

# Gender Distinction in Neural Discrimination of Sex Pheromones in the Olfactory Bulb of Crucian Carp, *Carassius carassius*

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## Abstract

Studies on projection of the sensory neurons onto the olfactory bulb in fish have revealed a clear subdivision into spatially different areas that each responded specifically to different classes of odorants. Amino acids induce activity in the lateral part, bile salts induce activity in the medial part, and alarm substances induce activity in the posterior part of the medial olfactory bulb. In the present study, we demonstrate a new feature of the bulbar chemotopy showing that neurons specifically sensitive to sex pheromones are located in a central part of the ventral olfactory bulb in crucian carp. Extensive single-unit recordings were made from these neurons, stimulating with four sex pheromones, 17,20 $\beta$ -dihydroxy-4-pregnen-3-one, 17,20 $\beta$ -dihydroxy-4-pregnen-3-one-20-sulfate, androstenedione, and prostaglandin F<sub>2 $\alpha$</sub> , known to induce specific reproductive behaviors in males of carp fish. All substances were applied separately to the sensory epithelium at a concentration of 10<sup>-9</sup> M. Of the 297 neurons recorded in males, the majority (236 or 79.5%) responded exclusively to one of the four sex pheromones and thus showed a high specificity. Of the 96 neurons recorded from the olfactory bulb in females, only 1 unit showed such a specific activation. These findings reflect remarkable differences between males and females in the discriminatory power of the olfactory neurons toward these sex pheromones. The gender differences are discussed in relation to behavior studies, expression of olfactory receptors, and the convergence of sensory neurons onto the secondary neurons in the olfactory bulb.

**Key words:** chemotopy, olfaction, olfactory bulb, specificity, sex

## Introduction

The initial events in olfactory processing occur at the apical surfaces of the olfactory receptor neurons (ORNs), where a large number of receptor types detect odorous molecules (Buck and Axel, 1991). The interaction of odorants with the olfactory receptors initiates a reaction cascade leading to an electrical signal traveling to the olfactory bulb as a first relay station in the brain. In teleosts, three morphological types of ORNs have been recognized within the olfactory epithelium, that is, ciliated ORNs, microvillous ORNs, and crypt cells (Ichikawa and Ueda, 1977; Thommesen, 1983; Hansen and Finger, 2000). The ORNs are distributed within each lamella so that any given restricted site in the glomerular layer in the bulb receives axons from ORNs widely scattered throughout the epithelium (Oakley and Riddle, 1992). A correlation between morphology, distribution, receptor expression, and G-proteins was recently established in goldfish, *Carassius auratus* (Hansen *et al.*, 2004).

Thommesen (1978) first established that ORNs responding to the same odorants project to restricted areas of the olfac-

tory bulb in fish. This was confirmed by surface electrode recordings (Døving *et al.*, 1980), single bulbar neuron recordings (Nikonov and Caprio, 2001, 2004; Hamdani and Døving, 2003), and optical imaging methods (Friedrich and Korsching, 1997, 1998). These studies substantiate the existence of spatial coding of olfactory information within the olfactory bulb, showing that responses to amino acids and bile salts were recorded from the lateral and medial parts of the olfactory bulb, respectively.

The correlation between ORN morphological phenotypes and their axonal projections has been demonstrated using a neural tracer, 1,1-dilinoleyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), in crucian carp, *Carassius carassius* (Hamdani *et al.*, 2001a; Hamdani and Døving, 2002), and has been confirmed in channel catfish, *Ictalurus punctatus* (Hansen *et al.*, 2003), and zebra fish, *Danio rerio* (Sato *et al.*, 2005). In crucian carp, ciliated ORNs project to the medial part, while the microvillous ORNs project to the lateral part of the olfactory bulb. The functional significance of these

projections becomes evident as the microvillous ORNs project to the bulbar neurons whose axons make up the lateral bundle of the olfactory tract, which mediates feeding behavior (Hamdani *et al.*, 2001b). Ciliated ORNs project to bulbar neurons whose axons make up the medial bundle of the medial olfactory tract, which mediates the alarm reaction (Hamdani *et al.*, 2000). In an adjacent study, we demonstrate that the crypt cells project to the ventral region of the bulb and make connections to the neurons that form the lateral bundle of the medial olfactory tract (IMOT) (Hamdani and Døving, 2006). This bundle of the olfactory tract mediates sex behavior in crucian carp (Weltzien *et al.*, 2003).

Sex pheromones, which in fish are gonadal steroids and/or prostaglandins (Dulka *et al.*, 1987; Sorensen *et al.*, 1988; Stacey *et al.*, 1989), affect sexual behavior. The connection between pheromone release and behavioral responses has been well established in the goldfish. In response to elevated temperature, females release two sets of pheromones: one preovulatory consisting of several steroids and one postovulatory consisting of prostaglandins (for review see Stacey *et al.*, 2003; Sorensen and Stacey, 2004). Three of the steroidal components of the preovulatory pheromone were shown to elicit distinctive behavior in males: 17,20 $\beta$ -dihydroxy-4-pregnen-3-one (17,20 $\beta$ P) induced a low level of chasing and nudging behavioral patterns that were long lasting, 17,20 $\beta$ -dihydroxy-4-pregnen-3-one-20-sulfate (17,20 $\beta$ P-S) induced more intense chasing and nudging that lasted only 5 min, and androstenedione (AD) elicited intense aggressive behavior among males (Poling *et al.*, 2001). The release of these steroids declines at ovulation, while prostaglandin F<sub>2 $\alpha$</sub>  (PGF<sub>2 $\alpha$</sub> ) and its metabolites are released as a postovulatory set of pheromones that affect male spawning behavior (Kobayashi *et al.*, 2002; Stacey *et al.*, 2003). In crucian carp, which is from the same subtribe, Cyprini, as goldfish and common carp, *Cyprinus carpio* (Rainboth, 1991), males showed short followings and inspections of the anal papillae of the PGF<sub>2 $\alpha$</sub> -injected females (Weltzien *et al.*, 2003). Steroids and prostaglandins are referred to as sex pheromones in the present study.

In the present experiments, we show that the nervous activity of the neurons in the central part of ventral olfactory bulb is specifically sensitive to sex pheromones. In addition, we found that there was a remarkable gender difference in the responses of the bulbar neurons toward the four sex pheromones that we used as stimuli.

## Materials and methods

### Animals and surgical procedures

Crucian carp (26–30 g body weight) were caught in a small lake (Tjersrud) just outside Oslo city borders, Norway, and transported to the aquaria facilities at the Department of Molecular Biosciences, University of Oslo. The fish were fed three times a week. The experiments were carried out

so as to minimize animal suffering and the number of fish used and are mainly similar to those described previously (Hamdani and Døving, 2003). All experimental procedures were made in accordance with national legislation and institutional guidelines at the University of Oslo. Fish were preanesthetized with benzocaine (45 mg/l) and given an intraperitoneal injection of Saffan (alphaxalon 0.9% and alfadolone acetate 0.3%) (24 mg/kg). Additional anesthesia with doses of Saffan was injected in the musculature. To avoid any unforeseen movement during the experiment, fish were wrapped in a wet cloth, adjusted in a cradle, and fixed belly down by two steel rods, which were fastened to the upper parts of the orbital bones taking care not to damage the olfactory epithelium. Fish were continuously irrigated through the mouth and over the gills by city springwater during the experiments. The skull above both the olfactory tract and the right olfactory bulb was removed under a stereomicroscope. The mesenchymal tissue around the olfactory tract was aspirated by gentle sponging, and the anterior part of the brain cavity was filled with paraffin oil. The fish remained in good condition for at least 8 h after surgery, as judged by the blood flow and the nervous activity recorded.

### Solutions

Sex pheromones (17,20 $\beta$ P, 17,20 $\beta$ P-S, AD, and PGF<sub>2 $\alpha$</sub> ) and bile salts (glycolic acid, glycolithocholic acid, taurocholic acid, and tauroolithocholic acid) were each prepared separately as stock solutions (600  $\mu$ l) at a concentration of 10<sup>-3</sup> M in dimethyl sulfoxide (DMSO). Amino acids (glycine, L-arginine, L-proline, and L-serine) were each prepared separately as stock solutions (600  $\mu$ l) at a concentration of 0.1 M in oxygenated artificial pond water (APW) (mg/l): NaCl (29), KCl (3.7), CaCl<sub>2</sub> (58), NaHCO<sub>3</sub> (16). All solutions were kept at -18°C. The final concentrations were made in APW (mg/l), prepared daily prior to each experiment. Substances were purchased from Sigma-Aldrich (Oslo, Norway).

### Experimental strategies

Bulbar areas where neurons are sensitive to amino acids, nucleotides, alarm substances, or bile salts have been mapped in different fish species (Nikonov and Caprio, 2001, 2004; Hamdani and Døving, 2003). In this study, we extended the investigations to include the neurons responding to sex pheromones. Three different types of stimuli were used: a mixture of four amino acids, each applied at a final individual concentration of 2.5  $\times$  10<sup>-4</sup> M (glycine, L-arginine, L-proline, and L-serine); a mixture of four bile salts, each at a final individual concentration of 2.5  $\times$  10<sup>-10</sup> M (glucolic acid, glycolithocholic acid, taurocholic acid, and tauroolithocholic acid); and a mixture of the four sex pheromones, each at a final individual concentration of 2.5  $\times$  10<sup>-10</sup> M (17,20 $\beta$ P, 17,20 $\beta$ P-S, AD, and PGF<sub>2 $\alpha$</sub> ). Where bulbar neurons were found to be exclusively sensitive to the solution of sex pheromones, single-unit discrimination ability was tested. In this

part of the study, the four different sex pheromones were applied separately (17,20 $\beta$ P, 17,20 $\beta$ P-S, AD, and PGF<sub>2 $\alpha$</sub> ) at a concentrations of 10<sup>-9</sup> M.

The olfactory organ ipsilateral to the recording site (the right side) was exposed to a continuous flow of oxygenated APW. The flow could be interrupted by a series of miniature valves to give exposure to one of the test solutions described earlier. The recordings were made from different regions in the bulb, as the electrode descended from the dorsal surface to the ventral region. When a spontaneously active unit was encountered, we began the application of chemicals to the anterior naris of the olfactory organ through a polyethylene tube with a flow of 0.3 ml/min with minimum mechanical stimulation of the olfactory receptor cells. The duration of each stimulus was 10 s, and the olfactory epithelium was not stimulated for a second time until the spontaneous activity returned to the prestimulus level. As the valves were arranged in series, the delay between valve opening and the arrival of the stimulus at the sensory epithelium varied between the stimuli. This delay was measured, and the stimulation bars used in the figures illustrate the actual period when the stimulus entered the sensory epithelium. Each unit was tested three times to ensure the reliability of the results.

#### Data analysis

Nervous activity recorded and displayed on the oscilloscope was converted to digital signals and stored on a personal computer for later analysis. The number of units encountered depended on the quality of the recording electrode. In our experiments, only electrodes detecting single or a few units were used. The electrical activity of single units was analyzed by the software program Spike 2 (version 4.04, CED, Cambridge, UK). The congruence of the spike activity from different units was verified by the overdraw function whereby all the spikes in a particular recording sequence are superimposed, as seen in the example in Figure 3, upper part. A positive response from a single unit upon stimulation of the olfactory epithelium with a particular substance was considered as excitatory only if the spike frequency increased concomitant with the arrival of stimulus and increased to be at least 10 times higher than the spontaneous activity. This software also distinguished between spikes from type I and type II units, which correspond probably to mitral cells and ruffed cells, respectively (Hamdani and Døving, 2003). The latter type constitutes a significant proportion of the cells in the mitral cell layer in teleost olfactory bulb (Kosaka, 1980).

#### Results

Each fish used in these experiments was chosen at random from the aquaria regardless of sex. At the end of each experiment, the sex was determined. The results are based on recordings from 22 males and 8 females. During the penetration of the electrode in the bulb, unit activity was encoun-

tered at two different regions corresponding to mitral cell layers: one in the dorsal and one in the ventral part of the olfactory bulb. In some recordings, distinct types of units were encountered simultaneously at a single recording site, that is, type I and type II units, displaying differences in form and spike duration (Zippel *et al.*, 1999, 2000; Hamdani and Døving, 2003). Type I units, believed to be mitral cells, displayed diphasic action potential with a raise time of about 1 ms. These units responded to stimuli by excitatory responses, and all units referred to in the present study are type I neurons.

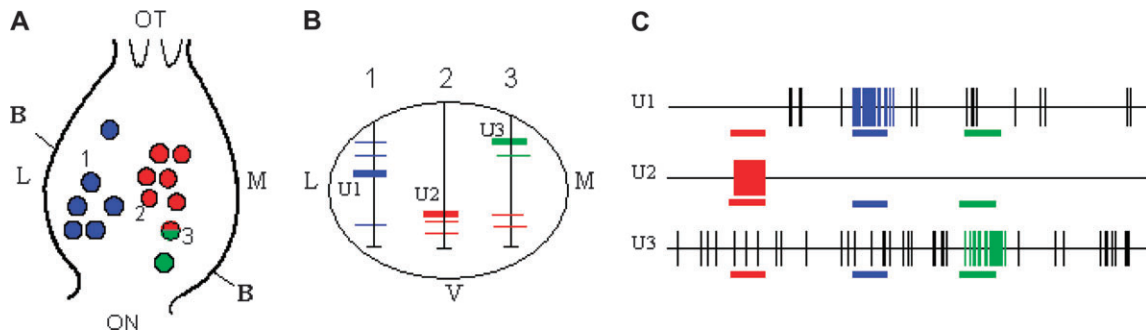
#### Chemotopy

Our previous studies on the chemotopy of the olfactory bulb of crucian carp have demonstrated a region in the posterior part of the medial bulb where the neurons respond to alarm substances present in the fish skin (Hamdani and Døving, 2003). The present study started with a series of experiments to find the region where neurons responded to sex pheromones, each known to induce specific behavior patterns in male goldfish. We performed experiments on both males ( $n = 4$ ) and females ( $n = 2$ ), in which the electrode penetrations were made in different areas of the bulb while exposing the olfactory epithelium to three mixtures: one with amino acids, one with bile salts, and one with sex pheromones. All stimuli were made with equal aliquots of four substances with the final concentrations described in Materials and methods. Preliminary experiments showed that the initial solvent DMSO applied to the olfactory epithelium at the same concentration range as that used to dissolve odors had no effect on the nervous activity recorded.

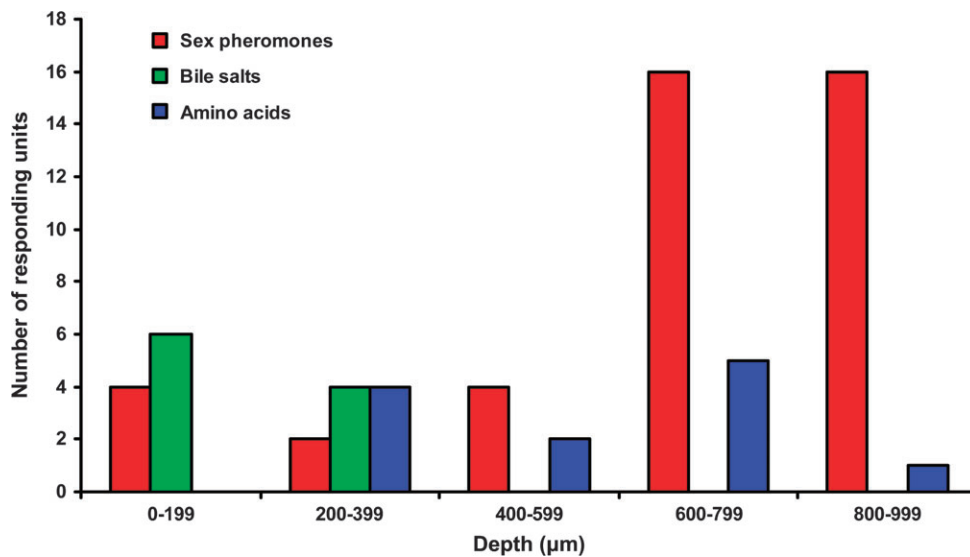
In total, 16 electrode penetrations were made in different parts of the bulb (Figure 1), where each mixture activated neurons in a restricted area. In trajectories made in the lateral part, the neurons encountered responded only to the mixture with amino acids, illustrated in blue. In trajectories made in the anterior part of the medial olfactory bulb, neurons responded to bile salts, illustrated in green. In the central part of the ventral olfactory bulb, neurons responded to sex pheromones, illustrated in red (Figure 1A). Examples of three trajectories are given in a bulbar cross section in Figure 1B, and three examples of representative responses of neurons to the three mixtures are given in Figure 1C. The depth distribution of selective neurons is given in the histogram in Figure 2.

#### Specificity and gender differences

The majority of the units reacting exclusively to sex pheromones by increased firing frequency were located in a region in the central part of the ventral olfactory bulb, about 300  $\mu$ m in length, 200  $\mu$ m in width, and at a depth between 600 and 900  $\mu$ m. To investigate the specificity of neurons in this region, we performed extensive single-unit recordings when applying the four sex pheromones separately to the olfactory epithelium.



**Figure 1** Regional distribution of neural response patterns across the olfactory bulb. **(A)** Dorsal view of the olfactory bulb showing the position of trajectories. The color of the circles indicates the selectivity of the neurons encountered in a single trajectory: blue, amino acids; red, sex pheromones; and green, bile salts. **(B)** Cross section of the olfactory bulb along the plane B–B in (A). The numbers 1, 2, and 3 refer to the corresponding trajectories in (A). **(C)** Examples of the responses from three units (U1, U2, and U3) demonstrating the selectivity of the neurons. ON, olfactory nerve; OT, olfactory tract; L, M, and V, lateral, medial, and ventral parts of the bulb.



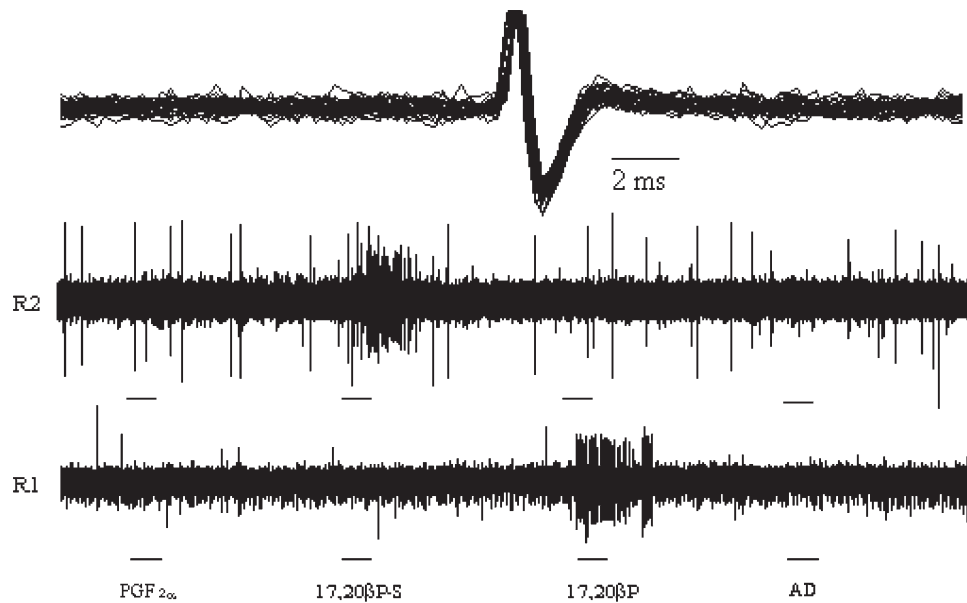
**Figure 2** Depth distribution of single units. Histogram showing the number of neurons responding to sex pheromones (red), bile salts (green), or amino acids (blue) with respect to depth, where 0 corresponds to the dorsal surface of the olfactory bulb.

The spontaneous activity of the neurons in this area varied without any obvious external cause and was in general low, less than 0.5 impulses/s. The characteristic pattern of discharge displayed by a given unit usually remained constant for observation periods of several hours. In a total of 393 single bulbar units encountered and examined in males ( $n = 18$ ) and females ( $n = 6$ ), 375 units responded with an increased activity upon stimulation by at least one of the four stimuli used. The stimuli-induced activity increased in conjunction with the arrival of the stimulus at the olfactory organ. This response depended upon the type of the stimulus and the location of the cell from which we recorded. In the example shown in Figure 3, the unit R1 displayed a low spontaneous discharge rate of  $\sim 0.3$  impulses/s. The stimulation with 17,20 $\beta$ P caused an increase in the activity to about 11 impulses/s.

### Males

We applied four different sex pheromones at a concentration of  $10^{-9}$  M. The most remarkable finding concerning the response of bulbar neurons in males was the high selectivity of neurons toward the sex pheromones. Typically, a single olfactory bulb unit in males was activated by only one of the four pheromones (Figure 3).

Sixty-seven units in the olfactory bulb of males were stimulated only by 17,20 $\beta$ P, 65 units were stimulated only by 17,20 $\beta$ P-S, 41 units were stimulated only by AD, and 63 units were stimulated only by PGF $_{2\alpha}$ . Thus, of the 297 units examined, 236 or 79.5% demonstrated such a high specificity. Only a few male units responded to more than one stimulus. Since we applied four stimuli and the type I units were excited by stimulation, there are 16 possible combinations of unit responses to these four substances, called response profiles.



**Figure 3** Examples of original extracellular recordings in males. Two examples of extracellular recordings (R1 and R2) from two different units in the ventral part of the right olfactory bulb upon exposure of the right olfactory organ to sex pheromones in males. The upper trace shows the superimposed spikes from the fired unit in R1. Note the increase of the activity in conjunction with the stimulation. Dashes, stimulation of 10 s. The two lower recording traces are 300 s in duration.

The number of units displaying the different response profiles is listed in Table 1.

Another noteworthy feature observed was that stimulation by 17,20βP induced sustained firing of the units, up to 60 s after the end of stimulation. An example is demonstrated by unit C4 in Figure 4. The behavior pattern evoked by 17,20βP is described as chasing and nudging at a low level but long lasting. Such persistent discharge of action potentials was not seen in female neurons.

### Females

In females, it was more difficult to encounter bulbar units that responded to sex pheromones than in males. However, a total of 96 units were detected due to their spontaneous activity, and their responses to the four sex pheromones were recorded. Only 1 unit responded exclusively to one of the four stimuli, 9 units responded to two stimuli, 20 units responded to three stimuli, and the majority, 61 units, were excited by all four (Figure 5, Table 1). This remarkable difference between males and females made it possible to correctly predict the sex of the fish on basis of the response profile of the bulbar neurons. However, no differences were noticed between males and females concerning the location of responsive neurons.

### Discussion

This study represents an extension of a series of investigations demonstrating that different types of odorants are processed within specific regions of the olfactory bulb in fish

(Thommesen, 1978; Døving *et al.*, 1980; Riddle and Oakley, 1991; Oakley and Riddle, 1992; Friedrich and Korsching, 1998; Nikonov and Caprio, 2001, 2004; Hamdani and Døving, 2003). We demonstrate here that the secondary neurons that send information about sex pheromones to higher centers in the brain are located within a limited area in the central part of the ventral olfactory bulb in the crucian carp. We further report that male secondary neurons are selectively tuned to one of the four tested stimuli, while those in females are not. To our knowledge, this is the first study describing how fish single bulbar neurons decipher, on sex-related basis, the information provided by sex pheromones.

### The functional organization of the olfactory system in fish

Soon after Døving and Selset (1980) first provided a strong argument for the concept of a spatial distribution of responses to odorants in the fish olfactory system by electrical stimulation of the olfactory tract in cod, *Gadus morhua*, many studies have focused on the functional organization of the olfactory system both in goldfish (Stacey and Kyle, 1983; Demski and Dulka, 1984; Sorensen *et al.*, 1991) and in crucian carp (Hamdani *et al.*, 2000, 2001b; Weltzien *et al.*, 2003; Hamdani and Døving, 2005). These findings demonstrate that each bundle of the olfactory tract mediates a specific behavior pattern and further implies that mitral cells forming a particular bundle of the tract receive distinct and specific information from the ORNs. The animal model chosen for these experiments thus provides several advantages. Besides its close relation to the goldfish, whose sexual behavior is the most thoroughly studied hitherto (see review Stacey *et al.*,

2003), the functional organization of the olfactory system in crucian carp is well established. In this species, we recently mapped the projections of two morphological different ORNs to the level of the olfactory bulb (Hamdani *et al.*,

**Table 1** Response profiles of bulbar neurons to the four sex pheromones

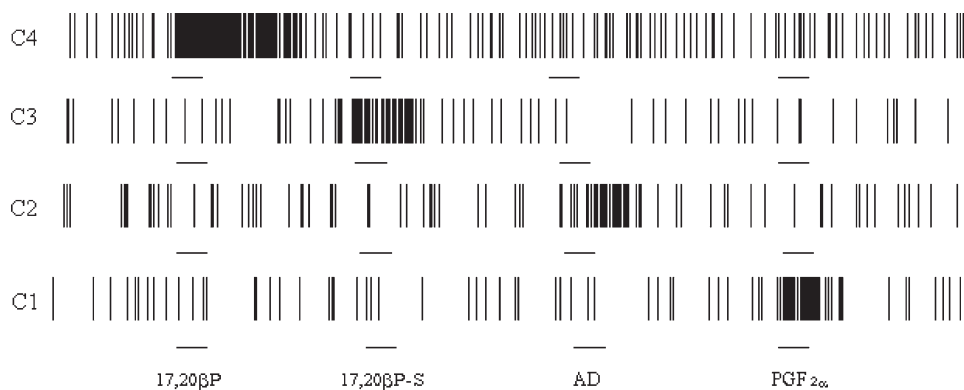
Substances inducing nervous activity in bulbar neurons	Number of units found for each response profile			
	Males		Females	
None	13	(4.3)	5	(5.2)
17,20βP	67		1	
17,20βP-S	65	(79.5)	0	(1.0)
AD	41		0	
PGF <sub>2α</sub>	63		0	
17,20βP + 17,20βP-S	4		3	
17,20βP + AD	7		1	
17,20βP + PGF <sub>2α</sub>	13	(11.8)	0	(9.4)
17,20βP-S + AD	3		2	
17,20βP-S + PGF <sub>2α</sub>	5		1	
AD + PGF <sub>2α</sub>	3		2	
17,20βP + 17,20βP-S + AD	2		8	
17,20βP + 17,20βPS + PGF <sub>2α</sub>	3	(2.0)	6	(20.8)
17,20βP + AD + PGF <sub>2α</sub>	1		3	
17,20βP-S + AD + PGF <sub>2α</sub>	0		3	
17,20βP + 17,20βPS + AD + PGF <sub>2α</sub>	7	(2.4)	61	(63.5)
Sum	297	(100)	96	(100)

The number in parentheses gives the distribution in percent of neurons grouped according to the number of substances that activated the units.

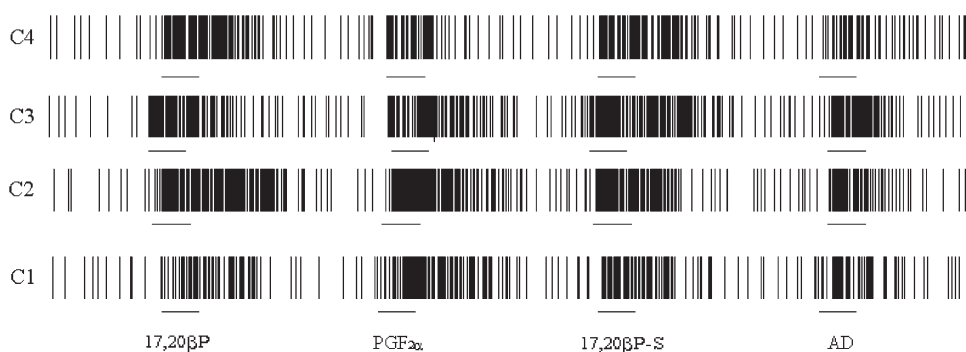
2001a; Hamdani and Døving, 2002). Thus, microvillous ORNs project to the lateral part of the bulb and mediate feeding behavior, while ciliated ORNs project to the medial part of the bulb and mediate alarm reaction. Reproductive behavior is mediated by the IMOT (Weltzien *et al.*, 2003), and the neurons that give rise to the axons in this part of the tract are found in the ventral region of the olfactory bulb. As shown in Hamdani and Døving (2006), the crypt cells converge to this region of the olfactory bulb and connect to the IMOT.

### Chemotopy within the olfactory bulb

The teleost olfactory bulb is clearly subdivided into spatially different structures that respond specifically to different classes of odorants. This concept has remained a central issue in olfactory research for a long time using surface electrode recordings (Thommesen, 1978), axon tracing experiments (Riddle and Oakley, 1991; Oakley and Riddle, 1992), and single-olfactory bulb unit recordings (Nikonov and Caprio, 2001, 2004; Hamdani and Døving, 2003). All these findings indicate that specific parts of the bulb are activated upon exposure of the olfactory epithelium to different classes of odorants. Amino acids induce activity in the lateral part of the bulb, bile salts induce activity in the medial part of the bulb, and alarm substances induce activity in a restricted zone in the posterior part of the medial olfactory bulb. Little is known, however, about how bulbar neurons process information provided by sex pheromones. Our present results clearly demonstrate that there is high specificity of neurons in the ventral olfactory bulb to sex pheromones. Friedrich and Korsching (1998), using voltage-sensitive dye techniques to study the glomerular responses in zebra fish, found that PGF<sub>2α</sub> and 17,20βP-S each induced a single focus of activity within the olfactory bulb. The response to prostaglandin was located in a single ventral glomerulus. These latter results are consistent with those of the present study.



**Figure 4** Male bulbar neuron responses to sex pheromones. Activity patterns of four different units (C1–C4) recorded extracellularly from the ventral part of the right olfactory bulb in male crucian carp when stimulating the olfactory organ with 17,20βP, 17,20βP-S, AD, and PGF<sub>2α</sub>. Note 1) the high specificity of the units toward the stimuli and 2) the long-lasting response of the unit C4 when stimulated by 17,20βP. Each recording lasted 300 s during which 10-s stimulations (dashes) were applied.



**Figure 5** Female bulbar neuron responses to sex pheromones. Activity patterns of four different units (C1–C4) recorded extracellularly from the ventral part of the right olfactory bulb in female crucian carp when stimulating the olfactory organ with 17,20βP, 17,20βP-S, AD, and PGF<sub>2α</sub>. Note the absence of specificity of the units toward the sex pheromones. Each recording lasted 300 s during which 10-s stimulations (dashes) were applied.

### Congruence with behavioral data

Studying behavioral reactions in male goldfish upon exposure to three steroidal components (17,20βP, 17,20βP-S, and AD) of the preovulatory pheromone (Poling *et al.*, 2001) showed that goldfish males performed different behaviors when exposed to each of these substances. This clearly indicates that the sex pheromones were distinguished by the olfactory system of the fish, which in turn implies that different sets of olfactory pathways were activated, including different subsets of ORNs, which project to different glomeruli. Our electrophysiological recordings from olfactory bulb neurons when stimulating with the same stimuli as in the behavioral study revealed that the male neurons responded with high specificity to these compounds. The majority of the bulbar neurons that responded to 17,20βP showed sustained activity that outlasted the cessation of the overt stimulus. This prolonged activity is congruent with behavioral observations as this particular stimulus elicited persisting effects on behavior.

There was a small number of units in the central part of the ventral bulb that did not respond to any of the four sex pheromones presented. These units were encountered due to their spontaneous activity, but none of the four substances induced changes in this nervous discharge. It should be recalled that there is a large number of substances in the body fluids that can act as sex pheromones. The identity of these substances is unknown, and the units that did not respond to the four substances used in the present study may be tuned to other sex pheromones.

### The significance of specific excitation

The fact that the majority of neurons in the male olfactory bulb respond to one, but not the other three, of these four pheromones demonstrates some unique properties and salient features of the olfactory system. It means that each one of the many thousands of sensory neurons converging onto a secondary neuron in this part of the bulb must express an odorant receptor that is activated by this substance. In

addition, it means that the receptors for these four pheromones must be highly specific. Our findings are consistent with the convergence pattern demonstrated in mammals by molecular biology (Ressler *et al.*, 1994; Vassar *et al.*, 1994), made possible by the identification of the olfactory receptors (Buck and Axel, 1991). What we demonstrate is appropriate of a system that in the fish will display different behavior patterns toward each of these four compounds. Such a segregation of responses could occur at a higher level in the nervous system. That it is already evident among the secondary neurons projecting to the telencephalon is a pertinent observation of the discriminatory power of the olfactory system.

### Gender distinction

The present study also demonstrates that olfactory neurons in female crucian carps, unlike those in males, do not discriminate between the four different sex pheromones. Consequently, either the olfactory receptors in females are differently tuned from those in the males or the convergence pattern of ORNs onto the secondary neurons is less distinct in females. It should be mentioned that most evidences for sex pheromones in fish are based on pheromones released by females (Stacey *et al.*, 2003; Sorensen and Stacey, 2004). Thus, the most illustrating example is the effect of the goldfish pre- and postovulatory pheromones. The ability of male *Tilapia*, *Oreochromis mossambicus*, to discriminate between pre- and postovulatory females (Miranda *et al.*, 2005) might also be based upon different sex pheromones emanating from the females and distinguished by the male olfactory system. There are, however, some studies that reported the effect of male pheromones on female behavior. Male black goby, *Gobius joso*, release a steroid that attracts females and induces them to oviposit in the absence of a male (Colombo *et al.*, 1980). This has also been confirmed in the round goby, *Neogobius melanostomus*, by Murphy *et al.* (2001), who showed that a male steroid, ethiocholanolone, increased ventilation rate in females. Whether female crucian carps discriminate between male pheromones is yet to be explored.

The difference between males and females in response to sex pheromones could be due to the fact that females ( $n = 6$ ) were undersampled, although this seems unlikely. A more finely tuned olfactory system in males could possibly be related to the crucial importance of male recognition of the female ovulatory status, while this information might be of less relevance to the females. The significance of the female detection of these sex pheromones is, however, unclear and needs further investigation. Nevertheless, it has been shown that androgen treatment can turn female goldfish into functional males that respond behaviorally to the 17,20BP pheromone (Stacey and Kobayashi, 1996). It would be interesting to study the neurophysiological changes in the bulb following such treatment. Could it be that the expression of olfactory receptors and/or wiring of the axons also change in connection with masculinization of females?

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