

Alarm Reaction in the Crucian Carp is Mediated by the Medial Bundle of the Medial Olfactory Tract

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Abstract

Experiments were performed to determine which bundles of the olfactory tracts were essential for mediating alarm reaction in crucian carp (*Carassius carassius* L.). The fish were maintained in physiological saline after surgery to preserve the remaining tracts and postoperative inspections revealed the functionality of the intact tracts. Operations on the tracts were performed symmetrically on both sides. Sham-operated and non-operated fish showed the typical alarm behaviour of fast swimming to the bottom, dashing movements and aggregation when exposed to skin extract which contain alarm substance. Fish with only the medial bundle of the medial olfactory tract intact also displayed the alarm behaviour upon exposure; however, these fish did not react to the amino acid, L-alanine with either feeding response or alarm reaction. Crucian carp which had the medial bundle of the medial olfactory tract cut, leaving both the lateral bundle of the medial olfactory tract and the lateral olfactory tract intact, did not display any alarm reaction to skin extract; however, these fish reacted to exposure to L-alanine with feeding behaviour. There were statistically significant differences between the behaviour scores for the fish subject to different treatments. The present study demonstrates that the medial bundle of the medial olfactory tract appears to be both necessary and sufficient for mediation of the alarm reaction. The results also show that the sensory neurons which respond to alarm substance terminate and make synaptic connections with the secondary neurons that make up the medial bundle of the medial olfactory tract; thereby demonstrating the specificity of the spatial aspect of olfactory processing. The results are discussed with respect to the spatial aspect of organization within the olfactory system, the pattern of generalization across orders of fish, and the functional implications of the spatial arrangement of information transmission between the peripheral olfactory organ and the brain.

Introduction

von Frisch (von Frisch, 1938, 1941) observed a particular fright behaviour in minnows when schools of fishes were exposed to water from injured minnows. He showed that an alarm substance was released from the skin of injured fishes and that the released substances induced rapid swimming into hiding places at the bottom, dashing movements, aggregation in a corner of the aquarium and, after some time, slow emergence from hiding places. Alarm substance is mainly found in species of the Ostariophysi (Pfeiffer, 1963) and is partly species-specific (Schutz, 1956). Although fright reaction is also found in species of other fish orders, for example the gadids, and the behaviour is most probably related to alarm, no substances have so far been found that induce fright in codfish.

The alarm reaction is mediated by the olfactory system (von Frisch, 1938, 1941) and, in cod, catfish and carp, the olfactory bulbs are placed close to the sensory epithelium. This arrangement leaves long olfactory tracts, which facili-

tates experimental manipulation. The olfactory tract in fish is subdivided into several discrete bundles (Sheldon, 1912). We will conserve the nomenclature used by Sheldon for goldfish and cod. The functional implications of the divisions of the olfactory tract were demonstrated for the first time by electrical stimulation of separate bundles of the olfactory tract in free-swimming cod (Døving and Selset, 1980). Electrical stimulation of the medial bundle of the medial olfactory tract (mMOT) induces lowering of the respiration rate and the appearance of dark regions on the sides and white spots on the dorsal region of the fish. This represents a typical reaction pattern of a frightened cod. On the other hand, stimulation of the lateral bundle of the medial olfactory tract (lMOT) induces quivering movements, a behaviour related to spawning. Finally, stimulation of the lateral olfactory tract induces food search behaviour: either restless swimming up and down and snapping, or bottom food search behaviour consisting of swimming

backwards, head down, trailing the barbels and the pelvic fins on the bottom. Based on these observations, it is evident that each bundle of the olfactory tract appears to mediate specific behaviour reactions. This proposition is further supported by the observation that courtship behaviour in goldfish is mediated by the medial olfactory tract (MOT) (Kyle *et al.*, 1982; Stacey and Kyle, 1983) and that sperm release is induced by stimulation of the medial tract (Demski and Dulka, 1984).

It is not known which part, or parts, of the olfactory tract transmits the information giving rise to the alarm reaction. In one study on tench, it was found that eight out of 15 fish with the lateral olfactory tract intact responded to skin extract, while seven fish did not (Pfeiffer *et al.*, 1984). In the same study, none of the 14 fishes with the medial bundle of the medial olfactory tract intact responded to the skin extract. In the present study, we made discrete transections of bundles of the olfactory tract in crucian carp. Special care was taken to maintain and control the function of the remaining olfactory tract fibres. We fulfilled these aims by preventing the brain of the fish from being exposed to fresh water, by keeping the fish in physiological saline and by inspecting the functionality of the olfactory tracts at the end of the experiments. In view of the findings in cod, we considered it probable that the medial bundle of the medial olfactory tract (mMOT) transmits the alarm reaction. We chose the crucian carp, because we are investigating the chemical nature of the alarm substance in this species and its possible involvement in the growth in body height observed in fish that are exposed to skin extracts (Stabell and Lwin, 1997).

Material and methods

Crucian carp (*Carassius carassius* L.) were caught in a small lake without predators just outside Oslo city border. They were transported to the aquarium facilities at the Department of Biology. The 14 crucian carp used for these experiments weighed between 15 and 22 g. The fish were anaesthetized with benzocaine (45 mg/l), placed in a stand with running water through the mouth and over the gills, and operated upon under a stereomicroscope. The skin just above the olfactory tracts was cut open and a portion of the dorsal cranium removed. The mesenchymal tissue in the brain case was aspirated and the meninges around the olfactory tracts were removed by using fine forceps. The olfactory tracts were clearly visible as three distinct bundles running from the olfactory bulb to the brain (Figure 1). The bundles were gently separated, taking care not to disrupt the blood vessels. When cutting a particular portion or portions of the olfactory tracts, care was taken to remove several millimetres of the fibre bundle to prevent regeneration. We visually inspected the fibre bundles to control possible regeneration. We cut the bundles of the olfactory tracts symmetrically on both sides as follows.

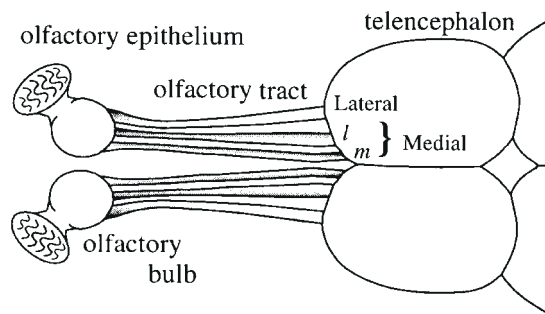


Figure 1 Schematic outline of the forebrain and the olfactory system in crucian carp. In carp the olfactory nerve leading from the sensory epithelium to the olfactory bulb is short and long olfactory tracts are present. The division of the olfactory tract into three discrete bundles is visible with the naked eye and further subdivision is seen with the aid of a dissecting microscope. Note that the medial olfactory tract is divided into a lateral (l) and a medial (m) part.

- Three fish were sham operated, i.e. the brain case was opened and the meninges were removed to see the olfactory tracts, and three fish were anesthetized only. These six fish together served as controls.
- In four fish, the mMOT was sectioned, and both the IMOT and LOT were left intact.
- In four fish, both the lateral bundle of the medial olfactory tract (IMOT) and the lateral olfactory tract (LOT) were sectioned, and the medial bundle of the medial olfactory tract (mMOT) was left intact.

After having cut the particular bundles, the cranial cavity was filled with a 2% solution of Agar-agar made up in physiological saline (g/l) NaCl (8.53), KCl (0.22), MgSO₄·7H₂O (0.25), CaCl₂·2H₂O (0.19).

The fish were transferred to physiological saline in 25 l aquaria (48 × 21 × 25; W, H, D) at 18°C. Fish that had been subjected to a similar type of treatment were grouped and placed in one out of three aquaria. Fish were fed commercial food twice a week. Oxygen was supplied by airstones, and only evaporated water was replaced during the experiments.

The behavioural experiments were performed 2 weeks after the surgical procedures. During the intervening time, the fish with the medial part of the medial olfactory tract intact responded poorly to food. When given food that floated on the water surface, the food flakes were still found present after a period of 2 h. This reluctant feeding behaviour during recovery was suspected also to influence the alarm behaviour, which follows the introduction of skin extract to the aquaria. In view of these considerations, we found it advisable to wait with exposure of all three groups of fish, until the fish with the medial part of the medial olfactory tract intact exhibited normal feeding behaviour to visible food at the surface.

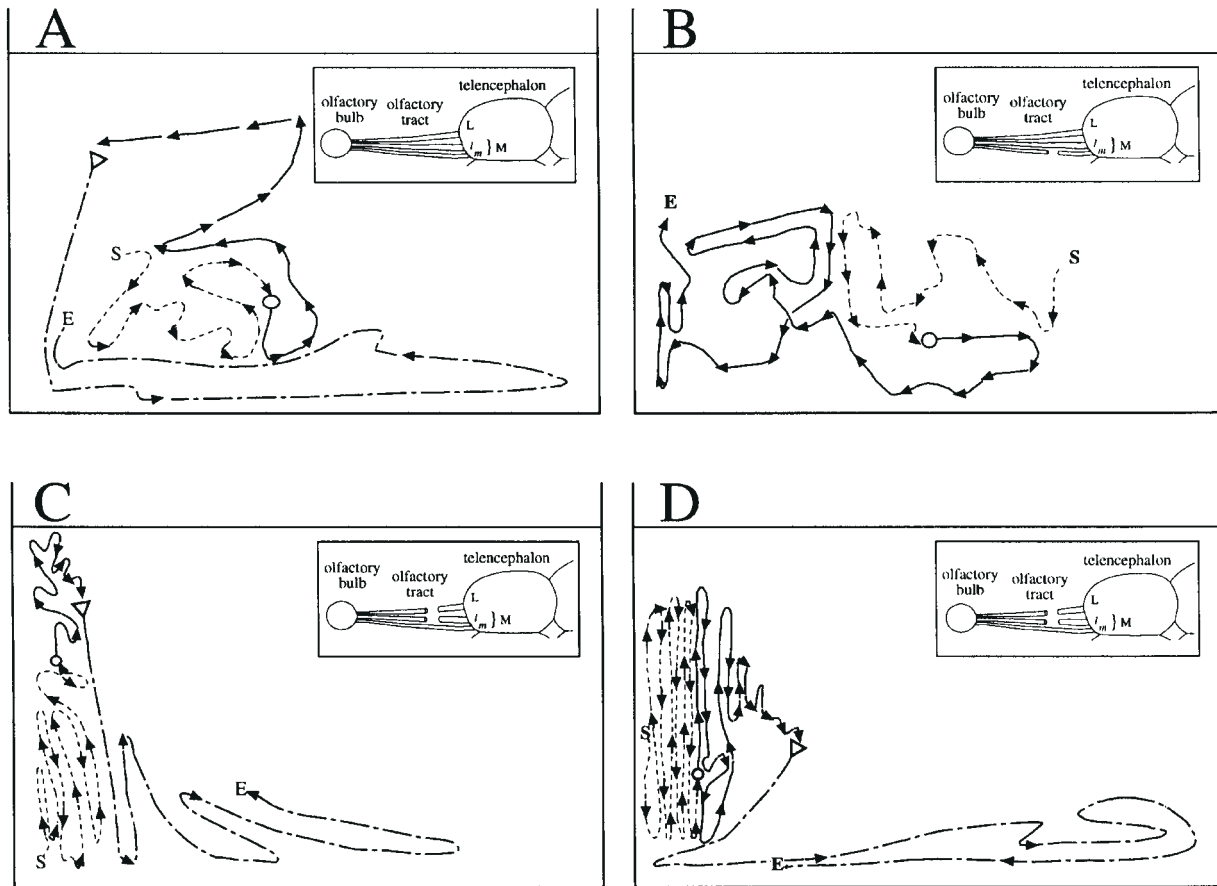


Figure 2 Movements of four crucian carp within the aquaria (width 48 cm) during the observation period. Paths are drawn from tracings of videotaped data. Insets are schematic representations of the right-hand side of the brain showing the divisions of the olfactory tract (M, medial olfactory tract; L, lateral olfactory tract; *m* medial bundle; *l*, lateral bundle). Each inset describes the various transections of olfactory bundles. The starting point of the observation period is represented by S and the intervals between each bold arrow along the line ($\blacktriangleright \bullet \bullet \bullet \blacktriangleright$) represent 5 s. The initial introduction of alarm substance into the tank is denoted by the open circle (O), with the path of the fish described by a solid line (—). The onset of the rapid bottom-seeking behaviour is denoted by the open triangle (\blacktriangleright), with the movements described by a short and long dashed line (---). The end of the observation period is denoted by E. **(A)** Sham operated with no olfactory tracts transected. The onset of the alarm reaction can be seen to occur in the upper left-hand portion of the panel, with a rapid translocation to the bottom of the tank. **(B)** Movement of crucian carp with the medial bundle of the medial olfactory tract transected and the fish exposed to alarm substance. The absence of the bottom-seeking behaviour that is associated with alarm substance exposure can be seen as the random appearing movements are maintained. **(C, D)** Response to alarm substance with the lateral olfactory tract and the lateral bundle of the medial olfactory tract transected. With only the medial bundle of the medial olfactory tract intact, the bottom-seeking behaviour as a consequence of alarm substance exposure can be seen in both fish.

Preparation of skin extract

The fish were killed by decapitation and skin was taken from their sides. Total weight was ~2 g. The skin samples were placed in 100 ml distilled water and homogenized in a blender. The homogenate was filtered through glass wool. From this extract we made a 1:10 dilution, which was used in the experiments.

Observation procedure

Monitoring of the fish during the experiments was by video camera, placed alternately in front of each of the three test aquaria. A screen prevented the fish from observing the experimenter. The stimuli introduced to the fish were either physiological saline (control), L-alanine (10^{-3} M) or skin

extract. The stimulus solution was added to the aquaria by the use of a peristaltic pump, at a flow of 5 ml/min for a duration of 1 min. When homogeneously distributed, the concentration of stimuli in the aquarium would represent a 5000 \times dilution of the introduced samples. The experiments were carried out either at *ca.* 06:00 or after 20:30, to prevent unforeseen disturbances of the experiments by daytime activity in the building.

Behaviour analysis

The video recordings were replayed and the movements of the fish were traced manually onto a transparent sheet mounted on the video screen (Figure 2). In addressing the methods for scoring we found it useful to divide the dif-

Table 1 The behaviour scores of the three groups of crucian carp observed when exposed to a control solution (physiological saline), to skin extract and to L-alanine

Behaviour type	Control fishes (6)			mMOT cut (4)			mMOT intact (4)		
	Control	Alanine	Skin extract	Control	Alanine	Skin extract	Control	Alanine	Skin extract
Feeding behaviour									
Bottom food search	19	11	7	9	5	7	10	9	7
Snapping	12	23	8	11	14	7	6	7	6
Swimming to the surface	4	20	0	1	1	1	5	0	2
Mouth opening	11	19	4	4	8	2	9	5	6
Exploring	9	19	6	6	15	4	8	3	5
Sum	55	92	25	31	43	21	38	24	26
Alarm behaviour									
Rapid swimming to the bottom	0	0	16	0	0	0	0	0	15
Dashing	2	1	24	0	0	0	0	0	16
Aggregation	9	5	25	5	0	3	0	5	13
Swimming against the wall	4	5	14	10	7	7	7	8	11
Sum	15	11	79	15	7	10	7	13	55

The observation time was 1 min during injection of a test solution into the aquarium.

ferent behaviours performed by the fish as belonging to either feeding behaviour or alarm reaction. The following feeding behaviours were scored: bottom food search, where the fish with an angle to the bottom head down were grasping objects on the bottom with repetitive mouth openings; snapping with the jaws; swimming to the surface; mouth opening in the midst of the water; and exploring, i.e. a restless search swimming in the water. The following behaviours were considered to be a part of the alarm reaction: rapid swimming to the bottom; dashing, vigorous staccato tail wiggling and swimming against the bottom, head down with short pauses; aggregation, where the fish gather close together, usually in a corner or towards a wall of the aquarium; and swimming against the walls, usually close to the bottom. The video recordings were run repeatedly for each fish to include all relevant classes of behavioural performance. The tapes were then replayed double blind for neutral observers (i.e. persons not otherwise participating in the experiments) to enable them to score the behaviour. There were only minor differences in the scores between the two groups of observers. For quantification, each type of behaviour was given a score of one every time it was performed by a fish. The behaviours were scored for a period of 1 min from the injection of stimulus into the aquarium.

The sums of the feeding behaviours and the alarm reactions for the different groups of fish were compared using the χ -square method with two degrees of freedom. The χ -square method is independent of the number of objects and observations within each aquarium.

Results

Control fish

The crucian carp that had the olfactory tracts intact (i.e. sham operated) reacted in a manner similar to non-operated fish (i.e. anesthetized only) to the presence of skin extract, displaying distinct behaviour patterns as described by von Frisch (von Frisch, 1941) for minnows. The crucian carp swam rapidly to the bottom and performed dashing movements with their noses down (Figure 2A). The dashing movements ceased after ~15 s, but the fish kept to the bottom and aggregated in a corner. These dashing and staccato movements with vigorous wiggling of the tail seem inappropriate when viewing the fish in an aquarium with a glass bottom; however, in an aquarium with a natural soft bottom this behaviour becomes conspicuously relevant and suitable. We set up one aquarium with such natural bottom to observe the behaviour of the crucian carp and found that the dashing behaviour led to agitation of debris on the bottom, followed by a swimming of the fish into the substrate to hide. This behaviour may confuse a potential predator and make the prey difficult to find. The fish exposed to skin extract did not leave their shelter to perform the usual swimming in the upper water column until at least 30 min after the stimulation.

The scores of different behaviours as a result of exposing the fish to a control solution, L-alanine or to skin extract, are summarized in Table 1. As seen in Table 1, the control fish reacted to L-alanine exposure with an increased level of feeding behaviours compared to those exposed to physio-

logical saline. The behaviours observed with L-alanine were snapping, swimming to the surface, mouth opening and exploring. However, L-alanine did not induce any increase in bottom food search behaviour compared to the control solution. The control fish reacted to the alarm substance with: rapid swimming to the bottom; dashing; aggregation; and swimming against the wall. These types of behaviours were seldom seen when exposing the fish to L-alanine or physiological saline. The dichotomy of the behaviour patterns elicited is striking.

mMOT cut

The carp with the medial bundle of the medial olfactory tract cut, and with both the lateral bundle of the medial olfactory tract and the lateral olfactory tract intact, did not display any reaction to skin extract (Figure 2B; Table 1). These fish continued to swim around in the upper part of the water column and displayed no apparent detection of the alarm substance.

The injection of L-alanine into the aquarium with the fish with mMOT cut induced feeding behaviour when compared to the control solution. The scores, however, were lower than found with the control fish (Table 1).

mMOT intact

The fish with the medial bundle of the medial olfactory tract intact reacted to skin extract in the same way as the sham-operated fish. In response to the skin extract, the fish displayed vigorous and fast swimming to the bottom, with all the fish reacting in a similar manner (Figure 2C,D). However, both the fish with the mMOT intact and the fish with IMOT and LOT sectioned reacted to L-alanine exposure with a lower level of feeding behaviour than the control fish (Table 1).

Spontaneous behaviour following disturbances

The fish with the mMOT intact, as well as the control fish, reacted to sudden illumination of the aquarium with fast swimming to the bottom and dashing movements. Approaching the aquarium and tapping the walls induced a similar alarm reaction, in both the control fish and the fish with the medial bundle of the medial olfactory tract intact.

The fish with the mMOT transected, however, did not react to sudden illumination of the aquarium room, but continued to swim slowly around. Neither did tapping of the aquarium wall induce any alarm reactions in these fish. These observations suggest that, in addition to conveying chemical alarm signals, an intact mMOT influences alertness in a general manner.

Comparing the different treatments

Comparisons between the groups of fish that had undergone different treatments were made by χ -square analysis and the results are given in Table 2. The scores for feeding behaviour were significantly different (lower) for the fish

Table 2 Comparisons between the summated behaviour scores for the three different groups of crucian carp

Groups compared	χ^2	Significance level
Feeding behaviour		
Control versus mMOT intact	17.70	$P < 0.001$
Control versus mMOT cut	2.86	not significant
mMOT intact versus mMOT cut	6.37	$P < 0.05$
Alarm behaviour		
Control versus mMOT intact	2.44	not significant
Control versus mMOT cut	21.63	$P < 0.001$
mMOT intact versus mMOT cut	22.16	$P < 0.001$

The numbers compared are the summated scores for feeding behaviour and alarm reaction. The entries are χ -square values, two degrees of freedom.

with the mMOT intact compared to controls, whereas no significant difference in scores between control fish and fish with the mMOT cut was found. These results imply that feeding behaviours persist after section of the mMOT.

Comparisons of the alarm reaction behaviour scores revealed that there was no significant difference between the control fish and the fish with the mMOT intact. However, there was a highly significant difference between the control fish and the fish with the mMOT cut (Table 2). These results strongly support the contention that the medial part of the medial olfactory tract is transmitting the alarm reaction.

Discussion

The results of the present study demonstrate for the first time that the medial bundle of the medial olfactory tract appears to be both necessary and sufficient for mediation of the alarm reaction. The data obtained are discussed with respect to: the spatial aspect of organization within the olfactory system; the functional implications of the spatial arrangement of information transmission between the peripheral olfactory organ and the brain; the participation of the olfactory system in modulation of alert behaviour; and the pattern of generalization across orders of fish.

The results of the present study imply that the sensory neurons expressing the receptors for the alarm substance terminate on the secondary neurons that send their axons in the medial bundle of the medial olfactory tract. It should be noted that the fish with only the medial bundle of the medial olfactory tract intact did not react to L-alanine with any alarm behaviour. Thus, the sensory neurons that react to L-alanine do not activate the neurons forming the medial bundle of the medial olfactory tract, neither directly nor indirectly. At present, it is a question of which type of sensory neuron (Hansen and Zeiske, 1998) and which type of receptor protein detect the alarm substance (Cao *et al.*, 1998). The information gained from the present experiments may prove useful in answering these questions.

In a study conducted on the tench in attempts to

Table 3 Summary of the behaviour reactions mediated by the different tract bundles in different species

Olfactory tract bundle	Behaviour	Species (source)
Medial bundle of the medial olfactory tract (mMOT)	alarm reaction	<i>C. carassius</i> (present paper)
Lateral bundle of the medial olfactory tract (lMOT)	alarm reaction	<i>G. morhua</i> (Døving and Selset, 1980)
	courtship	<i>C. auratus</i> (Stacey and Kyle, 1983)*
Medial bundle of the lateral olfactory tract (mLOT)	sperm release	<i>C. auratus</i> (Demski and Dulka, 1983)*
	quivering	<i>G. morhua</i> (Døving and Selset, 1980)
	feeding	<i>C. auratus</i> (Stacey and Kyle, 1983)*
	response to amino acids	<i>C. auratus</i> (von Rekowski and Zippel, 1993; Zippel <i>et al.</i> , 1993)*
Lateral bundle of the lateral olfactory tract (lLOT)	search/snapping	<i>G. morhua</i> (Døving and Selset, 1980)
	bottom food search	<i>G. morhua</i> (Døving and Selset, 1980)

In some cases the distinction between the different bundles within the medial or the lateral olfactory tracts has not yet been made, these are marked with an asterisk.

investigate which bundle of the olfactory tract transmit the alarm reaction, the results were not coherent (Pfeiffer *et al.*, 1984). Fish that had been treated in similar ways behaved differently. A possible explanation could be that the olfactory tracts and brain were exposed to fresh water after operation. It should be noted that this objection could also be relevant for other similar experiments made on the fish olfactory system. In preliminary studies we observed that in operated fish the olfactory tracts were destroyed and the blood flow to the olfactory bulb interrupted when the fish were transported to fresh water after the operation. Thus, experiments had to be repeated with more appropriate techniques, such as those we employed in the present study.

The organization of the sensory pathways from the sensory epithelium onto the olfactory bulb has been studied by application of various different methods. The spatial arrangement is evident in the electrical responses to stimulation of the olfactory organ with amino acids and bile salts, recorded on the olfactory bulb surface (Døving *et al.*, 1980). It has been shown that stimulation with amino acids induces complex combinatorial patterns of active glomerular modules that are unique for different stimuli and concentrations (Friedrich and Korsching, 1997). In the present paper we show that a particular behaviour pattern is released via information mediated through a single olfactory tract. There is a topographical projection from the olfactory bulb to the olfactory tract in both carp (Satou *et al.*, 1979) and tench (Dubois-Dauphin *et al.*, 1980). These studies demonstrate that the representation of odour quality is maintained in a specific spatial pattern that is further reflected in the transmission of information from the olfactory bulb to the brain.

It must be stressed that the olfactory tract bundles are subdivided into several fascicles. The medial bundle of the medial olfactory tract is distinct and appears uniform when observed with a stereomicroscope, but both the lateral bundle of the medial olfactory tract and lateral bundle of

the olfactory tract are divided into several smaller bundlets in goldfish (Sheldon, 1912), crucian carp, cod and burbot (Døving and Gemne, 1965). Recordings of the compound action potential and inspection of diameters using electron microscopy in burbot (Døving and Gemne, 1965) and other species (Døving, 1967) have shown that the bundles of the olfactory tract are composed of a wide variety of myelinated and unmyelinated fibres of different diameters. These variations in fibre diameter most probably reflect differences in function.

Our data also suggest that the neurons in the mMOT are important as general modulators of alert behaviour, because fish with the mMOT removed lose their alertness to non-chemical stimuli, while fish with the lMOT and the LOT removed do not. This may suggest that a background neural input via the mMOT is important for upholding normal responses to other sensory inputs that may represent danger. This proposal is supported by the fact that olfactory-intact crucian carp seem to increase their levels of alertness to non-chemical stimuli when continuously exposed to chemical alarm signals (O.B. Stabell, personal observation). The physiological and behavioural mechanisms underlying a tentative regulatory function of this kind seem intriguing.

Studies on goldfish have shown that the medial olfactory tract is essential for mediation of behaviour reactions that are related to courtship (Stacey and Kyle, 1983) and sperm release (Demski and Dulka, 1984). These authors, however, did not determine if it was the medial or the lateral bundle of the medial olfactory tract that is involved in that function. Feeding behaviour was seen when the lateral olfactory tract was left intact (Stacey and Kyle, 1983; von Rekowski and Zippel, 1993). In view of the results of the present study in crucian carp, and the induction of the quivering movements in cod when the lateral bundle of the medial olfactory tract is stimulated, it seems probable that both sperm release and courtship behaviour are mediated by the lateral bundle of the medial olfactory tract. As this bundle of the olfactory

tract is divided into different fascicles, it is further probable that sub-divisions of the olfactory tract bundles could mediate particular functions related to reproduction.

There is evidence that axons of the neurons forming the olfactory tracts could regenerate and restore the original functionality (von Rekowski and Zippel, 1993; Zippel, 1993; Zippel *et al.*, 1993). However, in the present study a piece of the olfactory tract was removed specifically to prevent regeneration during the experimental period. Visual inspection at the end of the experimental period confirmed that regeneration crossing the gap created by removing part of the olfactory tracts had not occurred.

A summary of the different behaviour patterns elicited through activity in particular bundles of the olfactory tract is given in Table 3. It should be noted that Stacey and Kyle (Stacey and Kyle, 1983) found that in goldfish with the lateral olfactory tract cut, there was only a marginal reduction of feeding response following exposure to a complex food odour. This finding is in contrast with the results of the present study, where L-alanine gave rise to feeding behaviour in fish with the lateral olfactory tract intact. Obviously, L-alanine alone does not represent any complex food odour and, accordingly, the question of whether the lateral olfactory tract alone mediates feeding behaviour seems to be a subject for further studies. It seems evident from Table 3 that corresponding bundles of the olfactory tract mediate similar behavioural reactions in different species of both cod and carp, indicating that there is congruence across the orders of fishes. In other words; the manner by which olfactory information is sorted and presented to the brain via the olfactory tracts appears to be homologous within different fish orders. Whether or not this is true for species of other orders (for instance catfish) has yet to be determined.

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