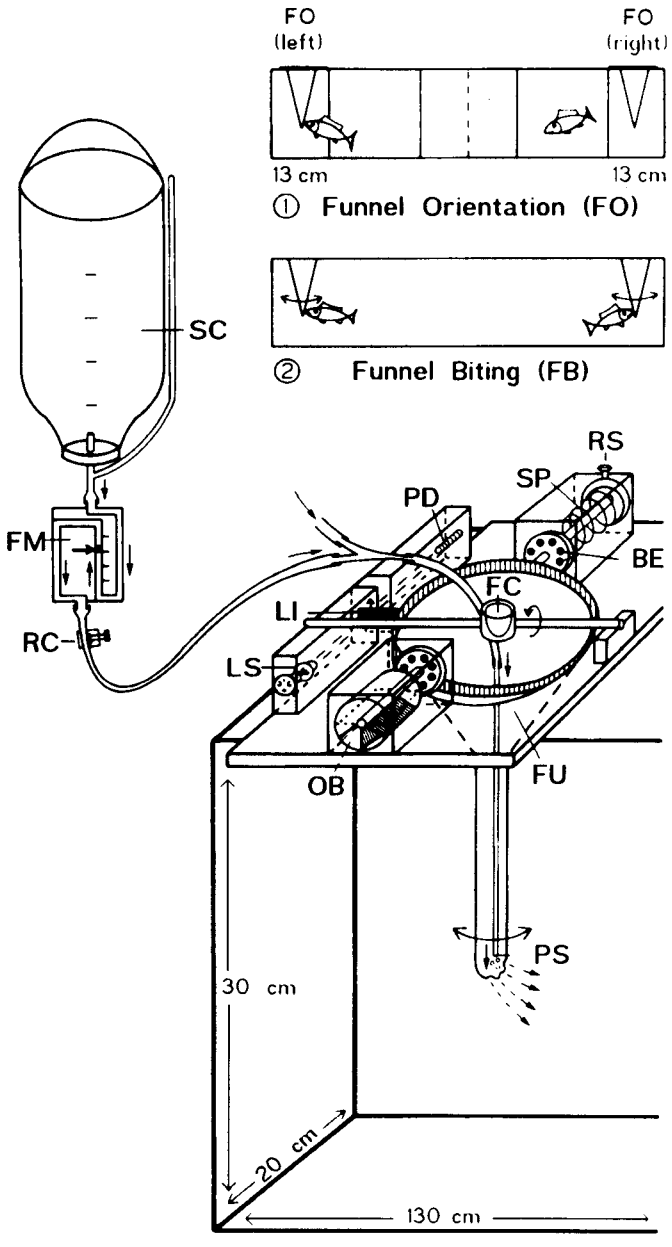

Fig. 1. Stimulus applications, recording technique and analysis of behaviour. *BE* (ball-) bearing; *FC* food container; *FM* flowmeter; *FU* funnel; *LI* light beam interruptor; *LS* light source; *OB* oil bath; *PD* photodiode; *PS* point of stimulus application; *RC* regulating clamp; *RS* regulating screw; *SC* stimulus container; *SP* spring. For analysis of behaviour see text

23 cm with aged tap water which dropped continuously from inlet tubes into each end and was maintained at a constant level by means of a centrally positioned outlet. For feeding, training and testing purposes, an opaque plastic funnel was suspended in the water at each end of the tank. The side of the funnel facing away from the end of the aquarium was perforated with 15 regularly spaced holes (diameter 2 mm) through which the fish could feed on *Tubifex* worms. Stimuli, non-familiar odours above and below the taste threshold, natural food extracts and amino acids were presented through the perforations in the funnels. Non-familiar stimuli and amino acids are listed in molar concentrations while the dilutions of *Tubifex* food extracts are presented as percent of 1 g wet weight. The stimulus concentrations quoted in the text refer to the undiluted values present at the tip of the funnel.

Recording technique and behavioural analysis. Before testing and shock-free training goldfish were kept in groups of 20–30 animals in large holding tanks for several months in order to acclimate to laboratory conditions. The training experiments were preceded by a habituation period of 4–5 weeks, during which time the fish were fed *Tubifex* worms daily through both funnels. A 2- to 3-week period of delayed feeding followed. The delay between the introduction of the funnels and the delivery of the food varied between 5 and 15 min. This procedure ensured that the fish maintained a high level of activity over the subsequent recording sessions. By the end of the habituation period, a complex behavioural repertoire directed towards one or both of the funnels developed. The following behavioural patterns (Fig. 1) were recorded: 1. funnel orientation (FO) – the time (in seconds) spent by the animals in the immediate vicinity of the funnels; 2. funnel biting (FB) – the number of bites at each of the funnels. (This behaviour may be interpreted as a specifically orientated food expectation.)

The behaviour patterns were recorded on a custom-built, electronic recording unit. FB was registered automatically by a photocell system (a light beam was interrupted during FB by a flat metal tongue affixed to the rim of the funnel), while FO was recorded manually by operating a time switch. The data were punched onto paper tape for final analysis by a PDP-11/34 computer.

Training and testing procedure. At the beginning of each session, a control registration (3 min duration) determined the animals' behaviour in response to the funnels in the absence of stimuli. In the immediately following test or training period (also of 3 min duration), the behaviour during stimulus application was recorded. At no time during or immediately following a *test* session was the animals' behaviour reinforced; the animals were fed on both sides of the aquarium at least 2 h after the last test on any particular day. In contrast, each *training* session was immediately followed by positive reinforcement; stimulus application was continued and the animals were fed with *Tubifex* worms through the appropriate funnel (interval reinforcement).

Discrimination training. The goldfish had to discriminate bilaterally presented stimuli. The reinforced stimulus was always applied through the spontaneously less preferred (control-negative) funnel and the non-reinforced stimulus was always applied through the spontaneously preferred (control-positive) funnel.

During control runs without stimulus, the animals preferred one funnel 60–70% of the time, and for the remaining 30–40% of the time they remained at the spontaneously disliked funnel, demonstrating a strong side preference during the time interval without stimulus. Strong side preference was frequently observed for the funnel through which the animals had been fed on the previous day. Thus, positive reinforced stimuli were applied through the non-preferred funnel during training. The absence of a behavioural standard distribution during control registration required non-parametric statistics (Wilcoxon rank test).

A training was considered successful if, in ten consecutive training runs, FO and FB were significantly different from the observations during the control sessions.

Threshold determinations. After successful training discriminative qualitative olfactory thresholds were determined by lowering stimulus concentrations. Non-olfactory "taste" thresholds were determined by increasing stimulus concentrations after bilateral elimination of olfaction. Olfaction was eliminated by the following methods: 1) axotomy of the olfactory nerves (Zippel 1970; Zippel et al. 1988; Hudson et al. 1990); 2) total olfactory bulbectomy (Zippel et al. 1981); 3) rostral and caudal olfactory bulbectomy (Zippel et al. 1988); and 4) squeezing or cutting the olfactory tract (Zippel et al. 1993).

Results

Spontaneous behaviour

Non-familiar chemicals. Amylacetate, α-ionone and β-phenylethanol elicited positive, but statistically not significant, responses in intact and anosmic animals only when offered at concentrations above 10^{-5} *M*, whereas a presentation at lower concentrations (10^{-5}–10^{-7} *M*) resulted in indifferent behaviour.

Tubifex-food-extracts. Olfactory and gustatory thresholds for food odours and their chemical fractions were determined in intact as well as in bulbectomized animals. In intact goldfish (Fig. 2), crude *Tubifex* extracts (concentrations based on 1 g wet weight of worms) elicited positive responses down to a dilution of 10^{-8} g · ml^{-1}, and the frequency and the persistency of the stimulus-orientated activity decreased uniformly from high (10^{-4} g · ml^{-1}) to low concentrations (10^{-8} g · ml^{-1}). The olfactory threshold determined was 10^{-8} g · ml^{-1}, although for some animals olfactory thresholds in some series of experiments were much lower (10^{-9} or 10^{-10} g · ml^{-1}), while the gustatory thresholds after bulbectomy were similar ($> 10^{-5}$ g · ml^{-1}). Below this concentration the animals' behaviour remained similar to that of the preceding control response. Thresholds for the dialysis products of *Tubifex* extracts, retentate (MW > 10000) or dialysate (MW < 10000) or a mixture of both components were similar to those determined for the crude extract. In bulbectomized animals thresholds increased to 10^{-5} g · ml^{-1} (gustatory threshold). Threshold for both FO and FB showed a parallel shift of roughly 2 log units. Lower values of FO and FB indicated the loss of olfactory input at lower concentrations resulting in a behavioural deficit.

Like the dialysis products, threshold concentration for a mixture of the five different MW fractions of *Tubifex* obtained from gel chromatography (Sephadex G–200) was similar to that for the crude extract described above. However, in the five single fractions the olfactory threshold decreased from higher (10^{-4} g · ml^{-1}) to lower (10^{-5} g · ml^{-1}) molecular weight fractions, while the gustatory threshold for all single fractions increased to 10^{-3} g · ml^{-1} after olfactory bulbectomy.

Amino acids. Goldfish spontaneously preferred higher concentrations of single amino acids ($> 10^{-5}$ *M*; Ala, Arg, Gln, Gly, Lys; Fig. 3A–E). Below 10^{-5} *M*, the responses became indifferent. These positive responses included taste responses which could also be elicited after bilateral transection of olfactory pathways. Responses to low (10^{-8}, 10^{-9} *M*) concentrations of mixtures of four or five amino acids (Fig. 3F, G) were only found in some groups of animals tested. These responses were found in goldfish which already responded positively to low (10^{-9}, 10^{-10} g · ml^{-1}) concentrations of *Tubifex* extracts. This preference for low concentrations of food extracts and amino acids may be caused by different prelaboratory experiences of the animals. As goldfish in Germany are not bred specifically for scientific purposes, the animals used in the experiments originated from different and unknown locations. The probability of different environmental experiences is further supported by the fact that different thresholds to non-familiar odours were never apparent in any group before, during or after training.

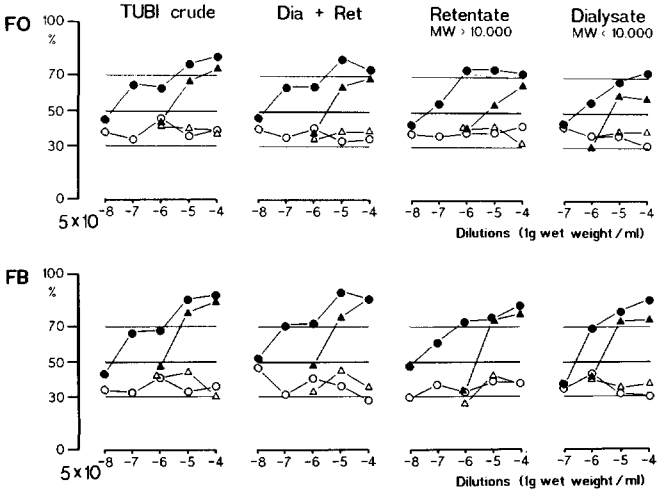

Fig. 2. Spontaneous responses to different concentrations of *Tubifex* food extracts and its dialysis products in the same goldfish (ten groups of two fish) before and after olfactory bulbectomy. The results are expressed in terms of percent responses during the control (without stimulus) sessions *(open symbols)* and during the test (with the designated stimulus) sessions *(closed symbols)*. Stimuli were presented through the spontaneously avoided funnel *(open symbols)*. Funnel orientation (FO) and funnel biting (FB) shown for the funnel through which *Tubifex* stimuli were offered . *Symbols:* control reactions ○–○ before bulbectomy △–△ after bulbectomy; test reactions ●–● before bulbectomy ▲–▲ after bulbectomy

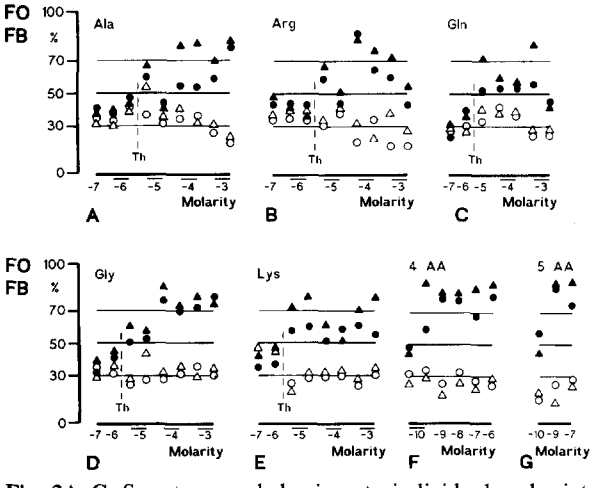

Fig. 3A–G. Spontaneous behaviour to individual and mixtures of amino acids. The data originate from different groups and were recorded before training or differentiation training. Thresholds (Th) indicated by *vertical dashed lines* **(A–E)** for positive spontaneous responses to individual amino acids were below 10^{-5} *M*, but drastically decreased in some groups when mixtures of four **(F)** or five **(G)** amino acids are presented. All data originate from 8–12 groups of two goldfish. See Fig. 2 for details. *Symbols:* FO ○ during control (negative side) ● during stimulus application (stimulus side); FB △ during control (negative side) ▲ during stimulus application (stimulus side). *Abbreviation:* Th, threshold

Olfactory training

Low ($< 10^{-5}$ *M*) concentrations of non-familiar stimuli and amino acids result in no behavioural responses in intact goldfish. Anosmic specimens do not react to, nor qualitatively discriminate, low concentrations of non-familiar stimuli and amino acids after prior discrimination training. These stimuli, at the stated concentrations, were therefore classified as olfactory stimuli.

Training and discrimination training to non-familiar odors. Training to a single synthetic odour required 40 training sessions to elicit constant positive behaviour (Zippel 1970; Zippel and Voigt 1982). Orientation to the reinforced funnel remained at approximately 60% and the corresponding FB at 70%. Furthermore, following training to a single stimulus, animals had considerable difficulty in qualitatively discriminating this stimulus when it was offered simultaneously with a competing odour (Zippel 1970). For this reason, discrimination training was immediately performed to two stimuli from the first training session onwards. Stimulus concentrations used during training to α-ionone versus amylacetate (10^{-5} *M*; Fig. 4) were only 1 log unit below the behavioural taste threshold. FO and FB responses to the reinforced stimulus increased extremely slowly and reached high, although fluctuating, values after 70 training sessions. Discrimination training with other combinations of synthetic stimuli yielded similar results.

During training and discrimination training to non-familiar odours, stimulus concentrations were increased above taste threshold either at the beginning (Fig. 5) or during some of the experiments to investigate the possibility that stimuli detectable with both chemical senses might have a positive influence on the long period necessary for olfactory training. The stimulus increase resulted in higher FO and FB values. However, after a decrease to olfactory concentrations FB values returned to the levels typical for the number of reinforcements during olfactory training, i.e. a period of stimulus increase had no influence on the duration of the olfactory training period. Olfactory discriminative memory for non-familiar stimuli following discrimination training was short. Behavioural responses remained positive for only 4 weeks, and after this time the fish became rapidly indifferent.

Amino acids. Training and discrimination training using amino acids was performed to determine whether goldfish could learn to discriminate amino acids, and whether

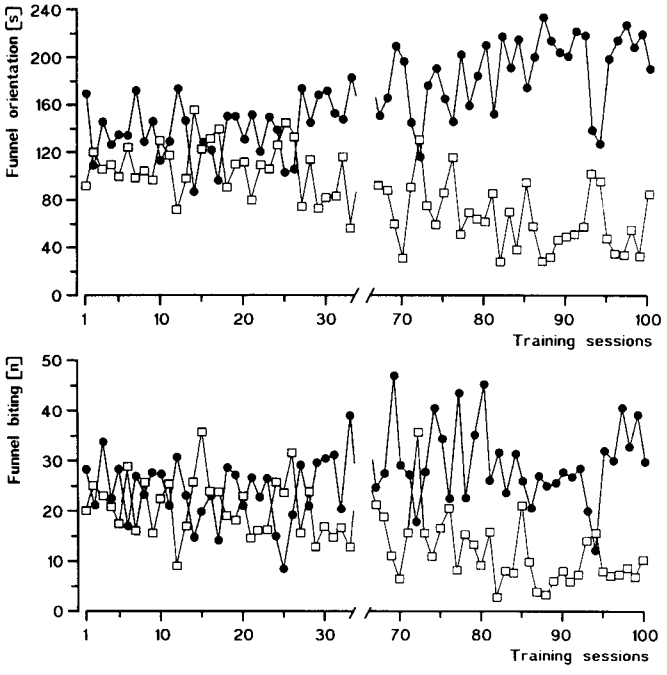

Fig. 4. Discrimination training to α-ionon (reinforced, *closed symbols*) versus amyl acetate (unreinforced, *open symbols*) 10^{-5} *M*. The reinforced stimulus was always applied through the control-avoided funnel, whereas the non-reinforced stimulus was presented through control-preferred funnel (not shown). The results during stimulus application are presented as raw data. Mean values for eight groups of two animals. The original data demonstrate that during 100 training sessions FO values were positive after 30 sessions and slightly increased during training sessions 70–100, whereas FB values after 30 sessions remained at a positive but fluctuating level of response. *Symbols:* ●–● α-Ionone 10^{-5} *M* (positive, reinforced stimulus); □–□ Amyl acetate 10^{-5} *M* (negative, not reinforced stimulus)

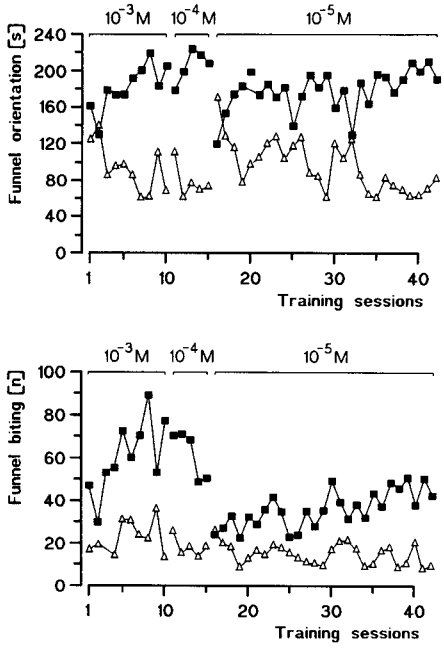

Fig. 5. Training to amyl acetate in above (10^{-3}–10^{-4} *M*) and below (10^{-5} *M*) taste threshold concentrations. Taste thresholds were determined after bilateral operative exclusion of olfaction. For application of stimuli and presentation of results see Fig. 4. Above taste-threshold concentrations, FO and FB values increase rapidly during training. After decreasing concentrations below taste threshold FO values remain stable, whereas FB values decrease to chance. However, FB values increase slightly during subsequent training and stabilize at a positive level that is typical for olfactory training to non-familiar stimuli (see Fig. 4). *Symbols:* ■–■ Amyl acetate (positive, reinforced stimulus); △–△ water without stimulus

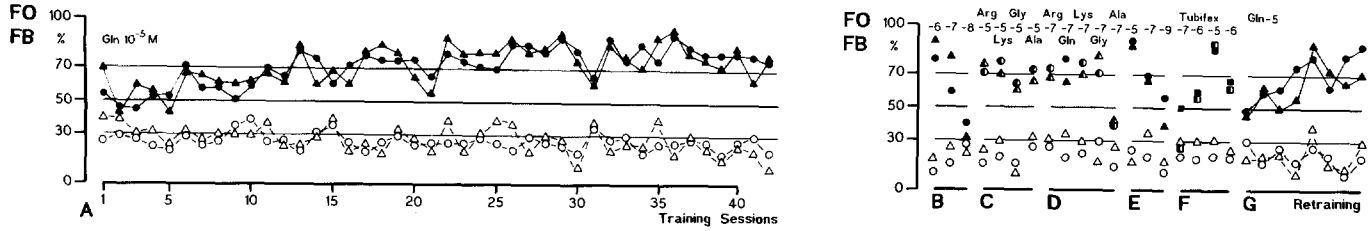

Fig. 6A–G. Training to 10^{-5} *M* glutamine (12 groups of two fish): **A** the learning curve for glutamine is shown over 42 training sessions. The results are expressed in terms of percent. 100% is the total number of reactions to both funnels. These reactions were not distributed equally to both funnels, rather the animals showed a consistent spontaneous avoidance for one funnel (*open symbols*). Only 30% of the reactions occurred at the avoided funnel. This is shown in this and subsequent figures by *thin solid line* at 30%. The avoided funnel was identified in control sessions in which no stimulus was applied in either funnel. Control session preceded each training session. During test or training session the stimulus was applied in the avoided funnel. The percent of reactions at this funnel (*closed symbols*) increased during training; **B** and **E** threshold determination (there was a minimal glutamine concentration necessary to increase the reactions at the control-avoided funnel); **C** and **D** generalisation to other amino acids (**C**: 10^{-5} *M*, **D**: 10^{-7} *M*); **F** reaction to *Tubifex* food extracts; **G** retraining to glutamine. *Symbols:* ○ FO △ FB Control without stimulus; ● FO ▲ Glutamine 10^{-5} *M*; ◐ FO ◭ FB other amino acids; ■ FO ◧ FB Tubifex food-extract

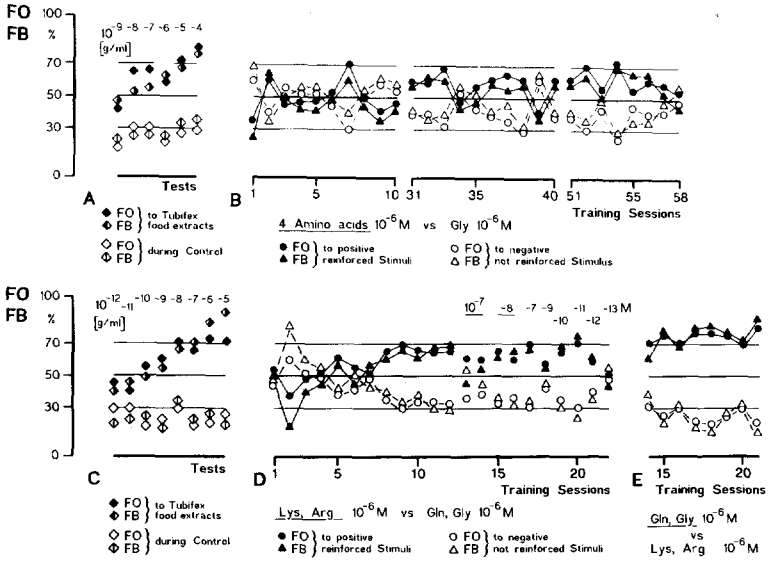

Fig. 7A–E. Discrimination training to a mixture of four amino acids (Ala, Arg, Gln, Lys) versus one (Gly), and two different amino acids versus two different amino acids: **A, B** ten groups of two goldfish; **C, D** 12 groups of two goldfish; **E** 14 groups of two goldfish. **A, C**: test reactions to various concentrations of *Tubifex* food extracts (*closed* and *partly closed symbols*) applied through the spontaneously avoided funnel (*open symbols*) before training. *Tubifex* stimuli in grams per millilitre concentrations. **B, D** and **E**: for discrimination training the percent of FO and FB reactions for the positive (rewarded, *closed symbols*) and the negative (concurrent, *open symbols*) stimulus during stimulus application are shown. The lines at the 30% and the 70% indicate percent reactions occurring at the avoided and the preferred funnel, respectively, during the preceding control session. **B**: discrimination training to a mixture of Ala, Arg, Gln, Lys (rein-forced, *closed symbols*) versus Gly (non-reinforced, *open symbols*). **D**: discrimination training to a binary mixture of Arg and Lys (reinforced, *closed symbols*) versus a binary mixture of Gln and Gly (non-reinforced, *open symbols*). Threshold investigations in sessions 13–22. **E**: discrimination training to a binary mixture of Gln and Gly (reinforced, *closed symbols*) versus a binary mixture of Arg and Lys (non-reinforced, *open symbols*). Sessions 1–13 not shown. For details see Fig. 6, inserts and text. *Symbols:* ◆ FO ◈ FB to *Tubifex* food extracts; ◇ FO ◈ during control; ● FO ▲ FB to positive reinforced stimuli; ○ FO △ FB to negative not reinforced stimuli

the learning and memory of these more natural stimuli are different from that observed for non-familiar odours described above.

Training to a single amino acid (Gln 10^{-5} *M*) resulted in positive responses after only ten sessions (Fig. 6A). During the next ten training sessions positive responses increased slightly to 70% for FO an FB, and with further training stabilized at 80%. After training, threshold was determined to be 10^{-8} *M* (Fig. 6B). After training, the animals also responded to other amino acids (Ala, Arg, Gly, Lys) at 10^{-5} *M* (Fig. 6C) and at 10^{-7} *M* (Fig. 6D). After presentations of *Tubifex* (Fig. 6F) a decrease of positive responses to glutamine (Fig. 6G) was observed. Four retraining sessions were necessary to elicit the positive responses observed during the initial training.

Training to amino acids and various mixtures of amino acids revealed a much greater variability than during training with synthetic stimuli. This variability probably depended on the animals' prelaboratory experience. Goldfish (ten groups of two animals) had difficulties in discriminating a mixture of four amino acids against one amino acid (Fig. 7B). Nearly 60 training sessions were

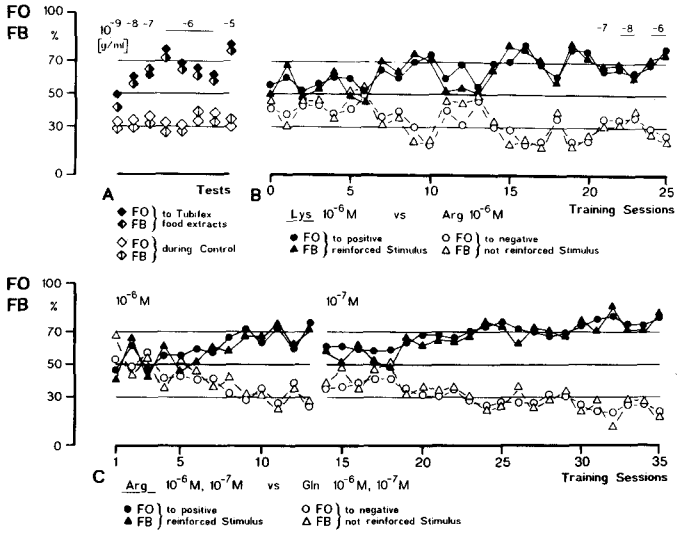

Fig. 8A–C. Discrimination training to two single amino acids: **A** reactions to various concentrations of *Tubifex* extracts applied before training (ten groups of two goldfish); **B** subsequent training to Lys versus Arg; **C** training to Arg versus Gln (training 1–14: 10^{-6} *M*; session 15–35: 10^{-7} *M*; 14 groups of two goldfish). For details see Figs. 6 and 7, inserts and text. *Symbols:* ● FO ▲ FB to positive reinforced stimulus; ○ FO △ FB to negative not reinforced stimulus

necessary before the animals showed stable positive responses at a low (60%) level.

In contrast, Fig. 7C represents 12 groups of 2 animals which spontaneously showed a significant response to extremely low concentrations of *Tubifex* 10^{-10} g · ml^{-1}, and a preference for even lower concentrations of *Tubifex* was noticeable but not significant. These animals (Fig. 7D) rapidly learned to discriminate different binary mixtures of amino acids (Arg + Lys versus Gly + Gln; each amino acid at 10^{-6} *M*) and reacted positively as early as the conclusion of the eighth discrimination training session. Threshold tests after the 12th training session even elicited positive responses using concentrations of 10^{-10}–10^{-12} *M*. In another series (Fig. 7E), reinforced (Gln + Gly) and non-reinforced (Arg + Lys) stimuli were exchanged. Here, positive responses to the training stimuli were established after only 13 days of discrimination training.

During discrimination training to single amino acids (Lys versus Arg, and Arg versus Gln) the animals' behaviour again reached positive levels after comparatively few training sessions (Fig. 8). Positive responses were elicited with amino acids at 10^{-6} *M* (Fig. 8B, C) after seventh training session. After decreasing the concentration to 10^{-7} *M* (Fig. 7C) only few reinforcements were necessary until the fish reached the 70% level again.

In general, amino acids were more easily learned and discriminated than non-familiar odours (Table 1). Thresholds in anosmic fish, however, were similar at 10^{-5} *M*. In some animal groups olfactory thresholds for amino acids were several log units lower than for non-familiar odours.

In a final series of experiments, ten groups of two animals were trained to discriminate amino acids applied

Table 1. Training and differentiation training using non-familiar odours and amino acids

Stimulus	Training	Discrimination Training	Concentration
Non-familiar odours			
Amylacetate	30–40 days		$10^{-5}M$
α-Ionon	30–40 days		$10^{-5}M$
β-Phenylethanol	30–40 days		$10^{-5}M$
α-Ionon vs β-Phenylethanol		70–80 days	$10^{-5}M$
β-Phenylethanol vs Amylacetate		70–80 days	$10^{-5}M$
Amino acids			
Gln	20 days		$10^{-5}M$
4 Amino acids vs Gly		50 days	$10^{-6}M$
Lys-Arg vs Gly-Gln		8 days	$10^{-6}M$
Gly-Gln vs Lys-Arg		10 days	$10^{-6}M$
Lys vs Arg		13 days	$10^{-6}M$
Arg vs Gln		7 days	$10^{-6}M$
Gln vs Arg		10 days	$10^{-6}M$

Training stimuli during discrimination training underlined. The number of days is the amount of training sessions necessary for 10 significant (Wilcoxonrank-test) subsequent positive responses

in high (10^{-4} *M*) concentrations (Fig. 9). It was expected that the spontaneously preferred amino acids would be easily learned and that the values might be as positively constant as during training to acetic acid (see Discussion). However, even after 22 training sessions (Fig. 9A) to glutamine (10^{-4} *M*; reinforced) versus arginine (10^{-4} *M*; non-reinforced) only a slight (20%), but not significant, preference for the reinforced stimulus was observed. FO and FB fluctuated at the 50% level (Fig. 8A). After the 22nd training session, stimulus concentrations were decreased (10^{-6} *M*) (Fig. 9B), and after only two reinforcements the same goldfish responded positively to the training stimulus (FO and FB stabilized at a 70% level).

This result suggests that learning occurred unconsciously during the previous taste discrimination training. Further decreases of glutamine concentration to 10^{-7}, 10^{-8} and 10^{-9} *M* after 37 sessions resulted in positive responses (Fig. 9C). The behavioural olfactory threshold was determined to be approximately 10^{-10} *M*. Increasing the concentration above the taste threshold to 10^{-4} *M* Gln now elicited positive responses. After bilateral tractotomy goldfish were no longer able to discriminate 10^{-7} *M* Gln from 10^{-7} *M* Arg (Fig. 9D), whereas responses to 10^{-4} *M* Gln and Arg, respectively, were comparable to preoperative responses.

Qualitative discrimination ability was tested 3 months after successful discrimination training. The behavioural data clearly demonstrated that long-term memory and threshold values were comparable with the results ob-

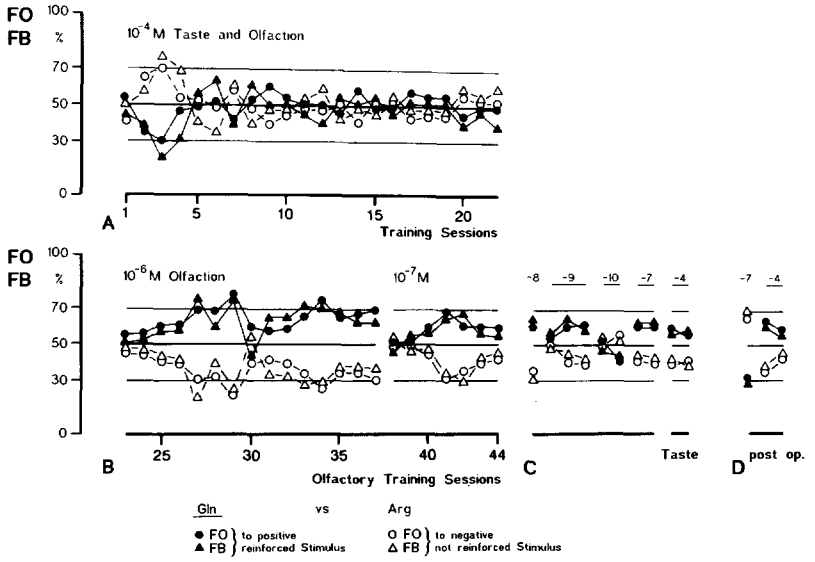

Fig. 9A–D. Discrimination of amino acids above and below olfactory concentrations (ten groups of two goldfish): **A** during 22 training sessions only slight increase (≈20%) in the reactions to the reinforced stimulus Gln (10^{-4} *M*) versus the non-reinforced stimulus Arg (10^{-4} *M*) was recorded; however, no significant preference for the training stimulus was observed in intact goldfish; **B** stimulus concentration decreased below taste threshold; positive discriminative responses after only two reinforcements; **C** threshold determinations; the animals responded positive when stimuli were applied at 10^{-4} *M*; **D** after bilateral tractotomy no response occurred to olfactory concentration (10^{-7} *M*), but significant positively values were elicited to stimuli above taste (10^{-4} *M*) threshold. For details see Figs. 6 and 7, inserts, and text. *Symbols:* ● FO ▲ FB to positive reinforced stimulus; ○ FO △ FB to negative not reinforced stimulus

tained during final discrimination training. From this, it is evident that after training memory for amino acids lasts at least three times longer than that for non-familiar stimuli.

Discussion

Responses of goldfish to chemosensory stimuli were tested using the same appetitive training and testing procedure, thus eliminating most of the stress factors associated with aversive training procedures, such as electric shock or frequent handling of the animals (Krinner 1935; Hafen 1935; Glaser 1966). Such traumatic procedures probably play some part in the unwanted suppression of behavioural responses either during the application of low stimulus concentrations or in the time after the surgical exclusion of olfaction during olfactory regeneration experiments. Furthermore, in previous reports different procedures used for taste and olfactory analyses in the same species preclude any common ground for data comparison (e.g. Herter 1953; Hara 1982). Experiments in which behavioural responses can be elicited from both external chemical senses, as seen in the regeneration and plasticity investigations in the olfactory system (Zippel et al. 1981, 1988; Hudson et al. 1990), require that the postoperative responses be tabulated on the basis of reproducible pretraining and training data as well as on threshold investigations.

Using goldfish has particular advantages for investigations of spontaneous pretraining behaviour, behavioural thresholds and for postoperative regeneration studies. Goldfish also become completely tame, lessening their susceptibility to trauma resulting from external effects which might otherwise alter behaviour. In both the classical and contemporary literature dealing with training experiments in fish, reports on spontaneous or pretraining behaviour is limited. However, failure to test pretraining behaviour carries the risk of ignoring an important source of variation. Pretraining preference or avoidance of a particular stimulus may facilitate or inhibit subsequent training.

Low stimulus concentrations used in olfactory training and discrimination training elicited no noticeable changes in spontaneous behaviour. However, the training data demonstrated that these spontaneously neutral stimuli, especially amino acids, could be discriminated when the biological significance became apparent by food reward. In some experiments, combinations of four or five amino acids elicited positive responses in low olfactory concentrations when presented to groups which had spontaneously reacted positively to low concentrations of *Tubifex* extracts, i.e. the biological importance of amino acids had been experienced in the natural environment.

"Gustatory" thresholds for all stimuli used for olfactory training were similar and were estimated at the 10^{-5} *M* level from studies in anosmic goldfish. Olfactory thresholds for the non-familiar odours were roughly 2 log units below those determined for anosmic goldfish, while olfactory thresholds for amino acids were 3–4 log units (in some cases even more) below thresholds determined after olfactory exclusion. Surgical removal of the taste system in goldfish is difficult and the destruction of the vagal lobes is also likely to result in general behavioural changes. Similar data obtained during training (see below) and spontaneous behaviour to "taste" stimuli in intact fish and after bulbectomy make a significant contribution of olfactory components on the discriminative ability very unlikely.

An early working hypothesis was that in goldfish gustatory discrimination training would be much easier than olfactory discrimination training. After having used amino acids, this hypothesis is no longer valid. Data presented for olfactory training using these more natural stimuli indicated that amino acids were not only learned more rapidly, but were also discriminated better than non-familar odours. It is highly probable that amino acids are biologically relevant for detection of food sources. The greater variabiliy in the training reactions

to natural stimuli, probably depending on different previous experiences, was described in the results. The funnel-oriented behaviour patterns exhibited during discrimination training in taste and olfaction were qualitatively and chronologically comparable. However, differences existed in their quantitative proportions as well as in the latency of their appearance.

Olfactory training to a single non-familiar stimulus was extremely difficult and required 40–45 rewards to reach a significant criterion level; for discrimination training 70–100 sessions were necessary. In both cases, recall lasted only 4 weeks. After this time, the stimuli learned over a long training period were rapidly forgotten.

The only major exceptions to this were in discrimination training to acetic acid and to other sour stimuli (Bieck and Zippel 1971; Zippel and Bieck 1971; Schoon and Zippel 1976, 1978; Zippel et al. 1978, 1981): A level of 80–100% correct responses is attained in five to ten sessions. Training to sodium chloride was also comparatively rapid, but lacked the enormous stability regularly recorded for sour stimuli. Training to the strongly avoided stimulus quinine was much more difficult and, as with NaCl, elicited less stable behavioural profiles. Discrimination training to sweet stimuli was only successful when the more strongly preferred monosaccharide (glucose) is reinforced (H.P. Zippel and H. Schoon, unpublished data). The obvious dominance of sour taste stimuli in appetitive learning through food rewards was further supported by the enormous 10-month memory capacity once a stable long-term memory had been established.

The effects of amino acids in olfaction and taste have been investigated in a number of fishes using different methods. As expected, physiological (Hara 1977; Caprio 1978, 1982; Kiyohara et al. 1981; Hara 1982; Caprio and Byrd 1984; Ohno et al. 1984; Kanwal et al. 1987; Marui et al. 1987; Johnsen et al. 1988; Caprio et al. 1989; Kohbara et al. 1990; Kumazawa et al. 1990; Sveinsson and Hara 1990a, b; Teeter et al. 1990; Wegert and Caprio 1991) and biochemical (Hara 1977; Novoselov et al. 1980; Brown and Hara 1981; Cagan 1981, 1986; Fesenko et al. 1983; Rhein and Cagan 1983; Saglio and Fauconneau 1985; Brand et al. 1987; Preston 1987; Bruch and Ruli 1988; Novoselov et al. 1988; Bryant et al. 1989; Kalinoski et al. 1989) studies demonstrate different sensitivities for various amino acids in different species. Also, the spontaneous behavioural response of different species to different amino acids varies (Carr et al. 1977; Goh and Tamura 1980; Pearson et al. 1980; Fuke et al. 1981; Cagan and Holland 1982; Holland and Teeter 1981; Mearns 1985; Ellingsen and Døving 1986; Johnsen and Adams 1986; Olsen et al. 1986; Rehnberg and Schreck 1986; Mearns et al. 1987; Zippel et al. 1988; Jones 1989). In the present investigations, the spontaneous response to low concentrations of non-familiar stimuli and single amino acids was indifferent. Both olfactory perception and the qualitative discriminative ability could, however, be demonstrated, especially for amino acids, during food-reward training.

The first sign of a positive response to the training stimulus is a slight shift of behavioural patterns to that stimulus. Thereafter, a stabilization of FO and an increase in the amount of FB (specifically orientated food expectation) occurred. In goldfish, sour "classical" taste stimuli were extremely effective during discrimination training. Spontaneous responses to high concentrations of other stimuli, recorded regularly during presentation, did not indicate that these stimuli were easily discriminated during taste training in anosmic fish (Zippel et al. 1981). The completely unexpected finding that intact goldfish were unable to discriminate amino acids applied at high (10^{-4} *M*) concentrations, whereas the same specimens easily learned to discriminate these stimuli after the stimulus concentrations were reduced to 10^{-6} and 10^{-7} *M*, warrants further investigations in anosmic fish. Valenticic and Caprio (1992, and personal communication) recently demonstrated that channel catfish trained to search the aquarium after the application of a particular amino acid did not show this discrimination subsequent to removal of both olfactory organs. In this case, the afferent pathway of this learned specific behaviour was olfaction and not by the highly sensitive and intact taste system. After bilateral tractotomy goldfish were not able to discriminate 10^{-7} *M* amino acids; however, behavioural discrimination by taste to 10^{-4} *M* amino acids was comparable to preoperative levels. The experimental differences observed between goldfish and catfish may be the result of species differences. Furthermore, anosmic goldfish also respond to non-familiar chemicals (Zippel et al. 1988) and *Tubifex* food stimuli when applied at concentrations above taste threshold.

The apparent behavioural discrepancy in chemosensory behaviour of catfish and goldfish may also result from slightly different experimental paradigms. Goldfish were "trained" to associate the funnels with food reward during the pretraining period. The increased search activity, FO and FB recorded during the habituation period was elicited by visual signals, i.e. the inserting of the funnels into the respective aquarium. Investigation of the pretraining spontaneous responses to chemical stimuli were only begun when FO and FB reached a constant and stabile level. During subsequent discrimination training, FO and FB values, therefore, were similar for the pretraining responses without stimulus and during the training sessions.

Prior to testing, goldfish frequently preferred the funnel through which they had been rewarded during the previous day. During food reward, the stimulus was continuously presented from the tip of the funnel until the animals had completed feeding on *Tubifex* worms. Successful discriminative behaviour of the various chemical stimuli used was attained when in at least ten successive sessions significant (<0.01, Wilcoxon-rank-test) positive responses were recorded. Whereas in the goldfish experiments the fish learned to respond with increased search activity by insertion of the funnels, catfish were trained to the conditioned stimulus with increased search activity by stimulus application.

Finally, spontaneously "ineffective" odour stimuli, like low concentrations of amino acids, could be discriminated during food-reward training, an observation

which indicates that goldfish perceived these stimuli. The biological importance of various olfactory stimuli can only be understood by examining the time period required for learning and the duration of the long-term memory. The ability to detect odour stimuli could probably be investigated more accurately by physiological or by biochemical methods, but the qualitative discriminatory ability and the biological importance of different olfactory stimuli could only be demonstrated in behavioural discrimination training.

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