BEHAVIORAL, ELECTROPHYSIOLOGICAL AND IMMUNOHISTOCHEMICAL INVESTIGATION OF THE OLFACTORY SUBSYSTEMS THAT MEDIATE THE DETECTION OF ODORANTS AND PHEROMONES IN SALMONID FISHES

by

FRÉDÉRIC LABERGE

A thesis submitted to the Faculty of Graduate Studies

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Zoology

University of Manitoba

Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Abstract

Pheromones and odorants modify behavior in fundamentally different ways. The reflex innate effects of pheromones contrast with the need for memory in learned odorant discrimination. Since both type of signals are detected by the olfactory system in fish, the present work attempted to highlight differences between reproductive pheromone and odorant signal processing by the early olfactory system. To this end, an investigation of the behavioral responses of salmonid fish exposed to putative reproductive pheromones, F-prostaglandins (PGFs), or food odors was performed as well as an electrophysiological study of the mechanisms involved in the detection of these olfactory signals. Additionally, an immunohistochemical study of the neurons activated by reproductive pheromones or food odors was attempted. The results show that PGFs and food odors trigger distinct behaviors in individually exposed fish. Further, the detection of PGFs is accomplished by a unique high affinity receptor, while several receptor types are involved in odorant detection. The second-order neurons integrating PGF or odorant information are specific for a chemical class and located in different regions. The PGF-responsive neuron population of lake whitefish does not exhibit the synchronized oscillatory activity common to odorant responses in the olfactory bulb, and remains activated well beyond stimulus duration. The PGF-responsive neuron population of lake whitefish does not express gonadotropin-releasing hormone and potentially receives direct input from olfactory sensory neurons. The results suggest that two distinct olfactory subsystems are responsible for pheromone and odorant signal processing in salmonid fishes. The pheromone subsystem could be a precursor of the vertebrate vomeronasal organ.

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Abbreviation list

17, 20P: 17α, 20β-dihydroxy-4-pregnen-3-one

Amino acids: Ala (L-alanine), Arg (L-arginine), Asp (L-aspartic acid), Cys (L-cysteine), Gln (L-glutamine), Glu (L-glutamic acid), Gly (L-glycine), His (L-histidine), Lys (L-lysine), Met (L-methionine), Ser (L-serine), Trp (L-tryptophan) and Tyr (L-tyrosine)

ANOVA: analysis of variance

BSA: bovine serum albumin

cAMP: cyclic adenosine monophosphate

cRNA: complementary ribonucleic acid

DL: dorsomedial bundle of the MOT

ECG: etiocholan-3α-ol-17-one glucuronide

EEG: electroencephalogram

EOG: electro-olfactogram

EPSP: excitatory post-synaptic potential

GABA: γ-aminobutyric acid

GnRH: gonadotropin-releasing hormone

GtH: gonadotropic hormone

ICI: independent component index

LOT: lateral olfactory tract

MDI: mixture discrimination index

MOT: medial olfactory tract

NMDA: n-methyl d-aspartate

NOR: nucleus olfacto-retinalis

OR: odorant receptor

ORN: olfactory receptor neuron

PBFS: phosphate-buffered fish saline

PGF: F-prostaglandin: $15K_{1\alpha}$ (15-keto-$PGF_{1\alpha}$), $15K_{2\alpha}$ (15-keto-$PGF_{2\alpha}$), $15Kdh_{2\alpha}$ (13, 14-dihydro-15-keto-$PGF_{2\alpha}$) and $dh_{2\alpha}$ (13, 14-dihydro-$PGF_{2\alpha}$)

POA: preoptic area

TCA: taurocholic acid

TN: terminal nerve

VM: ventromedial bundle of the MOT

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CHAPTER 1

Fish Olfaction: A Literature Review

1.1. Introduction

Sensory systems perceive important features of the internal and external environments. Contrary to terrestrial vertebrates, including humans, fish chemical senses detect only compounds dissolved in the surrounding water. Consequently, the substances perceived by fish chemoreception are mostly small molecules with high aqueous solubility. The chemical senses of fish consist of olfaction, gustation, and the less well defined senses represented by the general chemical senses and the solitary chemosensory cells (Hara, 1992; Kotrschal, 1995; Finger, 1997). Here, the focus will be on olfaction.

Olfaction plays a role in a variety of fish behaviors, including feeding (Atema et al., 1980; Kamchen and Hara, 1980; Valentinčič and Caprio, 1994; 1997), reproduction (reviewed in Sorensen, 1992), migration (Hasler and Scholz, 1983; Li et al., 1995), kin recognition and predator avoidance (reviewed in Myrberg Jr., 1980; Olsén, 1992; Smith, 1992). Behavioral studies of the role of olfaction have used lesion or blockage of the olfactory organ to create anosmic animals. Olfaction was deemed to mediate a response when anosmic fish did not react to an odor exposure previously demonstrated to be behaviorally effective in normal fish, or did not perform an olfactory discrimination task readily done by control fish. Another approach used to assess the role of olfaction in social interactions of fish has been to observe the performance of anosmic fish in a spawning situation. Anosmic males of kokanee salmon (*Oncorhynchus nerka*), rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) have lower sex hormone levels compared to intact males when allowed to

interact with females (Liley et al., 1993; Olsén and Liley, 1993; Olsén et al., 1998). These males also show behavioral deficiencies in spawning, which could be caused by the lack of detection of chemical cues from the female or the low sex hormone levels. These results, combined with the previous finding of Dulka et al. (1987) that female goldfish (*Carassius auratus*) release a steroid capable of increasing the milt response of males, demonstrate that olfactory signals can influence fish physiology as well as behavior.

One aspect of fish biology that has received considerable attention lately is the role played by pheromones in fish communication. Pheromones were first defined by Karlson and Lüscher (1959) as "substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behavior or a developmental process". This definition was intended to differentiate this class of biologically active substances from hormones, and other behavior 'releasers' such as visual, acoustic, electrical or mechanical stimuli. Wilson and Bossert (1963) introduced the traditionally used pheromone categories of 'primers', which primarily influence physiological processes, and 'releasers', which have immediate behavioral effects. Recent progress showing effects of goldfish female pheromones on both physiology and behavior raises the question whether the terms 'primers' and 'releasers' are adequate to categorize vertebrate reproductive pheromones (Sorensen et al., 1998). Sorensen and collaborators (1998) introduced a new definition that distinguishes pheromones from other substances that affect behavior, and distinguishes the pheromonal blend from its components. They defined pheromones as "substances, or specific mixtures of

substances, that are released by individual organisms into the environment, where they evoke specific and adaptive biological responses in conspecifics, and the expression of which does not require active learning".

Four main classes of chemicals are known to be detected by the fish olfactory sense: amino acids, gonadal steroids, bile acids and prostaglandins (Hara, 1994). Recent electrophysiological results showing that a zone of the channel catfish (*Ictalurus punctatus*) olfactory bulb is preferentially responsive to nucleotides suggest that these chemicals would form an additional odorant class (Nikonov and Caprio, 2001). The aforementioned compounds are non-volatile chemicals that humans typically do not smell. The four main chemical classes mentioned above are detected by separate olfactory receptors/transduction mechanisms (Hara, 1994; Michel and Derbidge, 1997). Electrophysiological evidence further suggests that multiple receptor types exist within all of these classes (amino acids: Caprio and Byrd Jr., 1984; Ohno et al., 1984; Caprio et al., 1989; Sveinsson and Hara, 1990b; Kang and Caprio, 1991, bile acids: Zhang and Hara, 1994; Li and Sorensen, 1997, prostaglandins: Sorensen et al., 1988, steroids: Sorensen et al., 1991b; 1995).

The majority of L-enantiomers of naturally occurring amino acids are effective olfactory stimulants in teleosts (Hara, 1994). Examination of the research done in many different fish species shows that amino acids are proposed to be involved in all of the aforementioned roles in fish behavior (see Hara, 1994). Because of the difficulty of isolating olfaction from gustation in behavioral studies of fish, the role of chemosensitivity to amino acids in fish behavior is not well understood. Strong evidence exists, however, that amino acids contribute to the stimulation of feeding

behaviors induced by food extracts in fish (Carr et al., 1977; Fuke et al., 1981; Ellingsen and Döving, 1986; Olsén et al., 1986; Mearns et al., 1987). The previous observation also applies to herbivorous fish (Johnsen and Adams, 1986). Experimentally induced anosmia results in the suppression of behaviors induced by food extracts in yellowfin tuna (*Thunnus albacares*) and lake whitefish (*Coregonus clupeaformis*) suggesting that the responses are mediated through olfaction (Atema et al., 1980; Kamchen and Hara, 1980). An amino acid-conditioned stimulus can trigger arousal and feeding behavior through olfaction in adult rainbow trout (Valentinčič and Caprio, 1997). Prior to the first exogenous feeding, salmonid alevins show a reflex biting/snapping behavior in response to amino acid stimuli that is mediated by the taste system (Valentinčič et al., 1999). The subsequent appearance of a full sequence of feeding behavior triggered by olfaction or vision is learned. The involvement of learning in food olfaction is indicated by experiments showing that maximal feeding responses are induced by food odors that fish have experienced, even if these odors are not from natural prey (McBride et al., 1962; Atema et al., 1980). It has also been demonstrated that fish can be conditioned to discriminate between amino acids through olfaction but not gustation (Little, 1981; Zippel et al., 1993; Valentinčič et al., 1994). As additional evidence that amino acids are involved in feeding behavior, it has been shown that the reappearance of olfactory sensitivity to amino acids is correlated with the return of behavioral responsiveness to food odors in goldfish with a regenerating olfactory system (Zippel et al., 1997a).

Some steroid hormones or their conjugates are detected by highly specific olfactory mechanisms in some species. Ovulatory female African catfish (*Clarias*

gariepinus) are attracted by a steroid glucuronide conjugate pheromone secreted by males (Resink et al., 1989). Additionally, 17α, 20β-dihydroxy-4-pregnen-3-one (17, 20P) is a female goldfish pre-ovulatory reproductive pheromone that enhances male milt production by triggering a gonadotropic hormone surge (Dulka et al., 1987). Interestingly, recent results show that the goldfish steroid pheromone system is more complex than originally believed. The female goldfish releases several pheromonally active gonadal steroids and their conjugates around ovulation in ratios that change with the ovulatory state of the female (Scott and Sorensen, 1994). Three steroids particularly effective as olfactory stimulants were shown to have different effects on male goldfish reproductive and aggressive behavior as well as gonadotropic hormone secretion (Poling et al., 2001). These results supported a previous suggestion that some fish pheromones could act on both reproductive physiology and behavior (Sorensen et al., 1998). Two species of carp closely related to goldfish also detect 17, 20P with a high sensitivity, suggesting that steroid olfaction could be a common feature among cyprinid fishes (Bjerselius and Olsén, 1993; Irvine and Sorensen, 1993). Gonadal hormones or their metabolites excreted through urine would signal the reproductive status of one fish to another through olfaction, from which a hypothesis of chemical spying has been proposed (Liley and Stacey, 1983; Sorensen and Scott, 1994).

The olfactory function of bile acids is not well understood, but it has been proposed that they may be involved in the migration of adult sea lamprey (*Petromyzon marinus*) to spawning streams (Li et al., 1995; Li and Sorensen, 1997). A recent report describes for the first time a bile acid acting as a reproductive pheromone in sea lamprey (Li et al., 2002). Note that sea lamprey only detect one naturally occurring

amino acid, arginine (Li and Sorensen, 1992); thus the use of bile acids in reproduction might be a specialization of early vertebrates and might not apply to teleosts. Some bile acids are also known to alter the behavior of many fish species when present in the surrounding water (see Hara, 1994). Zhang et al. (2001) recently demonstrated that several bile acids released by lake charr (*Salvelinus namaycush*) are extremely potent olfactory stimulants.

F-series prostaglandins (PGFs) are likely involved in triggering follicular rupture during ovulation in fish (Sorensen and Goetz, 1993; Goetz and Garczynski, 1997). A mixture of $PGF_{2\alpha}$ and 15-keto $PGF_{2\alpha}$ released into the water acts as a post-ovulatory pheromone in goldfish. PGFs stimulate principally male reproductive behavior in contrast to 17, 20P, which acts chiefly by priming the male reproductive system to increase available milt (Sorensen et al., 1989; 1998). A pheromonal action of PGFs is also strongly suspected in two other fish species. Female cobitid loach (*Misgurnus anguillicaudatus*) appear to release PGFs that induce male sexual behavior (Kitamura et al., 1994a). Mature male Arctic charr (*Salvelinus alpinus*) release PGFs to attract females and stimulate their spawning behavior (Sveinsson and Hara, 1995). It has also been reported that ovulatory female Atlantic salmon (*Salmo salar*) release PGFs in urine, enhancing the reproductive physiology of males (Moore and Waring, 1996). However, a recent report contradicts the previous finding by affirming that the priming chemical of Atlantic salmon is not a PGF (Olsén et al., 2002). The demonstration of PGF olfactory sensitivity in many other fish species suggest that these chemicals may be used commonly as reproductive pheromones in some fish groups (Stacey et al., 1994).

1.2. Anatomical features of the fish olfactory system

1.2.1. Peripheral olfactory organ

Before going further into mechanisms responsible for coding of olfactory information, I will give an overview of the anatomy of the fish olfactory system. In teleosts, a paired olfactory organ is located in the dorsal part of the snout. Each olfactory organ consists of an olfactory cavity (chamber) connecting with the exterior through two openings, the anterior and posterior nares, which direct a flow of water through the organ as the fish swims. The olfactory rosette, composed of a series of olfactory lamellae, is raised from the floor of the olfactory cavity. The rosette morphology and number of lamellae vary interspecifically. The lamellae consist of sensory and non-sensory epithelia. The olfactory receptor neurons (ORNs) are situated within the sensory epithelium. The sensory neuron extends a dendrite to the surface of the epithelium, bearing either cilia (ciliated olfactory neurons) or microvilli (microvillar olfactory neurons). The presence of these two neuron types is a characteristic feature of teleost fishes (see below). An additional cell type believed to be an ORN, the crypt cell, is present in the sensory epithelium of some fish groups (Hansen and Finger, 2000). The axons of the millions of ORNs converge to form the olfactory nerve, cranial nerve I (Yamamoto, 1982). It is worthy of mention that the ORNs are replaced continuously, or following chemical or axonal lesions (Evans and Hara, 1985; Saucier and Astic, 1995). In fish, as well as in other vertebrates, the ORN axon projects directly to the olfactory bulb without branching (sometimes over long distances) to synapse with dendrites of second-order olfactory neurons, mitral cells. Some species, like goldfish, have pedunculated

olfactory bulbs in close connection with the epithelium in the periphery. Other species, like salmonids, have sessile olfactory bulbs in close contact with the telencephalon. The ORN nerve terminals and the apical dendrites of mitral cells form compartmental structures, called glomeruli, in which integration of olfactory signals is thought to occur. Unlike higher vertebrates, the mitral cells of fish receive input from more than one glomerulus and can also contact diffuse ORN terminal fields (Satou, 1990; 1992). These aglomerular plexuses, also called brush-like terminals, seem to be unique to fish (Riddle and Oakley, 1992; Korsching et al., 1997). The fish pattern of mitral cell glomerular innervation is reminiscent of the mammalian accessory olfactory bulb where a single mitral cell contacts many glomeruli (Allison, 1953; Belluscio et al., 1999; Buck, 2000).

Another neuronal system that has been proposed to be chemosensory in fish is the terminal nerve (TN): a group of neurons with somata in the TN ganglion (or ganglia) situated in the ventral olfactory brain with an efferent connection to the olfactory organ that is not well defined. Its identification is subject to confusion. Not all ORN axons terminate in the olfactory bulb. In many fish species, a small subset of primary olfactory neurons project directly to the forebrain, entering the telencephalon in its ventromedial part (Bazer et al., 1987; Honkanen and Ekström, 1990; Riddle and Oakley, 1991; Szabo et al., 1991a; Becerra et al., 1994; Hofmann and Meyer, 1995). This extrabulbar primary olfactory pathway may previously have been mistaken for the projection fibers of the TN (Demski and Northcutt, 1983; Levine and Dethier, 1985). Both of these studies used two different sites of neurotracer application to reach their conclusions on the anatomy of the TN neurons. Tracer applied to the olfactory

epithelium did not label cell bodies belonging to the TN ganglion, whereas tracer applied to the optic nerve regularly filled cells in this ganglion. In fact, the TN does not appear to be a chemosensory structure, at least in the case of its rostral ganglion (Fujita et al., 1991). This implies that TN dendrites end in the basal membrane of the olfactory epithelium, not in contact with the external environment (see Demski, 1993). To further complicate the situation, it has been suggested that the TN complex could be composed of more than one ganglion of cells (Demski, 1993). One such ganglion is situated in the ventromedial olfactory bulb or the rostral telencephalon and has been referred to as the nucleus olfacto-retinalis (NOR), because it sends projections to the retina (Münz et al., 1981; Springer, 1983). However, some authors do not consider the NOR as part of the terminal nerve complex (Szabo et al., 1991b; see also Demski, 1993). Anatomical research suggests that neuron groups originally identified as part of the TN (maybe NOR?) receive input from primary olfactory fibers in the platyfish and three-spined stickleback (Schreibman and Margolis-Nunno, 1987; Honkanen and Ekström, 1990).

1.2.2. Forebrain projections of olfactory neurons

The projections of the second-order neurons of the olfactory bulb form the bulk of the olfactory tract, which is divided into the medial (MOT) and lateral (LOT) olfactory tracts. The LOT is mainly formed by projection fibers from the lateral part of the olfactory bulb, while the MOT carries most fibers from the medial olfactory bulb in carp, *Cyprinus carpio* (Sheldon, 1912). In agreement with the anatomical observations, neurons activated antidromically by LOT volleys are located mainly in the lateral part of the olfactory bulb, while those activated by MOT volleys are located mainly in the

medial part of the bulb (Satou et al., 1979; 1983). This has led to the concept of lateral and medial divisions of the olfactory system (reviewed in Satou, 1990). This division was also seen at the level of the olfactory epithelium. Some studies of other fish do not support this view (Riddle and Oakley, 1991; Ngai et al., 1993b; Hara and Zhang, 1996), while others point to a possible division within each rosette (Thommesen, 1982; 1983). The olfactory tract transmits odor information to the telencephalic hemispheres. Although there are some variations among fish species, the LOT usually projects to the dorsal part of the posterior telencephalon, with a smaller ramus sometimes running ventrally in the telencephalon (Levine and Dethier, 1985; Resink et al., 1989; Matz, 1995). The MOT has two major rami, the dorsolateral (DL) and ventromedial (VM) bundles. VM exclusively innervates the ventral telencephalon, preoptic area (POA) and hypothalamus. DL terminates in various regions of the dorsal and ventral telencephalon as well as the POA and hypothalamus. A significant portion of the olfactory tract fibers project contralaterally through different commissures. Centrifugal connections from the telencephalon to the olfactory bulb also run in the medial part of the MOT. Species with pedunculated olfactory bulbs have olfactory tracts that are readily accessible for lesion or recording. It has been demonstrated that lesions of the MOT prevent the effects of pheromone exposure in the African catfish and goldfish (Stacey and Kyle, 1983; Resink et al., 1989), while electrical stimulation or severing of the LOT affects feeding behavior (Döving and Selset, 1980; Stacey and Kyle, 1983).

The central neural pathways of the fish olfactory system are quite different from what is seen in mammals (see Eisthen, 1997 for a discussion of the evolution of the vertebrate olfactory systems). The existence of an anterior olfactory nucleus precursor

in fish is controversial (discussed in Satou, 1990); it may be represented by a population of cells in the deep layer of the olfactory bulb that project to the telencephalon (Matz, 1995). These third-order neurons in the olfactory bulb have not been clearly identified. Also, the relay between the amygdala and the hypothalamus is not necessary in fish where olfactory fibers project directly to the POA and hypothalamus. One common point between fish and mammals, however, is that little is known about how sensory information about different odorants is organized in the cortex (see Buck, 1996). The role of the rich olfactory pallial projections in teleost fishes, or the homologous regions they would represent in other vertebrates are also not yet known (Butler and Hodos, 1996). It is interesting that so far no thalamic relay of olfactory information has been demonstrated in fish.

1.3. Peripheral signal processing

1.3.1. Odorant receptors

Recently, genes thought to code for odorant receptors (ORs) have been cloned in rat and many other animals including fish (Buck and Axel, 1991; Ngai et al., 1993b; Weth et al., 1996; Cao et al., 1998; Naito et al., 1998; Sun et al., 1999; Yasuoka et al., 1999; reviewed in Mombaerts, 1999; Asano-Miyoshi et al., 2000). These OR genes expressed in ORNs represent the largest known multigene family of G-protein-coupled receptors and share sequence similarities with the metabotropic glutamate and vomeronasal receptors of mammals. OR genes are also divided into two groups in goldfish on the basis of sequence similarities. The goldfish OR genes seem to be distributed differentially between two cell types, the ciliated and microvillous ORNs, which could

be related to a functional difference between them as discussed below (Cao et al., 1998). The function of the product of these genes in odorant transduction has been demonstrated by expression in surrogate cells or the sensory neurons themselves (Raming et al., 1993; Zhao et al., 1998; Speca et al., 1999). An extensive review of the receptor transduction mechanisms has been made by Schild and Restrepo (1998). Briefly, ORN excitatory or inhibitory responses (Kang and Caprio, 1995a for fish) associated respectively with depolarizing or hyperpolarizing receptor potentials result from second messenger-coupled ionic permeability changes. Increased conductances to cations and chloride, gated by cAMP and calcium respectively, depolarize the ORN in freshwater. Note that fish are not likely to use chloride channels to depolarize the ORN in the high osmolar marine environment. The events leading to inhibition of ORN firing are not well known, but potassium permeability and different second messengers could be involved. The functional identification of a fish OR has recently been achieved by Speca and co-workers (1999). In this study, a goldfish OR was expressed in *Xenopus* oocytes and responded specifically to basic amino acids, potent odorants for fish. To obtain a response from the oocytes, these authors injected other cRNAs along with the one coding for the putative OR. Speca and colleagues injected the cRNAs for the subunits of G protein-gated inwardly rectifying potassium channels and the olfactory G protein, and then recorded ionic currents produced by the receptor's activation using the voltage clamp method.

Whether each ORN expresses single or multiple OR types has not been resolved. Gene expression studies in mammals and catfish suggest that only one OR type is expressed in an ORN (Ngai et al., 1993b; Buck, 1996). However, the existence

of more generally expressed odorant receptor genes in rat and goldfish raises the possibility that individual sensory neurons may bear different ORs (Speca et al., 1999; Rawson et al., 2000). An electrophysiological study on single neurons of the catfish olfactory epithelium also suggests that an olfactory neuron could express more than one odorant receptor (Kang and Caprio, 1995a).

1.3.2. Functional difference between ciliated and microvillar ORNs

As mentioned, two ontogenetically and morphologically different ORN types, ciliated and microvillar cells, exist in the olfactory epithelium of fish (Zielinski and Hara, 1988; Moran et al., 1992; Hara, 1993). The presence of these cell types and their evolutionary changes throughout the vertebrate radiation have been reviewed in detail (Eisthen, 1992; 1997). Here, I will briefly discuss the evidence for a possible functional difference between the two cell types. A segregated distribution of the two sensory cell types has been noted at the level of the lamella in salmonid fishes and catfish. Recording of the olfactory specificity in these areas has yielded mixed results. Electro-olfactogram (EOG) responses to bile salts in salmonids appeared correlated with a high density of ciliated sensory neurons, while microvillar cells could respond preferentially to amino acids (Thommesen, 1982; 1983). In catfish, this differential response to amino acids and bile salts in areas rich in one or the other sensory neuron type was not observed when EOG and multi-unit activity was recorded in the olfactory epithelium (Erickson and Caprio, 1984). Other investigators took advantage of the fact that ciliated receptor cells are replaced faster than microvillar cells after axotomy. It was shown that goldfish whose olfactory nerve had been cut recovered their olfactory sensitivity to

amino acids before pheromones (Zippel et al., 1997a; 1997b). Microvillar receptor cells were thus correlated with pheromone detection and ciliated cells with food odor sensitivity as was also demonstrated by behavioral assays. However, it was recently reported that amino acid odorants stimulate zebrafish microvillar olfactory neurons (Lipschitz and Michel, 2002).

When ORNs were backfilled by injection of a fluorescent tracer in the posterior olfactory bulb of catfish, different cell types were marked depending on whether the injection site was dorsal or ventral (Morita and Finger, 1998). Ciliated and microvillar ORNs were filled with tracer after injections in the ventral posterior bulb and the dorsal part of the posterior bulb, respectively. Surprisingly, the distribution of the labeled ORNs in each case was not concentrated in a particular area of the olfactory epithelium, even though a spatial distribution of the density of these cell types in the epithelium has been observed by electron microscopy in catfish (Erickson and Caprio, 1984). Morita and Finger (1998) suggested that ORN morphology could be involved in odorant coding because different parts of the olfactory bulb are thought to respond to different odorants. The two different cell types of this catfish study seem to have homologues in goldfish based on the same differential morphology of the cell bodies in the epithelium. Furthermore, these goldfish ORNs selectively express two different OR gene families (Cao et al., 1998). The goldfish cells that are probably equivalent to the ciliated ORNs express receptor genes related to the ORs of fish and mammals, whereas the cells that look like microvillar sensory neurons express OR genes similar to the putative pheromone receptors found in the vomeronasal organ of mammals. Note that fish do not possess a vomeronasal organ. Because of the paucity of studies on this topic,

generalizations cannot be made. However, it can be seen that in some species the ciliated and microvillar ORNs might be specialized to detect different odorant classes.

1.3.3. ORN distribution in the olfactory epithelium

Other methods have been used to assess the possibility of a topographical distribution of the olfactory elements in the epithelium. As mentioned above, OR gene expression has been mapped in goldfish (Cao et al., 1998), and these results show that the gene expression pattern seems to be random. The same random distribution appears to be present also in catfish (Ngai et al., 1993b). The situation is possibly different in zebrafish (*Danio rerio*) where putative OR genes were shown to be expressed in different concentric domains in the lamella. Apart from a role in early olfactory coding, it was suggested that this concentric distribution could be caused by a developmental requirement of the olfactory system of zebrafish (Weth et al., 1996). In zebrafish, the use of a tracer to backfill ORNs by retrograde axonal transport from injections made in small regions of the olfactory bulb, sometimes a single glomerulus, showed that labeled ORNs could be found throughout the whole olfactory epithelium (Baier et al., 1994). This even distribution of sensory neurons in the olfactory mucosa of zebrafish was postulated to maximize the probability of detecting odorants that activate the same glomerulus. It also suggests that segregation of OR genes in the zebrafish olfactory organ is probably not used in coding of olfactory information, because a single olfactory bulb glomerulus is likely to receive input from every part of the sensory epithelium. Riddle and Oakley (1991) used different neurotracers to label rainbow trout primary olfactory fibers. Lectins were also used by the same group to label ORNs and

their projections immunocytochemically (Riddle et al., 1993). These studies clearly show that subsets of ORNs projecting to restricted regions of the olfactory bulb are randomly distributed throughout the whole olfactory epithelium in rainbow trout. Biochemical differences in the ORNs appear to order the topography of the projection to the olfactory bulb.

Some electrophysiological evidence also suggests little spatial and functional segregation in the peripheral olfactory organ of fish (Chang and Caprio, 1996). Hara and Zhang (1996) have recorded electrical responses induced by different classes of odorants in the mucosa and olfactory bulb simultaneously. It was shown that lesions of different portions of the rosette prior to the recordings uniformly reduced both electrical responses. In a similar study done on Arctic charr, progressive removal of the lamellae uniformly diminished the olfactory epithelial and bulbar responses to a bile salt and an amino acid, even though some spatial segregation of the EOG response to these odorants exists at the level of the lamella (Thommesen, 1982). Overall, the idea that there is almost no spatial compartmentation in the peripheral olfactory system of fish is unusual when compared with other sensory systems. This suggests that the coding mechanisms in olfactory signal integration are probably very different from what has been discovered in other areas of sensory physiology.

Some features of the peripheral olfactory systems of other animals are of comparative interest here. Spatial segregation of OR expression has clearly been demonstrated in mammals where ORNs expressing a given OR are randomly dispersed within one of four broad zones, or domains, of the nasal epithelium (Ressler et al., 1993; Vassar et al., 1993). Neurons in different spatial domains of the epithelium

project their axons to different regions of the bulb. Thus, it appears that the entire fish olfactory epithelium may approximate a single compartment of the mammalian olfactory epithelium (Ngai et al., 1993a). Furthermore, gene expression experiments in *Drosophila* show that each OR is found in a clearly spatially restricted subpopulation of neurons in the antenna or the maxillary palp (Vosshall et al., 1999). These findings strengthen the idea that the fish peripheral olfactory organ is somewhat different from that of other animals, although one report shows that the bird peripheral olfactory system could resemble fish in that it forms a single compartment (Nef et al., 1996). The lack of a spatial distribution of ORNs in fish and possibly other animals like birds may be related to the inheritance of an ancestral condition. Alternatively, the requirement for life in an aquatic environment could be the driving force that shaped the fish peripheral olfactory system.

1.4. Coding in the olfactory bulb

1.4.1. Organization of the olfactory bulb

Although some of the anatomical aspects of the fish olfactory bulb have been introduced above, a more detailed examination of this structure is warranted before a discussion of what is known about the coding mechanisms of olfactory information is attempted. The incoming ORN axons run in the most superficial portion of the olfactory bulb and on the major part of its surface except for the most posterior part, forming the olfactory nerve layer of the bulb. Just below this layer is the glomerular layer, devoid of cell bodies in fish, where the primary olfactory fibers make synaptic contact with the distal dendritic tufts of the secondary mitral cells. The cell processes in

the glomerular layer often form spherical masses called olfactory glomeruli, but sometimes a lack of structure gives rise to an aglomerular plexus (Figure 1.1). Below the glomerular layer is the mitral cell layer containing the somata of secondary olfactory neurons, whose axons project to the telencephalon. These large projection neurons include both mitral and ruffed cells. The ruffed cells are special projection neurons that apparently do not receive direct ORN input and possess many pedunculated protrusions at the initial portion of their axons (Kosaka and Hama, 1979; Alonso et al., 1987; Zippel et al., 1999; also see Satou, 1990). The deeper portion of the fish olfactory bulb is the internal cell layer in which many cells of smaller size are densely packed. The internal cell layer contains many inhibitory interneurons called granule cells because of similarities with their mammalian counterpart (Oka et al., 1982; Satou, 1990). The granule cells lack axons and send dendritic processes into the mitral cell layer where they make reciprocal synaptic interactions with the dendrites, somata and initial axon segments of mitral cells. Those reciprocal synapses are made up of an excitatory portion, from mitral to granule cell, and an inhibitory portion, from granule to mitral. The involvement of the different cell types of the olfactory bulb and their synaptic interactions in sensory processing will be discussed after the evidence for the existence of functional divisions in this structure has been considered.

1.4.2. Glomerular map

Olfactory glomeruli within the zebrafish olfactory bulb form a map identifiable across other specimens of the species (Baier and Korsching, 1994). In the developing zebrafish, ORN axons grow directly toward their glomerular targets, following specific

cues in the olfactory bulb (Dynes and Ngai, 1998). Studies in the mouse suggest that the establishment of an olfactory sensory map is not dependent on activity-dependent processes or the presence of neuronal elements in the olfactory bulb (Bulfone et al., 1998). The migratory cues involved in ORN axon growth could come from glial cells. The ORN in search of a target in the bulb expresses odorant receptors in its growing axon terminals. It is believed that interactions of these receptors with migrating cues order the formation of the glomerular map (Mombaerts et al., 1996; Zippel et al., 1997b). This is an attractive model in which the odorant receptors confer the ability of functionally similar sensory neurons to order their projection onto a bulbar map. It was shown that the odorant response profiles of ORNs projecting to different parts of the mouse olfactory bulb are different (Bozza and Kauer, 1998). In the mouse, ORNs do not converge onto their glomerulus when their odorant receptor gene is deleted or altered by a nonsense mutation (Wang et al., 1998). Furthermore, the replacement of its OR gene with a different one diverts the ORN projection to a different glomerular target. These experiments were made possible by recent advances in genetic engineering. These methods, however, have not been applied to the fish olfactory system. It is likely that the mechanisms that have been discovered will be found to play a role in the establishment of topographic projections to the olfactory bulb in fish as well.

1.4.3. Functional topography within the olfactory bulb

Early anatomical and electrophysiological studies performed in rabbits suggest a possible role for the glomeruli as functional units of the olfactory system (reviewed in

Buck, 1996). Some recent work supports this idea by demonstrating that projection neurons that are in close contact in the glomerular layer are much more likely to have similar odorant response profiles than distant neurons since they would be connected to the same glomerulus (Buonviso and Chaput, 1990). The selective activation of regions of the olfactory bulb by different odorants has also been demonstrated by 2-deoxyglucose uptake, immediate early gene expression, functional magnetic resonance imaging, and optical imaging (see Buck, 1996 and Mori et al., 1999). It is interesting to note that a particular odorant activates many glomeruli and that this activation pattern varies with odorant concentration. The mapping of odor information by a glomerular code seems to be a common feature of the vertebrate and many invertebrate olfactory systems (see Hildebrand and Shepherd, 1997 and Strausfeld and Hildebrand, 1999). The existence of a functional topography in the olfactory bulb has also been demonstrated in fish. Using a voltage-sensitive axon tracer, responses to different odorants were recorded optically in the zebrafish olfactory bulb (Friedrich and Korsching, 1998). The results showed that different classes of odorants elicit optical responses in different parts of the bulb. The responses to amino acids and bile salts were thought to activate a large array of glomeruli, whereas the responses to putative pheromones were very restricted. Friedrich and Korsching (1998) showed that the response to $PGF_{2\alpha}$ was induced in a single ventral glomerulus. Their results strongly suggest that a spatial coding of odorant quality is also present in the fish olfactory bulb.

The importance of a functional topography in the fish olfactory bulb had been suspected for some time. Anatomical, behavioral and electrophysiological experiments showed a functional division between the lateral and medial parts of the olfactory bulb

(reviewed in Satou, 1990). Later, small medial regions of the goldfish bulb were shown to respond to reproductive pheromones (Fujita et al., 1991). Recent electroencephalographic (EEG) investigations of olfaction in salmonid fishes determined that amino acids activate the latero-posterior bulb, whereas a bile acid induced activity in a small region in the middle of the dorsal olfactory bulb (Hara and Zhang, 1996; 1998). The same method applied to goldfish also demonstrated a spatial patterning of responses to the different classes of odorants (Hanson et al., 1998). It is noteworthy that narrow foci of activity induced by steroid and prostaglandin pheromones could be detected in the goldfish, a cyprinid, but not in salmonids. It was suggested that the putative pheromone information could bypass the bulb through the extrabulbar primary olfactory pathway in salmonids (Becerra et al., 1994; Hara and Zhang, 1998). Recently, the spatial distribution of single olfactory neuron responses in the fish olfactory bulb was reinvestigated. Previous investigators seemed to have overlooked the spatial aspect of the olfactory response of fish bulbar neurons (Meredith and Moulton, 1978; Kang and Caprio, 1995b). Distinct functional zones activated by different biologically relevant odorants were identified in the channel catfish olfactory bulb (Nikonov and Caprio, 2001), while overlapping spatial maps of activity were found in goldfish (Masterman et al., 2001).

1.4.4. Temporal coding

In addition to spatial patterning of the activity, bulbar responses have been shown to comprise alternate periods of excitation and inhibition at varying frequencies. EEG recordings from the olfactory bulb surface first demonstrated the capacity of this

structure to generate oscillations upon stimulation (Adrian, 1942; 1950). It was shown that projection neuron population discharges oscillate at the frequency of the associated EEG (Freeman, 1975; Satou and Ueda, 1978). For most animals, it is still not known if this firing synchrony of groups of cells is involved in olfactory coding. In fish, the analysis of temporal patterns of single neuron responses in the olfactory bulb led to the proposal that odor quality and intensity may be encoded by neuron populations using a temporal code (Meredith and Moulton, 1978; Kang and Caprio, 1995b). The rhythmic EEG waves of the fish olfactory bulb induced by odor stimulation are slow (3-15 Hz) when compared to those in other animals. Variations in the wave amplitude have commonly been used to differentiate responses between chemical stimuli, but a possible role for specific frequency patterns has also been hypothesized (Hara et al., 1973; Kaji et al., 1975; Satou and Ueda, 1975). This idea stemmed from studies using band-pass filters and spectral analysis to extract the frequency components from the EEG waveform (Kudo et al., 1972). These analyses showed that some stimuli elicited a single dominant frequency in their response while responses to other stimuli contained more frequency components (Hara et al., 1973). Moreover, a temporal change in frequency components was also observed (Satou and Ueda, 1975). However, a recent study, using many odorant classes, found that the dominant frequencies of the EEG responses to different stimuli were similar (Hanson et al., 1998). It is clear that more experimentation, using precise methods of frequency component analysis, is required before a role for different oscillation frequencies in fish olfactory coding can be ascertained. Synchronized oscillations could have many other functions besides the one just mentioned. They could be involved in learning, in creating a periodic clock by

which neurons measure the phase in which they fire, in filtering noise, in refining the coding space of a response, or they may be epiphenomenal (discussed in Laurent, 1996). The possible temporal coding described would apply to the processing of information on the quality and/or intensity of a stimulus. The encoding of the temporal variations in odor exposure, as a result of odor plume characteristics, is a different issue that has been scantily studied in teleosts (see Bronmark and Hansson, 2000). It could be relevant to fish navigation where odorant concentration gradients would be sensed by the olfactory system and help direct the fish to its destination. The physiological basis of orientation in turbulent odor plumes has been studied in more detail in invertebrate animals (Moore and Atema, 1988; Leonard et al., 1994; Moore, 1994; Basil et al., 2000).

Important advances in understanding the role of synchronized oscillations in the olfactory system have been made in some invertebrates. A brief mention of these findings is warranted because they are by far the most complete reported to date. In locusts and honeybees, cellular network interactions in the antennal lobe (functional equivalent of the olfactory bulb of vertebrates) generate odor-induced field potential oscillations (Laurent, 1996). These oscillations are caused by transient firing synchronization of odor-specific neural assemblies. It was proposed that oscillations could contain stimulus information in the temporal sequence with which transient synchronization occurs within the neural assemblies (Wehr and Laurent, 1996). It is possible to eliminate synchronization across these neurons without altering the individual neuron's temporal response to an odor by applying the $GABA_A$ receptor antagonist picrotoxin to the antennal lobe (MacLeod and Laurent, 1996). This

pharmacological tool was used to identify the role of neural synchronization in honeybees trained to discriminate odorants (Stopfer et al., 1997). The blocking of the antennal lobe neurons' synchronization in honeybees prevented the discrimination between closely related odorants without altering the capacity to discriminate between structurally different ones. Stopfer and co-workers (1997) postulated that odor-induced synchronization could be used in fine-tuning between odorants that use overlapping spatial representations in the antennal lobe. These experiments demonstrated that an odor memory comprises information about synchronized oscillations in a group of neurons. In addition, neurons able to decode the synchronized oscillatory olfactory signals were found in a region influenced by antennal lobe inputs (MacLeod et al., 1998). These neurons lost their odor specificity when antennal lobe neurons were desynchronized by picrotoxin.

A similar effect has been observed in another invertebrate in which odor-induced oscillations can be manipulated while a correlated behavioral measure of olfactory discrimination is recorded. Terrestrial slugs can be trained to respond behaviorally to an odorant. When the slug's nose-brain preparation is manipulated *in vitro*, a strong nervous discharge in the external peritentacular nerve is obtained by stimulation with only this odorant, and not with a related odor to which this slug did not respond behaviorally. The application of an oscillation suppressor to the oscillation-generating area of the slug nervous system, the procerebral lobe, leads to a novel response to the unconditioned odorant (Gelperin, 1999).

So far, pharmacological tools that could disrupt the olfactory bulb oscillations in fish are not known. There is also no knowledge about which cell population(s) would

decode olfactory information. It is a matter of time and some effort before similar studies are made to determine if oscillations are used in fish olfactory processing. The possibility exists because many amino acids cause oscillations in large overlapping areas of the olfactory bulb and fish can discriminate behaviorally between amino acids or their mixtures (Hara, 1977; Little, 1981; Von Rekowski and Zippel, 1993; Valentinčič et al., 1994; 2000a; 2000b; Valentinčič and Koce, 2000).

1.4.5. Signal transmission in the olfactory bulb

A simplified diagram of the fish olfactory bulb cytoarchitecture is shown in Figure 1.2. A peculiarity of the fish mitral cells is that their contact with granule cells is limited to the mitral cell's proximal dendrite, soma and initial portion of the axon, as opposed to the widespread contacts on lateral dendrites in mammals (see Satou, 1990). The inhibitory granule cells have been proposed to mediate a form of lateral inhibition that would enhance the precision of glomerular activity between related stimuli (Yokoi et al., 1995; also discussed in Mori et al., 1999). This idea is based on the fact that the distal dendrites of mitral cells in some animals enter only one glomerulus, but have lateral processes contacted by granule cells that span a large area. This cannot apply to fish, where a mitral cell can receive ORN input from large portions of the glomerular layer, and granule cells contacts are found close to the cell body (see Satou, 1990). Thus, it seems possible that the granule cells' role in fish may lie in generating oscillation through the reciprocal interactions between the mitral and granule cells. A mitral cell's synaptic depolarization driven by the long-lasting EPSP from the sensory neurons depolarizes a granule cell dendrite, which in turn generates inhibition on the

mitral cell. This synaptic inhibition is mediated by local, graded responses in the granule cells. The decrease of this inhibition enables the mitral cell to recover its excitability, and the process goes on again if the ORN drive is maintained (Hasegawa et al., 1994). The ruffed cells also seem to have a role to play in the oscillatory responses found in the fish olfactory bulb. Their major synaptic input comes from granule cells that make reciprocal connections in the pedunculated processes of the initial unmyelinated portion of their axons. Anesthesia of the olfactory epithelium suppresses mitral cell activity, whereas ruffed cell activity increases with this treatment. It has been proposed that mitral cells suppress ruffed cells' activity via a granule cell-mediated lateral inhibition (Zippel, 1998; Zippel et al., 1999). This would result in opposing firing patterns for interconnected mitral and ruffed cells. Indeed, contrasting interactions between simultaneously recorded mitral and ruffed cells have been observed during odor stimulation in goldfish (Zippel, 1998; Zippel et al., 1999). The ruffed cells could also be part of a mechanism used to create oscillations when an odorant inhibits the basal firing rate of primary olfactory neurons. Disinhibited ruffed cells could activate a granule cell population through axo-dendritic reciprocal synapses. It would be interesting to see what role these two projection neuron types play in odor processing in fish, and if they have homologues in other animals. More experiments on the morphology and physiology of the ruffed cells are needed.

1.5. Output of olfactory signals to the forebrain

1.5.1. Nature of olfactory information

The axons of projection neurons reach the forebrain via the olfactory tracts. However, how an olfactory stimulus is defined by a pattern of action potentials spreading to different regions of the forebrain is currently unknown. It has recently been proposed that the application of information theory analyses could shed some light on how odors are represented by the nervous system (Alkasab et al., 1999). This statistical method is aimed at quantifying the properties of the olfactory system in order to highlight what information would be decoded. Recall that odors possibly comprise many features that could be used in a code. Variable size neural masses with spatial (odotopic or chemotopic) and temporal characteristics have been found. The spatial characteristics are likely to represent an identity code wherein the cell's identity conferred by the ORs it bears, and not its location, is important (discussed in Laurent, 1999). It was also found that overlapping combinations in neural identity and in time could be valuable information for the olfactory system. These combinatorial codes could result from multiple responses from the receptors or overlapping projections in the olfactory bulb (Friedrich and Korsching, 1998; Malnic et al., 1999).

Studies of insects (Stopfer et al., 1997; MacLeod et al., 1998) suggest that there is an identity component to the code as well as a temporal element brought about by neuronal synchronization of second-order olfactory neurons. Synchronization is most likely responsible for fine discrimination. These mechanisms are likely to be used also in fish since the same spatial (identity) and synchronized components have been

observed in their olfactory system (Hara and Zhang, 1996; 1998; Hanson et al., 1998; Nikonov and Caprio, 2001).

1.5.2. Decoding of olfactory information

The activation of neurons in the fish's early olfactory system (olfactory organ and olfactory bulb) influence neuron groups in the forebrain that are implicated in the generation and modulation of behavior. Some olfactory signal decoding mechanism must be present, and it would be linked functionally to memory systems where odors can be compared and assigned a hedonic value. Some pheromones might be an exception to this rule. But, where are these decoders? Currently, we have no idea. An interesting hypothesis has been proposed stating that the olfactory forebrain could use coincidence detector neurons to identify the various activity patterns observed in the bulb (Mori et al., 1999). In this model, as shown in Figure 1.3, the temporal summation of synaptic inputs from a specific set of glomeruli would drive a detector neuron to fire. EPSP synchronization would be required for spiking to occur. So, in that model, synchronized oscillations would be a mechanism whereby signals from different olfactory neurons are bound in time to be detected.

We know that the olfactory tracts transmit behaviorally relevant olfactory information to the forebrain through the LOT and MOT branches (Stacey and Kyle, 1983; Resink et al., 1989; Von Rekowski and Zippel, 1993). Unfortunately, it seems that most of the telencephalic ablation experiments done in the past date from a period when investigators used olfactory stimuli of questionable relevance for fish (Davis and Kassel, 1983). Reinvestigation of the telencephalic structures essential for olfactory

discrimination using behaviorally relevant odorants is clearly needed. It should focus on regions that are related to behaviors driven by olfaction, identified by electrical stimulation experiments, and known to receive olfactory inputs (Demski, 1983; Satou et al., 1984). The difficulty of working with fish could account for the paucity of data in the above-mentioned fields of study. Progress in these aspects of fish neurobiology would help us answer general questions on olfactory coding, olfactory memory formation and integration of olfactory information into behavioral and autonomic responses. The largest class of vertebrates needs more investigation if we really want to know how our nervous system evolved.

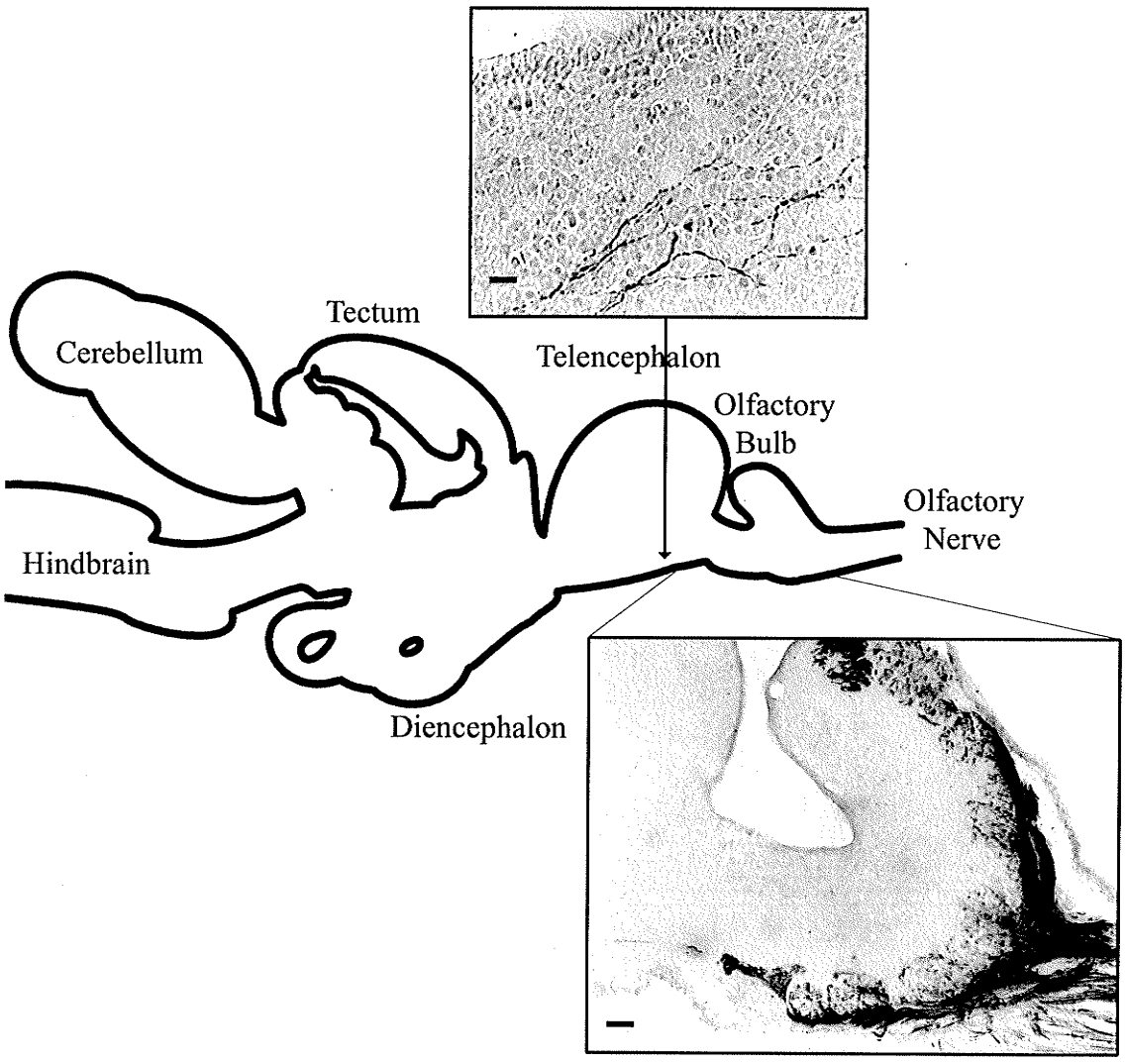

Tectum
Cerebellum
Telencephalon
Olfactory
Bulb
Olfactory
Nerve
Hindbrain
Diencephalon

Figure 1.1

Figure 1.1. Primary olfactory projections in rainbow trout (*Oncorhynchus mykiss*). The ORNs were labeled by application of 5% biotinylated dextran-amine to the olfactory epithelium. The lower micrograph shows a parasagittal section at the level of the olfactory nerve. Most primary olfactory fibers terminate in glomerular structures, some in an aglomerular structure (ventral OB on the left) and, some bypass the bulb (left). The extrabulbar primary olfactory fibers are shown in more detail in the upper micrograph. This is a horizontal brain section of the ventral telencephalon with the rostral side to the right and the third ventricle visible in the upper left corner. A drawing of a complete parasagittal section of the rainbow trout brain is shown to help localize the micrographs. Scale bar is 25 μm above and 100 μm below.

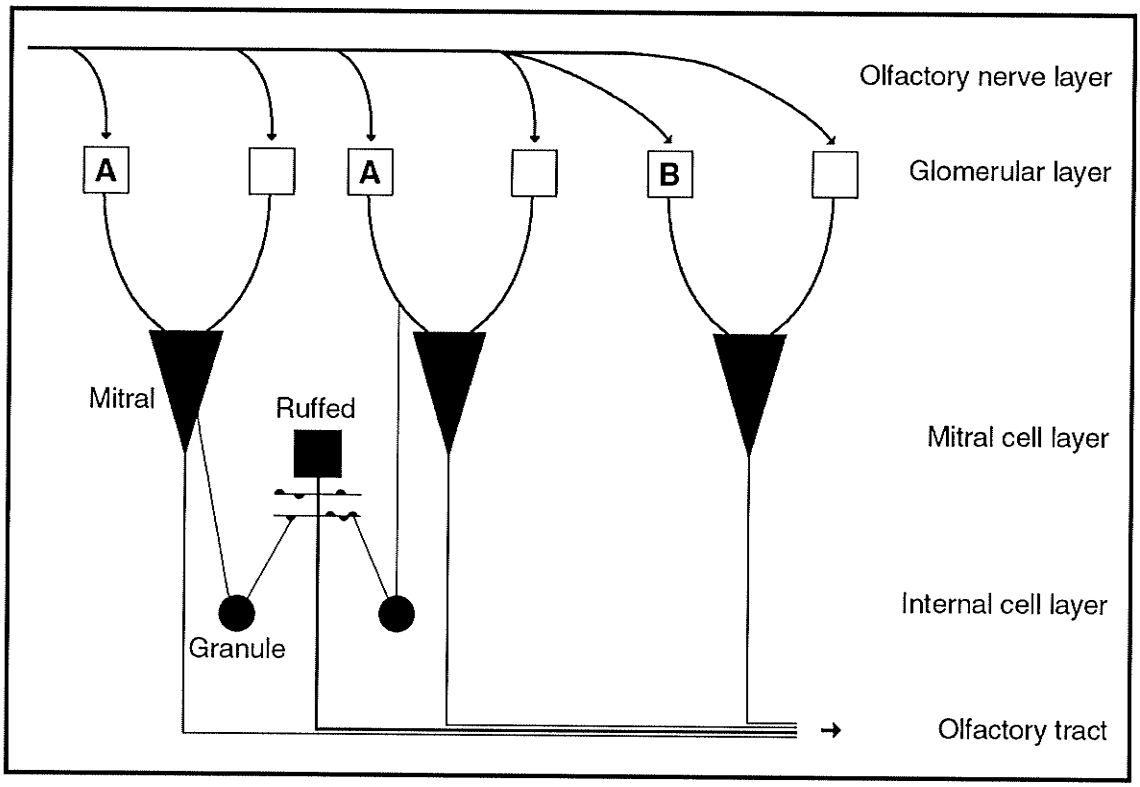


Figure 1.2. Cellular organization of the fish olfactory bulb. This simplified diagram shows how bulbar neurons are interconnected in fish. Each sensory neuron innervates a single glomerulus. Mitral cell dendrites reach the glomeruli and receive input from the olfactory nerve terminals. The ruffed cell dendrites look like glial processes in association with mitral cell dendrites. However, they make few synaptic contacts (only with granule cells) and thus, are omitted from this diagram to enhance clarity. The initial portion of the ruffed cell axon displays several protrusions that contact with neighboring granule cells' dendrites. Granule cells have no axon and contact both mitral and ruffed cells with their dendrites. The axons of mitral and ruffed cells form the olfactory tract, which projects to the forebrain. The letters A and B represent projection neurons in different regions of the OB.

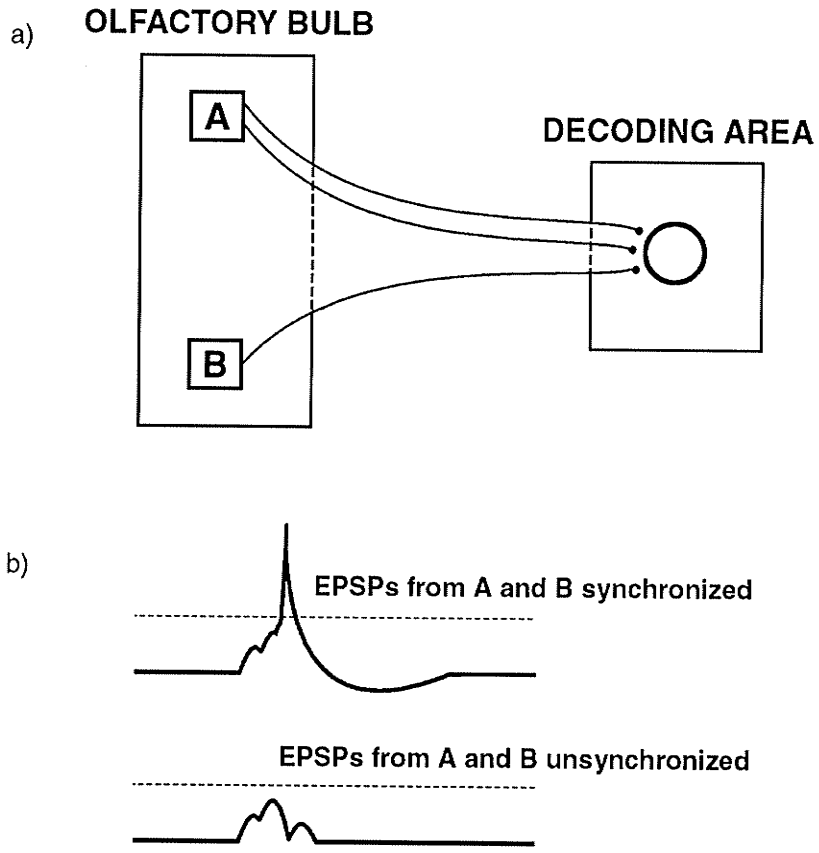

a)
OLFACTORY BULB
A
B
DECODING AREA
b)
EPSPs from A and B synchronized
EPSPs from A and B unsynchronized

Figure 1.3.

Figure 1.3. Coincidence detection hypothesis. (a) Areas A and B in the olfactory bulb (corresponding to the identified projection neurons of Figure 1.2) are shown projecting on a neuron (circle) located in a hypothetical region of the brain devoted to the decoding of olfactory signals. Synaptic input synchronization would be required for a coincidence detection neuron to reach firing threshold. The simultaneous activation of a combination of output cells in different areas of the OB could be necessary to activate a detecting neuron (A and B would have the same odorant specificity in that case). Another possibility is that A and B would respond to different odorants, coincidence detection being used to identify a mixture of two odorants. (b) Hypothetical intracellular recordings that would be obtained from the cell in the decoding region in (a) if input synchronization was relevant to its signal detection.

CHAPTER 2

Behavioral Responses to Food Odors and Reproductive Pheromone Candidates

2.1. Introduction

The family Salmonidae can be divided into three subfamilies: Salmoninae, Coregoninae and Thymallinae. Salmonine fish show a well-defined behavioral sequence at the time of spawning (Jones and Ball, 1954; Tautz and Groot, 1975; Stolz and Schnell, 1991; De Gaudemar and Beall, 1999). Most female salmoninae build nests once they have found a suitable spawning site in a stream, displacing the gravel substrate by digging with their tail. However, lake charr (Salmoninae) and the closely related lake whitefish (Coregoninae) do not build nests and spawn mostly in lakes. They leave their gametes in the water column to fall on a rocky substrate (Fabricius and Lindroth, 1954; Scott and Crossman, 1973). Fish constitute the largest class of vertebrates and show considerable diversity in reproductive strategies. However, there is limited knowledge about fish reproductive pheromone systems. The PGFs have already been shown to mediate pheromonal effects on the reproductive physiology of Atlantic salmon (Moore and Waring, 1996) and brown trout (Moore and Olsén, 2000), and the behavior of Arctic charr (Sveinsson and Hara, 1995). The aim of this study was to investigate the behavioral responses to food odors and an array of PGFs by three species, representing different genera of the Salmonidae family with different reproductive strategies. It was expected that fish using PGFs as reproductive pheromones would display reflex behaviors different from feeding behavior when exposed to these chemicals if the experimental parameters did not prevent the fish's responsiveness.

2.2. Materials and Methods

Brown trout (*Salmo trutta* L.) between three and four years old (length 26.8 ± 6 cm) at the time of the experiments were obtained at age one year from the Whiteshell Provincial Fish Hatchery, Manitoba and held in the laboratory. Lake whitefish (*Coregonus clupeaformis* Mitchill) were spawned from a wild stock (Clearwater Lake, Manitoba) four years before they were used. They were raised at the Freshwater Institute (length 24.1 ± 1.3 cm at the time of experiment). Rainbow trout (*Oncorhynchus mykiss* Walbaum, length 24.2 ± 2.1 cm) of the domestic Manx and Tagwerker strains were obtained from the Rockwood Aquaculture Research Centre, Freshwater Institute, Manitoba. The Manx fish were three to four years old whereas the Tagwerker fish were two to three years old. Each fish's sex was determined at the time of dissection. The experiments were conducted at different times of the year in order to detect a possible seasonal variation in behavioral responsiveness in our stocks. The fish were held in 50-l laboratory tanks with constant flowing aerated, dechlorinated Winnipeg city water (10.5-11.5°C). Light conditions were 12h on-12h off with dusk and dawn simulation accomplished by low intensity light bulbs on 30 min before and after the 12-hour illumination period. Fish were fed to satiation twice a week with floating commercial trout pellets (Martin Mills Inc. Canada; 41% crude protein, 11% crude fat, 3.5% crude fiber, 1% calcium, 0.85% phosphorus, 0.45% sodium, 6800 IU/kg vitamin A, 2100 IU/kg vitamin D, 80 IU/kg vitamin E and 50 ppm astaxanthin).

Fish were individually tested for their reactions to PGFs and to a food extract in a flow-through tank (8.3 l/min flow) shown in Figure 2.1. The tank dimensions were 90 × 30 × 32 cm and its bottom was covered with coarse gravel (2 to 4 cm diameter). The

fish were raised on the same food as was used to make the food extracts in order to induce behaviors related to feeding (Kamchen and Hara, 1980; Olsén et al., 1986). Commercial foodstuff contains free amino acids that induce heightened activity in exposed salmonid fish (Shparkovskiy et al., 1983; Mearns, 1985; Mearns et al., 1987). The test of a food extract was used to determine if the response to PGFs is different from a normal appetitive behavior. Note that the fish were not fed during the behavioral experiments (up to 8 days). Fish had no visual cue that a stimulus was present because only the food extract supernatant was pumped into the tank. The PGFs were continuously pumped into the tank at a calculated final concentration of 10^{-8} M after thorough mixing with the tank water. The ratio of water flows from the pump and the tank outflow was used to determine the concentration of the prostaglandin solution pumped into the tank. On an experiment morning, a frozen concentrated prostaglandin solution was thawed and diluted with distilled water. After four or five days of acclimation to the tank, behavioral measurements were made on the fish for the following three or four mornings. On an experimental day, fish behavior was recorded for a control period of 15 min followed by an exposure period of the same duration. Only one stimulant was tested on any given day and the same fish could be tested with each of the four stimuli in a random order. During control observations, distilled water was pumped at the same rate as the stimulants (1.25 ml/min). The number of turns (180^{o}), exaggerated buccal movements (opercula and mouth involved), and the occurrence of any other behaviors were recorded using a manual recording device. Also, the time spent in the right portion (downstream) of the tank was noted. Lines were drawn on the background so that the observer would know the portion of the tank

in which the fish was located. After observations, the fish were returned to a holding tank, and identified by their anatomical characteristics so that their sex could be assessed later.

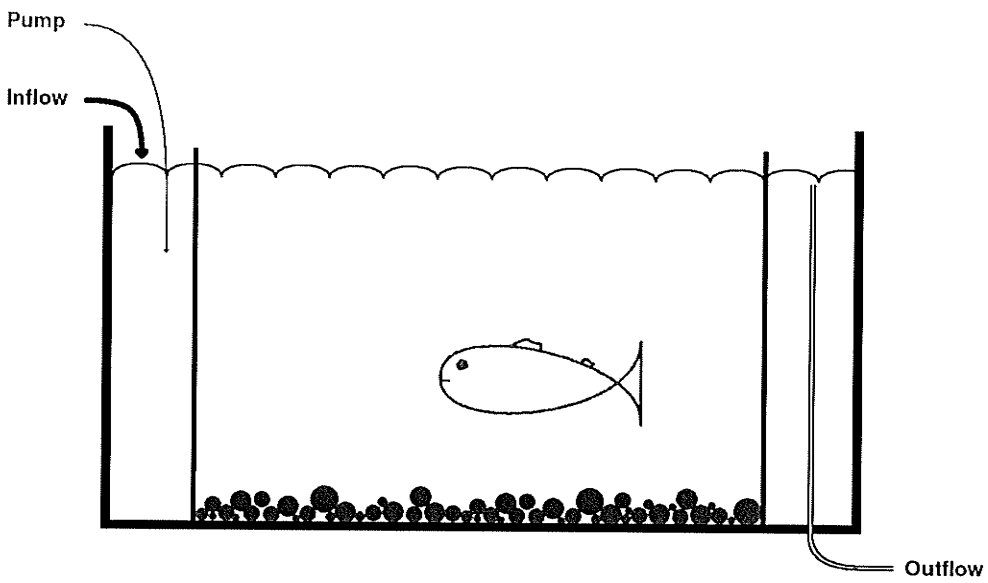
Stock solutions of test stimulants were prepared with distilled water. The three most stimulatory PGFs for these species (see Chapter 3) were tested: $PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$ ($15K_{2\alpha}$) and 13, 14-dihydro-$PGF_{2\alpha}$ ($dh_{2\alpha}$). Prostaglandins were purchased from Cayman Chemical (Ann Arbor, MI, USA). The food extract consisted of 10 4PT-size trout pellets (Martin Mills Inc. Canada) in 50 ml distilled water left overnight at room temperature.

Two-tailed paired t-tests were applied to the data to determine if exposure to the various olfactory stimulants affected the measured variables when compared to the control session.

2.3. Results

All three species significantly increased their number of turns upon infusion of a food extract (Fig. 2.2A). This measure was clearly related to locomotor activity. Brown and rainbow trout exposed to the food extract also searched with their snout through the gravel at the bottom of the tank, a behavior not observed during control sessions (Fig. 2.2B). Note that bottom searching was exhibited by rainbow trout of the Manx strain only, and was not statistically significant. Brown trout also increased their buccal movement frequency when exposed to the food extract (Fig. 2.3).

Brown trout increased significantly their turning activity in response to $PGF_{2\alpha}$ and $dh_{2\alpha}$, while lake whitefish increased their turning significantly when exposed to



Width 124 cm
Height 30 cm
Depth 32 cm

Figure 2.1. The tank used for behavioral experiments. This diagram shows the observer's view from his position 4 m away from the 119-l capacity tank. The tank is embedded in a chamber that allows observations to be made only from one side, minimizing visual stimuli accessible to the fish. The water inflow and outflow are in portions of the tank separated by solid plastic screens. The stimuli were pumped into the turbulence created by the water inflow for a rapid mixing. Thorough solution mixing within 2-3 min was observed visually by pumping methylene blue into the behavioral tank in preliminary experiments.

$PGF_{2\alpha}$ and $15K_{2\alpha}$ (Fig. 2.4A, B). Both sexes of brown trout and lake whitefish increased their turning activity in a similar way. There was no occurrence of searching through the gravel during PGF exposures in any of the three species used. Brown trout also significantly increased the frequency of buccal movements when exposed to $PGF_{2\alpha}$ (Fig. 2.5). In whitefish, a definite modification in the activity pattern was observed at the beginning of a PGF exposure. Turns were often not in the same direction and swimming against the window increased considerably, a behavior difficult to quantify. This swimming against the window without displacement did not contribute to an increase in the total number of turns but revealed an intense response of lake whitefish to PGF not seen with the food extract. Rainbow trout showed no reaction to PGF exposure (Fig. 2.4C and Fig. 2.5). Preference or avoidance of PGFs was not detected. No obvious variation in behavioral responsiveness to PGFs was observed when fish were tested at different times of the year.

The pre-spawning behaviors of digging and nest probing (cutting and feeling in Jones and Ball, 1954) occurred in four of the six female brown trout tested when exposed to 10^{-8} M $PGF_{2\alpha}$ (Fig. 2.6). Three of these females reacted during the observation period after a delay of several minutes (digging was restricted to the last 5 min of the 15-min stimulation period), while another began digging the gravel at the end of the stimulation period. This increase in digging behavior was not statistically significant ($P=0.0925$). However, out of a sample of only six, two females did not show that behavior. Also, a long delay before the onset of digging behavior diminished substantially the number of events included in the 15-min measurement period. Digging

was still observed two to three hours after the end of the stimulus in the four females that reacted with pre-spawning behaviors.

2.4. Discussion

Both brown trout and lake whitefish increased their turning activity when exposed to PGFs but their behavior changed in a manner distinct from responses to food extract; these observations militate against the possibility that PGFs induce a feeding response. The bottom-searching behavior was absent when the brown trout were exposed to PGFs, and lake whitefish behavioral responses to PGFs were subjectively considered more intense compared to the feeding response. Unfortunately, this difference could not be quantified because of the occurrence of frequent swimming against the window, which did not increase the number of turns. The increase in swimming activity upon exposure to $PGF_{2\alpha}$ and $15K_{2\alpha}$ has been noted in goldfish (Sorensen et al., 1988). The measure of locomotor activity used in the present study (the number of turns) has been used in studies of feeding behavior (Valentinčič and Caprio, 1994; 1997; Valentinčič et al., 2000a, b). It was also observed that the frequency of exaggerated buccal movements increased along with locomotor activity in brown trout exposed to PGFs or a food extract. This behavior termed "prey-shaking" in brown trout and Arctic charr has been deemed a displacement activity when executed in the absence of food (Fabricius, 1953). This behavior may be related to oxygenation of the gills as it correlates with locomotor activity. A transitory increase in ventilation frequency in response to putative steroidal pheromones was also observed in the round goby *Neogobius melanostomus* (Murphy et al., 2001). Preference or avoidance of PGFs was not detected. This was probably

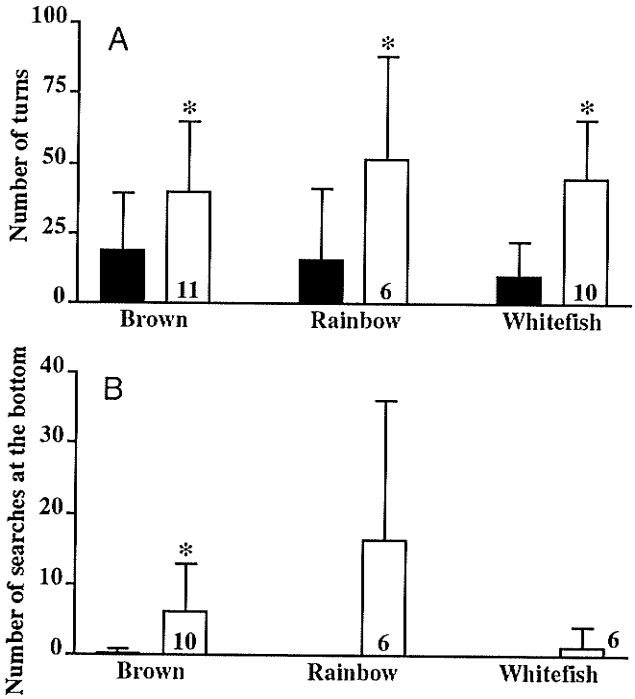


Figure 2.2. Number of turns (A) and bottom searches (B) in brown trout, rainbow trout (Manx strain) and lake whitefish exposed to a food extract (open bars) for 15 min compared to control (solid bars). Sample size is indicated inside the open bar. Vertical bars represent mean + standard deviation. Asterisks indicate a statistically significant difference from controls ($P<0.05$).

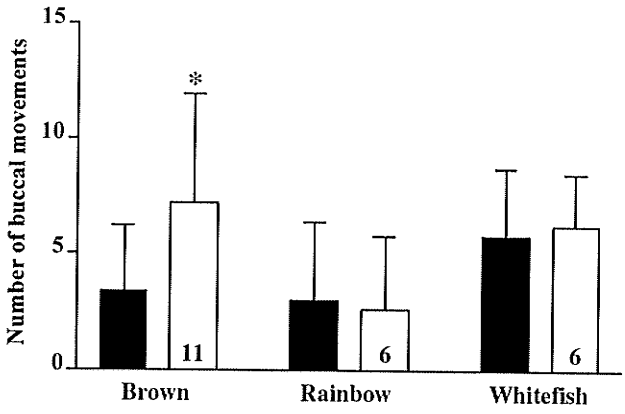


Figure 2.3. Number of buccal movements in brown trout, rainbow trout (Manx strain) and lake whitefish exposed to a food extract (open bars) for 15 min compared to control (solid bars). Sample size is indicated inside the open bar. Vertical bars represent mean + standard deviation. Asterisks indicate a statistically significant difference from controls ($P<0.05$).

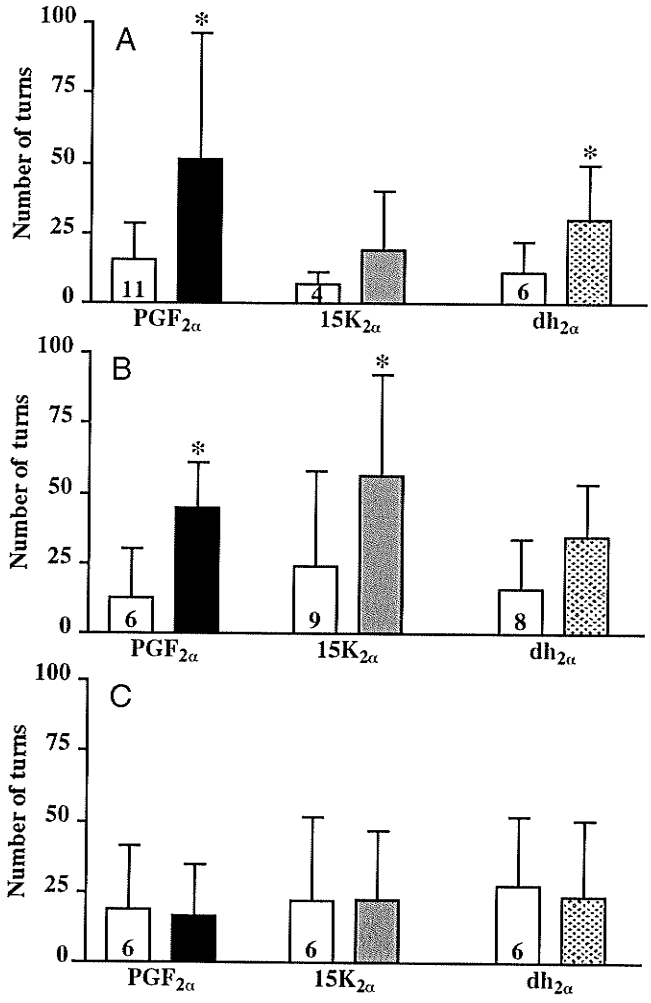


Figure 2.4. Number of turns of brown trout (A), lake whitefish (B) and rainbow trout (Manx and Tagwerker strains) (C) exposed to 10^{-8} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 15-keto-prostaglandin $F_{2\alpha}$ ($15K_{2\alpha}$) and 13, 14-dihydro-prostaglandin $F_{2\alpha}$ ($dh_{2\alpha}$) for 15 min. Sample size is indicated in the control (open) bars. Vertical bars represent mean + standard deviation. Asterisks indicate a statistically significant difference from controls ($P<0.05$).

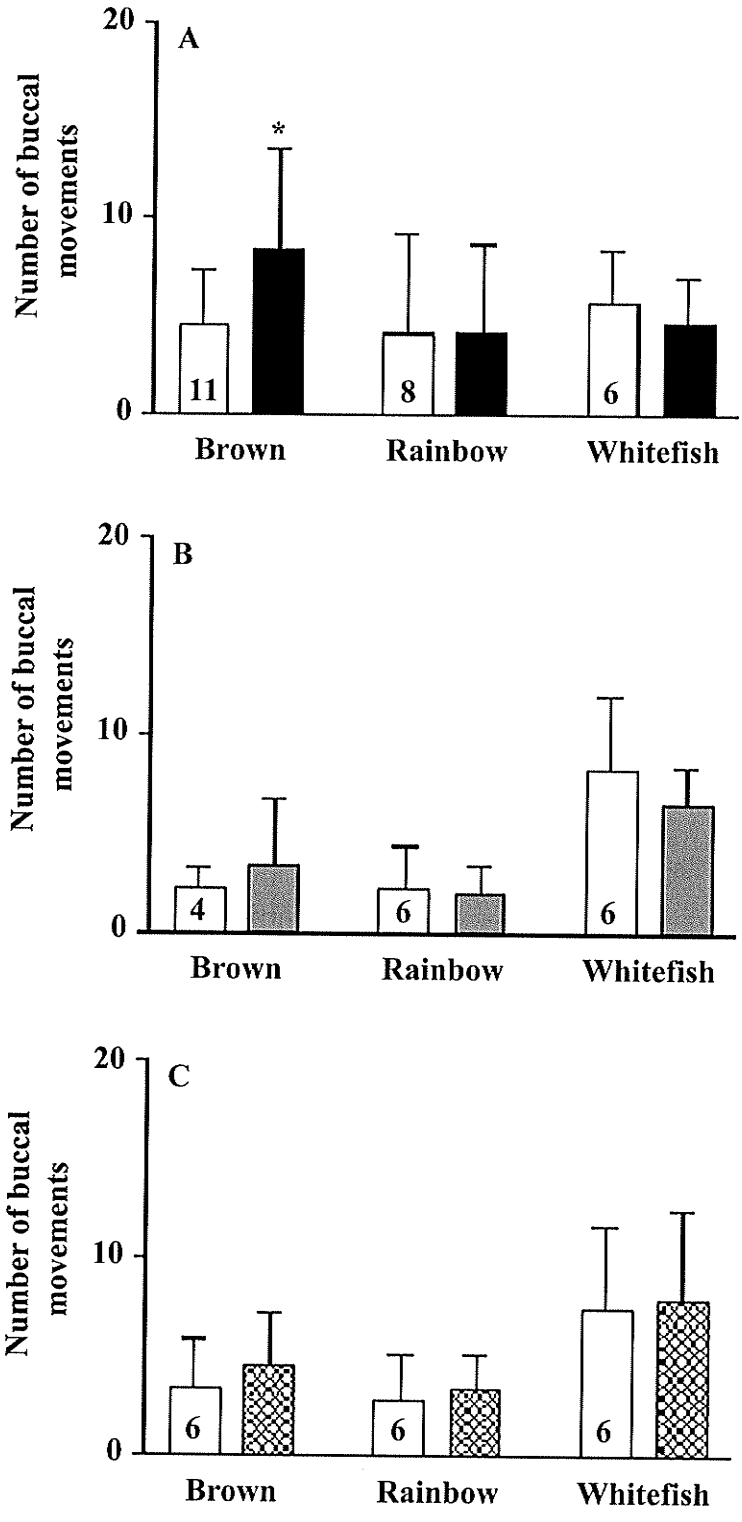

A
20
10
0
Number of buccal movements
*
11
8
6
Brown
Rainbow
Whitefish
B
20
10
0
Number of buccal movements
4
6
6
Brown
Rainbow
Whitefish
C
20
10
0
Number of buccal movements
6
6
6
Brown
Rainbow
Whitefish

Figure 2.5

Figure 2.5. Buccal movements in brown trout, lake whitefish and rainbow trout (Manx and Tagwerker strains) when exposed to 10^{-8} M (A) prostaglandin $F_{2\alpha}$, (B)15-keto-prostaglandin $F_{2\alpha}$ and (C) 13, 14-dihydro-prostaglandin $F_{2\alpha}$ for 15 min. Sample size is indicated in the control (open) bars. Vertical bars represent mean + standard deviation. The asterisk indicates a statistically significant difference from controls ($P<0.05$).

Figure 2.6. Number of digging attempts recorded during 15 min in brown trout exposed to 10^{-8} M prostaglandin $F_{2\alpha}$ (solid bars) compared to control (open bars). Sample size is 5 males and 6 females. Vertical bars represent mean + standard deviation.

because the pumped chemicals were homogeneously mixed quickly and the fish were not presented with a constant concentration gradient to explore.

Elements of the reproductive behavioral sequence of salmonine fish were observed in female brown trout exposed individually to 10^{-8} M $PGF_{2\alpha}$. Such spawning behaviors were previously observed in female Arctic charr exposed to PGFs (Sveinsson and Hara, 1995). Only two components of the pre-spawning behavioral sequence were observed in the present study: nest digging and probing of the nest depth (Fabricius, 1953; [cutting and feeling in Jones and Ball, 1954]; Tautz and Groot, 1975; De Gaudemar and Beall, 1999). Additional sensory cues from a male are probably needed for the female brown trout to execute a complete spawning act. The involvement of vibrational and visual cues has previously been demonstrated in the spawning act of landlocked sockeye salmon (Satou et al., 1987).

An important feature of the digging response of female brown trout observed in the present study is that it was delayed by several minutes after the beginning of PGF exposure. This delay in the onset of the pre-spawning behavior seems inconsistent with the concept of a releaser pheromone that triggers a behavioral reflex response. What could account for a quick increase in locomotor activity in female brown trout exposed to $PGF_{2\alpha}$ followed, after an extensive delay, by a behavioral switch to a lower activity and pre-spawning behaviors? The pre-spawning behaviors observed here could be triggered by an activation of the reproductive endocrine axis through the olfactory system. The activation of the hypothalamo-pituitary-gonad axis is involved in the olfactory PGF-mediated increase in sex hormones and expressible milt in male brown trout (Moore and Olsén, 2000). It is conceivable that a quick hormonal surge triggered

by $PGF_{2\alpha}$ could cause the pre-spawning behaviors in female brown trout. The presence of PGFs in female brown trout urine and ovarian fluid has been demonstrated recently (Moore and Olsén, 2000), but unfortunately males were not included in that study. If only the female releases PGFs to the water in brown trout, a new mechanism of pheromonal self-stimulation could account for the observed pre-spawning behaviors exhibited by females when exposed to $PGF_{2\alpha}$. Thus, the same prostaglandin pheromone could be involved in coordinating both reproductive behavior and physiology of male and female brown trout at spawning time.

2.5. Conclusion

The present results show that brown trout and lake whitefish are behaviorally responsive to a low concentration of some PGFs, and that the behaviors induced by PGFs are distinct from those induced by a food extract at least in brown trout. $PGF_{2\alpha}$ exposure by itself could trigger pre-spawning behaviors in female brown trout. These findings support a role for PGFs as reproductive pheromones in brown trout and lake whitefish.

CHAPTER 3

Olfactory Sensitivity to Food Odors and Reproductive Pheromone Candidates

3.1. Introduction

With the knowledge that reproductive pheromones are detected by the olfactory system, the EOG method has been used to screen the sensitivity of many fish species to putative pheromones (Sorensen and Goetz, 1993; Kitamura et al., 1994b; reviewed in Hara, 1994 and Stacey et al., 1994). This electrophysiological method measures the summated generator potentials of an activated population of olfactory receptors (Ottoson, 1971). Olfactory sensitivity to PGFs has been demonstrated in several members of the families Cyprinidae, Catostomidae and Salmonidae. Hara and Zhang (1998) previously demonstrated that the olfactory response to a high PGF concentration (10^{-7} M) is different among the three salmonid species used in the present study. Brown trout and lake whitefish were shown to be sensitive to $PGF_{2\alpha}$ and 15-keto-$PGF_{2\alpha}$, lake whitefish having the greatest response of all fish tested, and rainbow trout were found not to be responsive to these prostaglandins, as previously shown by Kitamura et al. (1994b).

Amino acids are behaviorally potent components of food odors (Carr et al., 1977; Fuke et al., 1981; Ellingsen and Döving, 1986; Johnsen and Adams, 1986; Olsén et al., 1986; Mearns et al., 1987). It has been demonstrated that multiple amino acid receptor types exist in the fish olfactory epithelium (Brown and Hara, 1981; Rhein and Cagan, 1983; Caprio and Byrd Jr., 1984; Ohno et al., 1984; Bruch and Rulli, 1988; Caprio et al., 1989; Sveinsson and Hara, 1990b; Kang and Caprio, 1991). Sorensen et al. (1988) suggested the existence of two receptor types for the detection of $PGF_{2\alpha}$ and

$15K_{2\alpha}$ in goldfish. The present study was aimed at identifying the most stimulatory PGFs to lake whitefish, brown and rainbow trout. Those PGFs could be considered pheromone candidates in these species. Additionally, the number and independence of olfactory receptive mechanism(s) for amino acids and PGFs were compared to highlight possible differences between food and pheromone signal detection.

3.2. Materials and Methods

See section 2.2 for a description of the fish used and their holding conditions. The experiments were conducted at different times of the year in order to detect a possible seasonal variation in olfactory sensitivity in our stocks. Two sexually undifferentiated fish (aged less than a year) were used to determine if olfactory sensitivity to PGFs is present in young brown trout. Behavior was observed (Chapter 2) on some of the fish used for EOG experiments. These fish were returned to a holding tank after observations and identified by their anatomical characteristics so that their sex could be assessed after they had been used for EOG recordings (a few days to a month later).

For EOG recordings, fish were tranquilized by exposure to water containing MS-222 (0.5 g/l), fully anesthetized by intraperitoneal injection of amobarbital (30 mg/kg body weight), and immobilized by intramuscular injection of Flaxedil (gallamine triethiodide; 3-5 mg/kg body weight, the higher dose range was used in lake whitefish) before being secured on a holder in a flow-through trough. The gills were continuously perfused (0.4 l/min) with dechlorinated water (10.5-11.5°C). Skin and cartilage covering the right naris were removed and the exposed naris was continuously perfused with dechlorinated water. EOGs were recorded using the methods of Evans and Hara

(1985) and Sveinsson and Hara (2000). The recording electrode's position above the olfactory epithelium was adjusted so that a maximal response to a standard stimulant, 10^{-5} M L-serine, was achieved. Electrical signals were amplified with a d.c. preamplifier (Type 7P1, Grass Instrument, West Warwick, RI, USA) and recorded on a polygraph (Model 79, Grass Instrument).

To perfuse the olfactory organ and deliver chemical stimuli, the method of Sveinsson & Hara (2000) was used. Briefly, a constant flow of dechlorinated water (10 ml/min) to the olfactory organ was maintained through a polyethylene tube leading to a glass capillary positioned near the rosette. During stimulant testing, the flow of dechlorinated water to the olfactory organ was instantly replaced with the stimulant solution for 10 s, and then dechlorinated water flow instantly resumed. A minimal recovery period of two minutes between each test stimulant was allowed. In the case of concentration-response relationships, stimulants were tested in ascending concentrations.

Cross-adaptation experiments were performed to determine if a single or multiple receptive mechanisms are involved in detection of amino acids and PGFs, and if these chemical classes share receptive mechanisms (Thommesen, 1982; Caprio & Byrd, 1984). The term "receptive mechanism" is used here to include both the receptor binding site and the transduction events following receptor activation that lead to the receptor generator potential. In cross-adaptation, the constant flow of dechlorinated water to the olfactory organ is replaced with an adapting solution. If the adapting stimulus concentration is high enough, the continuous presence of the adapting stimulus ensures saturation of the receptors for that stimulus while other receptors are not

affected. If a different stimulus competes for the same receptive mechanism, it cannot trigger an EOG response under cross-adaptation, or it will trigger a reduced response in the case of an adapting stimulus that does not saturate all receptors available. The opposite is true for a stimulus that does not compete with the receptive mechanism activated by the adapting stimulus, with possible variations in the degree of independence between the receptive mechanisms activated by the two stimuli. These experiments were performed in brown and rainbow trout for amino acids and in brown trout and lake whitefish for PGFs. The very small EOG responses to amino acids in lake whitefish precluded the performance of cross-adaptation experiments with these chemicals in this species. The adapting stimulus was perfused over the olfactory organ at a high concentration (10^{-5} or 10^{-3} M for amino acids and 10^{-8} M for PGFs) until the response declined and stabilized at the tonic level after 4-5 min. Then, various test stimuli dissolved in the adapting stimulus solution were applied at two-minute intervals (Sveinsson & Hara, 1990b). Chemicals representing additional odorant classes (steroid derivative and bile acid) were used in the cross-adaptation experiments to further examine the specificity of the olfactory receptive mechanism(s).

Amino acid binary mixture experiments were performed in brown and rainbow trout to confirm the cross-adaptation results. A trinary mixture of cysteine, arginine and serine was also tested in brown trout to evaluate the independence of each receptive mechanism for these stimulants. A binary mixture with the two most potent PGFs was also tested in lake whitefish. The binary mixture experiments consisted of EOG tests of two equipotent amino acid or prostaglandin solutions, a mixture containing the two stimulants in the same concentration as in the individual tests, and the tests of twice the

concentration of the mixture's components. The concentrations used were 10^{-6} M for Ser and Arg, 10^{-5} or 2×10^{-5} M for Glu, between 10^{-8} and 10^{-7} M for Cys, and 10^{-8} M for PGFs. The above variations in concentration show that some adjustments were needed for the solutions to have equivalent EOG responses in each fish. Each fish was tested at least 3 times with each solution to allow the measurement of potentially small EOG amplitude differences. In the case of the trinary mixture, three equipotent solutions were used along with tests of three times their concentration in the mixture. The method used in Caprio et al. (1989) was followed for calculation of the mixture discrimination index (MDI) and independent component index (ICI). MDI was measured by dividing the response to the mixture by the larger response to twice the concentration of one of its components. ICI was measured by dividing the response to the mixture by the sum of the responses to its components. Theoretically, if the stimulants in the mixture activate the same olfactory receptive mechanism, the MDI equals 1; the combination of two equipotent stimulants binding the same receptors will produce the same response as twice the concentration of either stimulant. Mixture suppression would be indicated by a MDI significantly less than 1, whereas mixture enhancement would be indicated by a MDI significantly greater than 1. Research on amino acid olfaction done in catfish showed no evidence of mixture suppression and concluded that the mechanism for mixture enhancement is the simultaneous activation of multiple types of receptor binding sites by the different components of the mixture (Caprio et al., 1989; Kang and Caprio, 1991). Thus, I expected to observe a mixture enhancement with amino acid pairs that activate different receptive mechanisms and an even larger mixture enhancement, as reflected by a large MDI, with the trinary mixture of amino acids if

this mixture activated more receptive mechanisms than a binary mixture. An ICI equal to 1 would mean that the components of a mixture are independent. The lower the ICI value is, the more related the mixture components are.

Stock solutions of test stimulants were prepared with distilled water. The free-acid form of $PGF_{2\alpha}$ and three steroids were initially dissolved in a small volume of pure methanol before addition of the distilled water. Stock solutions were stored at 4°C and aliquots (10 or 100 µl) of stock solutions were diluted with 10 ml of dechlorinated water immediately before testing. Six F series prostaglandins were tested: $PGF_{2\alpha}$, $PGF_{1\alpha}$, 15-keto-$PGF_{2\alpha}$ ($15K_{2\alpha}$), 15-keto-$PGF_{1\alpha}$ ($15K_{1\alpha}$), 13, 14-dihydro-$PGF_{2\alpha}$ ($dh_{2\alpha}$) and 15-keto-13, 14-dihydro-$PGF_{2\alpha}$ ($15Kdh_{2\alpha}$). The structure of the PGFs used in the present study is illustrated in Appendix A. All prostaglandins were purchased from Cayman Chemical. Two steroids and a steroid derivative were also tested because of the pheromonal action of these odorants in other fish species: etiocholan-3α-ol-17-one glucuronide (ECG), etiocholan-3α-ol-17-one and 17α-hydroxy-20β-dihydroprogesterone. The bile acid taurocholic acid (TCA) along with the several L-amino acids used was from Sigma Chemical Co. (St. Louis, MO, USA). The methanol control never gave any EOG response.

The EOG response threshold was considered to be the lowest tested concentration at which the averaged EOG measure and its standard deviation stood outside zero. Possible sex differences in EOG response magnitude to the more potent PGFs in brown trout and lake whitefish were tested by a two-way ANOVA for the factors sex and concentration. Unpaired t-tests at every concentration were additionally performed after a significant ANOVA result for the factor sex to determine at which

concentration(s) the responses between sexes differed. A one-way ANOVA followed by a Tukey post hoc test upon significance was performed on the percentage of unadapted responses obtained in the cross-adaptation experiments. A significant response in the Tukey test between the stimulation with the chemical used as adaptant and another stimulant was taken as evidence of the use of distinct receptive mechanisms. Finally, a two-tailed paired t-test comparing the EOG response magnitudes of the mixture and twice (three times for the trinary mixture) the concentration of its most potent component was performed to determine if they differed.

3.3. Results

3.3.1. Odorants

The EOG concentration-response relationships for the four amino acids used in brown trout cross-adaptation and mixture experiments are shown in Figure 3.1. Note that the cysteine response is very large and that the response threshold is around 10^{-8} M for all amino acids used. These curves helped to determine the equipotent amino acid concentrations for the mixture experiments in brown trout. Previously obtained amino acid concentration-response curves resembling those obtained here in brown trout were used for this purpose in rainbow trout (T. J. Hara, unpublished results).

The results from brown trout cross-adaptation experiments are shown in Figure 3.2. Cross-adaptation with cysteine (Fig. 3.2A), the most potent amino acid, did not suppress significantly the responses to $PGF_{2\alpha}$, TCA and arginine. Additionally, the response to 10^{-6} M glutamic acid under cysteine adaptation was significantly greater

than the response to the adapting stimulus, but the same is not true of glutamic acid tested at 10^{-5} M. There is no evidence from the cross-adaptation results that serine and cysteine activate different olfactory receptor types. Reciprocal cross-adaptation with the other amino acids suggested that all the stimulants, except serine and cysteine, activated different receptor types. The amplitudes of adapted responses for the odorants representing the chemical classes of prostaglandins and bile acids were always indistinguishable from their unadapted responses. Figure 3.3 shows the results of rainbow trout cross-adaptation experiments using adapting solutions of high concentration (10^{-3} M). It shows that only TCA had a significant response under cysteine adaptation while the response to arginine and lysine, although not significant, is not suppressed totally. The small response to glutamic acid under cysteine adaptation was the result of a response in only one of four fish and was not significant. Adaptation with arginine totally suppressed the response to arginine itself, lysine and glycine only.

An example of a binary mixture experiment in brown trout with cysteine and arginine (Figure 3.4) clearly demonstrates that the amplitude of the EOG response to the mixture of these amino acids is greater than the response to twice the concentration of either of its components. In brown trout, every binary mixture of amino acids used in this study produced a significantly greater response than did its more potent component when doubled in concentration, except in the case of the cysteine-glutamic acid mixture (P=0.07) (Table 3.1). The calculated values of MDI are all larger than 1, supporting the existence of mixture enhancement for the selected amino acids. Also shown in Table 3.1 are the values of ICI for these mixtures. The trinary mixture of Cys, Ser and Arg also reveals a statistically significant mixture enhancement, along with a large MDI and

a small ICI (Table 3.2). The same binary mixtures were also tested in rainbow trout along with the mixture of arginine and lysine (Table 3.3). The results are similar to those obtained in brown trout except that the cysteine-serine mixture enhancement is not significant. The mixture of arginine and lysine did not yield any mixture enhancement and the measured ICI for this amino acid pair is the smallest found in this study.

3.3.2. Pheromones

The EOG concentration-response relationships of PGFs and ECG for brown trout and lake whitefish are shown in Figure 3.5 (the data and sample sizes are listed in Appendix B). In brown trout, $PGF_{2\alpha}$ was the most potent, detected at the threshold concentration of 10^{-11} M. Lake whitefish were characterized by a very high response magnitude to PGFs relative to the standard L-serine, resulting in a high percentage PGF versus standard response magnitude. $15K_{2\alpha}$ was the most potent PGF for lake whitefish, with a threshold concentration of 10^{-10} M. Rainbow trout did not respond significantly to any of the PGFs tested, except $PGF_{2\alpha}$ at 10^{-6} M, where small responses were recorded from only two fish (Table 3.4). Of the steroids tested, only ECG gave some EOG responses in brown and rainbow trout. There was no obvious differences in EOG responses of fish tested at different times of the year.

Figure 3.6A shows the EOG responses to $PGF_{2\alpha}$ for male and female brown trout. Males had higher response magnitudes, especially at high concentrations ($P<$

0.05). A similar sexual difference in EOG responses to PGFs was found in lake whitefish (Fig. 3.6B shows the example of $15K_{2\alpha}$).

All PGF responses were suppressed by adaptation with 10^{-8} M $PGF_{2\alpha}$ in brown trout (Fig. 3.7). ECG and L-serine responses were not suppressed by the adapting solution. The same type of experiment was performed in lake whitefish using 10^{-8} M of both $15K_{2\alpha}$ and $dh_{2\alpha}$ as adapting stimuli (Fig. 3.8). Adaptations suppressed strongly responses to all PGFs tested in both sexes. The standard responses were not suppressed.

An example of EOG responses of lake whitefish to a binary mixture of 10^{-8} M $15K_{2\alpha}$ and 10^{-8} M $dh_{2\alpha}$, its components alone, and twice the concentration of its components is shown in Figure 3.9. The response magnitude to twice the concentration of the more potent component of the mixture and the mixture itself are not significantly different. The calculated MDI and ICI are shown on the table inserted in Figure 3.9.

3.4. Discussion

3.4.1. Odorants

In the present study, monophasic negative voltage changes with a rapid phasic component followed by a tonic component were obtained, characteristic of EOGs reported in salmonid fish species (Evans and Hara, 1985; Hara and Zhang, 1998). Previous electrophysiological studies done in fish highlighted the presence of several partially independent receptor types for amino acids (Caprio, 1982; Hara, 1982; Ohno et al., 1984; Caprio et al., 1989; Sveinsson and Hara, 1990b) and bile acids (Zhang and

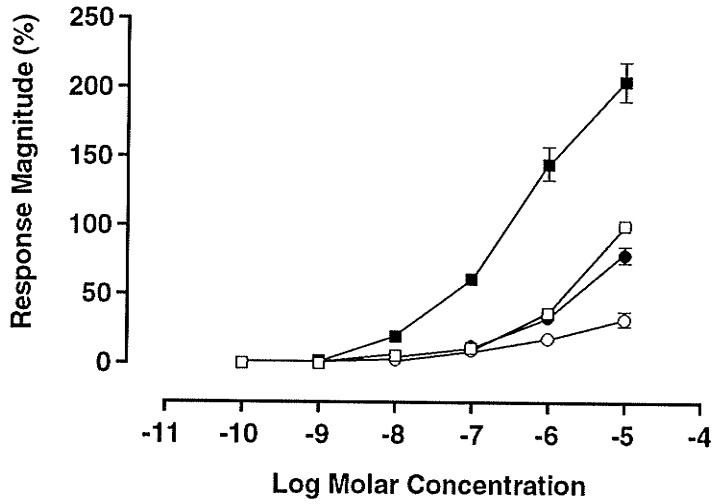


Figure 3.1. EOG concentration-response relationships in brown trout for selected amino acids. Response magnitude is expressed as a percentage of the response to 10^{-5} M L-serine. Stimulants: L-cysteine (solid squares), L-serine (open squares), L-arginine (solid circles), and L-glutamic acid (open circles). Each point represents mean ± standard deviation (n=4).

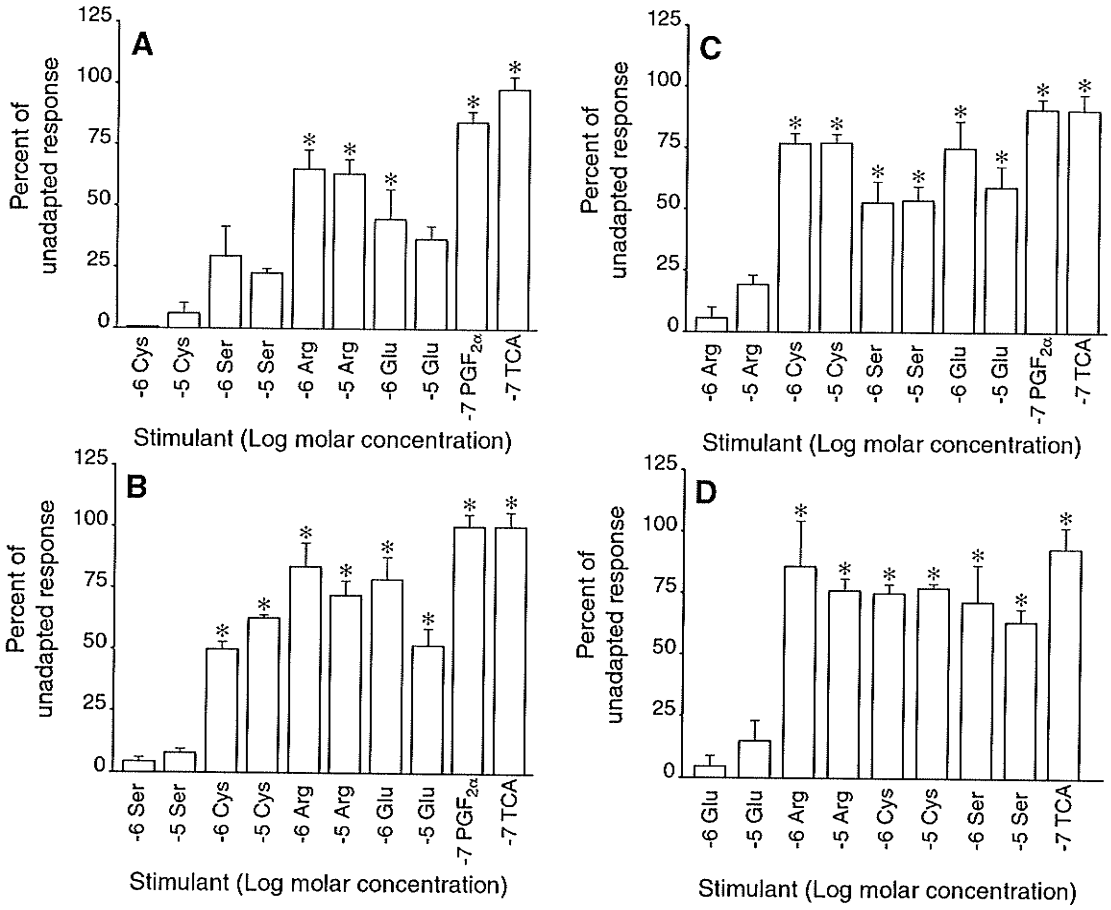


Figure 3.2. EOG responses of brown trout to selected chemicals during adaptation to 10^{-5} M L-cysteine (A), 10^{-5} M L-serine (B), 10^{-5} M arginine (C), and 10^{-5} M L-glutamic acid (D). Sample size is 5 in A, B, C, and 3 in D. Asterisks indicate a statistically significant difference when compared to the response to the adapting stimulus. Vertical bars represent mean + standard deviation.

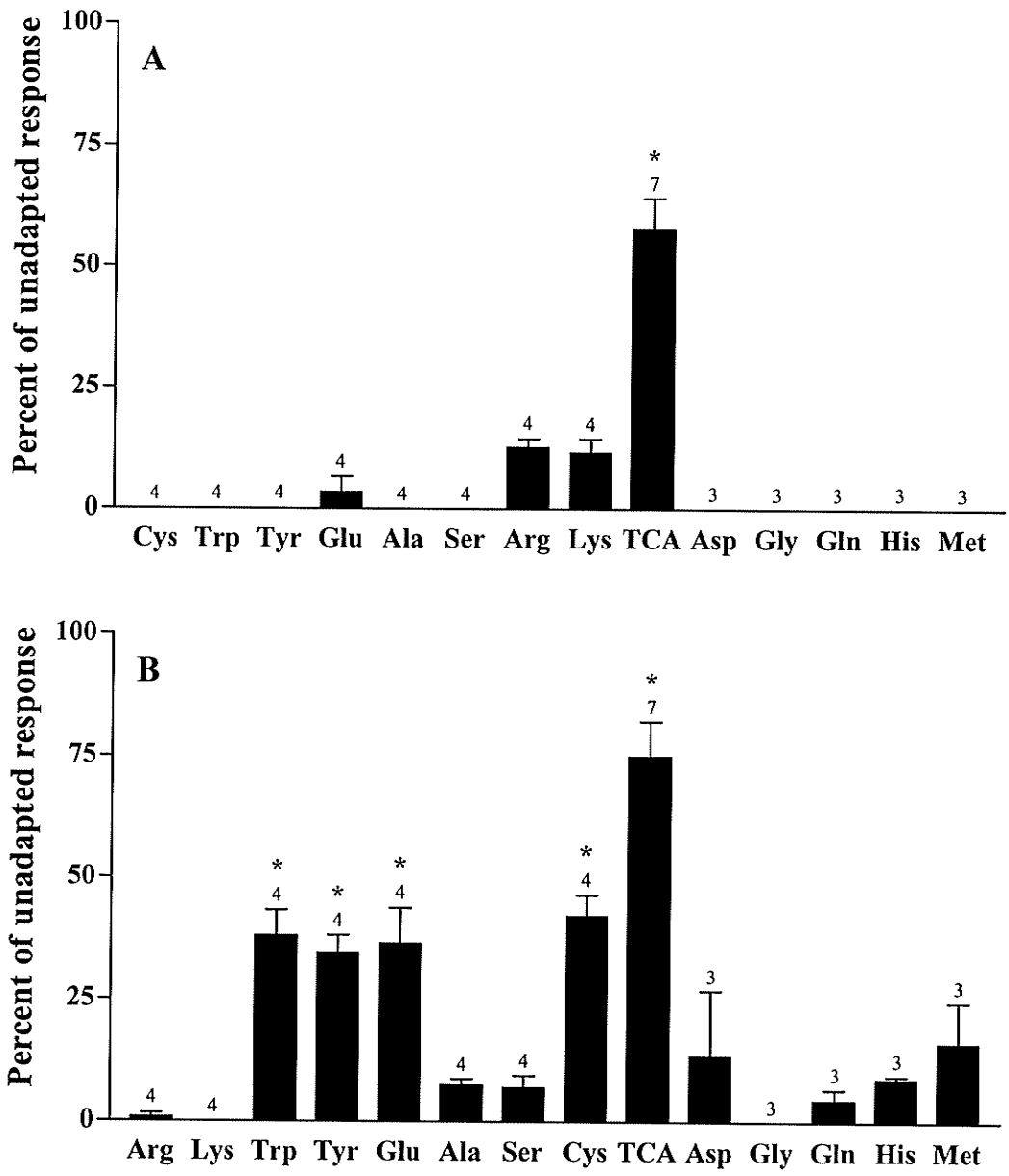


Figure 3.3. EOG responses of rainbow trout to selected amino acids tested at 10^{-5} M and taurocholic acid at 10^{-7} M during adaptation to 10^{-3} M L-cysteine (A) and 10^{-3} M L-arginine (B). Sample size is indicated above the bars. Asterisks indicate a statistically significant difference when compared to the test of the adapting stimulus. Vertical bars represent mean + standard deviation.

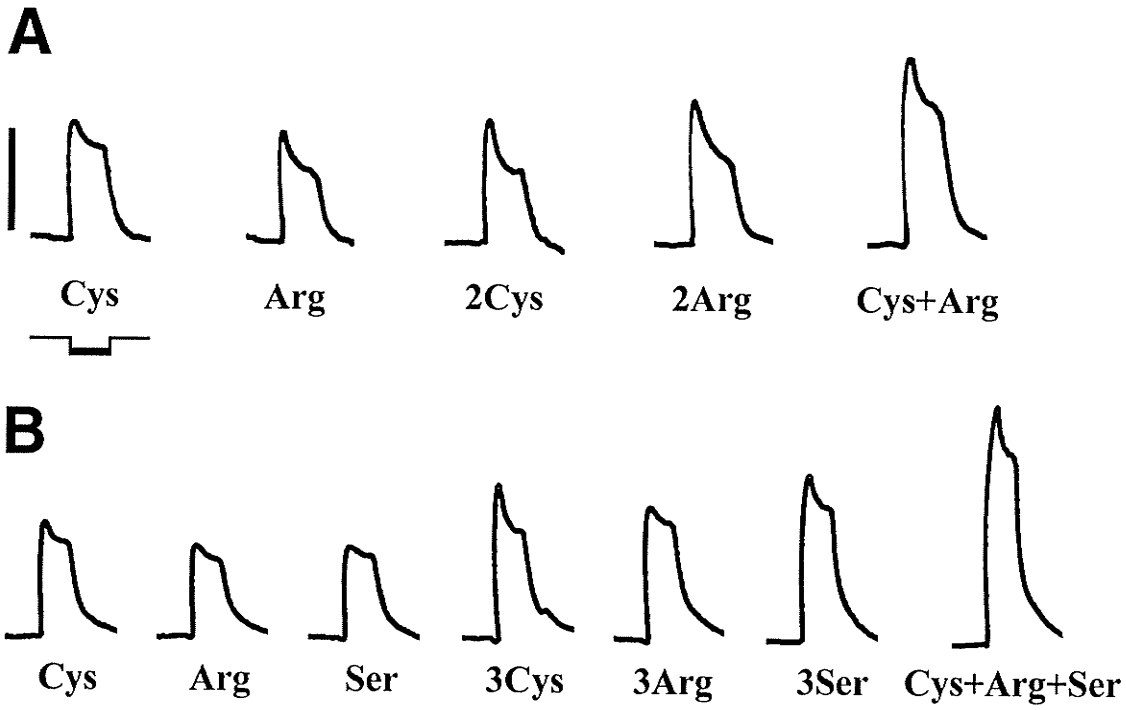


Figure 3.4. Example of EOG responses of brown trout to (A) a binary mixture and (B) a trinary mixture of amino acids. Traces obtained with 2×10^{-8} M L-cysteine (Cys), 10^{-6} M L-arginine (Arg), 10^{-6} M L-serine (Ser) and their mixture are shown as well as the responses to twice (A) and thrice (B) the concentration of each of the mixture's component. The vertical bar on the left in A represents 1 mV and the thick horizontal bar shows the 10-sec stimulus duration.

Table 3.1. Results from the binary mixture experiments in brown trout.

Mixture (n)	*MDI = AB/(2A or 2B)	†ICI = AB/(A+B)	‡AB (2A or 2B)
Glu-Arg (4)	1.203	0.772	yes
Glu-Cys (4)	1.141	0.806	no
Glu-Ser (4)	1.298	0.949	yes
Arg-Ser (6)	1.277	0.903	yes
Arg-Cys (6)	1.228	0.805	yes
Ser-Cys (6)	1.239	0.850	yes

* MDI= mixture discrimination index

† ICI= independent component index

‡ A paired t-test was performed to determine if the amplitude of the mixture response differed significantly from the largest response to twice the concentration of one of its component.

Table 3.2. Results from the trinary mixture experiment in brown trout.

Mixture (n)	*MDI = ABC/(3A, 3B or 3C)	†ICI = ABC/(A+B+C)	‡ABC (3A, 3B or 3C)
Arg-Ser-Cys (5)	1.343	0.691	yes

* MDI= mixture discrimination index

† ICI= independent component index

‡ A paired t-test compared the response amplitude obtained with the mixture to three times the concentration of its most stimulatory component.

Table 3.3. Results from the binary mixture experiments in rainbow trout.

Mixture (n)	***MDI = AB/(2A or 2B)**	**†ICI = AB/(A+B)**	**‡AB (2A or 2B)**
Glu-Arg (5)	1.336	0.856	yes
Glu-Cys (5)	1.220	0.887	yes
Glu-Ser (5)	1.297	0.815	yes
Arg-Ser (5)	1.266	0.849	yes
Arg-Cys (5)	1.224	0.894	yes
Arg-Lys (4)	1.011	0.646	no
Ser-Cys (5)	1.180	0.792	no

* MDI= mixture discrimination index

† ICI= independent component index

‡ A paired t-test was performed to determine if the amplitude of the mixture response was significantly different from twice the concentration of its most stimulatory component.

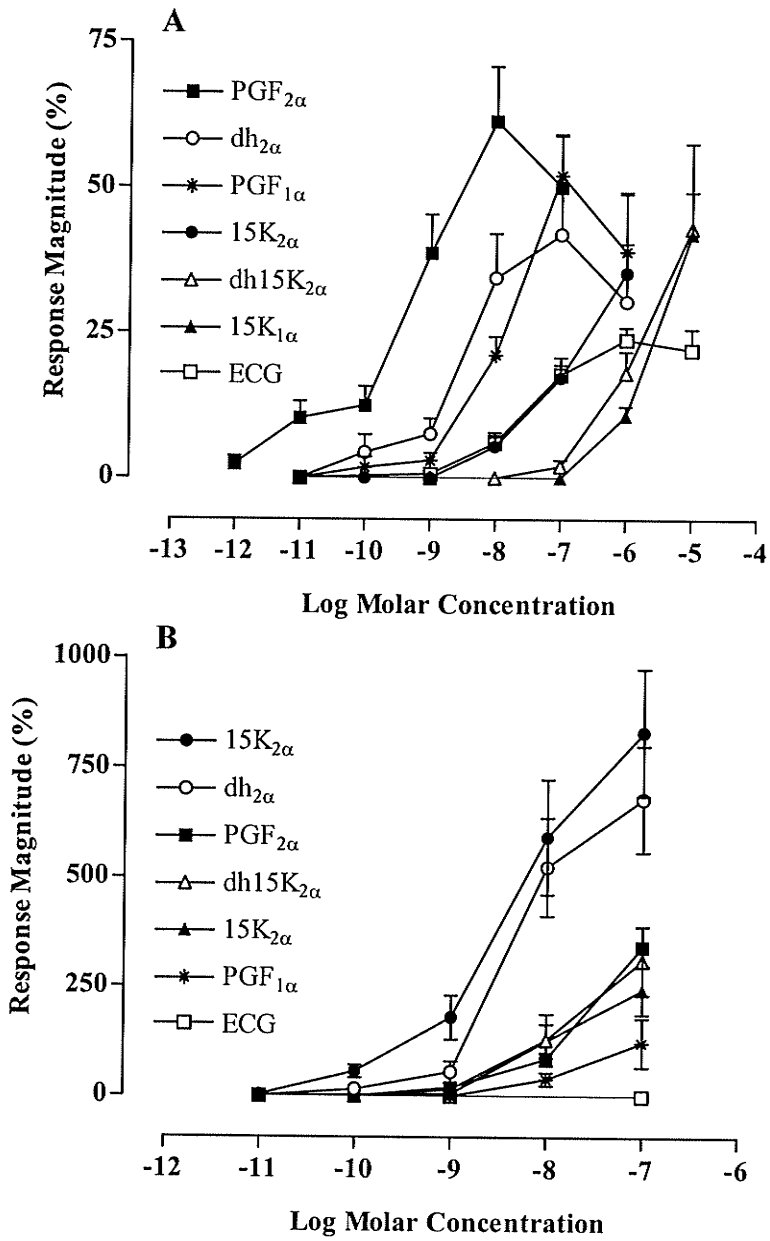


Figure 3.5. EOG concentration-response relationships of brown trout (A) and lake whitefish (B) for six F-prostaglandins and the steroid derivative ECG. Response magnitude is presented as the percentage of the response to the standard 10^{-5} M L-serine (2.96 ± 1.04 mV for brown trout and 0.35 ± 0.11 mV for lake whitefish). Each point represents mean ± standard deviation. Sample size is between 3 and 12 (see Table 3.4 for more details). Responses of both sexes are combined in this graph for clarity.

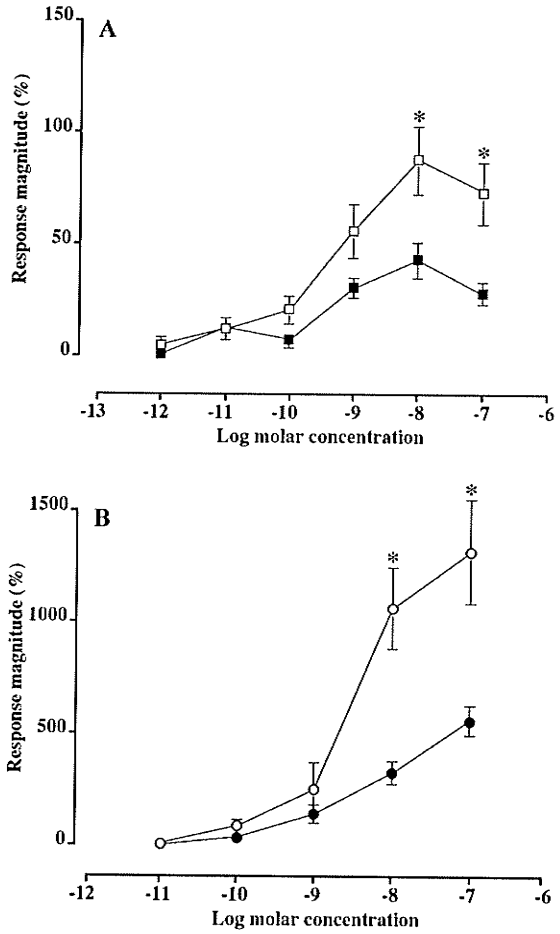


Figure 3.6. Sex differences in EOG responses to F-prostaglandins. (A) EOG response profiles of male (open squares) and female (solid squares) brown trout for prostaglandin $F_{2\alpha}$. The sample size is 5 except at 10^{-7} M where 4 females were tested. (B) EOG response profiles of male (open circles) and female (solid circles) lake whitefish for 15-keto-prostaglandin $F_{2\alpha}$. The sample size is 4 males and 7 females. Each symbol represents mean ± standard deviation. The response magnitude is expressed as percentage of the standard 10^{-5} M L-serine response. Asterisks indicate a statistically significant difference ($P<0.05$).

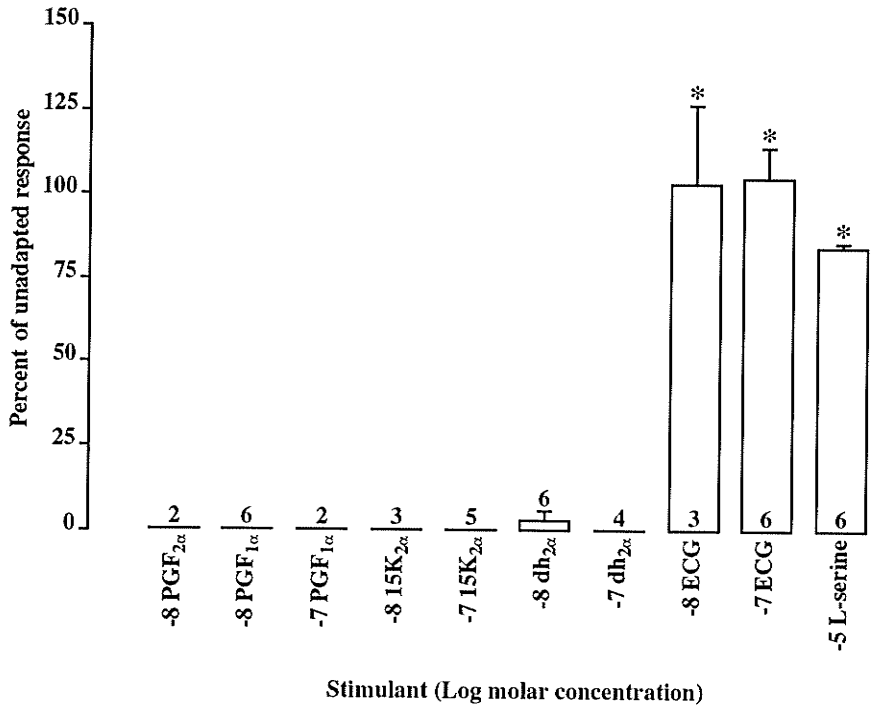


Figure 3.7. EOG responses of brown trout to selected chemicals during adaptation to 10^{-8} M prostaglandin $F_{2\alpha}$. Sample size for each stimulant is indicated at each bar. Asterisks indicate a statistically significant difference ($P<0.05$) when compared to the response to 10^{-8} M prostaglandin $F_{2\alpha}$ with a Tukey post hoc test. Vertical bars represent mean + standard deviation. Responses of both sexes are combined in this graph.

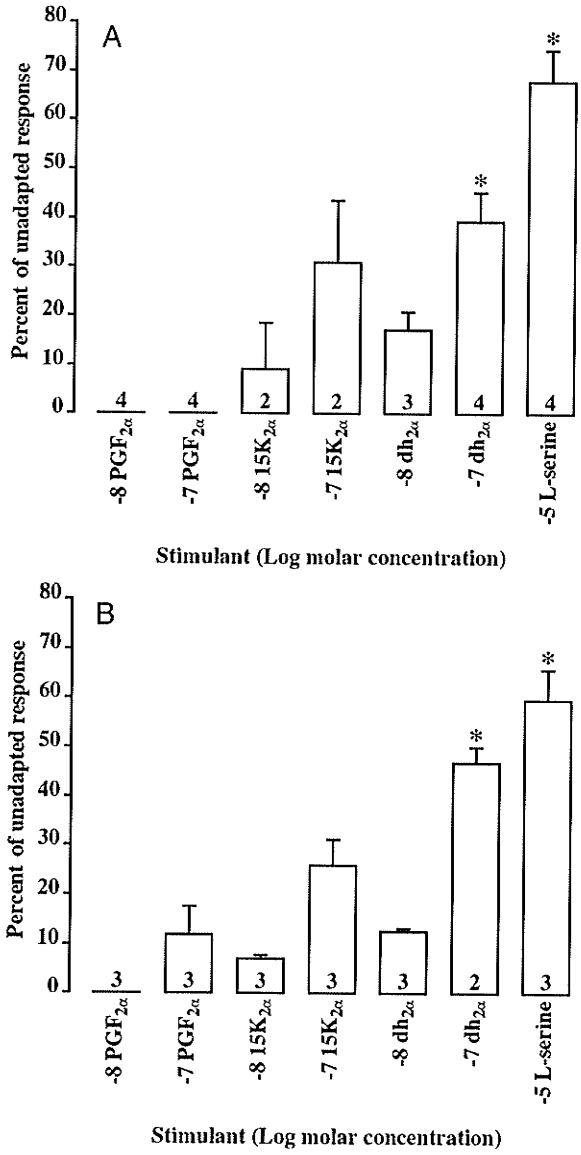


Figure 3.8. EOG responses of lake whitefish to selected chemicals during adaptation to 10^{-8} M 15-keto-prostaglandin $F_{2\alpha}$ (A) and 10^{-8} M 13, 14-dihydro-prostaglandin $F_{2\alpha}$ (B). Sample size for each stimulant is indicated at each bar. Asterisks indicate a statistically significant difference ($P<0.05$) when compared to the test of the adapting stimulus with a Tukey post hoc test. Vertical bars represent mean + standard deviation. Responses of both sexes are combined in this graph.

MDI = AB/2A or 2B	ICI = AB/A + B	N
0.95 ± 0.03	0.68 ± 0.12	5

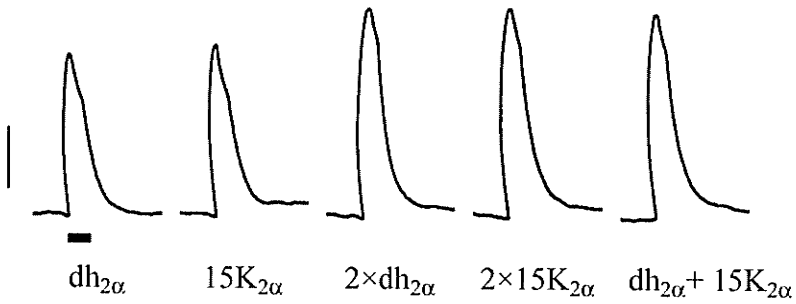


Figure 3.9. Example of EOG responses of lake whitefish to a mixture of 15-keto-prostaglandin $F_{2\alpha}$ and 13, 14-dihydro-prostaglandin $F_{2\alpha}$ at 10^{-8} M. The vertical bar is 1 mV and the horizontal bar shows the 10-sec stimulus duration. The table inserted on top shows the calculated values of mixture discrimination index (MDI), independent component index (ICI) and the sample size (N).

Hara, 1994; Li and Sorensen, 1997; Michel and Derbidge, 1997) in the olfactory epithelium. My cross-adaptation and mixture results confirm that multiple receptor types are involved in amino acid detection in brown and rainbow trout without major differences observed between the two species. The extent to which the response to one odorant is suppressed by adaptation of the olfactory organ with a second odorant could represent a measure of independence of the receptor sites binding the two odorants. For example, from Figure 3.2A, one might hypothesize that serine and cysteine activate similar receptor populations, while cysteine and arginine would activate a large quantity of independent receptors and share some receptors between them. $PGF_{2\alpha}$ or TCA would activate a receptor population independent of the one activated by cysteine. The large MDI value seen in the trinary mixture experiment is in accordance with the reciprocal cross-adaptation results showing that each amino acid used in this study activates an independent receptor type. The receptor sites commonly activated by different amino acids could be explained by the existence of one or some widely expressed odorant receptor(s) sharing affinity for several amino acids. This receptor(s) could detect common features of amino acid molecules rather than the functional group of each. This possibility is suggested by the present observation that only the glycine response was totally suppressed by adaptation with both Cys and Arg at 10^{-3} M in rainbow trout (Fig. 3.3). Glycine is the smallest amino acid with hydrogen as its functional group. Alternatively, the partially independent nature of amino acid olfactory receptor responses observed with the EOG could derive from the expression of more than one receptor type on each olfactory sensory neuron. These multiple receptor types on a single ORN could share the same transduction mechanism or their ionic responses may

not be perfectly additive; resulting in a lower EOG amplitude generation than when independent receptors are activated. A goldfish odorant receptor detecting basic amino acids is widely expressed in the olfactory epithelium, supporting the above idea (Speca et al., 1999). Also, glutamate metabotropic receptors expressed along with odorant receptor candidates in the same olfactory sensory neurons in catfish suggest that these neurons could bear more than one receptor type (Medler et al., 1998).

The existence of a receptor type for an acidic amino acid (glutamic acid) is demonstrated for the first time in a salmonid fish. Interestingly, the response to glutamic acid under cysteine adaptation is only significant at a low concentration (10^{-6} M). A mixture of glutamic acid at 10^{-5} M and cysteine did not yield a significant enhancement in the present study, and a previous cross-adaptation investigation in Arctic charr using high concentrations of the same stimulants could not demonstrate an independent receptor type for glutamic acid (Sveinsson and Hara, 1990b). This hints at a concentration dependence of the activation of some receptor types by glutamic acid. In brown trout, a significant mixture enhancement was observed for the pair cysteine-serine but not cysteine-glutamic acid, even when the response to glutamic acid was less suppressed than serine under cysteine adaptation. This could be because the concentration of glutamic acid used in the binary mixture experiment with cysteine was 10^{-5} M instead of 10^{-6} M. At that concentration, glutamic acid might activate more receptors with cysteine affinity and mask the difference between the electrophysiological responses to cysteine and glutamic acid. The idea that higher amino acid concentrations activate more receptor types is supported by my observation that binary mixtures of high amino acid concentrations do not produce a clear mixture

enhancement in brown trout. Further supporting this view is my result that cross-adaptation with a high concentration of cysteine suppresses completely the response to most amino acids in rainbow trout. A gradual loss of specificity in the activity patterns of olfactory bulb afferents stimulated with increasing amino acid concentrations in zebrafish also supports the above idea (Fuss and Korsching, 2001). These findings imply that the identity of an olfactory signal could be lost or change with increasing signal concentration.

3.4.2. Pheromones

The EOG concentration-response curves obtained for six PGFs and ECG differed greatly among the three species. Generally, the EOG response amplitude increased with stimulant concentration as expected from the linear receptor model (see Sveinsson and Hara, 1990a). In brown trout, the response amplitude to PGFs peaked at 10^{-8}-10^{-6} M, and declined with further increase in concentration. This phenomenon could be observed on most of the dose-response curves and was present in both sexes of brown trout. At present, it is not known why the responses to PGFs declined at high concentrations, but it could be caused by a negative cooperativity, where the binding of one odorant molecule to a receptor reduces the affinity for the next odorant molecule. The existence of very large EOG responses to PGFs in lake whitefish suggests that these odorants may play an important role in its life cycle.

The PGF response threshold values in the present study are similar to those found in Atlantic salmon and Arctic charr (Moore and Waring, 1996; Sveinsson and Hara, 2000). The response threshold for $PGF_{2\alpha}$ in brown trout is also consistent with

another EOG study (Moore and Olsén, 2000). Values of PGF detection thresholds of 10^{-11} M for two catostomid species (Cardwell et al., 1992), and 10^{-10} M and 10^{-11} M, respectively for the cyprinids common carp and goldfish are also reported (Sorensen et al., 1988; Irvine and Sorensen, 1993). The males of *Puntius schwanenfeldi*, a cyprinid, detect $15K_{2\alpha}$ at concentrations varying from 10^{-10} M to 10^{-12} M, depending on the maturity stage of the fish (Cardwell et al., 1995). It has also been shown that the male cobitid loach *Misgurnus anguillicaudatus* detects $15K_{2\alpha}$ and $15Kdh_{2\alpha}$ at the extremely low concentration of 10^{-13} M (Kitamura et al., 1994a).

Contrary to what was claimed before for brown trout (Essington and Sorensen, 1996), sex differences in the EOG responses to PGFs were found in both brown trout and lake whitefish. The difference lies in the fact that males have a larger EOG response magnitude than females at high PGF concentrations. The response thresholds are not different across sexes. Similar EOG results in response to $15K_{2\alpha}$ have been found in the common carp (Irvine and Sorensen, 1993). Male goldfish also have higher response amplitudes and lower detection thresholds to PGFs compared to females (Sorensen and Goetz, 1993). Could the higher EOG response magnitude of males be of any significance? It would be interesting to determine wether the olfactory system differs anatomically between males and females of a species in which a difference in the response to PGFs exists, because higher response magnitudes could simply reflect a larger olfactory epithelium/organ.

Previous work showed that the amplitude of the olfactory response to PGFs varies seasonally in Atlantic salmon (Moore and Waring, 1996) and with reproductive state in the cyprinids *Puntius* and crucian carp (Bjerselius and Olsén, 1993; Cardwell et

al., 1995). It is unlikely that PGF responses vary greatly with reproductive state in other salmonid fishes because immature Arctic charr and sexually undifferentiated brown trout have a strong EOG response to PGFs (Sveinsson and Hara, 2000; present study). Since the fish were kept under a 12h light/12h dark photoperiod all the time, seasonal EOG response variations were unlikely to be revealed in this study.

The present cross-adaptation experiments suggest that only one PGF-receptive mechanism exists in the olfactory receptor neurons of brown trout and lake whitefish. A binary mixture experiment conducted with the two most potent PGFs for lake whitefish, $15K_{2\alpha}$ and $dh_{2\alpha}$, shows that these two chemicals, the only two legitimate candidates that could possess specific detection mechanisms in view of the cross-adaptation results, activate the same receptor population. Moore and Waring (1996) also suggested from cross-adaptation data that $PGF_{2\alpha}$ and $PGF_{1\alpha}$ shared the same olfactory receptor in Atlantic salmon. Barring discovery of new PGFs, it appears that goldfish possess both $PGF_{2\alpha}$ and $15K_{2\alpha}$ receptor types, while salmonids possess only one receptor type as shown by the exclusive presence of the $PGF_{2\alpha}$ receptor in brown trout and the $15K_{2\alpha}$ receptor in lake whitefish. The presence of two PGF receptor types in the same species could be a characteristic of Cypriniformes as shown by the different shapes in their $PGF_{2\alpha}$ and $15K_{2\alpha}$ EOG concentration-response curves and cross-adaptation experiments (Sorensen et al., 1988; Cardwell et al., 1992).

3.5. Conclusion

The extreme sensitivity of the brown trout and lake whitefish olfactory system to some PGFs further supports a role of these chemicals as reproductive pheromones. The

detection of amino acids in salmonids is accomplished by several receptor types with overlapping affinities. On the other hand, brown trout and lake whitefish appear each to possess a single olfactory receptor type devoted to the detection of PGFs. The presence of multiple amino acid receptor types is compatible with the view that signal discrimination plays a critical role in learning different food odors, while the salmonid pheromone is detected by a high specificity detector of a chemical type of strict structure that elicits programmed innate behavioral/endocrine changes.

CHAPTER 4

Second-order responses to Odorants and Reproductive Pheromone Candidates

4.1. Introduction

Research shows that primary olfactory neurons found in large areas of the olfactory epithelium converge on specific areas of the olfactory bulb glomerular layer according to the receptor type they express (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996; Wang et al., 1998). Therefore, a spatial pattern of activity observed in the olfactory bulb upon stimulation with an odorant could represent which receptor type(s) is activated. This organization is not restricted to vertebrates as results in *Drosophila* show (Gao et al., 2000; Vosshall et al., 2000).

Imaging of primary olfactory neurons' synaptic terminals in the olfactory bulb has been used successfully to localize afferents activated by different odorants in zebrafish and mouse (Friedrich and Korsching, 1997; Friedrich and Korsching, 1998; Fuss and Korsching, 2001; Wachowiak and Cohen, 2001). Different odorants produce specific patterns of activation in bulbar afferents, even if overlap of responses to related odorants occur. Research in zebrafish has shown that despite specific bulbar activation patterns for single amino acids demonstrated by imaging studies (Friedrich and Korsching, 1997; Friedrich and Korsching, 1998; Fuss and Korsching, 2001), individual mitral cell responses are not specific to a single amino acid (Friedrich and Laurent, 2001), suggesting a combinatorial code for amino acid quality. Friedrich and Laurent (2001) further suggested that temporal patterning of mitral cell population responses during the course of a stimulus increase the distinctiveness of amino acid representations in zebrafish. In other fish species, most single olfactory bulb neurons

also respond to several amino acids (MacLeod, 1976; Bodznick, 1978; Meredith, 1981; Kang and Caprio, 1995b). Recent results also show that most olfactory bulb neurons are not specific to an odorant class in goldfish (Hanson and Sorensen, 2001; Masterman et al., 2001).

A chemotopy of odor representations in the olfactory bulb has recently been demonstrated in channel catfish through the use of both EEG and single unit recording (Nikonov et al., 2000; Nikonov and Caprio, 2001). Responses to mixtures of amino acids, bile acids and nucleotides, respectively, were found in different areas of the catfish olfactory bulb. These results showed for the first time that the selectivity of single olfactory bulb neurons underlies the specific foci of EEG activity obtained with different odorant classes in teleosts. EEG recording of neural activity induced by stimulation with different odorant classes demonstrated a topography of olfactory projections to the olfactory bulb in salmonid fishes (Thommesen, 1978; Döving et al., 1980; Hara and Zhang, 1996; Hara and Zhang, 1998). However, no EEG responses to putative pheromones were recorded in the salmonid bulb, suggesting that a distinct signal-processing mechanism exists for pheromones (Hara and Zhang, 1998). Unlike many vertebrates, fish do not possess a distinct vomeronasal organ (Eisthen, 1992), but it has been hypothesized that pheromonal pathways of teleosts could be functional correlates of the tetrapod vomeronasal systems (Dulka, 1993). Electrophysiological and optical imaging studies show that pheromonal information appears to be processed by an olfactory subsystem in fish (Fujita et al., 1991; Sorensen et al., 1991a; Friedrich and Korsching, 1998).

In the present study, bulbar EEG and single neuron responses to odorants and

pheromone candidates were recorded. The correlation between the established topography of population and single neuron responses in salmonid fishes was of interest as well as the neural pathways through which putative pheromonal signals are transmitted to the central nervous system. Additionally, the neuron response characteristics within a zone of the olfactory bulb responsive to amino acids were investigated to examine if the relative independence of amino acid receptor mechanisms found in the olfactory epithelium might be maintained in the response profiles of olfactory bulb neurons.

4.2. Materials and Methods

Brown trout (*Salmo trutta*) one to three years old at the time of the experiments were originally obtained from the Whiteshell Provincial Fish Hatchery, Manitoba. Lake whitefish (*Coregonus clupeaformis*) were spawned from a wild stock (Clearwater Lake, Manitoba) three to five years before they were used. They were raised at the Freshwater Institute, Winnipeg. Rainbow trout (*Oncorhynchus mykiss*) of the Tagwerker strain were obtained from the Rockwood Aquaculture Research Centre, Freshwater Institute. Rainbow trout were three to four years old at the time of the experiments. The fish's sex could only be determined at dissection time. Some young brown trout were sexually undifferentiated. For a description of the animal holding conditions see Section 2.2.

For electrophysiological recordings, fish were prepared as described in Section 3.2. The gills were continuously perfused (0.4 l/min) with dechlorinated water. The roof of the skull was opened, and cartilage and mesenchymal tissues removed to expose

the dorsal brain from the olfactory nerves to the telencephalon. Unit recording was performed with tungsten or stainless steel microelectrodes (impedance, 5 MΩ, World Precision Instruments, Sarasota, FL). Electrical signals were amplified (model P511, Grass Instruments, Quincy, MA; ½ amplitude lower than 30 Hz and higher than 3 kHz) and fed to a slope/height window discriminator (Frederick Haer and Co., Bowdoinham, ME) to isolate single units. A constant auditory monitoring of raw signal neural activity was maintained with an audio output to a speaker. The window discriminator permitted the setting of three amplitude threshold levels on the oscilloscope monitor. It was thus possible to manipulate the amplitude window size so that only a spike of a specific height would trigger an output response from the discriminator. The shape and sound of the firing units could also be used to judge the presence of one or more units. A typical recording comprised several units firing well above noise level. In order to be confident that the output of the window discriminator represented the activity of a single unit, the position of the recording electrode was adjusted so that an easily isolable unit could be seen on the oscilloscope. The output was then recorded on a polygraph (model 79, Grass Instruments). A reference electrode was placed on the dorsal skin of the snout. Electrode surface location and depth were noted for every recording.

A bipolar platinum-iridium electrode (tip diameters 50 μm, separated by a gap of 0.3 mm, WP Instruments) was used to record surface and intra-bulbar EEGs. Electrical signals were amplified (model 7P3 A, Grass Instruments; ½ amplitude lower than 0.3 Hz and higher than 75 Hz) and recorded on a polygraph. To visualize the PGF response that I could hear but not see in the EEG trace in lake whitefish, audio outputs of EEG responses to olfactory stimulants in some experiments were transformed into

waveforms by using version 1.2.4 of the Canary program (Laboratory of Ornithology, Cornell University). Briefly, EEG sound recordings taken in the vicinity of the audio speaker monitoring neural activity were transformed from analog to digital format at a sample rate of 44.1 kHz and a sample size of 16 bits without frequency filtering. The resulting graphs were plots of signal amplitude versus time. The Canary program converted the signal amplitude from millivolts to micropascals according to built-in calibration.

Microinjections of methylene blue were originally performed in three lake whitefish to identify the general location of the PGF-responsive neurons. First, the stereotaxic location of a PGF responding neuron was identified and the electrode removed from the brain. Using the same micromanipulator, a 10 μl Hamilton syringe was lowered to the same spot, and 0.2-0.3 μl of methylene blue solution was injected for 10 min. Prior to dissecting the brain, the fish was deeply anesthetized with MS-222, and then perfused through the heart with 30 ml of fish saline (0.1 M phosphate buffer; 0.725% NaCl; pH 7.4) followed by 250 ml of 4% paraformaldehyde in fish saline. The brain was left in the fixative solution overnight, and then cryoprotected in 30% sucrose fish saline for a day. Sagittal sections 40 μm thick were cut on a cryostat (American Optical Corp.) and mounted on gelatin-coated glass slides. To mark more precisely the recording locations in the PGF-responsive region, a direct current (10 V-10 s, 25 V-5 s and 25 V-10 s) was passed through the stainless steel recording microelectrodes in three lake whitefish. The Prussian blue reaction (Gomori, 1936) with Nuclear Fast Red counterstain was used to reveal iron deposits on sagittal sections obtained as described above.

The delivery of chemical stimuli has already been described in Section 3.2. Note that longer recovery periods (5-15 min instead of 2 min) between stimulations were allowed when sustained responses were encountered in lake whitefish PGF neurons. Preparation of stock solutions has been described in Section 3.2. Test chemicals used in brown trout were the amino acids L-arginine, L-cysteine and L-serine at 10^{-5} M, the bile acid TCA at 10^{-7} M and $PGF_{2\alpha}$ at 10^{-8} M. The three most potent PGFs for lake whitefish, as demonstrated by previous EOG results (Chapter 3), were used: $15K_{2\alpha}$, $dh_{2\alpha}$ and $PGF_{2\alpha}$. Other stimulants used with lake whitefish were the amino acids L-cysteine and L-arginine at 10^{-5} M, and the bile acid TCA at 10^{-7} M. The test chemicals used in the four rainbow trout tested for unit responses were the amino acids L-arginine, L-cysteine, L-serine, L-glutamic acid and L-lysine at different concentrations (between 10^{-8} and 5×10^{-5} M) as well as the bile acid TCA at 10^{-7} M. PGFs were purchased from Cayman Chemical, and the other chemicals were from Sigma Chemical Co.

Even though most single neuron response types could be determined visually, statistical analysis was required to determine response threshold concentrations or the significance of small responses. Analysis of variance was calculated on single trials. For this purpose, a neuron activity record was divided into 30-sec pre-stimulus, 10-sec stimulus, and 30-sec post-stimulus periods. The stimulus period was further divided into two for analysis of neurons suspected to be of the phasic response type. Also, additional 30-sec post-stimulus periods were used when dealing with possible sustained responses. The periods were divided into 3-sec (pre-stimulus and post-stimulus) or 2.5-sec (stimulus) time bins and spikes were counted in each time bin. The 2.5-sec time bin

counts were multiplied by a factor of 1.2 to make them equivalent to the 3-sec time bins. Upon a significant ANOVA result ($P<0.05$), Tukey post hoc tests were done to determine which period(s) were responsible for the significant increase or decrease in spike frequency.

4.3. Results

4.3.1 Responses to odorants

Responses of 84 olfactory bulb neurons were recorded to representative test chemicals from three odorant classes (amino acid, bile acid and prostaglandin) and a water control in 8 brown trout. Cysteine, arginine and serine were selected to represent stimulants within the amino acid odorant class. These amino acids were chosen to represent odorants that activate similar (cysteine and serine) or independent but overlapping receptive mechanisms (cysteine and arginine) according to the previous EOG results (Chapter 3). Forty-one neurons responded specifically to one odorant class, 2 neurons responded to all the stimulants tested and 41 neurons did not respond to any of the stimulants tested. Additional neurons responded to the water control, presumably because of mechanical disruption of the flow to the naris, and are excluded from the present analysis. The response profiles encountered in brown trout bulbar neurons are listed in Table 4.1.

For the purpose of this analysis, the olfactory bulb was divided into dorsal and ventral halves at a depth of 1000 μm from the surface (recordings showed that the maximal depth of the olfactory bulb was around 2000 μm). Further, these halves were divided into a rostral zone and two medial and lateral zones at the middle and posterior

levels of the bulb for a total of ten bulbar regions. Figure 4.1 illustrates the general regions where olfactory bulb neurons responsive to amino acids and TCA were found. Bile acid-responsive neurons were concentrated in the dorsal part of the middle olfactory bulb, with a limited number of neurons found in neighboring rostral and posterior regions. Amino acid-responsive neurons were found mainly along the dorsoventral extent of the latero-posterior bulb. Some neurons responsive to amino acids were also found in the ventral part of the medio-posterior olfactory bulb and occasionally in the middle of the bulb. Also shown in Figure 4.1 are some examples of neuron responses recorded from the olfactory bulb. The proportion of neurons of the posterior olfactory bulb responding to the selected stimuli used in this study was greater in the lateral part than in the medial part (79% of 33 neurons, lateral; 41% of 22 neurons, medial).

Distribution of the surface positions of single neuron recordings did not reveal differences related to cell response specificity within the amino acid-responsive zone. Therefore, neurons with different response specificities were divided into 5 groups and graphed according to their recording depth in order to see if different layers of the olfactory bulb could be responsible for most responses of one given type. The 5 groups were: Cys(+) Ser(+) Arg(+); Cys(+) Ser(+); Cys(+); neurons of the posterior bulb showing no response; and a complex group comprising all neurons showing inhibitory responses, two Arg(+) neurons and a Ser(+) neuron. Figure 4.2 shows that there is no clear relationship between recording depth distribution and neuron response profiles in the brown trout bulb.

Responses of single olfactory bulb neurons to odorants were also recorded in

lake whitefish (Table 4.2). However, the number of neurons responsive to odorants is limited to 15 because the focus of the investigation in this species was the PGF-responsive neurons. With the exception of one generalist neuron, the amino acid- and TCA-responsive neurons of whitefish were also specific for an odorant class and are found in regions corresponding to those previously defined in brown trout. Several inhibitory responses were obtained with amino acid stimulation in this species.

Rainbow trout were used to investigate the effect of amino acid concentration on the response of single olfactory bulb neurons because brown trout were not available at this stage of the investigation. The single neuron response profiles in rainbow trout are listed in Table 4.3. Four of the 9 amino acid-responsive neurons recorded in rainbow trout were held long enough for the determination of concentration response thresholds. The results show that a single neuron responsive to multiple amino acids can have varied response thresholds to different amino acids. Thus, these neurons gain responses to new amino acids with increases in stimulant concentration.

EEG responses to amino acids could be easily obtained in the dorsal olfactory bulb of the three species used. EEG responses to cysteine were also observed in the ventral bulb of lake whitefish. These responses are generated by synchronized oscillatory activity of the activated bulbar neurons. An example of EEG response to cysteine and its sound waveform clearly shows the population firing by bursts at a constant frequency during odorant stimulation (cf. Fig. 4.5).

4.3.2 Responses to reproductive pheromone candidates

A total of 96 single neurons from 17 lake whitefish (8 males and 9 females) were studied. The neuron response profiles are listed in Table 4.2. With the exception of two neurons, all neurons recorded from the olfactory bulb proper responded only to amino acids or TCA. Of the 64 neurons recorded from the ventromedial posterior region linking the olfactory bulb to the telencephalon, 21 neurons increased firing (excitatory) and 7 neurons decreased firing (inhibitory) specifically in response to PGFs (Fig. 4.3). Two neurons in this region responded only to amino acids and one was inhibited by all stimuli. Thirty-three neurons in this zone did not respond to any test chemicals used. The basal firing rate of the neurons studied in this zone was 0.57±0.63 (range 0-2.27) spikes/sec. No neurons located more posteriorly in the ventral telencephalon responded to the olfactory stimuli used. Several neuronal response patterns were observed upon stimulation with PGFs (Fig. 4.4A). In addition to the phasic or tonic activation during stimulus application, approximately a third of the neurons showed sustained activity after stimulus termination, sometimes for up to three minutes. Some neurons displayed an excitatory response delayed by several seconds after the onset of stimulation. These sustained excitatory responses were characteristic of some PGF neurons, and were not observed in neurons responsive to other odorant classes.

Firing frequency in relation to PGF concentration was studied in 6 neurons (an example is shown in Fig. 4.4B). The response frequency increased with $15K_{2\alpha}$ concentration in some neurons, while it peaked at 10^{-9} or 10^{-8} M in others. Response thresholds for $15K_{2\alpha}$ were between 10^{-10} (or lower —10^{-11} M not tested) and 10^{-8} M, while they were 10^{-8} M or higher for $dh_{2\alpha}$. Some neurons responsive to low $15K_{2\alpha}$ concentrations gained significant sustained responses with increases in the stimulant

concentration. The general response pattern of PGF neurons did not appear to change with time or repeated stimulation (Fig. 4.4C).

After the PGF-responsive neuron population had been localized in the ventromedial bulb-telencephalon transition region, EEGs were recorded to determine if it showed synchronized oscillatory responses. No EEG responses were induced by PGF stimulation in 11 whitefish tested, while the same fish had EEG responses to amino acids in the dorsal olfactory bulb (Fig. 4.5A). However, high-frequency neural responses to PGFs were clearly detected by audio monitoring of the EEG output. To illustrate this point, tape recordings of a PGF and an amino acid response were transformed into sound waveforms. Unlike low frequency bursting induced by amino acids in the dorsal olfactory bulb, PGF responses in the bulb-telencephalon region produced firing of high frequency (Fig. 4.5B); note that the response to $15K_{2\alpha}$ persists after stimulus termination.

The recording sites were marked with iron deposits and dye injections. The Prussian blue reaction for iron helped locate the PGF-responsive neuron population (Fig. 4.6). PGF-responsive neurons were found in the ventromedial tissue strip that connects the olfactory bulb to the telencephalon. A fiber bundle was seen running through the middle of the PGF-responsive nervous mass, presumably the medial olfactory tract.

Surprisingly, neurons specific for $PGF_{2\alpha}$ could not be found in brown trout despite robust EOG responses to this chemical class (Hara and Zhang, 1998 and Chapter 3). The telencephalon and preoptic area were also unsuccessfully probed for

PGF responses in brown trout. This stands in marked contrast to the finding of a PGF-specific neuron population in the lake whitefish olfactory system.

4.4. Discussion

4.4.1. Odorants

Neurons specifically responsive to TCA or amino acids were distributed in different regions in the brown trout olfactory bulb resembling those found in a previous EEG investigation of salmonid bulbar responses (Hara and Zhang, 1998). The TCA-responsive region is centered in the middle of the dorsal olfactory bulb and the amino acid-responsive region in the lateral part of the posterior bulb. Amino acid-responsive neurons were found in the ventromedial but not the dorsomedial part of the posterior bulb. The amino acid and bile acid zones are not totally exclusive since a few amino acid-responsive neurons were found in the TCA region and a TCA-responsive neuron was found in the dorsal posterior bulb. Overall, the bulk of neurons responsive to each odorant class are segregated into different bulbar regions. The regions responsive to amino acids and bile acids in brown trout differ from their equivalent regions in catfish and zebrafish. In catfish and zebrafish, the amino acid-responsive region is found more rostrally than in salmonids, and the presence of bile acid-responsive neurons in the ventral olfactory bulb is only predominant in catfish (Friedrich and Korsching, 1998; Nikonov and Caprio, 2001).

Most amino acid-responsive neurons in brown trout showed increased firing when stimulated with all three amino acids used or the most potent amino acid alone,

Table 4.1. Response specificity of recorded single neurons in brown trout.

Response type	Number of neurons
Cys(+) Ser(+) Arg(+)	13
Cys(+)	7
Cys(+) Ser(+)	6
TCA(+)	6
Arg(+)	2
Cys(+) Ser(-) Arg(+)	2
Cys(+) Ser(-) Arg(-)	1
Cys(-) Ser(+)	1
Cys(-) Ser(-)	1
Ser(+)	1
Arg(-)	1
Cys(+) Ser(+) Arg(+) TCA(+) PGF(+)	1
Cys(-) Ser(-) Arg(-) TCA(-) PGF(-)	1
No Response	41

Response type: (+) excitatory and (-) inhibitory.

Symbols are Arg: L-arginine; Cys: L-cysteine; PGF: prostaglandin $F_{2\alpha}$; Ser: L-serine; TCA: taurocholic acid.

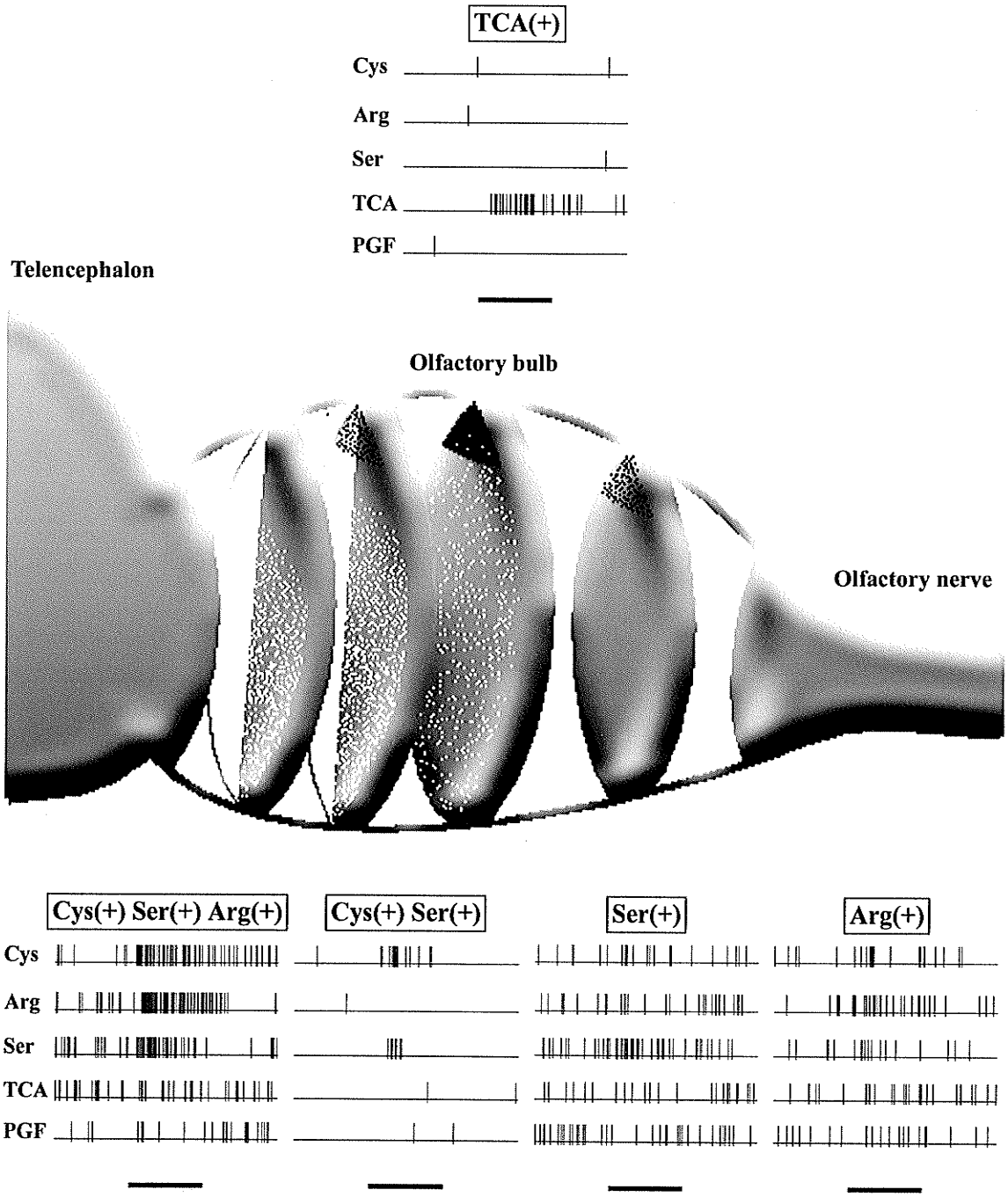

TCA(+)
Cys
Arg
Ser
TCA
PGF
Telencephalon
Olfactory bulb
Olfactory nerve
Cys(+) Ser(+) Arg(+)
Cys(+) Ser(+)
Ser(+)
Arg(+)
Cys
Arg
Ser
TCA
PGF

Figure 4.1

Figure 4.1. General distribution of amino acid- (white) and bile acid- (black) responsive neurons in the brown trout olfactory bulb. The drawing represents sections of the left olfactory bulb. Dorsal is up, lateral is away from the viewer, the olfactory nerve is to the right and the telencephalon is to the left. The activity of five representative single neurons is also shown above and below the bulb drawing. The traces are pulse trains from a window discriminator. The neuron response type is specified in a box above the traces and the bar under the traces represents the stimulus duration. The stimuli tested for each neuron are (from top to bottom) L- cysteine, L-arginine, L-serine, taurocholic acid, and prostaglandin $F_{2\alpha}$.

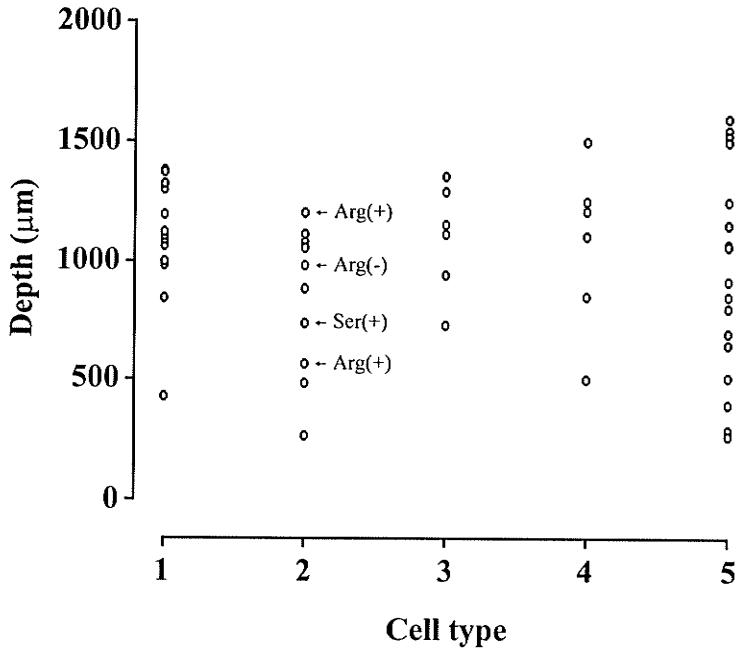


Figure 4.2. Depth distribution of brown trout single neurons according to their response types. Groups are: 1: Cys(+) Ser(+) Arg(+) neurons, 2: all neurons showing inhibitory responses, two Arg(+) neurons and a Ser(+) neuron, 3: Cys(+) neurons, 4: Cys(+) Ser(+) neurons, 5: neurons of the posterior bulb showing no response. Arrows show the Arg-specific neurons and the Ser-specific neuron.

Table 4.2. Response specificity of recorded single neurons in lake whitefish.

Response type	Number of neurons
Cys(+) Arg(+)	2
Cys(-) Arg(-)	2
Cys(+) Arg(-)	1
Cys(-) Arg(+)	1
Cys(-)	3
Arg(-)	3
TCA(+)	2
PGF(+)	23
PGF(-)	7
Cys(-) Arg(-) TCA(-) PGF(-)	1
No Response	51

Response type: (+) excitatory and (-) inhibitory.

Symbols are Arg: L-arginine; Cys: L-cysteine; PGF: prostaglandin $F_{2\alpha}$; Ser: L-serine.

Table 4.3. Responses of 9 recorded single neurons in rainbow trout.

Response type
(neurons' response thresholds)
Cys(+) 10^{-6} Ser(+) 10^{-5} Arg(+) 10^{-5}
Cys(+) 10^{-6} Ser(+) 10^{-6} Arg(+) 10^{-5} Glu(+) 10^{-5}
Arg(+) 10^{-5} Lys(+) 10^{-5}*
Arg(-) 10^{-7} Cys(+) 10^{-5} Ser(+) 5×10^{-5}
(neurons tested at 10^{-5} M only)
Cys(+) Ser(+) Glu(+)
Cys(+) Ser(+)
Cys(+)
Arg(+)
Ser(-) Glu(-) Arg(-)

*L-lysine was only tested with this neuron.

The concentrations used were between 10^{-8} and 5×10^{-5} M.

Response type: (+) excitatory and (-) inhibitory.

Symbols are Arg: L-arginine; Cys: L-cysteine; Glu: L-glutamic acid; Ser: L-serine.

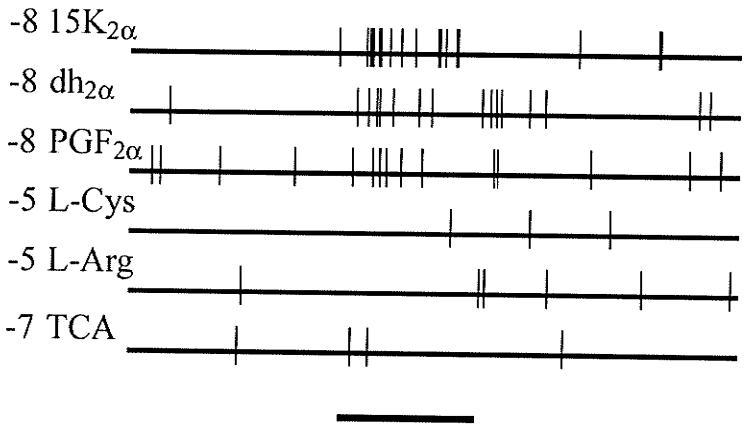


Figure 4.3. Representative example of a neuron that responds only to F-prostaglandins. The stimulant and its log molar concentration are indicated on the left of each record. The bar under the records shows the 10-sec stimulus period. The traces are pulse trains from a window discriminator.

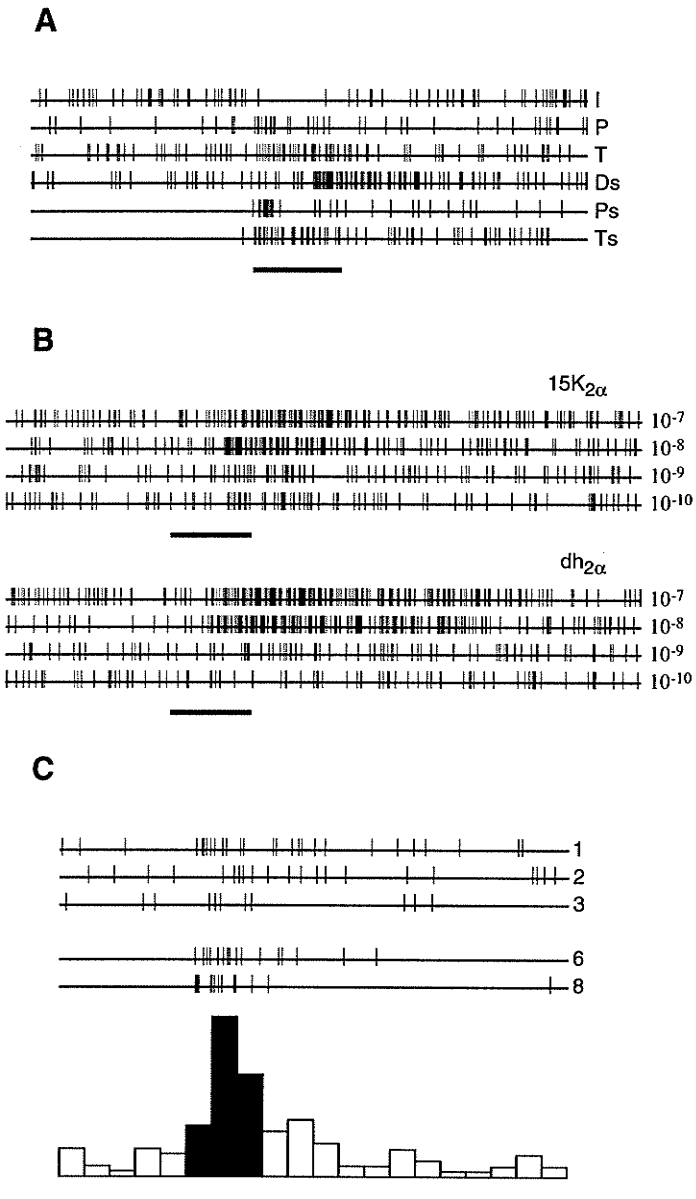

A
I
P
T
Ds
Ps
Ts
B
15K2α
10-7
10-8
10-9
10-10
dh2α
10-7
10-8
10-9
10-10
C
1
2
3
6
8

Figure 4.4

Figure 4.4. Response characteristics of lake whitefish F-prostaglandin neurons. (A) Six different response pattern combinations were observed. I: inhibitory, P: phasic, T: tonic, D: delayed, s: sustained. The bar under the traces in A and B shows the 10-sec stimulus period. (B) Effect of successive ten-fold increases in 15-keto-prostaglandin $F_{2\alpha}$ and 13, 14-dihydro-prostaglandin $F_{2\alpha}$ concentration on the response frequency of a neuron. (C) Effect of repeated stimulation with 10^{-8} M 15-keto-prostaglandin $F_{2\alpha}$ on a neuron. Stimuli 1, 2 and 3 were applied successively at short intervals (2 min or more), while stimuli 6 and 8 were applied approximately one hour later. A frequency histogram for the eight responses (3-sec time bins) is shown at bottom. The black columns show the stimulus period. The traces are pulse trains from a window discriminator.

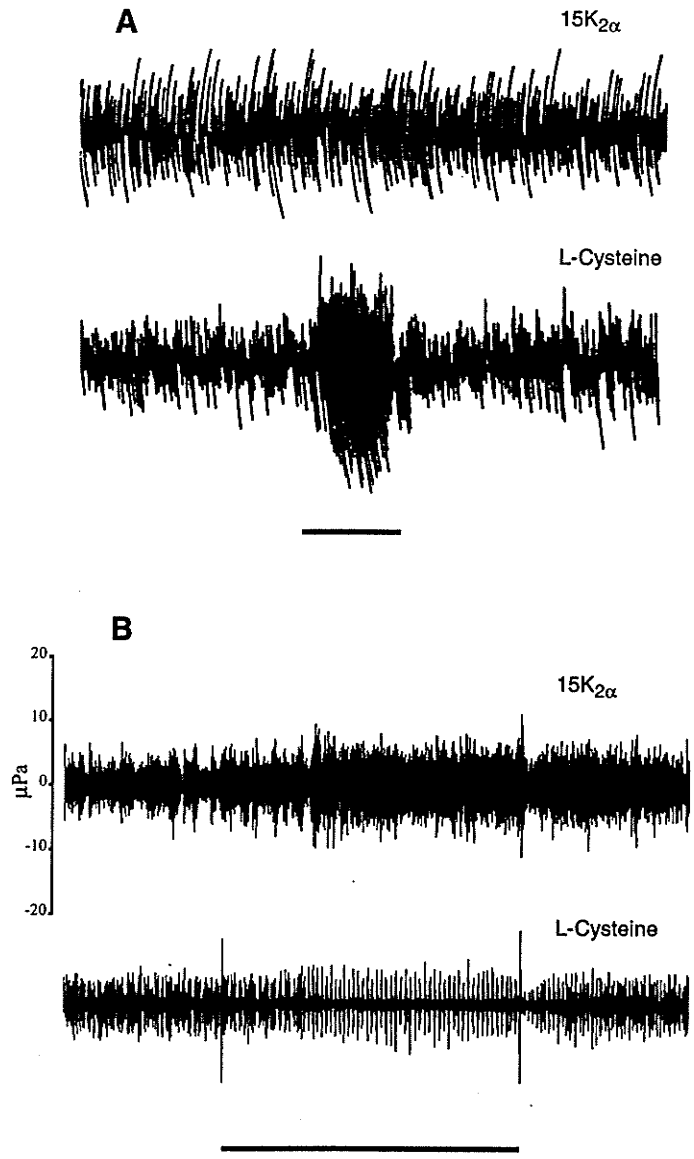


Figure 4.5. Activation of the lake whitefish F-prostaglandin (PGF)-sensitive neuron population does not induce an electroencephalographic (EEG) response. (A) Stimulation of the PGF-responsive region with 10-7 M 15-keto-prostaglandin $F_{2\alpha}$ fails to produce an EEG response (top), whereas 10-5 M L-cysteine induces a normal EEG response in the dorsal olfactory bulb. (B) Sound waveforms of the same responses shown in (A). Note that the bars under the records showing the 10-sec stimulus period are not the same in A and B.

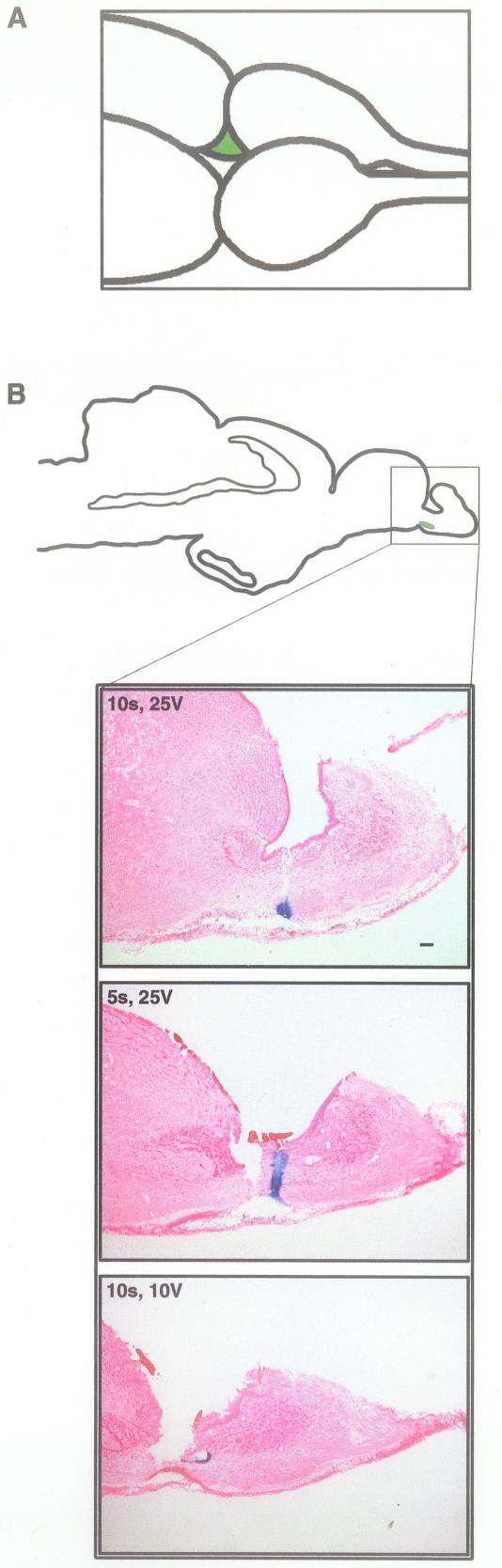


Figure 4.6

Figure 4.6. Location of the F-prostaglandin-responsive neuron population in lake whitefish. The PGF-responsive region (green) is shown on a schematic tilted dorsal view of the brain (A) and a diagrammatic parasagittal section of the brain (top in B). Three PGF neuron locations are identified by the Prussian blue reaction showing the site of iron deposited through the recording electrode (bottom in B). The lesion parameters are shown in the upper left corner of each picture. Scale bar is 100 μm.

cysteine. However, complex response patterns including inhibition, possibly explained by intra-bulbar connections between neurons, and responses restricted to arginine or serine alone were observed. The demonstration of bulbar responses restricted to arginine or serine confirms the mixture results that independent receptor types for basic (arginine) and more than one short-chain neutral (serine and cysteine) amino acids exist in the brown trout olfactory epithelium. As an example, the observed excitatory response to serine only cannot be explained by intra-bulbar processing because the EOG characteristics of the serine response are close to cysteine, but serine is less potent than cysteine. Primary input to the bulb has to be the source of the specific serine response. Not enough amino acids were tested here to make the claim that these bulbar neurons are specific for an amino acid or a group of closely related amino acids, but the selectivity revealed in a limited number of neurons seems responsible for the uniqueness of related amino acid representations by olfactory bulb neurons. These results agree with the largely overlapping nature of fish amino acid receptors. The existence of non-specific bulbar neurons in goldfish points to species differences in olfactory coding strategies used at the bulb level (Hanson and Sorensen, 2001; Masterman et al., 2001). Thus, the finding that stimulus-specific temporal patterns of firing may be used in amino acid signal optimization by zebrafish mitral cells (Friedrich and Laurent, 2001) could derive from the absence of selective bulbar neurons like those observed in brown trout.

The EOG results of Chapter 3 suggested that higher amino acid concentrations activate more receptor types. This idea is reinforced by the observation that some rainbow trout olfactory bulb neurons gained responses to new amino acids with

increases in stimulant concentration. This loss of amino acid specificity in single neurons parallels the gradual loss of specificity observed in the activity patterns of zebrafish olfactory bulb afferents stimulated with increasing amino acid concentrations (Fuss and Korsching, 2001). Thus, the olfactory receptor properties related to stimulant concentration appear to be projected without modification in the olfactory bulb.

4.4.2. Pheromone candidates

The single neuron recordings clearly show the high specificity of the PGF neurons to this chemical class in lake whitefish. This is in marked contrast with recent results obtained from goldfish indicating that most olfactory bulb neurons responsive to reproductive pheromones also respond to other odorant classes (Hanson and Sorensen, 2001; Masterman et al., 2001). Observations in zebrafish are more comparable with the present results in lake whitefish (Friedrich and Korsching, 1998). In zebrafish, a glomerulus specific for $PGF_{2\alpha}$ exists in the ventral olfactory bulb. It remains to be seen how variable will be the schemes used to encode pheromone information in different fish species.

Many PGF-specific neurons exhibited responses that continued for long periods after stimulus termination, despite the transient nature of EOG responses. This unique response pattern is evoked only by PGFs in lake whitefish. These PGF neurons could represent a central pattern generator for the production of a behavioral or endocrine pheromone response. It has previously been suggested that a central pattern generator controls the releaser pheromone-induced spawning behavior of Pacific herring

(Carolsfeld et al., 1997). The presence of PGF neurons with different response types could be explained by the existence of third, fourth or higher order synapses within the PGF neuron population possibly coordinating different aspects of a response.

Bulbar EEG responses (like the induced wave in response to amino acids in Fig. 4.4A) have been commonly used as an indicator of olfactory responses (Adrian, 1950; Hara, 1975). The EEG response is generated by summated periodic synchronous synaptic activation of the granule cells' dendrites, and secondary neuron discharges are synchronized with the EEG (Satou and Ueda, 1978; Freeman and Skarda, 1985; Yamaguchi et al., 1988; Hasegawa et al., 1994). Synchronized oscillations are involved in fine olfactory discrimination in some invertebrates (reviewed in Gelperin, 1999; Friedrich and Stopfer, 2001; Laurent et al., 2001; Laberge and Hara, 2001). Because the EOG cross-adaptation and binary mixture experiments suggest the existence of a single PGF receptive mechanism in the lake whitefish olfactory epithelium (Chapter 3), fine olfactory discrimination of PGFs using synchronized oscillations may not be required. Alternatively, encoding the precise dynamic features of a pheromone odor plume could create a need to process PGF olfactory information without oscillations in whitefish, as seen in the male moth *Manduca sexta* (Christensen et al., 2000; Vickers et al., 2001). Note that lake whitefish are schooling fish, and that fall spawning migrations to shallow waters are known to occur in this species (Scott and Crossman, 1973). Thus, there exists a possibility that odor plumes from conspecifics could be used in lake whitefish school maintenance or migration. The lack of oscillatory discharges here could also be likened to a response to electrical stimulation demonstrated in the guinea-pig anterior accessory olfactory bulb (Sugai et al., 1997). The guinea-pig accessory olfactory bulb is

divided into two functional subdivisions. The posterior part shows clear oscillatory responses when stimulated, whereas oscillations in the anterior part are weak and of short duration. The accessory olfactory bulb of mammals is known to process pheromone chemosensory information, so the anterior accessory olfactory bulb could be another instance where oscillatory discharges are not involved in pheromone signal coding. Interestingly, research in the mouse has shown that the anterior accessory olfactory bulb is involved in the processing of chemosensory cues detected in a sexual context, while the posterior part of this structure is involved in male-to-male interactions (Kumar et al., 1999).

The present study detected no specific responses to PGFs in the olfactory bulb of brown trout. One possible explanation is that PGF-responsive neurons are present but so few in number that they were missed. Alternatively, the responsiveness of the PGF neurons could be seasonal, a possibility that was not studied since the fish were not exposed to seasonal cycles (12h light-12h dark photoperiod) and did not reproduce in captivity. Another possibility is that the responsiveness of PGF neurons could be activated by intra-specific interactions. More research is needed in brown trout because of the recent demonstration of the potential for $PGF_{2\alpha}$ to be a reproductive pheromone acting on both sexes of that species (Chapter 2; Moore and Olsén, 2000).

4.5. Conclusion

Odorant response topography in the olfactory bulb of brown trout is similar whether mapped by EEG or single unit recording. Information from the small number of independent receptor types distinguishing different amino acid signals is represented in

the olfactory bulb in a limited number of neurons with distinct response profiles. The observation that some rainbow trout olfactory bulb neurons gained responses to new amino acids with increases in concentration suggest that information distinguishing different amino acids is more distinct at low concentration. Thus, it is not known if the high amino acid concentrations (10^{-5} M and higher) used in single neuron recording studies, including this one in brown trout, enable us to study biologically relevant olfactory codes. Indeed, it is not known if fish can discriminate amino acids in high concentrations (Zippel et al., 1993 showed that goldfish could not), with the possible exception of conditioned cardiac responses obtained in channel catfish (Little, 1981).

A neuron population responsive to PGFs was identified in the lake whitefish ventromedial brain, at the transition between the olfactory bulb and telencephalon. In contrast to the bulbar responses to other odorant classes, this neuron population responded without synchronous oscillatory discharges when the olfactory organ was stimulated with PGFs, putative pheromones. Some of the neurons responsive to PGFs also showed responses sustained beyond stimulus duration.

CHAPTER 5

Immunohistochemical Investigation of the Neurons Activated by Food Odors and Reproductive Pheromone Candidates in Lake Whitefish

5.1. Introduction

An investigation of cellular and molecular characteristics of the neuron population responsive to PGFs in lake whitefish was undertaken to elaborate the knowledge available of this specialized olfactory neuron group. Previous findings of primary olfactory projections to the olfactory bulb-telencephalon transition region have been reported in two fish species: three-spined stickleback, *Gasterosteus aculeatus* (Honkanen and Ekstrom, 1990) and platyfish, *Xiphophorus maculatus* (Schreibman and Margolis-Nunno, 1987). Anterograde tracing of the primary olfactory projections in lake whitefish was done in order to demonstrate a possible direct link between the ORNs and the PGF-responsive neurons.

The immediate early gene *c-fos* is a known metabolic marker of neuronal activity (Sheng and Greenberg, 1990; Morgan and Curran, 1991; Herrera and Robertson, 1996). It was reported that a polyclonal antibody against mammalian fos could be used as a marker of neuronal activity in teleost fish (Bosch et al., 1995). Two *c-fos*-related genes were subsequently identified in rainbow trout and their induction by neural activation was confirmed (Matsuoka et al., 1998). The rainbow trout *c-fos*-related genes have two highly conserved regions (amino acids 1-15, leucine zipper and amino acids 130-151, zinc finger) among all vertebrate species studied. The above studies suggested that the *c-fos* method could be used to label the active PGF neurons in lake whitefish and thus reveal the boundaries of this neuron population in detail.

Immunohistochemistry to fos was used here in an attempt to visualize the neurons activated by PGF or food odor exposure in lake whitefish.

The PGFs are reproductive pheromone candidates in lake whitefish. It follows that the neurons activated by PGF stimuli might utilize a chemical messenger potentially capable of triggering a quick reproductive response. Some important messengers present in the brain regions controlling reproduction are dopamine, γ-aminobutyric acid (GABA) and gonadotropin-releasing hormone (GnRH) (Kah et al., 1993), three forms of which are found in the lake whitefish brain (Adams et al., in press). GnRH is a well-known regulator of gonadotropic hormone (GtH) secretion, which impacts greatly on fish reproductive physiology and behavior (Liley and Stacey, 1983). It is now known that salmonids have two GtHs: GtH I and GtH II which are considered homologues of the follicle-stimulating hormone and luteinizing hormone of higher vertebrates respectively (Prat et al., 1996; Saligaut et al., 1999). Dopamine is reported to maintain an inhibitory tone on GtH II secretion during vitellogenesis in salmonids (Billard et al., 1984; Van Der Kraak et al., 1986; Saligaut et al., 1999). GABA has also recently been shown to be involved in the modulation of GtH secretion in salmonids (Mananos et al., 1999; Trudeau et al., 2000). Immunohistochemistry to GnRHs, tyrosine hydroxylase (an enzyme of the dopamine synthesis pathway), and the neurotransmitter GABA was performed in order to find out if neurons positive for these messengers could be found in the PGF-responsive region of the lake whitefish brain.

5.2. Materials and Methods

Lake whitefish (*Coregonus clupeaformis*) were spawned from a wild stock (Clearwater Lake, Manitoba) three to five years before they were used. They were raised at the Freshwater Institute, Winnipeg. The fish holding conditions were as described in Chapter 2. Each fish's sex was determined at the time of dissection.

Labeling of primary olfactory projections was carried out to characterize the olfactory input to the lake whitefish PGF neuron population. Briefly, 5% biotinylated dextran-amine (Sigma Chemical Co.) was applied for 15 min to the olfactory organ of anesthetized fish. The fish were killed 35 days later. This survival period was necessary for the tracer to reach the olfactory bulb and forebrain as determined in preliminary experiments. Prior to dissecting the brain, the fish were deeply anesthetized with MS-222, and then perfused through the heart with 30 ml of phosphate-buffered fish saline (PBFS) (0.1 M phosphate buffer; 0.725% NaCl; pH 7.4) followed by 250 ml of 4% paraformaldehyde in PBFS. The brain was left in the fixative solution overnight, and then cryoprotected in 30% sucrose PBFS for a day. Sagittal sections 40 μm thick were cut on a cryostat (American Optical Corp.). The biotin moiety of the neurotracer was detected by immunohistochemistry on the free-floating brain sections. Briefly, the sections were incubated in PBFS + 0.3% hydrogen peroxide for 30 min to inhibit endogenous peroxidase activity. After 3×10 min rinses in PBFS, the sections were incubated with avidin-biotin: peroxidase + 1% bovine serum albumin (BSA) + 0.4% TRITON-X for 120 min (ABC method). After 3×10 min rinses in PBFS, the chromagen solution (100 mM $NiSO_4$, 125 mM acetate, 10 mM imidazole, 0.03% diaminobenzidine, 0.003% H_2O_2) was applied to the sections for 2-15 min. The sections

were finally mounted, dried, dehydrated in graded alcohols, cleared in toluene and coverslipped. The chemical supplies were from Sigma Chemical Co. The ABC kit was from Vector Laboratories (Burlingame, CA, USA).

The detection of fos activation as a marker of neuronal activity was attempted in lake whitefish exposed to food odors or PGFs. The fish were acclimated to the behavioral tank, exposed to the olfactory stimulants and their behaviors were recorded as in the behavioral experiments described in Chapter 2. After a 15 min control observation period, a food extract (described in Chapter 2), 10^{-8} M $15K_{2\alpha}$, 10^{-8} M $dh_{2\alpha}$ or distilled water was pumped in the tank for 15 min. The fish were allowed 60, 90, 120 or 180 min before sacrifice in order to detect the peak of fos expression in lake whitefish. The preceding time values were chosen to comprise the early and late phase of *c-fos*-related mRNA expression in rainbow trout injected with kainic acid (Matsuoka et al., 1998). Brain tissue was obtained as described above and immunohistochemistry to fos using three different primary antibodies was performed on free-floating sections. The primary antibodies were from Chemicon International (Temecula, CA, USA; # AB1584, sheep anti-fos polyclonal antibody raised against amino acids 4-17 of human fos), Santa Cruz Biotechnology (Santa Cruz, CA, USA; # sc-253-G, goat anti-fos polyclonal antibody raised against a highly conserved portion of human fos), and Oncogene Research Products (Cambridge, MA, USA; # PC38, rabbit anti-fos polyclonal antibody raised against amino acids 4-17 of human fos). The sections were incubated in PBFS + 4% normal donkey serum + 0.4% TRITON-X + 1% BSA for 20 min (a blocking step with normal serum from the species in which the secondary antibody is raised), followed by incubation with the first antiserum diluted 750, 1000 or

2500 times in PBFS + 0.4% TRITON-X + 1% normal serum + 1% BSA at 4°C overnight. On the next day, the sections were rinsed 10 × 10 min in PBFS + 0.02% TRITON-X + 0.25% BSA, followed by incubation with the biotinylated secondary antibody diluted 1000 times in PBFS+ 0.02% TRITON-X + 1% BSA for 60 min. The antigens of the biotinylated secondary antibodies were whole IgGs of sheep (Chemicon), goat (Santa Cruz) or rabbit (Oncogene). The sections were rinsed again 2×15 min in PBFS + 0.25% BSA, followed by incubation with avidin-biotin: peroxidase + 1%BSA + 0.4% TRITON-X for 120 min. After 3×10 min rinses in PBFS, the chromagen solution (100 mM $NiSO_4$, 125 mM acetate, 10 mM imidazole, 0.03% diaminobenzidine, 0.003% H_2O_2) was applied to the sections for 2-15 min. The sections were finally mounted on gelatinized glass slides, dried, dehydrated in graded alcohols, cleared in toluene and coverslipped. The secondary antibodies and normal serum were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and the ABC kit was from Vector Laboratories. Additional chemical supplies were from Sigma Chemical Co.

Labeling of GnRH-positive neurons in the lake whitefish brain was also performed in order to determine if these neurons could be part of the PGF-responsive region. Two antisera with different affinities for the three forms of GnRH found in the lake whitefish brain were used: Snoopy-10/7CR-10 and GF-6. Table 5.1.1 shows the affinities of both antisera for the three GnRH forms present in lake whitefish, while Table 5.1.2 lists the peptide sequences of those GnRH forms. Brain sections were obtained as described above. Immunohistochemistry was performed on free-floating sections using the same method as described above for the detection of fos. The

primary antisera (Rabbit anti-GnRH) were diluted 2500 or 5000 times. The secondary antibody was biotinylated Donkey anti-Rabbit (antigen was whole rabbit IgG). Controls were performed by adding 100 μg of whitefish or salmon GnRH peptide to the primary antisera solutions 30 min prior to the application to the sections. The primary antisera and GnRH peptides were kind gifts of Dr. N. Sherwood (University of Victoria, BC).

Labeling of tyrosine hydroxylase-positive neurons in the lake whitefish brain was also attempted using the same method as in GnRH immunohistochemistry. The primary antibody was from Chemicon International (# AB151, rabbit anti-tyrosine hydroxylase polyclonal antibody).

Labeling of GABA-positive neurons in the lake whitefish brain was also attempted. For neurotransmitter immunohistochemistry, the fish were prepared as described above and then perfused through the heart with 30 ml of a solution of 0.1 M cacodylate acid and 1% $Na_2S_2O_5$ at pH 6.2 followed by 250 ml of a solution of 2.5% glutaraldehyde, 0.1 M cacodylate acid and 1% $Na_2S_2O_5$ at pH 7.5. The dissected brains were left in the glutaraldehyde solution for 120 min, then washed 4 times in a solution of 0.1 M PBFS and 0.725% $Na_2S_2O_5$ at pH 7.5, and cryoprotected in a solution of 0.1 M PBFS, 0.725% $Na_2S_2O_5$ and 30% sucrose at pH 7.5 for a day. Sagittal sections 40 μm thick were cut on a cryostat (American Optical Corp.). The neurotransmitter immunohistochemistry was performed on free-floating brain sections. Briefly, the sections were incubated in PBFS + 0.725% $Na_2S_2O_5$ + 4% normal donkey serum for 60 min (blocking step), followed by incubation with the primary antibody (Chemicon International # AB131, rabbit anti-GABA polyclonal antibody) diluted 1000 times in PBFS + 0.725% $Na_2S_2O_5$ + 0.4% TRITON-X + 1% normal serum at 4°C overnight. On

the next day, after 10×10 min rinses in PBFS + 0.725% NaCl at pH 7.5, the sections were incubated with the secondary antibody (biotinylated anti-whole rabbit IgG) diluted 1000 times in PBFS + 0.725% NaCl + 1% normal serum for 90 min. After 3×10 min rinses in PBFS + 0.725% NaCl, the sections were incubated in a solution of avidin-biotin: peroxidase + 1% normal serum for 120 min (ABC method). After 2×10 min rinses in PBFS+ 0.725% NaCl, followed by 2×10 min rinses in a solution of 175mM acetate and 10mM imidazole at pH 7.2, the chromagen solution (100 mM $NiSO_4$, 125 mM acetate, 10 mM imidazole, 0.03% diaminobenzidine, 0.003% H_2O_2) was applied to the sections for 3 min. The sections were washed and then mounted as described above. The secondary antibody and normal serum were from Jackson ImmunoResearch Laboratories and the ABC kit was from Vector Laboratories. Additional chemical supplies were from Sigma Chemical Co.

5.3. Results

The labeling of lake whitefish primary olfactory neurons with the neurotracer biotinylated dextran-amine showed that axons of these neurons pass horizontally near the PGF-responsive zone at the level of the olfactory nerve and show varicosities suggestive of synaptic contacts in this region (Fig. 5.1).

Immunohistochemistry to GnRH revealed the existence of neurons positive for this neuropeptide in several regions of the lake whitefish brain. Figure 5.2, 5.3, 5.4, 5.5, and 5.6 show representative examples of GnRH-positive neurons in the olfactory nerve-olfactory bulb region, olfactory bulb-telencephalon region, ventral telencephalon, preoptic area and midbrain respectively. Both Snoopy-10/7CR-10 and GF-6 antisera

labelled a similar number of neurons in all regions except the midbrain, where only Snoopy-10/7CR-10 did label neurons (Table 5.2). The addition of salmon GnRH peptide to the Snoopy-10/7CR-10 primary antibody solution abolished all labeling, whereas the addition of whitefish GnRH peptide had no effect. The addition of whitefish GnRH peptide to the GF-6 primary antibody solution abolished all labeling. The GnRH neurons of lake whitefish were of three types: 1) large darkly stained neurons with extensive arborization in the ventral telencephalon and preoptic area; 2) small fusiform neurons found around the olfactory bulb and sometimes in the preoptic area; and 3) large lightly stained neurons with a limited arborization in the midbrain. Some GnRH-positive neurons in the ventral telencephalon appear to be in contact with each other as shown in Figure 5.4. Figure 5.7 shows the general distribution of fibers labeled by the two antisera used in the present study. The distribution of labeled fibers was similar with both antisera used. However, fibers appeared to be less abundant around the midbrain when GF-6 was used. The pituitaries were rarely maintained attached to the brain in the present study preventing the investigation of GnRH neuron projections to this structure.

A population of GnRH-positive neurons was found in the region where PGF-responsive recording sites were labeled with iron deposits in the tissue strip that connects the olfactory bulb and telencephalon (Figs. 4.6 and 5.3). From serial sagittal brain slices stained with thionin blue, I determined that the PGF neurons are present in a position medial to the olfactory nerve level. This position is approximately 120 μm laterally away from a GnRH-positive ganglion of cells found at the medial extreme in this region of the lake whitefish brain (Fig. 5.8).

The three fos antibodies used in this study did not label any neuron or other cellular element in the brain of lake whitefish.

The tyrosine hydroxylase and GABA antibodies used in this study did not label any neuron or other cellular element in the brain of lake whitefish. The use of glutaraldehyde in neurotransmitter immunohistochemistry resulted in a significant background staining.

5.4. Discussion

Previous reports of synaptic contacts from primary olfactory neurons in the olfactory bulb-telencephalon region of platyfish and three-spined stickleback hinted at a possible olfactory role for this part of the brain, but physiological investigations have not been conducted to confirm this role (Schreibman and Margolis-Nunno, 1987; Honkanen and Ekstrom, 1990). Note that the previous studies affirmed that the terminal nerve complex cells are the ones contacted by primary olfactory fibers. Interestingly, it was hypothesized that the responsiveness of the terminal nerve complex cells to olfactory inputs could vary with sex and stages of the reproductive cycle, enabling them to respond only during a restricted period (Schreibman and Margolis-Nunno, 1987; Honkanen and Ekstrom, 1990; Flynn et al., 1997; Flynn et al., 1999). The presence of varicose fibers from primary olfactory neurons in the olfactory bulb-telencephalon region of lake whitefish supports the idea that the PGF-responsive neurons are second-order olfactory neurons contacted directly by ORNs.

It was unfortunate that all of the antibodies used in the present study did not detect the fos-related product in lake whitefish. The 4-17 amino acid sequence of

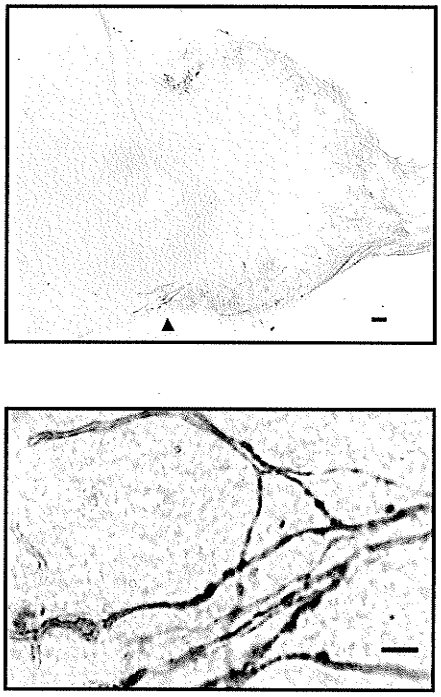


Figure 5.1. Primary olfactory projections in lake whitefish. Top: Low magnification of the olfactory bulb shows the organization of olfactory projections (scale bar is 100 μm). Bottom: The fibers running through the olfactory bulb-telencephalon region (arrowhead in A) have many varicosities suggesting synaptic contacts (scale bar is 1 μm).

Table 5.1.1. Gonadotropin-releasing hormone (GnRH) antisera cross-reactivities.

Antiserum	GnRH form	Percent cross-reactivity	Reference
GF-6	mammalian	100	Sherwood et al., unpublished data and Lescheid et al., 1997
	whitefish	124	Sherwood et al., unpublished data
	salmon	68.8	Lescheid et al., 1997
	chicken-II	3.9	Lescheid et al., 1997
Snoopy-10/7CR-10	mammalian	<0.03	Lescheid et al., 1997
	whitefish	<0.04	Sherwood et al., unpublished data
	salmon	84.8	Lescheid et al., 1997
	chicken-II	100	Sherwood et al., unpublished data and Lescheid et al., 1997

Table 5.1.2. Gonadotropin-releasing hormones peptide sequences.

GnRH form	Peptide sequence	Reference
chicken-II	pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2	King and Millar, 1982
salmon	pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2	Sherwood et al., 1983
whitefish	pGlu-His-Trp-Ser-His-Gly-Met-Asn-Pro-Gly-NH_2	Adams et al., in press

Table 5.2. Presence of labeled neurons in different brain regions in lake whitefish.

Antiserum	ON-OB	OB-Tel.	VT	POA	Midbrain
Snoopy-10 (n=6)	+ (5 of 6)	+ (3 of 6)	+ (6 of 6)	+ (5 of 6)	+ (4 of 6)
Snoopy-10 + 100 μg wfGnRH (n=2)	+ (1 of 2)	+ (2 of 2)	+ (2 of 2)	-	+ (1 of 2)
Snoopy-10 + 100 μg sGnRH (n=2)	-	-	-	-	-
GF-6 (n=4)	+ (2 of 4)	+ (3 of 4)	+ (4 of 4)	+ (2 of 4)	-
GF-6 + 100 μg wfGnRH (n=2)	-	-	-	-	-

The symbols + and - indicate the presence or absence of labeled neurons respectively. The numbers in parentheses indicate the number of fish in which the presence of neurons was observed.

Other symbols: n: sample size; wfGnRH: whitefish form of gonadotropin-releasing hormone; sGnRH: salmon form of gonadotropin-releasing hormone; ON-OB: olfactory nerve-olfactory bulb region; OB-Tel.: olfactory bulb-telencephalon transition region; VT: ventral telencephalon; POA: preoptic area.

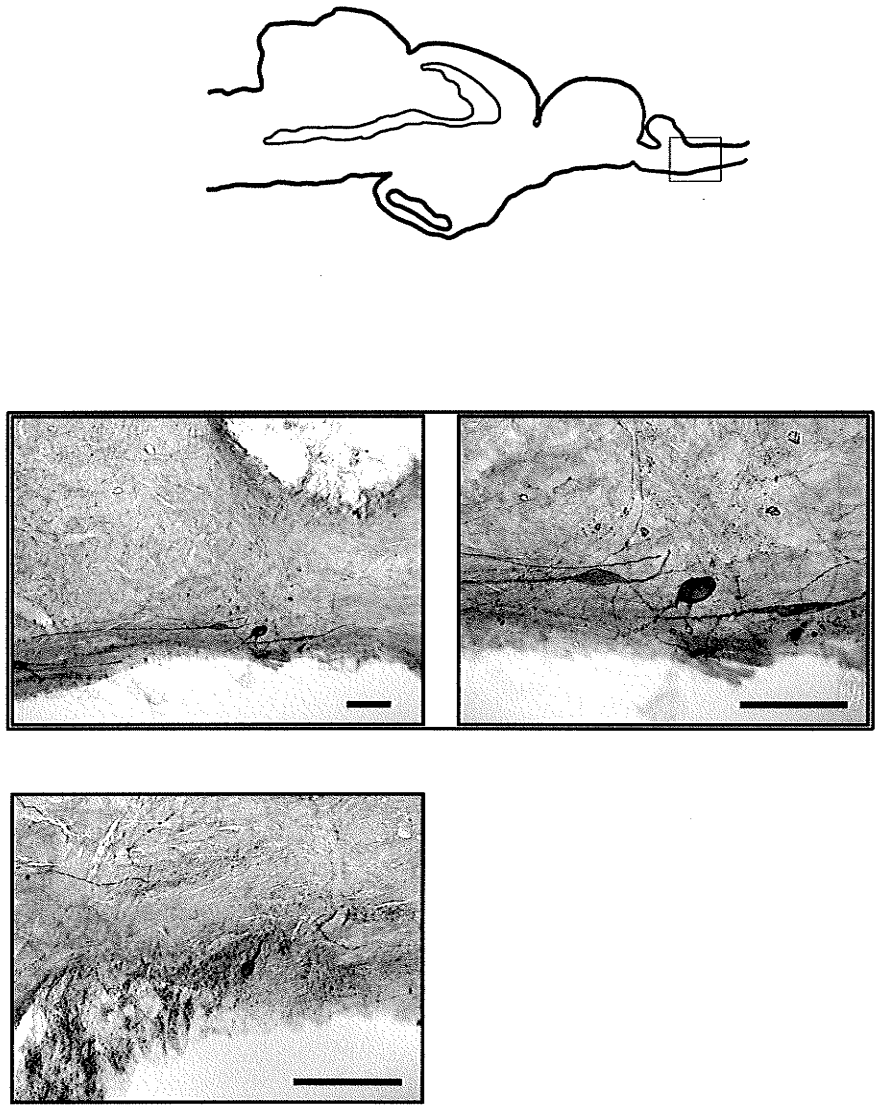


Figure 5.2. Gonadotropin-releasing hormone (GnRH)-positive neurons found at the olfactory nerve-olfactory bulb junction in lake whitefish. The topmost micrographs show different magnifications of the same area. A GnRH neuron in the same region in another fish is shown below the top micrographs. A drawing of a complete medial parasagittal section of the lake whitefish brain is shown to help localize the micrographs (top). Scale bars are 100 μm.

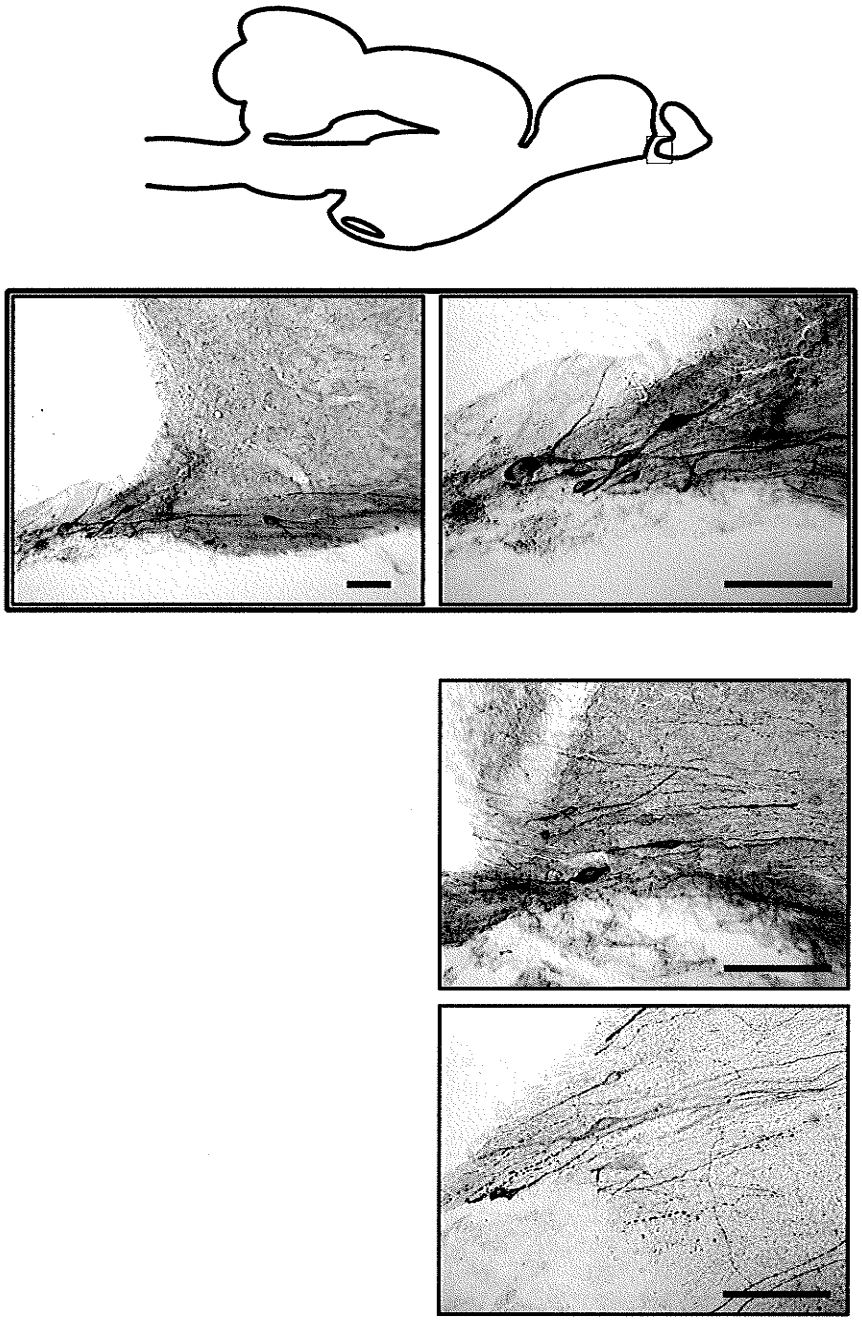


Figure 5.3

Figure 5.3. A gonadotropin-releasing hormone (GnRH)-positive neuron population is found at the medial extreme of the olfactory bulb-telencephalon transition region in lake whitefish. The topmost micrographs show different magnifications of the same area. GnRH neurons in the olfactory bulb-telencephalon region in two additional whitefish are shown below the top micrographs. A drawing of a complete medial parasagittal section of the lake whitefish brain is shown to help localize the micrographs (top). Scale bars are 100 μm.

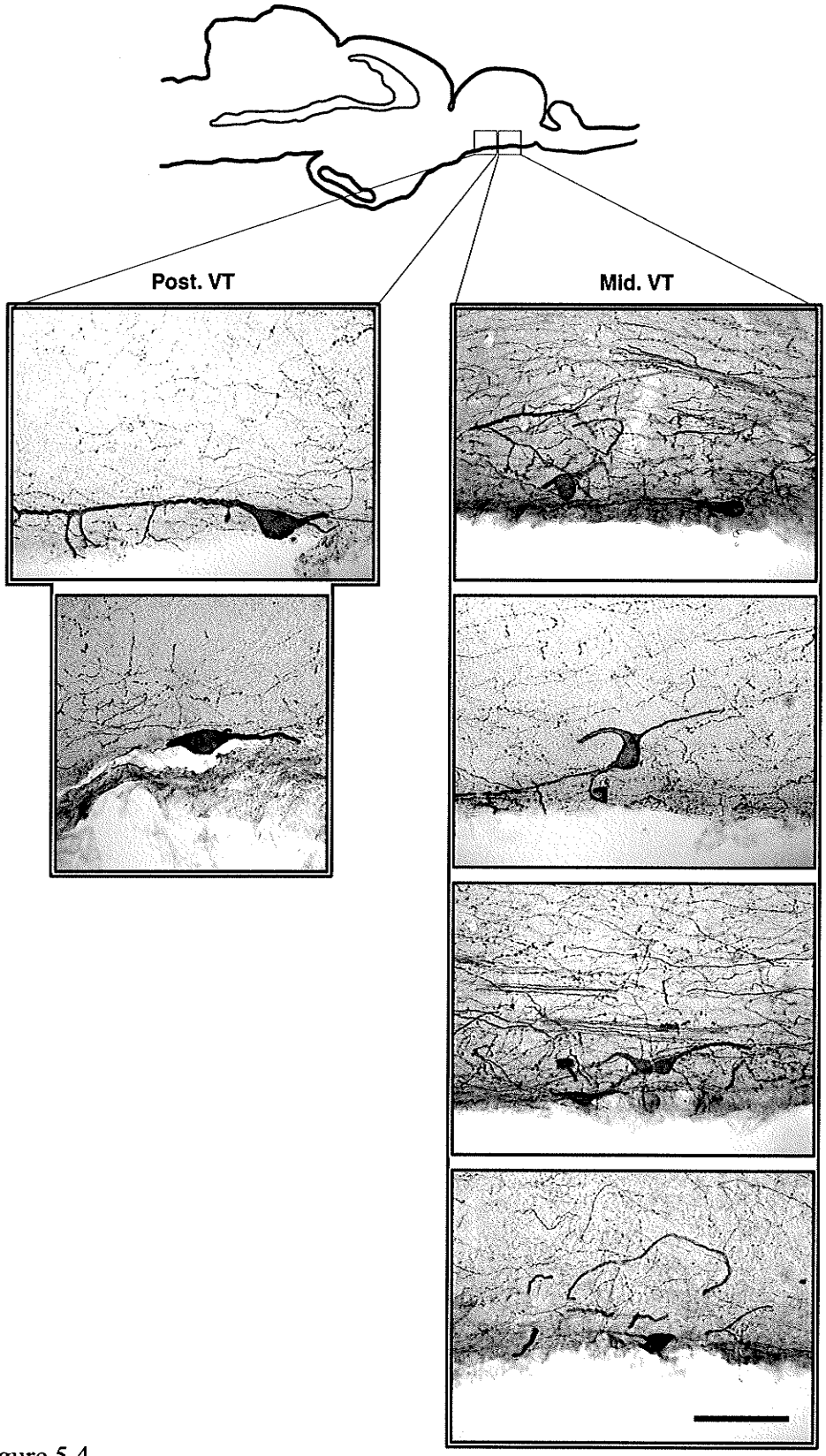


Figure 5.4

Figure 5.4. Gonadotropin-releasing hormone (GnRH)-positive neurons found in the ventral telencephalon in lake whitefish. Examples of GnRH neurons found in the posterior (Post. VT) and middle (Mid. VT) ventral telencephalon are shown in the micrographs on the left and right respectively. A drawing of a complete medial parasagittal section of the lake whitefish brain is shown to help localize the micrographs (top). The scale bar is 100 μm and applies to all micrographs in this figure.

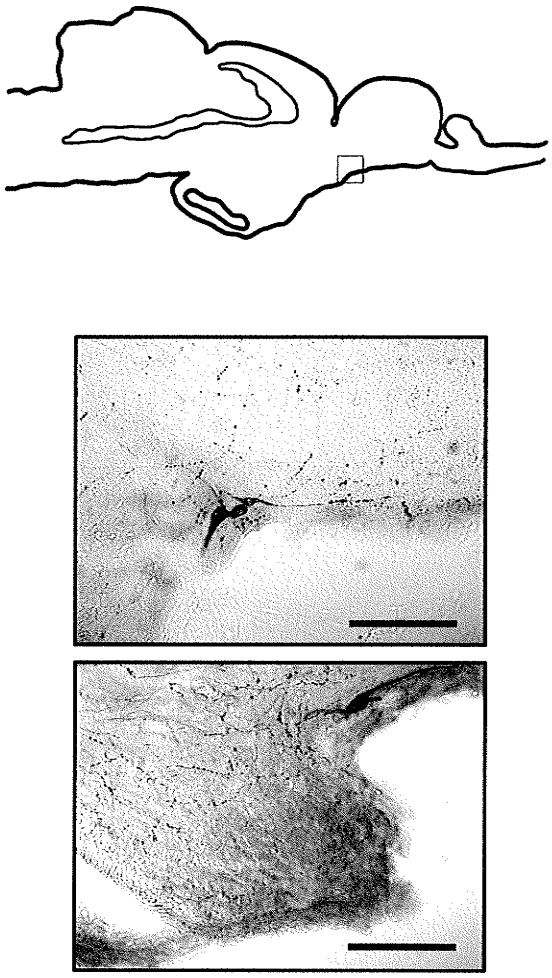


Figure 5.5. Gonadotropin-releasing hormone (GnRH)-positive neurons found in the preoptic area in lake whitefish. A drawing of a complete medial parasagittal section of the lake whitefish brain is shown to help localize the micrographs (top). Scale bars are 100 μm.

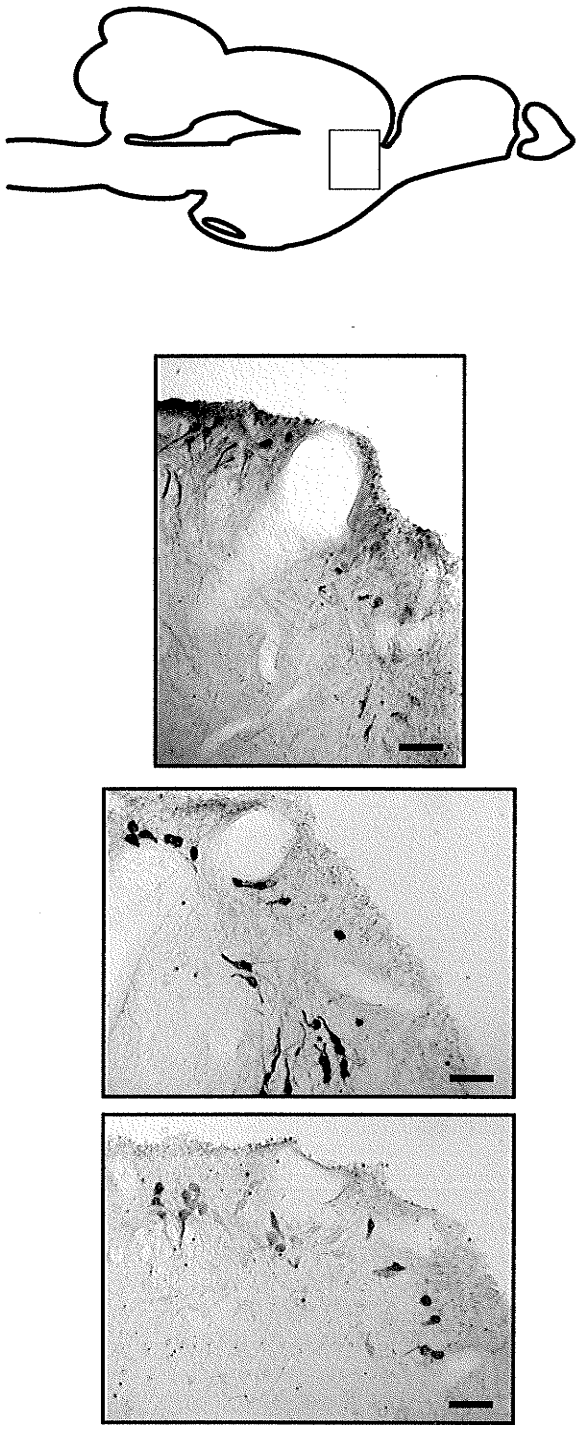


Figure 5.6

Figure 5.6. Gonadotropin-releasing hormone (GnRH)-positive neurons found in the midbrain in lake whitefish. A drawing of a complete medial parasagittal section of the lake whitefish brain is shown to help localize the micrographs (top). Scale bars are 100 μm.

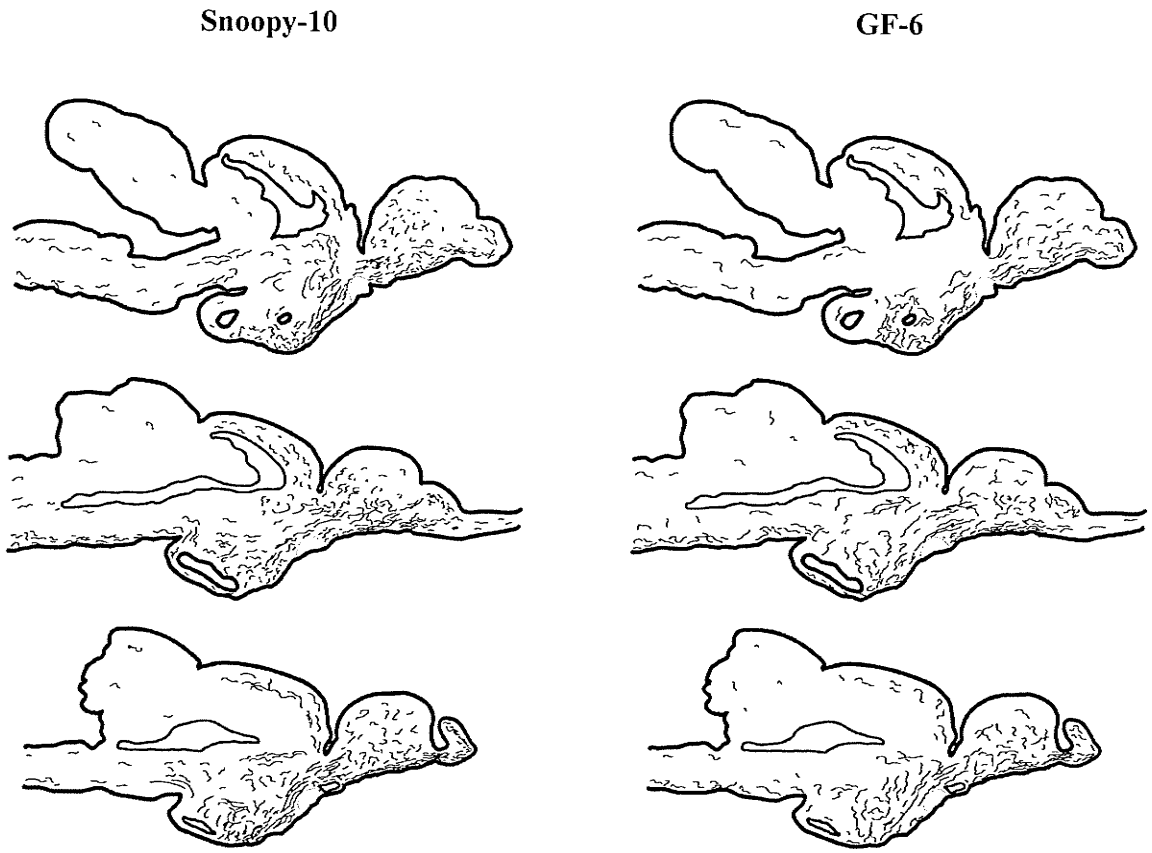


Figure 5.7. General distribution of gonadotropin-releasing hormone (GnRH)-positive fibers labeled by the antisera Snoopy-10/7CR-10 and GF-6 in lake whitefish. Three parasagittal sections of the lake whitefish brain from lateral to medial levels (top to bottom) are used in these examples.

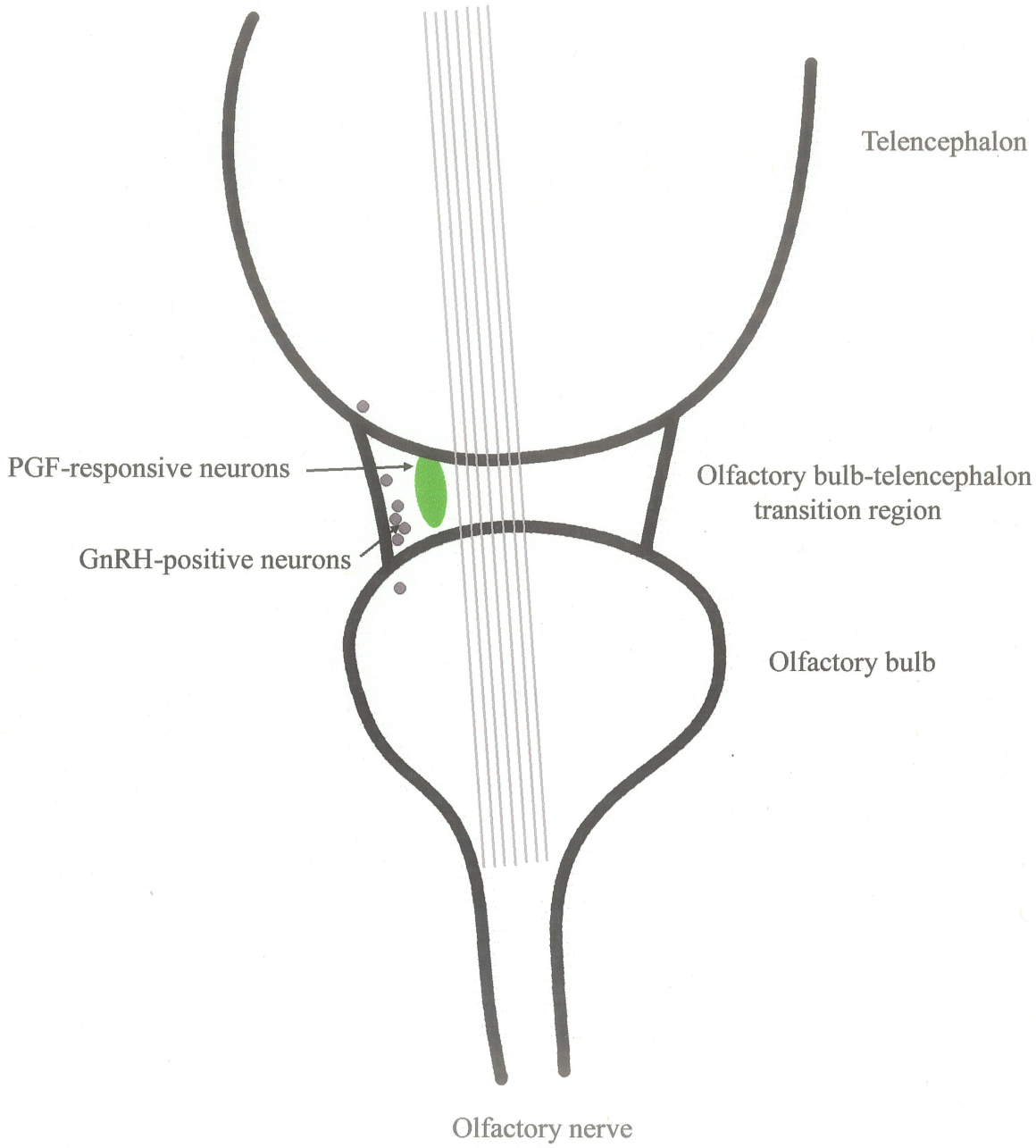

Telencephalon
PGF-responsive neurons
Olfactory bulb-telencephalon transition region
GnRH-positive neurons
Olfactory bulb
Olfactory nerve

Figure 5.8

Figure 5.8. Schematic representation of the location of the F-prostaglandin (PGF)-responsive and gonadotropin-releasing hormone (GnRH)-positive neuron populations in the lake whitefish brain. The drawing is a dorsal view of the brain with medial to the left and caudal on top. The gray lines represent the olfactory nerve level in the parasagittal plane. The figure shows that the PGF-responsive neuron population is found in a medial position to the olfactory nerve level while the GnRH-positive neurons are found at the medial extreme of the lake whitefish brain.

human fos protein differs at only two positions when compared to the fos-related sequence in rainbow trout (Matsuoka et al., 1998).

The results of the experiments described in Chapter 4 showed that the tissue strip that connects the ventromedial olfactory bulb to the telencephalon contains neurons specifically responsive to putative reproductive pheromones in lake whitefish. This region of the fish brain was previously thought to contain only the olfactory tract and cells of the nucleus olfacto-retinalis, part of the terminal nerve complex. A ganglion of GnRH-positive neurons, thought to be the nucleus olfacto-retinalis, is present medially between the olfactory bulb and the ventral telencephalon in many fish species (Münz et al., 1982; Oka and Ichikawa, 1990; Amano et al., 1991; Bailhache et al., 1994; Andersson et al., 1995; Nevitt et al., 1995; Stefano et al., 2000; Gonzales-Martinez et al., 2001). A ganglion of GnRH-positive neurons is also present in the same region of the lake whitefish brain (Fig. 5.3). However, these GnRH-containing neurons are found at the medial extreme of the brain close to the nervous tissue surface, while the PGF-responsive neurons are located just medial to the olfactory nerve level, approximately 120 μm lateral to the GnRH ganglion. Therefore the data suggest that the PGF-responsive neurons described here are not part of the terminal nerve complex, but represent a new type of olfactory neuron in fish.

There are three forms of GnRH found in the lake whitefish brain (Adams et al., in press). Their localization could help elucidate their function. The chicken GnRH-II form is restricted to the posterior brain in all vertebrate gnathostomes studied (Muske, 1993; Lescheid et al., 1997; Oka, 1997; Parhar, 1997; Stefano et al., 2000; Dubois et al., 2001), except goldfish where the chicken-II form is also expressed in some neurons

in the forebrain (Kim et al., 1995). Therefore, the use of two antisera with affinities to the whitefish and salmon GnRH forms (GF-6) and the salmon and chicken-II forms (Snoopy-10/7CR-10) had the potential to reveal if a forebrain region expresses the whitefish or salmon GnRH form in lake whitefish. Only Snoopy-10/7CR-10 labeled neurons in the midbrain, suggesting that these neurons contain chicken GnRH-II. It was surprising that both antisera used in the present study labeled a similar number of neurons in the four other brain regions containing GnRH neurons. Both antisera used detected salmon GnRH. Therefore, it cannot be ascertained if the neurons in the different forebrain regions expressed salmon or whitefish GnRH. The salmon and whitefish GnRH forms could be expressed in the same neurons or the number of neurons containing whitefish GnRH could be small. Recent studies of the European sea bass (*Dicentrarchus labrax*) suggest that the distribution of cells expressing salmon or seabream GnRH overlap in the olfactory bulb, ventral telencephalon and preoptic area (Gonzalez-Martinez, 2001; 2002). The situation could be similar in lake whitefish where both salmon and whitefish GnRH-containing neurons could be present around the olfactory bulb, in the ventral telencephalon and in the preoptic area.

5.5. Conclusion

The PGF-responsive neuron population of lake whitefish potentially receives direct input from olfactory sensory neurons and is not positive for GnRH despite the presence of a ganglion of GnRH-positive neurons in the same brain region.

CHAPTER 6

General Conclusion

Research on mammalian chemical communication shows that most reproductive pheromones are detected by specific neurons present in the vomeronasal organ (Döving and Trotier, 1998; Keverne, 1999; Holy et al., 2000; Leinders-Zufall et al., 2000). The vomeronasal neurons project to the accessory olfactory bulb, which in turn projects to regions of the brain involved in the control of behavioral and endocrine responses, bypassing cortical integration (reviewed by Buck, 2000; Dulac, 2000). A lack of cognitive influences clearly distinguishes the vomeronasal from the main olfactory system. Two parallel olfactory systems are also found in insects. Some insects have a broad range (generalist) receptor system with its central connections for discrimination of odor signals, and a comparatively narrowly tuned (specialist) high sensitivity system associated with the male macroglomerular complex of the antennal lobe for detection of dilute reproductive pheromonal signals (O'Connell, 1986; Hildebrand and Shepherd, 1997; Sorensen et al., 1998).

Dulka (1993) concluded that the goldfish olfactory system comprises anatomical and functional subdivisions that resemble those associated with the main and accessory olfactory systems in tetrapods, and proposed that a specialized subsystem detecting pheromones could be present in a form not yet recognized in fish. This idea derived from research showing that only the medial olfactory tract conveys pheromone information to the central nervous system in fish (Stacey and Kyle, 1983; Resink et al., 1989; Sorensen et al., 1991a). The existence of an extrabulbar primary olfactory pathway projecting to reproductive regions of the brain in salmonids prompted the

suggestion that it might mediate the effects of reproductive pheromones (Becerra et al., 1994). In support of this hypothesis, Hara and Zhang (1998) observed that no EEG response to putative pheromones could be recorded in the salmonid olfactory bulb. The present results show that neurons responsive to reproductive pheromone candidates are present in the olfactory bulb-telencephalon region of lake whitefish, arguing against a direct role for the extrabulbar primary olfactory pathway in the mediation of pheromone effects. However, there exists the possibility that the fibers of the extrabulbar primary olfactory pathway contact the PGF-responsive neurons and then continue on their way into the forebrain, preventing the definitive exclusion of this extrabulbar pathway in PGF detection. This pheromone subsystem of fish could represent a precursor of the vertebrate vomeronasal system. I propose a schematic model of the primary olfactory pathways of lake whitefish in Figure 6.

The finding that the population of PGF neurons of lake whitefish responds without inducing an oscillatory EEG wave is the first report of a non-oscillatory olfactory bulb response to a biologically relevant stimulant in vertebrates. It was also demonstrated in Chapter 3 that lake whitefish likely possess a single type of PGF olfactory receptor. In comparison, results in goldfish suggest the existence of two types of PGF olfactory receptors (Sorensen et al., 1988) and show that bulbar EEG responses to these chemicals can be recorded (Fujita et al., 1991; T.J. Hara, unpublished observations). I suggest that, as in insects, oscillations in the early olfactory system of fish play a role in fine olfactory discrimination. Lake whitefish have no need to discriminate different PGFs as they all activate the same receptor; therefore there is no need for oscillations. However, the role of oscillations in goldfish is problematic. If one

accepts that learning is not involved in pheromone responses, then the most popular hypothesis stating that oscillations are involved in learning cannot apply. It follows either that oscillations in the early olfactory system could have two different roles depending if odorants or pheromones are considered, or that oscillations are not involved in olfactory learning. A preliminary report of Hanson and Sorensen (2001) suggests that two populations of PGF neurons exist in the goldfish olfactory bulb, one specific for PGFs and another also responsive to other pheromone or odorant classes. Could only the non-specific population cause the generation of EEG waves? This question deserves further investigation because the role of oscillations in the central nervous system is a contentious point in brain research at this moment.

Another issue raised by this study concerns the representation of amino acid information in the olfactory bulb. The present results show that in salmonids, unlike goldfish, the majority of neurons are specific for an odorant class. Combinatorial codes that use many receptor types to represent a stimulus (by distinct inputs to a glomerulus or the use of many glomeruli in encoding of a signal) could be used in salmonids, especially at high concentration, but are they biologically relevant? From the result that some olfactory bulb neurons have responses restricted to some amino acids, it can be envisioned that in salmonids the quality of an olfactory stimulus of amino acid at a low concentration can be encoded in a non-combinatorial manner. Fortunately for fish, in nature the total amino acid concentrations in water may be 10^{-7} M or lower (Gardner and Lee, 1975), where discrimination of some amino acids may be possible. Because amino acid-responsive neurons respond to additional amino acid types with increasing concentration, more research is needed to elucidate if fish can be conditioned to

discriminate specific amino acids at high concentration to determine if combinatorial codes are used in fish olfactory coding.

It is necessary to point out the major shortcomings of this study. There is a need to study PGF release in lake whitefish to confirm the role of these chemicals as reproductive pheromones. Similarly, the male or female origin of released PGFs that would trigger female brown trout reproductive behaviors needs to be characterized. Also, the inability to find second-order neurons specific for PGFs in the brown trout early olfactory or central nervous system is surprising. These neurons could have been missed in the present investigation if they occupy a small region, if their responsiveness varies seasonally, or if they are activated by intra-specific interactions (e.g. a priming pheromone in the opposite sex). Anatomical methods to reveal neuronal activity might be more successful than electrophysiology in locating these neurons. Finally, the anatomy and neurochemistry of the PGF-responsive neurons of lake whitefish deserve further investigation.

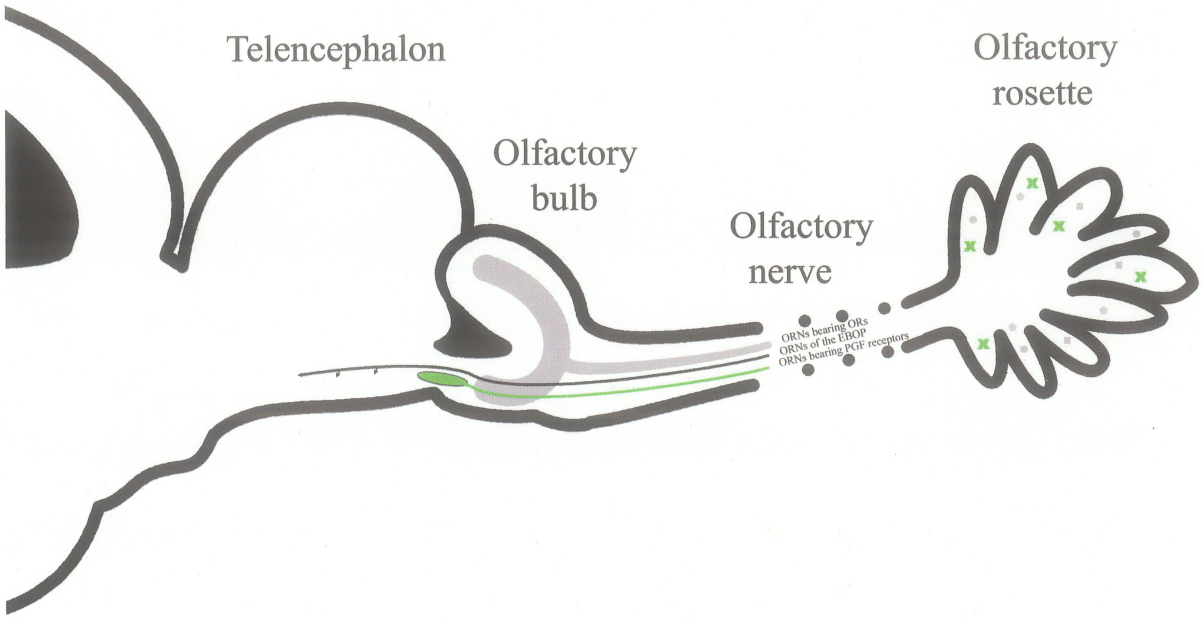


Figure 6. Proposed schematic model of the primary olfactory pathways of lake whitefish. Multiple odorant receptor (OR) types (circle, square and pentagon symbols) and a single F-prostaglandin (PGF) receptor type (X symbol) are randomly distributed in the sensory epithelium of the olfactory organ. Axons of olfactory receptor neurons (ORNs) bearing ORs, PGF receptors or unknown receptors in the case of the extrabulbar primary olfactory pathway (EBOP) travel through the olfactory nerve to terminate in the olfactory bulb (ORNs bearing ORs), the olfactory bulb-telencephalon transition region (ORNs bearing PGF receptors), and the forebrain (ORNs of the EBOP). Olfactory neurons detecting odorants and those detecting pheromones represent two distinct subsystems.

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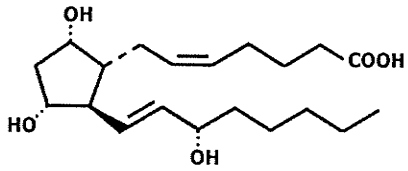
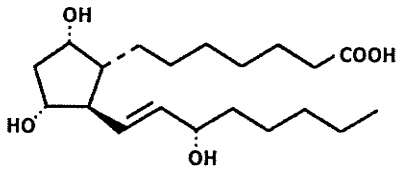
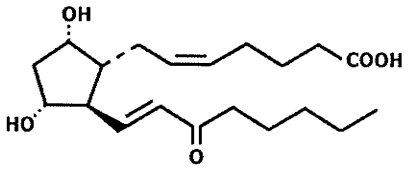
Appendix A. Structure of the F-prostaglandins used in the present study.



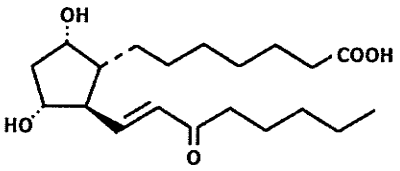
Prostaglandin $F_{2\alpha}$



Prostaglandin $F_{1\alpha}$



15-keto-Prostaglandin $F_{2\alpha}$



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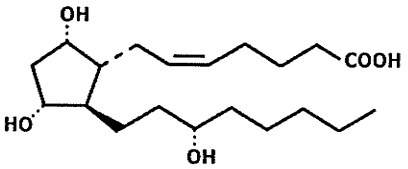
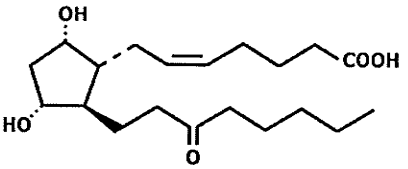


13, 14-dihydro-Prostaglandin $F_{2\alpha}$



13, 14-dihydro-15-keto-Prostaglandin $F_{2\alpha}$

Appendix B. EOG responses to PGFs: data.and sample sizes.

Brown trout

Stimulant concentration (Log molar)		10^{-12}	10^{-11}	10^{-10}	10^{-9}	10^{-8}	10^{-7}
$PGF_{2\alpha}$	Males	4.7±7.2† (5)*	11.7±10.8 (5)	20.7±13.5 (5)	55.9±26.2 (5)	87.5±33.6 (5)	72.2±31.6 (5)
	Females	0.9±2.1 (5)	12.1±11.3 (5)	6.9±6.9 (5)	30.3±10.1 (5)	43.0±18.3 (5)	27.9±10.3 (4)
	Undifferentiated	0 (2)	2.1±3.0 (2)	6.0±0.2 (2)	16.5±2.7 (2)	42.5±1.7 (2)	39.6±0.8 (2)
$PGF_{1\alpha}$		n.t. (not tested)	0 (10)	1.8±4.8 (7)	3.0±4.4 (11)	21.2±10.7 (11)	52.1±22.8 (11)
$dh_{2\alpha}$		n.t.	0 (9)	4.4±7.0 (5)	7.6±9.0 (11)	34.5±24.2 (10)	42.0±26.6 (11)
$15K_{2\alpha}$		n.t.	0 (10)	0 (3)	0 (10)	5.5±5.6 (9)	17.3±11.8 (11)
$dh15K_{2\alpha}$		n.t.	0 (9)	n.t.	0 (11)	0 (3)	2.1±3.7 (11)
$15K_{1\alpha}$		n.t.	0 (9)	n.t.	0 (11)	n.t.	0 (11)
ECG		n.t.	0 (10)	n.t.	0.7±2.4 (11)	6.1±5.4 (9)	17.9±5.5 (11)

†Response amplitude is expressed as a percentage of the response to 10^{-5} M L-serine.

*Sample size is indicated in parentheses.

Appendix B. EOG responses to PGFs: data.and sample sizes.

Lake whitefish

Stimulant concentration		10^{-11}	10^{-10}	10^{-9}	10^{-8}	10^{-7}
$15K_{2\alpha}$	Males	10.3±20.6 (4)	85.7±58.5 (4)	248.2±246.3 (4)	1060.1±365.4 (4)	1309.1±467.0 (4)
	Females	0 (7)	37.5±28.8 (6)	142±103.1 (7)	324.9±133.7 (7)	556.3±178.8 (7)
$dh_{2\alpha}$	Males	0 (4)	16.6±33.2 (4)	85.4±116.9 (4)	835.6±200.1 (4)	1111.8±301.8 (4)
	Females	0 (7)	14.9±17.2 (4)	39.0±58.0 (7)	275.0±217.0 (5)	431.3±166.5 (7)
$PGF_{2\alpha}$		0 (11)	0 (6)	20.0±31.7 (11)	85.5±48.6 (11)	341.5±152.2 (11)
$PGF_{1\alpha}$		0 (7)	n.t.	0 (7)	39.7±29.6 (4)	122.3±147.7 (7)
$dh15K_{2\alpha}$		0 (6)	0 (4)	16.3±28.3 (6)	129.7±143.0 (6)	310.5±191.6 (6)
$15K_{1\alpha}$		0 (7)	n.t.	5.8±15.4 (7)	126.7±53.8 (2)	243.1±146.7 (7)
ECG		0 (3)	n.t.	0 (4)	n.t.	0 (6)

†Response amplitude is expressed as a percentage of the response to 10^{-5} M L-serine.

*Sample size is indicated in parentheses.

Appendix B. EOG responses to PGFs: data.and sample sizes.

Rainbow trout

Stimulant concentration	10^{-11}	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}
$PGF_{2\alpha}$	0 (6)	n.t.	0 (6)	n.t.	2.9±4.7 (6)	8.65±2.9 (2)	0 (4)
$dh_{2\alpha}$	0 (5)	n.t.	0 (6)	n.t.	3.4±5.6 (6)	n.t.	n.t.
$15K_{2\alpha}$	0 (5)	n.t.	0 (6)	n.t.	1.0±2.4 (6)	n.t.	n.t.
$PGF_{1\alpha}$	0 (5)	n.t.	0 (6)	n.t.	2.3±3.7 (6)	n.t.	n.t.
$dh15K_{2\alpha}$	0 (5)	n.t.	0 (6)	n.t.	0 (6)	n.t.	n.t.
$15K_{1\alpha}$	0 (5)	n.t.	0 (6)	n.t.	0 (6)	n.t.	n.t.
ECG	0 (6)	0 (2)	0 (6)	0 (3)	12.0±11.3 (6)	23.2±13.5 (3)	40.6±1.7 (2)

†Response amplitude is expressed as a percentage of the response to 10^{-5} M L-serine.

*Sample size is indicated in parenthes