Olfactory processing of sex and alarm cues in the crucian carp *Carassius carassius*

by

Stine Lastein

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Preface

To all of you who have inspired me during my time at the University of Oslo.

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And last but not least, my life outside the walls of Blindern; a big thanks to my friends and my parents for all encouragement.

-Stine-

Abstract

The olfactory organ in fishes is organized in parallel pathways; sub-populations of neurons respond to biologically different types of odorants, and there is a direct link between activation of the different neuronal pathways and the specific behaviors induced. The aim of my thesis has been to study how the crucian carp processes odors involved in sexual behaviors and in the fright reaction, a stereotypic avoidance behavior. Two types of odors have been applied. The first, female sex pheromones, are hormones released from the female during sexual maturation. The second, extracts of fish skin, are complex odors which contain alarm substances inducing the fright reaction, but also a multitude of other chemicals.

To investigate how behaviors related to, or influenced by sex hormones, may be reflected in the olfactory system, I have studied how olfactory bulb neurons respond to female sex pheromones (Paper I), seasonal variations in the olfactory epithelium (Paper II) and whether responses to alarm odors vary in relation to reproductive status (Paper III).
To investigate how skin extracts from different species induce behavioral and nervous responses, I have studied the behavioral and neural responses to alarm odors from conspecifics and cross-order species (Paper IV) and how olfactory bulb neurons respond to complex odors (Paper V).

The results show that the neurons of the olfactory bulb in males, but not in females, very precisely discriminate female sex pheromones, indicating importance for males of knowing the exact time of ovulation (Paper I). Olfactory sensory neurons believed to respond to these odors varied in number throughout the year, being abundant in the summer and almost absent during winter months (Paper II). Furthermore, the fright reaction is suppressed in females in the final stages of sexual maturation, which can be related to altered levels of sex hormones, indicating increased risk-taking during mating (Paper III).

Neurons sensitive to alarm substances did not show the same clear cut discriminative properties as neurons sensitive to the sex pheromones, but did however distinguish better between odor cues from conspecific and heterospecific skin extracts, than between odor cues from two heterospecific skin extracts (Paper IV and V). This can be seen in relation to the general message of these stimuli; the presence of a predator, which does not necessitate an extremely accurate interpretation about the exact nature of the victim. Furthermore, extract of skin which is used as stimuli, is a complex mixture, containing food-related odor as well as pheromones, in addition to the alarm substances (Paper V). These odors may have

complimentary functions but their importance is so far unclear, they could play a role in acquired responses to heterospecific skin extracts.

The present findings show interesting aspects of the properties of the fish olfactory system. Large variance in the discriminatory capacity was observed, which related to the function of the detected odor. Also, a seasonally dependent expression of one type of sensory neurons and the adaptation of some behavioral responses according to sexual maturation indicates a highly flexible and adaptable sensory system in this animal group.

Introduction

In fishes, olfaction is central to reproduction and predator avoidance. Behaviors related to these essential life processes may be induced exclusively by activation of the olfactory system. The different types of odors involved carry very specific information about particular situations, which requires accurate detection mechanisms. The importance of an odor may also be context-dependent, and proper interpretation is a necessity. The olfactory system detects odorant and processes the sensory input, enabling each individual to make appropriate decisions, thereby increasing its own probability of survival and reproductive success.

The fish olfactory system

The organization of the olfactory system in fishes is based on similar principles as in other vertebrates. Odorants are detected by sensory neurons at their apical ending, which is in physical contact with the external environment. The neurons are located in the olfactory epithelium and their axons make up the olfactory nerve, terminating in the olfactory bulb. Here, they transmit the sensory input to the secondary neurons in the olfactory bulb. The latter have axons which make up the olfactory tract, ending up in the telencephalon (comparable to the forebrain/cortex in mammals) and other higher brain centers.

Some fishes, the gadids (cod fishes), the sillurids (catfishes) and many of the cyprinids (carp fishes), have a unique anatomical appearance; the olfactory bulbs are located close to the olfactory epithelia, whereas the olfactory tracts are long and easy to recognize (Figure 1). Species with this characteristic have proven advantageous for studying how the olfactory system functions due to the easy access of the secondary neurons.

The sensory neurons

The sensory neurons are situated in the olfactory epithelium; paired structures in the nasal cavities located on the front of the head, separated from the mouth and the respiratory organs. In many fishes these organs are shaped to form rosettes, consisting of lamella grouped together to form a leaf-like structure. The gross morphological shape varies largely among species, although a general property is a large surface-to-volume ratio, a formation which provides space for a vast number of sensory neurons and increases the probability of odorant detection.

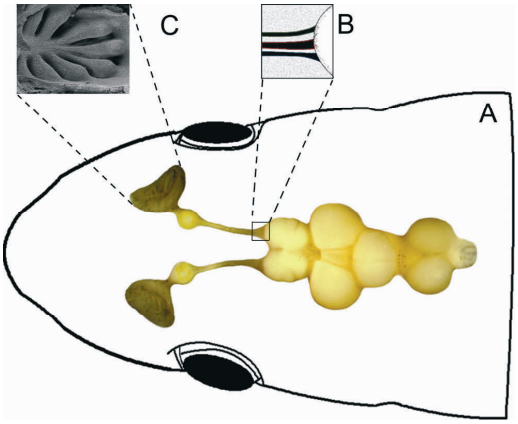

Figure 1. Overview of the fish brain. **A**. Dorsal view of the head of a crucian carp showing the brain and the olfactory system. **B.** Schematic drawing of the olfactory tract as it enters the telencephalon (the brain), demonstrating the three distinct bundles. **C.** Scanning micrograph of the olfactory epithelium. From Hamdani and Døving (2007).

The cell bodies of the sensory neurons in fishes are situated with varying distance from the apical surface. Three different morphological types have been identified; ciliated cells, microvillous cells and crypt cells (Ichikawa & Ueda, 1977; Thommesen, 1983; Hansen & Zeiske, 1998). The ciliated cells have cell bodies deep down in the epithelium with dendrites reaching the apical surface where they bear cilia. The microvillous cells have cell bodies located at an intermediate depth in the epithelium with dendrites bearing microvilli at the surface. The crypt cells have cell bodies located near the apical surface bearing both cilia and microvilli (Hansen & Zeiske, 1998; Morita & Finger, 1998; Hamdani *et al.*, 2001a; Hamdani & Døving, 2002, 2006). Thus, the three types of sensory neurons have cell bodies arranged in different vertical layers of the olfactory epithelium with respect to morphology.

Odorant receptors

Odorants are detected by odorant receptors which are molecules located in the membrane of the cilia and the microvilli of the sensory neurons. They belong to a large family of 7-trans-membrane G-protein coupled receptors (Buck & Axel, 1991; Ngai *et al.*, 1993b) and are activated when an odor binds to the binding site.

The olfactory system is capable of detecting and discriminating between thousands of odorants, which is possible because the odorant receptors function in a combinatorial way. One receptor may be activated by a range of certain odorants, whereas one odorant may activate different receptors. Thus, each odorant activates a unique combination of receptors. A

sensory neuron expresses only one (or few) receptors, meaning that each odorant activates a unique sub-set of neurons (Ngai *et al.*, 1993a; Malnic *et al.*, 1999).

The secondary neurons

The secondary olfactory neurons are mitral cells, named from their characteristic shape in mammals, and are located in the olfactory bulb (Figure 2). This organ is the first relay center of the olfactory system, where the olfactory information is initially processed. Two sub-types of mitral cells have been classified in teleosts (Alonso *et al.*, 1988; Fuller *et al.*, 2006) with respect to differences in morphology and location, indicating that their physiological functions also are partly segregated. There are, in addition, minor morphological variations between different species (Alonso *et al.*, 1989)

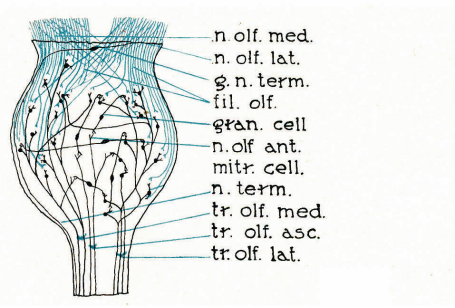

Figure 2. Schematic illustration of the olfactory bulb in common carp. The incoming axons of sensory neurons make synaptic contacts with the mitral cells. *n.*, nervus, *olf.*, olfactorius; *med.*, medialis, *lat.*, lateralis; *g. n. term.*, ganglion cell of the nervus terminalis; *fil. olf.*, fila olfactoria; *gran.*, granule., *ant.*, anterior; *mitr.*, mitral., *tr.*, tractus; *asc.*, ascendens. From Sheldon (1912).

The axons of the sensory neurons make synapses to the mitral cells with very high convergence, in an estimated ratio of approximately 1000:1 (Allison & Warwick, 1949; Gemne & Døving, 1969). These synapses are located in the glomeruli, structures which are considered to be the functional units of the olfactory bulb based on findings in rodents, where sensory neurons expressing the same receptor protein converge to one glomerulus. The arrangement of this high convergence enables the detection of odors in extremely low concentrations.

The axons of the mitral cells ascend along the olfactory tracts, which terminate in the telencephalon, and project further to higher centers of the central nervous system (Sheldon, 1912). In crucian carp, where the olfactory tracts are long, these may be divided in three separate bundles; the lateral olfactory tract (LOT), the lateral bundle of the medial olfactory tract (lMOT) and the medial bundle of the medial olfactory tract (mMOT). A similar

characteristic is also observed in other fish species with the same anatomical structure of these neurons.

Other neurons in the fish olfactory bulb

Several other types of neurons are observed in the olfactory bulb (Figure 3). One of them, the granule cells (Sheldon, 1912), make reciprocal synapses with the dendrite of mitral cells, suggesting a two-way reciprocal communication. Granule cells are also innervated by efferent fibers; the centrifugal fibers (Ichikawa, 1976), which originate from the telencephalon and the contra-lateral olfactory bulb (Sheldon, 1912; Oka, 1980; Bass, 1981; von Bartheld *et al.*, 1984). Ruffed cells, another cell type (Kosaka & Hama, 1979), exclusively found in fishes, also make synaptic contacts with the granule cells (Kosaka & Hama, 1982).

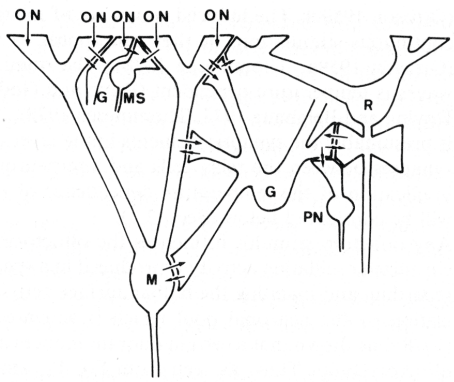

Figure 3. The cellular organization of fish olfactory bulb neurons. The axons of the sensory olfactory neurones, constituting the olfactory nerve, make synapses with mitral cells. Mitral cells and ruffed cells, both make synapses with the granule cells, and have axons projecting to the olfactory tract, but the ruffed cells do not make synapses with the sensory neurons. ON, olfactory nerve; M, mitral cell; G, granule cell; R, ruffed cell; MS, mixed-synapse cells; PN, perinest cells. Arrows indicate synapses. From Kosaka and Hama (1982).

Granule cells and ruffed cells apparently do not make synaptic contact with axons of the sensory neurons. However, their organization suggests influence on the mitral cell activity and in the processing of the sensory input. Contrasting interactions between two distinct types of neural activity, believed to represent the activation of mitral cells and ruffed cells respectively, have previously been described (Zippel *et al.*, 2000; Hamdani & Døving, 2003). Combination of histological and electrophysiological approaches to verify this has so far not been done. Other bulbar neurons with possible influence on mitral cell activity are short axon cells, perinest cells and mixed synapse cells (reviewed by Satou, 1990).

The functional organization of the olfactory organ

Morphology – function relationship

Sensory neurons expressing the same type of odorant receptor, thereby responsive to the same odorant(s), are widely dispersed within the olfactory epithelium (Ressler *et al.*, 1994b; Weth *et al.*, 1996; Hansen *et al.*, 2004). In mammals, the spatial termination of sensory neurons expressing a given odorant receptor terminates at one or few glomeruli (Ressler *et al.*, 1994a; Vassar *et al.*, 1994). Consequently the olfactory bulb is chemotopically organized and there is a spatial segregation of the sensory input; each glomerulus is activated by specific odorants (Figure 4).

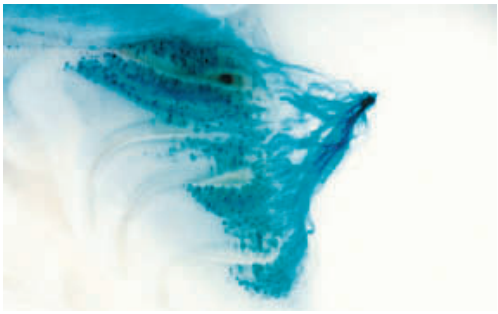

Figure 4. Projections of sensory neurons to glomeruli in mouse olfactory bulb. Sensory neurons expressing the same odorant receptor gene, MOR23, visualized in transgenic mice. The gene is tagged by homologous recombination with an IRES-taulacZ cassette, which enables co-expression of the receptor with a histological marker. These sensory neurons are widely distributed in the olfactory epithelium, with axonal projection to one of their two target glomeruli in the olfactory bulb. From Vassalli and co-workers (2002).

A similar organization is found in fishes. The morphology of a sensory neuron is also correlated to its spatial termination in the olfactory bulb (Thommesen, 1982, 1983; Morita & Finger, 1998). In crucian carp, the axons of the microvillous receptor cells project to the lateral part, axons of the ciliated receptor cells to the medial part and the axons of crypt cells project to the ventral part of the olfactory bulb (Hamdani *et al.*, 2001a; Hamdani & Døving, 2002, 2006). Furthermore, ciliated and microvillous cells have been demonstrated to express separate classes of odorant receptors (Hansen *et al.*, 2005). This strongly suggests that there is a correlation between the morphology of a sensory neuron, the odorant receptor it expresses, and to which class of odorants it responds (Hansen *et al.*, 2003; Hamdani & Døving, 2007). Thus, the relationship between the odorant receptor expressed and the target in the bulb, probably obeys to rules similar to those observed in mammals.

The functional properties of sensory cells are most likely related to morphology, although investigations show somewhat inconsistent results. Initially, microvillous and ciliated cells were suggested to respond to amino acids (food-related) and bile salts (pheromones), respectively (Thommesen, 1982, 1983). Later studies found correlations

between ciliated cells and amino acid sensitivity, as well as between microvillous cells and sex pheromone sensitivity (Zippel *et al.*, 1993; Zippel *et al.*, 1997b). Subsequently, it was shown that microvillous cells also respond to amino acids, while the ciliated respond both to amino acids and pheromones (Sato & Suzuki, 2001; Lipschitz & Michel, 2002; Schmachtenberg & Bacigalupo, 2004). Crypt cells were recently found to respond to amino acids, and not to bile salts, however sex pheromones was never tested as stimuli (Schmachtenberg, 2006). These discrepancies show that further investigations are required to determine the function of each cell type.

Spatial specificity of secondary neurons

Since the sensory neurons with identical odorant receptors project to the same glomeruli, each odor activates specific parts of the olfactory bulb. In fishes, many biologically relevant odors are known, an advantage which facilitates the study of the olfactory system. Applications of electrophysiological techniques have enabled the mapping of separate regions of the olfactory bulb, each responding by increased nervous activity to a distinct group of odors. Generally, odors related to feeding behavior, such as amino acids, nucleotides and polyamines activate neurons in the lateral part of the olfactory bulb. Odors with pheromone-like functions, such as bile salts and alarm substances, activate neurons in the medial part (Thommesen, 1978; Døving *et al.*, 1980; Nikonov & Caprio, 2001; Hamdani & Døving, 2003).

Animals may respond with different behaviors to odors with similar biological function (Valentincic *et al.*, 2000; Poling *et al.*, 2001). This distinction is reflected in the activation of secondary neurons, which also can discriminate them, for instance two structurally related amino acids (Nikonov & Caprio, 2004). However, this ability largely varies from neuron to neuron and depends on its glomerular connection. The organization of a secondary neuron, i.e. the number of glomeruli it contacts, has influence on its discriminatory power: a high number of innervated glomeruli lower the discrimination.

The bundles of the olfactory tract originate from different parts of the olfactory bulb, and terminate in different parts of the telencephalon, and each of the bundles has been shown to have a distinct function. Investigations performed on Atlantic cod (*Gadus morhua*) showed that electrical stimulation of a separate bundle induced a specific behavior (Døving & Selset, 1980). In the crucian carp, ablation of the LOT resulted in loss of feeding behavior, the lMOT in loss of sexual behavior, and the mMOT in loss of avoidance behavior (Hamdani *et al.*, 2000; Hamdani *et al.*, 2001b; Weltzien *et al.*, 2003). In goldfish *Carassius auratus*, ablation

of the lateral and medial bundles resulted in reductions in feeding and reproductive behavior, respectively (Stacey & Kyle, 1983). The results from the ablation experiments were in congruence with the electrical stimulations on cod. This means that each bundle in the olfactory tract, and thereby each region of the olfactory bulb, mediates a distinct behavioral pattern. Furthermore, the organization seems to be conserved in distantly related species.

An important conclusion to be drawn from these studies is that differential activation of the secondary neurons induces characteristic behaviors in fishes, demonstrated both upon electrical and chemical stimulation. The activation of the olfactory bulb neurons therefore correlates to the behavioral response to an odor; different sub-populations of secondary neurons are inducers of distinct behavioral patterns. Simultaneous activation of functionally different neurons is, on the other hand, poorly investigated. Previously, secondary neurons were proposed to be organized in a hierarchical manner with respect to which type of information they transmit to the higher brain centres (Døving, 1966; Døving & Hyvarinen, 1969). This aspect still remains to be more deeply investigated.

Higher brain centers

Studies on how odor information is processed after being handled in the fish olfactory bulb are scarce. Extracellular recordings in the telencephalon (forebrain) of channel catfish (*Ictalurus punctatus*) upon stimulation with odorants (Nikonov *et al.*, 2005; Nikonov & Caprio, 2006), demonstrated that the chemotopy of the olfactory bulb is partly conserved with respect to food-related odors and bile salts. This finding is in congruence with studies on mammals, where odors which activate spatially separated regions in the olfactory bulb, activate specific, but partly overlapping regions in the olfactory cortex (Zou *et al.*, 2001). Although anatomical comparisons between the telencephalon in fishes and cortex in mammals are controversial due to divergence in early embryonic development, the mechanisms for the processing of the bulbar output seem to have some functional elements in common.

The fright reaction

Among social animals it is a common strategy to warn conspecifics of potential dangers. Such warnings can be transmitted by many different forms of communication, and will often lead to diverse anti-predator behavior. Fishes use odors to warn others about dangerous situations. In fish skin there is a substance or a set of substances eliciting stereotypic avoidance behavior,

the fright reaction, upon detection. This particular behavior was discovered by von Frisch (1938; 1941), who observed that European minnows (*Phoxinus phoxinus*) fled and disappeared when they were exposed to damaged fish skin, but only when their olfactory system was intact.

The fright reaction is widespread among fishes, particularly within the ostariophysian super order (Schutz, 1956), although the response varies from species to species. Typical behavioral patterns are rapid darting movements (escape-like swimming), dashing against the substrate, seeking down to the bottom and resting in an immobile posture. Shoaling and aggregation in a corner of the aquarium are characteristic collective reactions. The behaviors make the fright reaction suitable as a experimental model in studying Pavlovian conditioning and learning (Kimbrell *et al.*, 1970; Chivers & Smith, 1994; Ferrari *et al.*, 2005; Ferrari & Chivers, 2006).

The chemical identity of alarm substances is unknown in spite of extensive investigations, although recent studies indicate nitrogen-oxide to be a functional group on alarm molecules in ostariophysean species (Brown *et al.*, 2000). Alarm substances are believed to be stored in specialized epidermal cells, the club cells (Pfeiffer, 1963). These are non-secretory, which means that mechanical damage of the skin is necessary for the alarm substance to leak out and be detectable to other individuals. In both laboratory and field experiments, extract of fish skin is used to induce the reaction.

Neural pathways

The crucian carp has been applied as a model organism to identify the olfactory pathway involved in the fright reaction. Ablation of one specific bundle of the olfactory tract, the mMOT, results in loss of the fright reaction upon exposure to skin extract, the same bundle which in cod mediates defensive behavior when electrically stimulated (Døving & Selset, 1980). The fright reaction remains intact when the mMOT is left undamaged while the other parts of the olfactory tract, lMOT or LOT, are ablated (Hamdani *et al.*, 2000).

The mMOT gathers axons of secondary neurons with their cell body located in the medial part of the olfactory bulb. The posterior region is only activated by skin extracts, and thus believed to be sensitive to alarm substances (Hamdani & Døving, 2003). Furthermore, the sensory neurons projecting to this region are ciliated cells (Hamdani & Døving, 2002), which indicates that odorant receptors sensitive to alarm substances are expressed in this morphological type of neuron.

Species specificity

Fishes do not only react to skin extracts from conspecifics, but from other species as well (Schutz, 1956; Pfeiffer, 1963; Smith, 1982; Mathis & Smith, 1993; Mirza & Chivers, 2001), although extract of skin from conspecifics is usually more efficient than skin extracts from other species (Schutz, 1956; Kasumyan & Ponomarev, 1986; Døving *et al.*, 2005). The ability to detect heterospecific alarm substances may be advantageous to sympatric species with common predators.

The species specificity in the fright reaction indicates that there are several different substances eliciting the reaction and/or that the chemical structures varies from species to species. It also means that there are different types of odorant receptors involved. The behavioral responses to skin extract exposure should be reflected in the activation of secondary neurons.

The skin extract as odor stimuli

Physiological studies applying complex odor stimuli are scarce. To apply crude skin extract in investigations on the properties of the olfactory system is a realistic approach, mimicking what happens in nature when a fish detects odors from the injured individual. The skin contains a multitude of substances, including amino acids and steroids (Hay *et al.*, 1976; Saglio & Fauconneau, 1985; Ali *et al.*, 1987). These usually initiate behaviors not related to avoidance when introduced alone, but could have other functions when detected in concert with alarm substances.

Hormones as pheromones – inducers of sexual behaviors

Plasma levels of sex hormones vary throughout the reproductive season. In fishes, some of these have functions as pheromones as well, first proposed as a theory suggesting that hormones could be released and/or leak out to the surroundings, thereby informing other individuals about the reproductive status of the sender (Døving, 1976). It is now well established that gonadal steroids and prostaglandins function as sex pheromones (Dulka *et al.*, 1987; Sorensen *et al.*, 1988; Stacey *et al.*, 1989), and have influence on sexual behavior upon olfactory detection.

Hormones implicated in late stages of sexual maturation in goldfish, a close relative to the crucian carp, are particularly well studied. Females release hormones which can be

distinguished as two sets of pheromones related to reproduction; one pre-ovulatory and one post-ovulatory. The pheromones provide the male with important information about the ovulatory phase of the female. Three of the preovulatory pheromones induce distinctive courtship behavior in males upon olfactory detection (Poling *et al.*, 2001). One induces a low level of long-lasting chasing and nudging behavioral patterns, another induces more intense chasing and nudging lasting only about five minutes, and a third elicits intense aggressive behavior among males. The release of the pre-ovulatory steroids declines at ovulation, replaced by release of prostaglandins, which affect male spawning behavior (Kobayashi *et al.*, 2002; Stacey *et al.*, 2003). In accordance, male crucian carp show short followings and inspections of the anal papillae of $PGF_{2\alpha}$-injected females (Weltzien *et al.*, 2003). The different behavioral responses upon detection of female sex pheromones, requires an olfactory system in males finely tuned to respond specifically to each odor.

Other types of chemical substances work as pheromones as well. Bile salts have previously been proposed to be involved both in migration and reproduction (Døving *et al.*, 1980; Li *et al.*, 1995), as shown in sea lamprey (*Petromyzon marinus*), which are anadromous. Male individuals arriving at spawning areas send out these chemical cues to attract the females, guiding them upstream (Li *et al.*, 2003).

Sensitivity towards sex pheromones

Males and females show different responsiveness towards sex pheromones released by females. For instance in goldfish (Sorensen & Goetz, 1993), red fin shark (*Epalzeorhynchus frenatus*) and tinfoil barb (*Barbonymus schwanenfeldii*) (Cardwell *et al.*, 1995), males are more sensitive than conspecific females to prostaglandins. The same studies showed that both genders exhibit similar electro-olfactogram (EOG) responses to steroids, whereas only males have been reported to respond behaviorally to these odors. However, androgen-treatment of female round goby (*Neogobius melanostomus*) induces male-typical behavioral distinction/responses to steroids (Murphy & Stacey, 2002) which normally are absent. The behavioral responsiveness towards these pheromones therefore seems to be in correlation with plasma levels of male sex hormones.

Female sensitivity towards odors from males is poorly investigated, but was recently studied in three-spine sticklebacks (*Gasterosteus aculeatus*) and brook sticklebacks (*Culaea inconstans*) (McLennan, 2003). The behavioral response to olfactory cues from males varied in correlation to the ovulatory phase of the female (McLennan, 2005); ovulating females

showed increased responsiveness to odors from reproductively active males. The physiological mechanisms involved in increased sensitivity are unknown, but probably involve hormonal effects on the brain and/or the olfactory system.

Effect of sex hormones on non-reproductive physiology and behavior

During reproductive periods, alterations of plasma levels of sex hormones have impact on the behavioral patterns related to courtship. These variations may also affect behaviors not related to reproduction. Studies on mammals show that both males and females show less fear during mating season, which can be related to altered levels of androgens and/or estrous hormones (Boissy & Bouissou, 1994; Aikey *et al.*, 2002; Koss *et al.*, 2004; Lunga & Herbert, 2004). Increased risk-taking, such as ignoring potential predators, may be considered a trade-off where the balance between survival and reproduction is optimized.

Effect of sex hormones on non-reproductive behavior in fishes is poorly investigated; however, androgens seem to have direct impact on components related to the fright reaction. The epidermal cells (club cells) containing the alarm substances (Pfeiffer, 1963) have in several species been reported to be lost or be decreased in sexually mature males and to decrease in some females during spawning season (Smith, 1976). Histological studies indicate that testosterone reduces the amount of club cells (Smith, 1973) and thereby loss of alarm substances, meaning that the skin no longer induces fright reaction upon olfactory detection. Apparently, this loss takes place in species displaying aggressive behaviors during mating season, such as abrasive contact and fighting that could easily lead to damage of skin. Therefore, the loss of alarm substance prevents sending false information about potential predators, which could lead to interruption of spawning behavior.

Aim of thesis

The aim of my thesis has been to study how the crucian carp handles odors that are involved in two essential life processes; reproduction and predator avoidance. I have focused on two main approaches:

I

Plasma levels of sex hormones vary throughout the reproductive season, and have effect on maturation of gonads and sexual behavior. In fishes, these substances also function as pheromones, informing about the reproductive state of the sender. This has been particularly well studied in goldfish (a close relative to the crucian carp), where the female releases several pheromones, with each inducing distinct behaviors in males upon olfactory detection. The sensitivity towards sex pheromones released from individuals of the opposite sex has been suggested to vary in relation to the receiver's reproductive state or sex hormone levels. Although poorly investigated in fishes, sexual maturation may in addition have effect on behaviors not related to reproduction, as shown in many mammals. To investigate how behaviors related to, or influenced by sex hormones, may be reflected in the olfactory system, I have studied:

1) how olfactory bulb neurons respond to and discriminate between female sex pheromones, comparing males and females (Paper I)
2) seasonal variations in the olfactory epithelium by looking at the number of crypt cells, sensory neurons believed to respond to sex pheromones (Paper II)
3) whether behavioral responses to alarm odors vary in relation to reproductive status such as maturation of gonads and altered plasma levels of gonadal steroids (Paper III)

II

The fright reaction, avoidance behavior induced by olfactory detection of injured conspecifics, is widespread among teleosts. This response is elicted by alarm substances in the skin, and extracts made of skin are frequently applied to induce the behavior. Many fishes also respond to skin extracts from other species, but with varying intensity. This could be related to similar, but non-identical, chemical structures of alarm substances and different types of odorant receptors involved. The skin extracts contain in addition several odors normally involved in behaviors not related to the fright reaction. The species specificity of the

behavioral response to such a complex mixture of odors indicates precise coding of the olfactory input. This should be reflected in the nervous activity in the olfactory bulb, since the activation of the secondary neurons induces behaviors. To investigate how skin extracts from different species induce behavioral and nervous responses, I have studied:

4) the behavioral responses to skin extracts from conspecifics and cross-order species, and the correlation with activation of olfactory bulb neurons sensitive to alarm substances (Paper IV)
5) how olfactory bulb neurons respond to the different classes of odors in skin extract from conspecifics and other carp fishes (Paper V)

Methods

Experimental animals

Crucian carp (6-45 g body weight) were caught by traps in Tjernsrud and Langmyr, two small lakes 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 through-flowing freshwater, and maintained under a photoperiod of light:dark (LD) 12:12 and a temperature of 10 ^{o}C. The fish were fed three times a week with commercial pelleted feed (Modulfôr, Ewos, Norway). All experimental procedures were made in accordance with national legislation and institutional guidelines at the University of Oslo.

Single unit recordings of odor-induced activity in the olfactory bulb

The behavioral responses to odors are related to properties of the olfactory system, and should be reflected in nervous activity. Single unit recordings permit to investigate the activation of olfactory bulb neurons upon stimulation of the sensory neurons with odors, and odor-selectivity and discriminatory power can be determined. For the investigations in this thesis, the recordings were applied to compare the activation induced by different stimuli, i.e. how many units were activated by stimulus A compared to stimulus B, and how many units could distinguish between the two.

The stimulus was delivered through a polyethylene tube placed into the right anterior naris. There was a continuous flow of artificial pond water, which could be replaced by the odor solutions using miniature valves connected to the tube. Recordings were made from single units, referring to the activity of one or few neurons, in the olfactory bulb using microelectrodes made from tungsten wire (125 µm, impedance 1-2 MΩ, 1 kHz), prepared as described by Hubel (1957). The microelectrode position was adjusted by an electrical micromanipulator (SD Instruments MC 1000, CA, USA), and the signals led to a differential amplifier (DP 301, Warner Instrumental, Corp., 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 using a software program (Spike 2, version 4.04, CED Cambridge, UK).

Single unit activity represents the activation of a single neuron or few neurons with identical activity pattern. The visual appearance on the computer of the amplitude and shape of the action potentials vary with respect to the position of the electrode. The Spike program allowed sorting them out based on these parameters, thereby distinguishing between different units at the same electrode position.

The units in the olfactory bulb can be divided into two categories, type I and type II units respectively (Hamdani & Døving, 2003). In brief, type I units are believed to correspond to the activity of mitral cells, and respond by excitation; a burst of impulses, concomitant to stimulus arriving at the olfactory epithelium. Only type I units responding to at least one of the stimuli are included in the analysis, and response to a skin extract was categorized as response judged by the appearance of a burst of impulses, or no response.

Neurotracing

Neurotracing can be applied to visualize cells in the olfactory epithelium, and the different morphological types of sensory neurons can be distinguished and quantified.

Transcardial perfusions were performed with 4 % buffered paraformaldehyde. The fish were decapitated and the heads were placed in fixative (paraformaldehyde). After two days, the olfactory organ was dissected out and small crystals of DiI (1,1-dilinoleyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes, Eugene, OR, USA) were inserted by a sharp needle into the olfactory bulb. The preparations were placed in buffered paraformaldehyde and kept in dark at room temperature for five weeks. 50 µm sections were made on a Vibratome. Sections obtained were inspected with fluorescence (550 nm excitation, 565 nm emission) in a fluorescent microscope (Olympus BX50WI) and photographed by an Olympus digital camera (DP50). Some preparations were also examined in a confocal microscope (Olympus FluoView 1000, BX61W1).

Behavioral studies

The components of the behavioral response to conspecific skin extracts are well known in crucian carps. The fright reaction in this species is therefore a suitable model investigating influence of sexual maturation on non-reproductive behavior. Also, the response to skin extracts from other species can be applied to investigate the species specificity of this behavior, comparing it to nervous activity in the olfactory bulb.

Fish were transferred from the holding tank to observation aquaria (25 L) where they were kept in isolation. The experiments were performed the day after insertion, to allow the fish to acclimate to the new environment. Black curtains covering the sides of the aquarium minimized the risk of visual disturbance from the experimenter, and the fish were videotaped through a small opening in the curtain. The fish were exposed to skin extract (2 mg skin/L), injected into the water through a polyethylene tube.

Measurement of sex steroid hormones

The major sex steroid hormones in mature male and female teleost fish are 11-ketotestosterone (11-KT) and testosterone (T), and T and 17*β*-estradiol (E_2) respectively (Borg, 1994). The plasma levels of 11-KT, T and E_2 were measured by specific radioimmunoassay (RIA) according to Pall *et al.* (2002). In brief, individual plasma samples (15-50 µL) were diluted with 300µL in RIA buffer, heated at 80 °C for 60 min. The samples were centrifuged at 13000 rpm for 15 min, after which the supernatant was extracted and stored at 4 °C until being assayed. To incubation vials was added 50 µL aliquots of the sample together with 50 µL RIA buffer, 50 µL of the radiolabelled steroid (^{3}H-T and ^{3}H-E_2 was purchased from Amersham International, and ^{3}H 11-KT was a gift from Dr. A.P. Scott, CEFAS, UK) (30-35,000 dpm/50 µL) and 200 µL of steroid antiserum (a gift from Dr. Helge Tveiten, University of Tromsø). All samples were run in duplicate. The vials were vortexed and incubated overnight at 4 °C. Free, unbound steroid were separated from bound steroids with dextran charcoal suspension (Activated charcoal and Dextran T-70). Following 5 min centrifugation at 3900 rpm (GPR Centrifuge, Beckman), the supernatant was poured into scintillation vials containing 4 mL scintillation fluid (OptiPhase Hi Safe II, LKB Wallac) and run for 5 min in the counter (1214 Rackbeta liquid scintillation counter, LKB Wallac). The detection limit for the assay was c. 2 ng/mL, and the intra- and interassay coefficient of variance was 5.4 and 7.0 % respectively.

Synopsis of results

Paper I

Gender distinction in neural discrimination of sex pheromones in the olfactory bulb of crucian carp, *Carassius carassius* (2006)

Authors: Stine Lastein, El Hassan Hamdani and Kjell B. Døving

Chemical senses 31(1) p.69-77

The olfactory bulb shows a clear chemotopic organization with spatially distinct regions each responding specifically to odors with similar biological function. We demonstrate a new feature of the bulbar chemotopy in fishes showing that neurons specifically sensitive to sex pheromones released by the female are located in a central part of the ventral olfactory bulb in crucian carp. The majority of the units recorded from males responded exclusively to one of the four sex pheromones, and thus showed a very selective tuning. In females, only one unit showed such a specific activation. These findings reflect remarkable differences between males and females in the discriminatory power of the olfactory neurons towards these sex pheromones.

Paper II

Seasonal variation in olfactory sensory neurons − Fish sensitivity to sex pheromones explained? (2008)

Authors: El Hassan Hamdani, Stine Lastein, Finn Gregersen and Kjell B. Døving

Chemical senses 33 p.119-123

Crucian carps were caged in an outdoor pond exposed to the natural environment. Fishes were sampled every month and the olfactory organ was stained with the neurotracer DiI, permitting visualization of the sensory cells. The number of crypt cells in the olfactory epithelium was drastically reduced during the period from August to December from several hundred to less than 10 per olfactory rosette. When the number of crypt cells increased in March many were found close to the basal lamina of the sensory epithelium, indicating that they were not exposed to the environment. These findings demonstrate a variation in appearance of the olfactory sensory neurons in vertebrates that has not been observed before.

Paper III

Risk it all: Female crucian carp (*Carassius carassius*) lose predator avoidance when getting ready to mate

Authors: Stine Lastein, Erik Höglund, Ian Mayer, Øyvind Øverli and Kjell B. Døving

In June, crucian carps were exposed to conspecific skin extract, followed by behavioral analysis and determination of gonadal maturation. The majority of individuals not responding to alarm substances with a fright reaction were sexually mature. In females, mean plasma concentrations of 17β-estradiol and testosterone, gonadal steroids known to decrease during the later stages of sexual maturation, were lower in the individuals not responding with a fright reaction compared to those responding. In males, there were no differences between responsive and non-responsive individuals in mean plasma levels of androgens involved in spermatogenesis and male sexual behavior, testosterone and 11-ketotestosterone. As the fright reaction in crucian carp consists of behavior incompatible with spawning behavior, we hypothesize that this short-term suppression of the fright response has evolved so that spawning can occur uninterrupted.

Paper IV

Exposure of crucian carp (*Carassius carassius*) to skin extracts from conspecifics and cross-order species: Correlation between behavioral and neurophysological responses

Authors: Stine Lastein, Ole B. Stabell, Helene K. Larsen and Kjell B. Døving

We exposed crucian carp to skin extract from conspecifics and three cross-order species of fish (brown trout, perch, and pike), followed by behavioral analysis (in February) as well as single unit recordings from olfactory bulb neurons. Skin extract from cross-order species of fish induced behavioral fright reactions, but less frequent than conspecific skin extract. The difference in behavioral responses obtained with skin extracts correlates with a variation in the activation of olfactory bulb neurons located in the region involved in the fright reaction. In addition, we found several neurons responding to heterospecific, but not to conspecific skin extract. This indicates that crucian carp are able to detect and distinguish between alarm substances with different chemical structures, and recognize these olfactory cues as potential danger.

Paper V

Deciphering complex odors in the fish olfactory bulb

Authors: Stine Lastein, El Hassan Hamdani and Kjell B. Døving

Fish skin contains odors that induce the fright reaction. Additionally, other odors related to feeding and sexual behaviors are present in the fish skin. Responses of olfactory bulb neurons to different types of odors in skin extracts from conspecifics and three other species from the carp family (common carp, tench and bream), were compared. Recordings from single units were made in three functionally distinct regions of the olfactory bulb; the alarm region, the pheromone region and the food-related region, where the skin extracts activated units in all regions. The majority of units responding only to one of the skin extracts were sensitive to conspecific skin extract. Furthermore, the discrimination between conspecific skin extract versus skin extracts from other species was in general better than between skin extracts from two heterospecifics. The results demonstrate that pheromones and food related odors from the skin are detected in addition to the alarm substances, possibly functioning as complementary odors. The diluted conspecific skin extract was well distinguished from the other species in all the three regions of the bulb. This demonstrates that pheromones and food related odors from the skin are detected in addition to the alarm substances, possibly functioning as complementary odors. The identification of injured fishes may therefore be based on different functional groups of odors.

Discussion

Distinction between functionally related odorants

Chemical communication between individuals demands great accuracy in the process of interpreting messages. Distinguishing one important odor from another is often advantageous; individuals with this property will be favored from an evolutionary point of view. Male fishes with a high discriminatory power between sex pheromones released by females (Paper I) have probably had higher reproductive success than males lacking this quality. Knowing exactly when the females release eggs in the water enables appropriate timing of their own reproductive behavior. The benefit of a higher chance of fertilizing eggs has exceeded the cost of developing and maintaining this discriminatory capacity in the olfactory system. The females on the other hand, which do not discriminate as well between female pheromones (Paper I), have had no advantage of developing such a characteristic. Their ability to detect pheromones from other females have been suggested to be related to synchronization of reproduction, assumingly reducing the predation risk (Lima & Dill, 1990). The findings regarding sex pheromone discrimination can be seen in context with previous studies on the goldfish pheromone system, where the reproductive status of the female has influence both on the maturation of male gonads and male behavior related to courtship (Poling *et al.*, 2001; Kobayashi *et al.*, 2002; Stacey, 2003).

A good discriminatory capacity signifies importance of a precise processing of the messages. However, not all important messages are subject to extreme distinction. The same level of precision was not observed in crucian carp when exposing the fish to skin extracts from different species (Paper IV and V). The unique neurons encountered (responding to only one of the skin extracts tested) could play a key role in recognizing conspecifics from other sympatric species. Nevertheless, the majority of neurons responded to more than one skin extract. The alarm substances, although probably with different chemical structures, carry the same message; potential danger. The phylogenetic relationship between donor and receiver is of little significance, as long as the threat is relevant. Less discriminatory receptor neurons may in fact be an advantage; increased survival. More broadly tuned odorant receptors means that the alarm neurons will be activated by several types of alarm substances, regardless of the species. Thus, excellent discrimination is not necessarily favorable.

Deciphering complex odors

The use of crude skin extract as stimuli in single unit recordings probably mimics the actual events taking place in nature. Here, the fish is exposed to a composite mixture of odors, resulting in activation of different parts of the olfactory bulb. The response in regions involved in reproduction and feeding is noteworthy (Paper V), demonstrating detection of compounds similar to the steroids and amino acids known to be present in the fish skin (Hay *et al.*, 1976; Saglio & Fauconneau, 1985; Ali *et al.*, 1987). The widespread activation of bulbar neurons upon skin extract exposure shows that these odors are present in significant amounts and thus detected simultaneously and in parallel with the alarm substances.

What does it mean that several regions of the olfactory bulb are activated simultaneously? In this concert of olfactory cues, the fish is still able to sort out the essential message. The characteristic organization of the olfactory system might enable the fish to take advantage of the various components of the skin. Odors normally related to non-predator situations may function as complementary odors to the alarm substances, for instance informing about the diet of the donor, as previously proposed (Saglio & Blanc, 1989). The functional significance of this is possibly most evident when detecting skin from other species. Behavioral responses to predators may be acquired or learned by previous experiences, enabling the association of odors from another prey fish with a threat (Brown & Smith, 1998; Chivers *et al.*, 2002; Darwish *et al.*, 2005; Ferrari *et al.*, 2005).

Physiological studies on vertebrates using natural complex stimuli are scarce. In recent studies on rodents, Lin and co-workers applied natural blends of odors, such as urine and food to investigate neural responses in the main olfactory bulb (2005; 2006). Both extracellular recordings and imaging-techniques were applied in combination with gas chromatography (GC), enabling the separation and identification of the single odors in the stimuli and their respective activation pattern. The authors conclude that the bulbar response to a complex mixture in general reflects the sum of responses to each of the components. Whether this also is representative for the detection of the skin extracts in the fish olfactory bulb, remains to be shown. The resemblance of the organization of the olfactory system among vertebrates suggests that the mechanisms of decoding and processing complex odors are similar, even though the fish olfactory system differs from terrestrial animals.

In attempt to identify the alarm substances of fish, a similar approach to studies performed with rodents have been conducted with skin extract, recording from the alarm region of the olfactory bulb in crucian carp (Brondz *et al.*, 2004). The nature of the stimuli required high-performance liquid chromatography (HPLC), which is more complicated and

long lasting compared to than the GC, but demonstrated the presence of unknown substances with very high ability to activate neurons; potentially the alarm substances. In recent investigations, we further approached this by applying HPLC to fraction the different components of highly concentrated skin extracts, and behavioral tests indicate that there may be different chemical compounds needed to induce a fully expressed fright reaction (Antonietta Labra and Tobias Beckström; personal communication). To find the chemical identity of alarm substances would enable examination of how this important predation cue is detected and interpreted, and thereby also help understanding the function of other odors involved the fright reaction.

Finally, studies on the auditory and visual cortex show that complex and/or natural stimuli do not necessarily reflect the sum of responses to simple stimuli (reviewed by Kayser *et al.*, 2004; Nelken, 2004). This demonstrates that interpretation of composite sensory input takes place in higher brain centers. The same feature might be valid for olfactory cortex or equivalent brain centers in fishes, pointing toward the high relevance of using natural stimuli in physiological and behavioral studies.

The activation of olfactory bulb neurons

Many biologically relevant odors are known in fishes, and have enabled mapping the chemotopic organization of the olfactory bulb (Figure 5). Extracellular recordings from the region responding to sex pheromones was a contribution to the series of investigations on the unitary responses upon stimulation of the olfactory epithelium (Nikonov & Caprio, 2001; Hamdani & Døving, 2003; Nikonov & Caprio, 2004). The neurons showed unique properties not previously observed. The outstanding distinction between female sex pheromones in the male neurons is exceptional compared to the discriminatory capacity of other known odorants. Even though the glomerular organization is morphologically less defined (Oka, 1983; Riddle & Oakley, 1992) than in higher vertebrates, this demonstrates that the fish olfactory bulb neurons are capable of passing on information to the brain in an accurate way.

The known chemotopy of the fish olfactory bulb is a valuable tool in investigations of secondary neuron activity. This allows comparing the activation induced by crude skin extract and activation induced by pure and known odorants (Paper V). No obvious differences were observed between the activity patterns induced by the two types of stimuli, thus, it is possible to roughly predict the amount of the different food-related and pheromone-related odors in the skin.

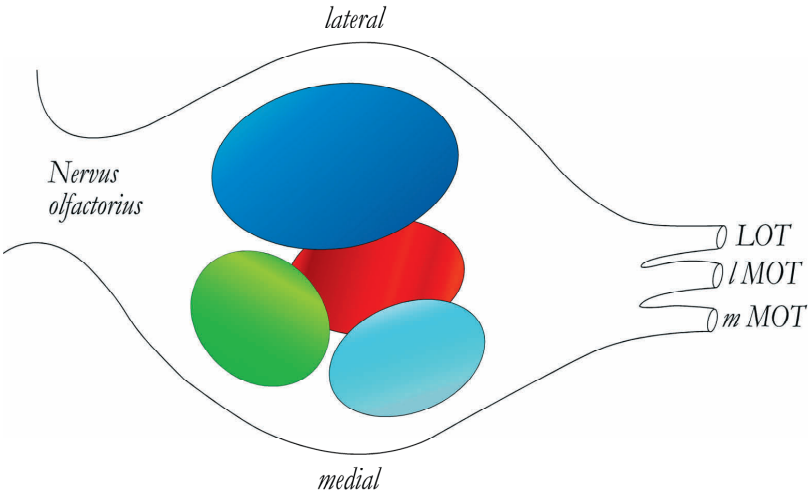

Figure 5. Functional distinct regions in the olfactory bulb of crucian carp. Dorsal view of the olfactory bulb in crucian carp, showing regions responding specifically to blue, food-related odors; red, sex pheromones; green, bile salts; and light blue, alarm substances. LOT, lateral olfactory tract; lMOT, lateral bundle of the medial olfactory tract; mMOT, medial bundle of the medial olfactory tract.

Extracellular recordings reveal two distinct types of neural activity in the olfactory bulb. These have previously been described in detail as type I and type II units respectively (Zippel *et al.*, 2000; Hamdani & Døving, 2003). Type I units are believed to be activity of the mitral cells, the secondary cells. The type II units are believed to be activity of the ruffed cells, and work in contrasting interactions with type I units. Recorded simultaneously from one electrode position, type I units are active when type II units are silent, and vice versa. It was previously proposed that type II units on type I activity could be significant, possibly modulating responses to odorants. For instance, they may interfere with the duration of the induced activity in type I units, which ranged between 5 and 70 s upon stimulation, which always lasted 10 s (Paper I, IV and V). Modifying the mitral cell firing patterns could have significant impact on behavioral responses, as the activation are inducers of behaviors (Døving & Selset, 1980).

Whether there are distinctions between secondary neurons in functionally different parts of the olfactory bulb, remains to be investigated. There are morphological variations between mitral cells in the medial and lateral part. Possibly, there are electrophysiological differences between sub-populations as well, related to their spatial location and function. No obvious differences were observed during recordings in the olfactory bulb of crucian carp, however, such a possibility should not be excluded (Paper V).

Variations in the olfactory system

A response to an odor is often context dependent and determined by several aspects in attempt to maximize fitness (Lima & Dill, 1990). Behavioral patterns such as feeding, reproduction, migration and avoidance may be induced directly by odors, but their presence do not always lead to the same response. Some species responding with a fright reaction upon detection of conspecific skin extract as juveniles, lose this behavior on a permanent basis as adults (Marcus & Brown, 2003; Harvey & Brown, 2004). The loss of response to the skin extract in crucian carp related to ovulation and altered plasma levels of female sex hormones is probably temporary, reflecting that this species has an advantage of the fright reaction under non-reproductive periods, but not during courtship behavior (Paper III).

Risk taking behavior seems unfavorable from an evolutionary point of view, since predation eliminates all future chances of producing offspring. However, behaviors related to reproduction are probably incompatible with the fright reaction, as release of alarm substance during male competition could interrupt the spawning. Thus, during the latest phases of sexual maturation of female crucian carps, the behavioral response to potential dangers is supressed in order to fulfill an essential biological process – reproduction.

How does reproductive status have effect on the behavioral responses to odors related to danger? Central mechanisms are likely to be involved in increased risk-taking during mating season. Expression of neurotransmitters and receptors in the mammalian brain, which are involved in anxiety-related behavior, are under influence of steroids (Biegon & McEwen, 1982; Neumann *et al.*, 2000; Wood *et al.*, 2001; Bowman *et al.*, 2002; Isgor *et al.*, 2003; Lunga & Herbert, 2004; Douglas *et al.*, 2005). Alterations in plasma levels of female sex hormones in fishes could have similar effects on the crucian carp in late maturation stages.

Sensory neurons are rapidly replaced in fishes (Zippel *et al.*, 1997a), and recruitment or functional changes of new sensory neurons could possibly account for altered behavioral response to an odor. The remarkable variations in expression of one type of the sensory cells in crucian carp, numerous in the summer while almost absent during the wither months, is so far a unique feature not shown in any other adult vertebrates (Paper II). These cells are probably involved in detection of sex pheromones in this species. Application of the neurotracer DiI on the ventral part of the olfactory bulb resulted in a staining both of the crypt cells and of the axons in lMOT (Hamdani & Døving, 2006), the bundle of the olfactory tract mediating sexual behavior. However, studies on species phylogenetically distant to crucian carp indicate other functions as well. In the marine fish Pacific jack mackerel (*Trachurus symmetricus*) crypt cell were found to respond to amino acid solutions, although steroids and

prostaglandins were not tested as stimuli (Schmachtenberg, 2006). Furthermore, their presence in juvenile thornback ray (*Raja clavata*) may indicate functions related to non-reproductive behavior (Ferrando *et al.*, 2007).

What happens to the secondary neurons wired to sensory neurons that are temporarily lost? The olfactory bulb is a composite network of different types of neurons, where plasticity could account for behavioral changes upon odor detection. Re-wiring of the synaptic connections between sensory and secondary neurons, or re-arrangement of interneurons, may take place. Whether the other sensory cell types undergoes similar cyclical changes as the crypt cells, remains to be shown. A similar event might for instance take place upon the loss of the fright reaction during ovulation. Altered sensitivity and behavioral responses to odors might implicate both peripheral and central components, and may be more important than previously considered.

Conclusion

A large majority of olfactory bulb neurons in males responded specifically to only one of four sex pheromones released by females, a property not observed in neurons in females. In females, sexual maturation seems to increase risk-taking behavior, as the behavioral response to alarm odors is suppressed. Hormonal variations may play a major role, with influence on both central and peripheral mechanisms, such as the alterations in crypt cell expression. Furthermore, the discrimination in the olfactory bulb between skin extracts from different species varied largely between neurons, and could be seen in relation to behavioral responses, indicating species specific alarm substances and odorant receptors. Odors in the skin normally involved in feeding and reproduction were detected, and could possibly function as complementary odors.

The present findings point towards several important features of the fish olfactory system. The neurons are tuned to respond to different odorants but the discrimination between related messages is highly varying. Simultaneous detection of multiple odorants mimics what happens in natural environments, and shows that the fish is able to sort out the most essential information, according to the context, from a complex mixture. Although these detections involve functionally distinct bulbar regions, a final cognitive process in the higher brain regions is able to adequately interpret the messages and adapt the behavior. Finally, structural modulations in the olfactory system, such as the expression of sensory cells, and adaptation of behavioral responses to some significant odors, show that this plasticity is central in this sensory system, which therefore seems highly adjustable to different phases in the life of an adult fish.

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Corrigenda

Page 20 and Paper III. Title and reference:

Seasonal variation in olfactory sensory neurons − Fish sensitivity to sex pheromones explained? (2008). *Chemical senses* **33,** 119-123.

Page 35 and Paper IV. Reference:

Nikonov AA & Caprio J. (2004)….*Journal of neurophysiology* **92,** 123-134.

Paper V. Page 10:

In sub-set 1…and in sub-set 2 from less than 0.03 to 1.6 spikes per second…

Page 12:

Pheromone region… activated units (n = 51) was considerably lower…