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Single unit responses to skin odorants from conspecifics and heterospecifics in the olfactory bulb of crucian carp *Carassius carassius*

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SUMMARY

Injured fish skin leaks alarm substances that induce the fright reaction upon olfactory detection. The skin also contains a multitude of other odorants traditionally related to other behaviors, but to what extent they are detected upon injury is unknown. We have performed single unit recordings in the olfactory bulb (OB) of crucian carp while exposing the olfactory epithelium to skin extracts from conspecifics and three other species of the carp family, common carp, tench and bream. The aims were to investigate whether neural activity may be induced by different types of skin odorants and how well the odorants from injured conspecifics are distinguished from other species. The OB of crucian carp shows a clear chemotopy as units located in different regions respond to either food-related odorants, to pheromones or to alarm odorants respectively. Units in all regions responded to skin extracts, which indicate the detection of odorants usually involved in reproduction and feeding, in addition to the alarm substances. Among OB units responding to only one of the skin extracts, most were sensitive to conspecific skin extract. Furthermore, pair-wise comparisons showed that the discrimination between conspecific skin extract and skin extract from another species was in general better than the discrimination between skin extracts from two heterospecifics. The findings suggest that identification of injured fishes may be based on different groups of odorants and that the crucian carp olfactory system discriminates well between odorants from conspecifics and those from other fish species.

Key words: complex odors, olfactory bulb, species specificity, skin extract, alarm substances, pheromones, fright reaction, olfaction, teleost.

INTRODUCTION

Natural odors are complex stimuli containing a multitude of odorants. Yet, the majority of physiological studies of the vertebrate olfactory system used single or 'pure' odorants in an attempt to understand how the neurons respond to stimuli. In contrast to this tendency, two recent studies in rodents demonstrate how glomeruli in the olfactory bulb (OB) respond to complex stimuli containing social signals and food odors, and that each of the single compounds contributed to the overall response (Lin et al., 2006; Lin et al., 2005). In fishes, alarm substances from injured skin induce fright reaction (reviewed by Chivers and Smith, 1998; von Frisch, 1938), but the skin also contains a multitude of other odorants. The present study concerns the effect of different groups of odorants in skin extracts from different fish species on single unit activity of OB neurons of crucian carp *Carassius carassius*.

Sensory neurons responsive to a particular odorant are widely dispersed within the olfactory epithelium (Ressler et al., 1994b; Weth et al., 1996), and their axons project to the OB, the first processing center of olfactory input, forming synapses with a small number of secondary neurons within glomeruli. In mammals, sensory neurons expressing a given odorant receptor terminate at one or a few glomeruli forming a chemotopic organization. Here, a spatial representation of sensory input occurs where each glomerulus responds specifically to functionally related odorants (Ressler et al., 1994a; Vassar et al., 1994). A similar organization of the OB is found in fishes (see Hamdani and Døving, 2007) in which many biologically relevant odorants are known. The chemotopy of the fish OBs has also been mapped (Friedrich and Korsching, 1997; Hamdani and Døving, 2003; Lastein et al., 2006; Nikonov and Caprio, 2001).

In many fish species, projections of secondary neurons to higher brain centers form long olfactory tracts that are separated into distinct bundles. Electrical stimulation of separate bundles in Atlantic cod *Gadus morhua* lead to distinct behaviors (Døving and Selset, 1980). These findings are congruent with studies in the crucian carp where ablation of each bundle resulted in loss of a distinct behavior (Hamdani et al., 2001; Hamdani et al., 2000; Weltzien et al., 2003). A topological relation between the OB and the olfactory tract also exists in fish (Dubois-Dauphin et al., 1980; Satou et al., 1979), i.e. each bundle, and each corresponding region of the OB, is activated by functionally distinct odorant groups that mediate distinct behaviors.

Many fish species respond to olfactory cues from injured skin of conspecifics by performing stereotypic avoidance behavior (e.g. the fright reaction) (von Frisch, 1938). Injured skin from other species may cause the same response, but is usually less effective than that from conspecifics (Mathis and Smith, 1993; Mirza and Chivers, 2001; Smith, 1982). Extracts of fish skin are commonly used to study the fright reaction, and odorants inducing this behavior (the alarm substances) are believed to be stored in specialized epidermal cells (Pfeiffer, 1963). In addition, skin extracts contain a multitude of other odorants, such as amino acids and steroids (Ali et al., 1987; Hay et al., 1976; Saglio and Fauconneau, 1985). Applied as pure compounds, these odorants were shown to be involved in feeding and sexual behavior, respectively. Usually, when exposed simultaneously with alarm substances present in the skin, food- and

sex-related stimuli do not induce their 'typical' behaviors, but to what extent they activate olfactory neurons is unknown. However, when the fright-mediating part of the olfactory tract is ablated, crucian carp perform feeding behavior (Hamdani and Døving, 2000). This indicates activation of several types of neurons upon skin extract detection.

In the present study, we stimulated the olfactory epithelium of the crucian carp with skin extracts from four species, crucian carp, common carp *Cyprinus carpio*, tench *Tinca tinca*, and bream *Abramis brama* while recording from single OB units. We took advantage of the clear OB chemotopy where regions responsive to food-related odorants, pheromones and alarm odorants, respectively, are reliably distinguishable. The aims were to examine neural activity induced by different types of skin odorants and to investigate how well injured conspecifics are distinguished from other species.

MATERIALS AND METHODS

Animals and surgical procedures

Crucian carp *Carassius carassius* (Linnaeus) (Cyprinidae, Cypriniformes) (25–45 g body mass) were caught by traps in a small lake on the outskirts of the city of Oslo, Norway, and transported to the aquaria facilities at the Department of Molecular Biosciences, University of Oslo. The fish were kept in 1000 l aquaria supplied with flowing freshwater, and maintained under a photoperiod of 12 h:12 h light:dark and a temperature of 10°C. The fish were fed three times a week with commercial pelleted feed (Modulfôr, Ewos, Norway). All experimental procedures were carried out in accordance with national legislation and institutional guidelines at the University of Oslo.

Twenty-two fish were initially anesthetized with benzocaine (45 mg l^{-1}) and subsequently given an intraperitoneal injection of Saffan (alphaxalon 0.9% and alfadolone acetate 0.3%, 24 mg kg^{-1}; Shering-Plough Animal Health, Welwyn Garden City, UK). Additional Saffan (24 mg kg^{-1}) was injected intramuscularly when the duration of the experiment exceeded 5 h. To avoid unintended movements during the experiment, fish were wrapped in a wet cloth, adjusted in a cradle, and fixed belly down by two steel rods towards the upper parts of the orbital bones. Fish were continuously irrigated through the mouth and over the gills by city spring water during the experiments. The right olfactory tract and the right OB were exposed by removing the skull roof under a stereomicroscope. The mesenchymal tissue around the olfactory tract was aspirated by gentle use of a moist sponge, and the anterior part of the brain cavity was filled with paraffin oil. The preparation allowed recording of nervous activity for at least 8 h after the surgery.

Preparation of skin extracts

Skin was removed from previously frozen crucian carp, common carp, tench, and bream. Approximately 2 g of skin was homogenized manually with 100 ml distilled water in a mortar. The homogenate was filtered through glass wool and frozen immediately at –20°C in 1 ml aliquots. Freezing does not interfere with the functional properties of alarm substances (Lawrence and Smith, 1989; Stabell and Lwin, 1997). The procedure might alter the proportions of odorants in the skin and/or cause loss of certain odorants; however, the extracts are prepared in the same way as to those we have used in our behavioral studies. In addition, this substantially reduced the number of individuals needed to produce skin extracts.

At the day of experiment the filtrates were diluted in oxygenated artificial pound water (APW) to a final concentration of 10^{-2} or 10^{-4} from the stock solutions. APW was prepared by adding the following chemicals to distilled water (mmol l^{-1}): NaCl (0.5), KCl (0.05), $CaCl_2$ (0.52), $NaHCO_3$ (0.19).

Test solutions of commercial chemicals

Four test solutions were prepared for the identification of units in the pheromone region and the food region of the OB. Solution 1: the sex pheromones 17,20β-dihydroxy-4-pregnen-3-one, 17,20β-dihydroxy-4-pregnen-3-one-20-sulfate, androstenedione, and prostaglandin $F_{2\alpha}$ (2.5×10^{-10} mol l^{-1} each). Solution 2: the bile salts; glucocholic acid, glucolithocholic acid, taurocholic acid, and taurolithocholic acid (2.5×10^{-10} mol l^{-1} each). Solution 3: the amino acids glycine, L-arginine, L-proline, and L-serine (2.5×10^{-5} mol l^{-1} each). Solution 4: the polyamine spermine (1.0×10^{-5} mol l^{-1}). All solutions were made in APW and applied in concentrations as described above.

The sex pheromones and bile salts were separately prepared as stock solutions (600 µl) at a concentration of 10^{-3} mol l^{-1} in dimethyl sulfoxide. Amino acids and spermine were separately prepared as stock solutions (1 ml) at a concentration of 0.1 mol l^{-1} in APW. All solutions were stored at –20°C. Chemicals were purchased from Sigma-Aldrich, St Louis, MO, USA.

Stimulation of the olfactory epithelium

A polyethylene tube was placed into the right anterior naris, exposing the olfactory epithelium to a continuous flow of APW (1.2 ml min^{-1}). Without changing the flow rate, the APW was replaced by odorant solutions using miniature valves connected to the tube. This installation minimized the risk of mechanical stimulation and prevented the epithelium from drying. Between each exposure, a flow of APW was directed to the olfactory epithelium to ensure that all stimulating substances were washed out from the olfactory capsule. The olfactory epithelium was not stimulated for a second time until the nervous activity returned to the prestimulus level.

Single unit recordings

Nervous activity was investigated by extracellular recordings from single OB units using microelectrodes made from tungsten wire (125 µm, impedance 1–2 MΩ, 1 kHz), prepared as described previously (Hubel, 1957). The microelectrode position was adjusted by a motorized micromanipulator (SD Instruments MC 1000, Grant Pass, OR, USA), and the signals led to a differential amplifier (DP 301, Warner Instrumental, Hamden, CT, USA). The reference electrode was positioned on the border of the brain cavity. The bandwidth was adjusted to 0.3–3 kHz, and a notch filter of 50 Hz was activated. Signals from the amplifier were displayed on an oscilloscope (Tektronix 565; Portland, OR, USA). The nervous activity was digitalized with an A/D converter (µ1401; CED, Cambridge, UK), stored and later analyzed by software (Spike 2, version 4.04; CED, Cambridge, UK).

The distance between the electrode and the neuron determines the amplitude and shape of action potentials as it appears on the computer. The Spike program allowed the sorting of action potentials based on these parameters, thereby distinguishing between different units at the same electrode position.

Experimental procedure

The alarm, the pheromone and the food regions can be reliably distinguished in the crucian carp OB, where neurons are selectively activated by alarm odorants, pheromones and food-related odorants, respectively (Hamdani and Døving, 2003; Lastein et al., 2006).

Recordings were made from units in all regions upon exposure to skin extracts. The electrode was introduced from the dorsal side, and the position of each electrode trajectory was marked on a schematic drawing of the OB (Fig. 1).

To identify units in the alarm region, conspecific skin extract was used in either the high or low concentration (10^{-2} or 10^{-4} of stock solution). When a unit was exited by a stimulus, recordings were made upon exposure to the three other skin extracts tested at equivalent concentrations. Commercial chemicals were applied to identify units in the pheromone region and the food region. Solutions of putative pheromones, a sex pheromone solution and a bile salt solution were applied while recording the activity in the pheromone region. Solutions inducing feeding behavior, an amino acid solution and a spermine solution, were applied while recording the activity in the food region. When a unit responded to a stimulus, recordings were subsequently made upon exposure to all four skin extracts, either in the high or in the low concentrations.

Analysis of single unit activity

Units can be divided into two categories, type I and type II (Hamdani and Døving, 2003; Zippel et al., 2000). In brief, type I units are assumed to correspond to the activity of mitral cells and respond with a burst of impulses concomitant with the stimulus arriving at the olfactory epithelium. Their responses to odorants reflect the chemotopic organization of the OB. Type II units are believed to correspond to the activity of the ruffed cells. These neurons lack a clear chemotopic organization found in the mitral cells (S.L. and E.H.H., personal observation). In the present study, only type I units that responded to at least one of the stimuli were included in the analysis. Previous studies show that the spontaneous and the interstimulus activity of type I units varied between 0.01 and 4 spikes s^{-1} (Hamdani and Døving, 2003; Zippel et al., 2000). However, most type I units sensitive to skin extracts had a very low spontaneous and interstimulus activity, all responding with increased activity upon stimulation (Hamdani and Døving, 2003).

In the present study, single unit recordings were performed to compare nervous activity induced by different stimuli. Only type I units that responded to at least one of the stimuli are included in the analysis. The effect of a skin extract was categorized as a response (+) if there was a burst of impulses, or as a no response (0). In congruence with our previous studies, no inhibitory responses were observed. The response profiles where collected in a data sheet from units in each region, and separate data sheets were made for stimuli with the high and low concentrations of the skin extracts. The ability to discriminate between the skin extracts was investigated by pair-wise comparisons. A unit was considered to discriminate between skin extracts from two different species when responding to only one of the two extracts. A unit was considered not to discriminate between skin extracts from two different species when responding to both or none.

Validation of response analysis

The burst of impulses observed upon stimulation is easily recognizable, however, to verify that the increased activity was statistically significant, a sub-set of 40 units was randomly chosen. The spontaneous activity was assessed from an interstimulus time interval of 30 s, which was divided in six periods of 5 s. The number of spikes in each period was counted and the mean and standard deviation was calculated. These values were compared to the number of spikes during a 5 s response period during stimulation.

The induced activity always exceeded the mean interstimulus activity, and all nervous activity considered a response was more than a multiple of 3.85 standard deviations from mean interstimulus activity (range 3.85–81.24). Thus, the measured increase in activity (burst) upon stimulation was always statistically significant (confidence interval, $P<0.001$). The mean interstimulus activity and the induced activity are plotted for each unit (Fig. 2).

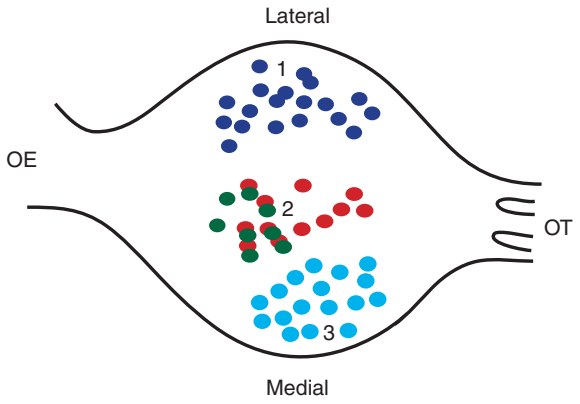

Fig. 1. Dorsal view of the right olfactory bulb showing the position of the electrode trajectories. Each circle represents an electrode trajectory. The colors represent the selectivity of the neurons encountered: dark blue (1), amino acids and spermine; red (2), sex pheromones; green (2), bile salts; and light blue (3), alarm substances. OE, olfactory epithelium; OT, olfactory tract.

To ensure that these units were typical for the entire population of tested units, a second sub-set was randomly chosen, and for the two sub-sets the interstimulus activities were compared by a χ^2-test (Microsoft Excel 2003). The units were divided into the following intervals (in spikes s^{-1}); less than 0.1, 0.1–0.2, 0.2–0.3, 0.3–0.6 and more than 0.6. In sub-set 1 the frequencies ranged from <0.03 to 1.3 spikes s^{-1}, and in sub-set 2 from <0.03 to 1.6 spikes s^{-1}. There was no significant difference between these two sub-sets (χ^2-test, $P=0.59$), and one sub-set may therefore be regarded as typical for the entire population of units.

RESULTS

Nervous activity induced by skin extracts

Data were obtained from 406 single units at 283 electrode positions in 52 electrode trajectories (Fig. 1). In the alarm region, we recorded from units located between 0.07 and 0.6 mm from the dorsal side. In the pheromone region, we recorded from units located between 0.08 and 0.9 mm from the dorsal side. In the food region, we recorded from units located between 0.1 and 1.0 mm from the dorsal side. The skin extracts induced activity in all regions (Fig. 3), and responses lasted from 5 s to over 1 min, appearing as a sudden burst of nerve impulses. None of the type I units was observed to respond with inhibition to skin extract.

Often more than one unit was encountered at a single electrode position and the units that did not respond to the first stimulus tested, but to at least one of the following stimuli were included in the analysis. Several units in the alarm region did not respond to conspecific skin extract, but were excited by at least one of the heterospecific skin extracts. Also, several units in the pheromone and the food region responded only to the skin extracts, but not the solutions of the commercial chemicals. These units could not be distinguished from other units in their respective regions with respect to spike activity.

We frequently encountered responding units at two successive electrode positions that were separated by less than 100 µm. If two or more such units evoked identical response profiles and shape of

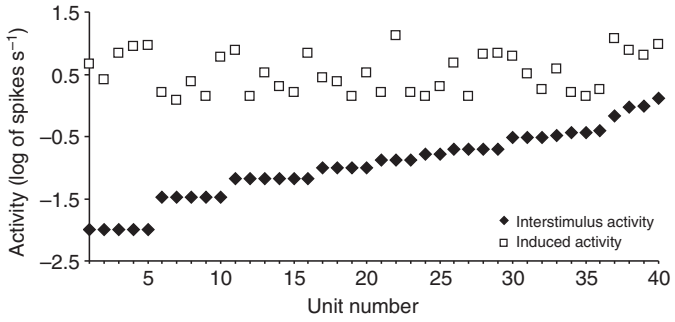

Fig. 2. Interstimulus and odor-induced activity of single units in the olfactory bulb. Scatter plot of activity (spikes s^{-1}) of units in sub-set 1. The logarithm to the interstimulus activity is plotted against the logarithm to the odor-induced activity. Units are sorted and numbered by their interstimulus activity; the unit with the lowest frequency corresponds to number 1 and the unit with the highest frequency corresponds to number 40.

action potential, only one was kept for the subsequent analysis. In total, 104 units were discarded by this procedure. The results from the analysis of the two data sets were similar (data not shown). Also, in numerous trajectories (not shown), units were discovered as a result of their spontaneous activity, but did not respond to any of the stimuli used in the present study. These units were not included in the calculations as the aim was to investigate units responding to skin extracts.

Units responding to different skin extracts in different OB regions

The number and percentage of units responding to each skin extract is presented for each OB region (Fig. 4). In the alarm region, the number of units activated was lower when stimuli were applied at low concentrations compared to high concentrations for all skin extracts except tench. Units in the pheromone region were in general more frequently activated by the low than by the high concentrations of skin extracts. A notable exception was the percentage of units activated by the conspecific skin extract; which was 72.7% at the high and 19.6% at the low concentration. Units in the food region were more frequently activated by the high than by the low concentrations of skin extracts. At both concentrations the conspecific skin extract activated fewer units than those from heterospecifics.

Unique units

Of all units (N=406) 70, (17.2%) were excited by only one of the four different skin extracts. Of these units, 43 (61.4%) responded to conspecific skin extract. As illustrated in Fig. 5, there was a prevalent occurrence of unique units in the alarm and pheromone region. In the alarm region, the number of unique units was higher when skin extracts were applied in low concentrations. In the pheromone region, the number of unique units was lower when the low concentrations were applied, and none of the units responded uniquely to the conspecific skin extract.

Discrimination between skin extracts from different species

A unit was considered to discriminate when it was excited only by one of two skin extracts from different species (i.e. crucian carp–common carp, crucian carp–tench, crucian carp–bream, common carp–tench, common carp–bream and tench–bream; pair-wise comparison, see Materials and methods). The number and percentage of these units is presented for each region separately (Table 1; Fig. 6).

In general, the units discriminated better between skin extract from conspecifics and another species than between skin extracts from two heterospecifics. In the alarm and food regions this was prominent when applied at low concentrations. In the pheromone region this did not seem to be correlated to the concentration of the stimuli.

When applied at low concentrations, the units in the alarm region showed increased discrimination between the conspecific skin extract and the heterospecific skin extracts, compared with high concentration applications. This tendency was also observed for the units that discriminated between skin extracts from common carp and bream. In the pheromone region there was a reduction in discrimination between the conspecific skin extract and two heterospecific skin extracts, tench and bream, when applied at low concentrations. For the other pair-wise comparisons, increased discrimination was observed. In the food region, there was an increase in the percentage of units that discriminated between the low concentration applications compared to the high concentration applications.

DISCUSSION

The present study concerns the effect of skin extracts from four different species of the carp family on single OB unit activity in crucian carp. Skin extracts mimic natural stimuli, and are relevant for studying how the bulbar neurons respond to the various odorants that may emanate from the skin of injured fish. The OB in crucian carp demonstrates a clear and known chemotopy which provided a means of investigating how single units respond to different types of skin odorants. We discuss the neural activation in the different regions, difference between high and low concentration of skin extracts, and recognition of conspecific odorants.

Neural activation induced by various odorants present in the skin extracts

Many units were activated both by solutions of commercial chemicals and by skin extracts in the pheromone and the food regions. According to previous studies on the functional

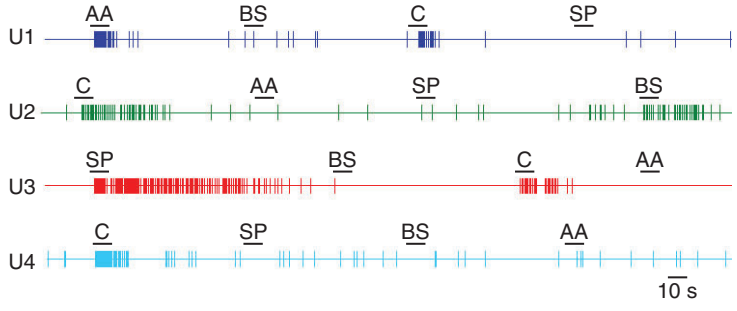

Fig. 3. Neural responses recorded from single units in the olfactory bulb upon odorant stimulation. Examples of the responses in units located in the food region (U1), the pheromone region (U2, U3) and the alarm region (U4), upon stimulation of the olfactory epithelium with amino acids (AA), bile salts (BS), sex pheromones (SP) and skin extract from crucian carp in the high concentration (C). Bars above each trace indicate time course of exposure of the olfactory epithelium with the given stimuli. The duration of each raster trace is 300 s.

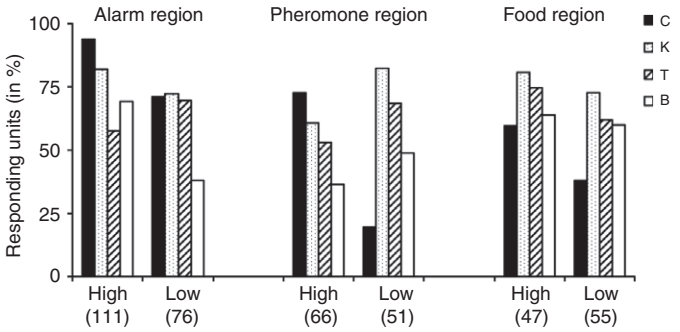

Fig. 4. Regional differences in single unit responses towards skin extracts from different species. The percentage of neurons activated by each skin extract in the alarm region, the pheromone region and the food region, at the high and the low concentrations of stimuli (see Materials and methods). C, crucian carp; K, common carp; T, tench and B, bream. Numbers in brackets represents the total number of units tested for the corresponding region and stimulus concentration.

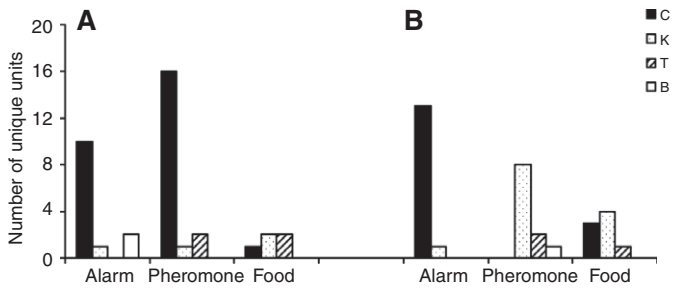

Fig. 5. Distribution of unique units. The number of unique units, i.e. units responding only to one of the four skin extracts, in the different regions of the olfactory bulb, when applied at (A) the high concentrations and (B) the low concentrations. C, crucian carp; K, common carp; T, tench and B, bream.

organization of the olfactory organ, OB units are sensitive to specific odorants with approximately the same functions. Thus, the induced neural activation suggests that odorants normally related to reproduction and feeding are present in detectable amounts in the fish skin. The detection of these odorants in skin extract might be biologically relevant. It may be possible for an odorant to have more than one function depending on context and/or concentration. Pheromone-like properties of amino acids in fish skin were previously suggested (Saglio and Blanc, 1989), but this has never been confirmed. Steroids and bile salts are more likely to have putative functions providing information about the injured fish. We speculate that this is more important when detecting skin odorants from other species than from conspecifics. Behavioral responses to dangers may be acquired by previous experiences, enabling the association of odors from another prey fish with predators (Brown and Smith, 1998; Chivers et al., 2002; Darwish et al., 2005; Ferrari et al., 2005).

All skin extracts applied induced nervous activity in the alarm region. This could mean that these units respond to and discriminate between alarm substances from different species. The axons of neurons in the alarm region form the bundle of the olfactory tract (mMOT) which mediates the fright reaction and it is probably that these are activated by odorants inducing the behavior. We can still not say with certainty whether all the units are sensitive to alarm substances or whether some respond to other unknown odorants in the skin extracts.

Activation of OB neurons with comparable stimuli to those in the present study, was investigated in rodents (Lin et al., 2005), where a combination of single unit recordings and gas chromatography (GC) was applied. Similar approaches could help identifying potent alarm substances in fish skin. Previously, we performed a combination of single unit recordings in the alarm region of the OB in conjunction with high performance liquid chromatography (HPLC) analysis. This enabled us to identify a highly potent fraction of the skin extract, probably containing the alarm substances (Brondz et al., 2004). Although such experimental procedures are possible, they are hardly practical, since it is difficult to analyze the different components detected by HPLC. Also a single HPLC run, which would be required for each single unit recording, takes about an hour. Thus, such an approach would be very time consuming.

The mitral cells located in the lateral part of the teleost OB are morphologically different from those in the medial part (Alonso et al., 1988; Fuller et al., 2006), which suggests physiological and functional distinctions. However, no differences between the medial

Table 1. Pair-wise comparison of discrimination between skin extracts in different topographic regions of the olfactory bulb

		Olfactory bulb region											
		Alarm				Pheromone				Food			
Pairs		+ +	+ 0	0 +	0 0	+ +	+ 0	0 +	0 0	+ +	+ 0	0 +	0 0
High	C–K	86	18	5	2	28	20	12	6	26	2	12	7
	C–T	62	42	2	5	23	25	12	6	24	4	11	8
	C–B	72	32	5	2	15	33	9	9	20	8	10	9
	K–T	56	35	8	12	30	10	5	21	31	7	4	5
	K–B	70	21	7	13	22	18	2	24	29	9	1	8
	T–B	49	15	28	19	20	15	4	27	29	6	1	11
Low	C–K	35	19	20	2	9	1	33	8	16	5	24	10
	C–T	32	22	21	1	10	0	25	16	16	5	18	16
	C–B	17	37	12	10	10	0	15	26	14	7	19	15
	K–T	45	10	8	13	32	10	3	6	30	10	4	11
	K–B	27	28	2	19	23	19	2	7	30	10	3	12
	T–B	29	24	0	23	22	13	3	13	29	5	4	17

C, crucian carp; K, common carp; T, tench; B, bream.

Responses: ++, number of units responding to both skin extracts; +0, the number of units responding to the first but not the second skin extract; 0+, the number of units responding to the second but not the first skin extract; 0 0, number of units not responding to any of the two skin extracts.

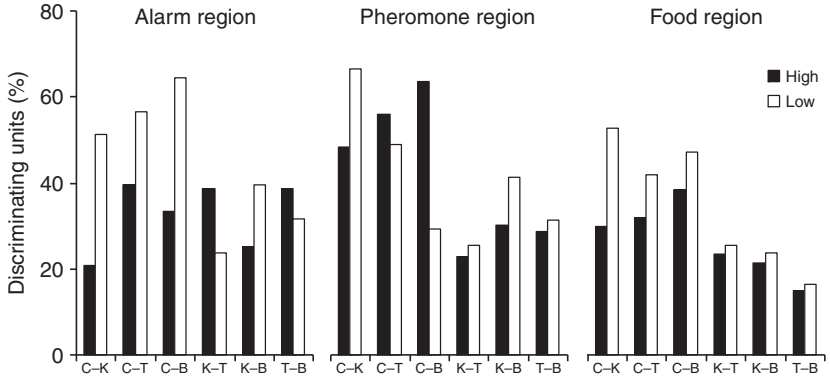

Fig. 6. The ability of single units to discriminate between skin extracts. The percentage of neurons discriminating between the skin extracts from different species (C, crucian carp; K, common carp; T, tench and B, bream). Pair-wise comparisons are presented for high and low concentrations, in which units were considered to discriminate between two species when responding to only one of the two, and to not discriminate when responding to both or none.

and the lateral OB units with respect to neural responses were observed in the crucian carp. Still, different functions related to skin extract identification cannot be excluded.

The skin extracts were taken from skin of previously frozen fish. This gives potent extracts which induce the fright reaction; however, this procedure might alter the proportions of odorants in the skin and/or cause loss of certain odorants. Nevertheless, the present method is the most suitable for studies on single unit activation and reduces the number of individuals needed for making the skin extracts, as application of fresh skin would require a new specimen of each species for each experiment.

Differences between responses to high and low skin extracts

The low concentrations of skin extracts correspond to what we commonly use in behavioral studies with crucian carp and the single unit responses probably reflect bulbar activation in free-swimming animals. Interestingly, both in the food and in the pheromone regions of the OB the conspecific skin extract activated considerably fewer units than did skin extracts from other species. Lower sensitivity towards conspecific odorants and/or lower (sub-threshold) content of potent odorants in conspecific skin compared to the other species could account for these observations. However, we find it unlikely that the sensitivity towards conspecific skin odorants is lower compared with heterospecific skin odorants. A suppression of neural activity in OB regions not activated by alarm substance when a stimulus is applied in relevant concentrations could be a more reasonable possibility. Such a process was also suggested previously (Lin et al., 2006).

Suppressing activity in other regions of the olfactory bulb that mediate messages not related to potential danger could reinforce the alarm signal. This could prevent contradictory information from reaching higher brain centers where sensory inputs are integrated, which may be advantageous. Contrasting interactions between type I and type II units were demonstrated in goldfish *Carassius auratus* (Zippel et al., 2000) and in crucian carp (Hamdani and Døving, 2003). Zippel and co-workers suggested that the type I units are mitral cells and that type II units are the ruffed cells. A possible scheme could be that the ruffed cells (type II), which are believed to lack input from the sensory cells (Kosaka and Hama, 1979), suppress the activity of mitral cells (type I). Another possibility is that the nervous activity, when reaching the brain, activates centrifugal fibers. Centrifugal activity can be induced by odorants as well as non-odorant stimuli, and may influence the activity of the secondary neurons in the olfactory system (Døving, 1966; Døving and Gemne, 1966).

When applied in high concentration, the conspecific skin extract induced responses in a majority of the units, thus, there was no apparent modulation of activity. Stimulating the olfactory epithelium with an excessive amount of skin extract could potentially activate sensory neurons that normally are silent.

Discriminating between conspecific and heterospecific skin extracts

Paired comparisons showed that OB neurons in general had a better ability to discriminate between skin extracts of crucian carp and another species, than between skin extracts of two other species. Previous observations showed that skin extracts from heterospecifics induced fright reactions with lower intensities than skin extracts from conspecifics (Schutz, 1956), which was proposed to be based on differences in the chemical structure of alarm substances; however, the present study shows that involvement of other odorants could be significant.

The differences observed in discriminatory ability are probably related to properties of the odorant receptors, such that odorants from conspecifics activate other groups of sensory neurons than odorants from other species. There was also a surprisingly high number of single units in the alarm and the pheromone regions that were activated only by skin extract from crucian carp. Thus, the OB appears to have two sets of units sensitive to conspecific odorants, one informs about danger, the other informs about presence, but not necessarily danger.

Since conspecific skin extract was used to localize units in the alarm region we might potentially have missed units sensitive only to heterospecific skin extracts. Previous attempts to identify units with heterospecific skin extracts were, made but with little success, and none were located in trajectories without ‘conspecific-sensitive’ units. We therefore assume that most, if not all units in the alarm region only sensitive to heterospecifics are located near units sensitive to conspecifics.

The difference between neural activation caused by the four skin extracts indicates that several types of odorants may be used to distinguish between the species. Several previous investigations focused on mate recognition among fishes based on olfactory cues (McKinnon and Liley, 1987; McLennan, 2003). For instance, female swordtails (*Xiphophorus nigrensis* and *X. pygmeaus*) showed preference for conspecifics based on olfactory, but not visual cues (Decaprona and Ryan, 1990), and a Lake Malawi cichlid fish (*Pseudotropheus emmiltos*) showed preference for conspecifics only when olfactory cues were present (Plenderleith et al., 2005). These studies and the present findings demonstrate the central role of olfactory cues in identification of other individuals.

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