THE UNIVERSITY OF MANITOBA

F-TYPE PROSTAGLANDINS AS REPRODUCTIVE PHEROMONES IN ARCTIC CHAR (*Salvelinus alpinus*): BIOCHEMICAL, ELECTROPHYSIOLOGICAL, AND BEHAVIOURAL STUDIES

By

TORARINN SVEINSSON

A thesis submitted to the Faculty of Graduate Studies in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Winnipeg, Manitoba
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ISBN 0-315-77749-4

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ABSTRACT

In this dissertation, I present a study on biochemical, electrophysiological, and behavioural aspects of the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) as a reproductive pheromone in Arctic char, *Salvelinus alpinus*. Electrophysiological investigation suggested a receptor with high specificity for $PGF_{2\alpha}$ which transduces information to be transmitted through the olfactory nerve to the brain. The electroolfactogram (EOG) threshold of the responses to $PGF_{2\alpha}$ was 5-10 pM. Cross-adaptation experiments provided evidence for the independence of the receptor for $PGF_{2\alpha}$ from receptors for other known olfactory stimuli in char. The responsiveness of the olfactory system was similar for both sexes and for different maturational stages, with the exception of a few post-ovulatory females which responded very poorly. The concentration-response curve of the EOG data seems to indicate a receptor with a high affinity for its ligand. However, classical ligand-binding studies failed to identify such a receptor. Several reasons could account for this: a lower affinity of the receptor for the ligand than expected from electrophysiological data; a switch from high- to low-affinity state of the receptor when exposed to ligand; a need for the ligand to be enzymatically modified before it can bind to the receptor; or a low density of receptor molecules. Immunoassay revealed F-type PGs in water inhabited by char of both sexes performing spawning-related acts. High concentrations of immunoreactive PGFs seemed to coincide with spawning activity of the males. Males kept isolated in tanks produced much more odour (as assayed by EOG) than either females or immature char. When males received chemical cues from ovulated females, they increased their release of odorants, including F-type PGs, into the water. $PGF_{2\alpha}$ at

concentrations of 0.1 nM attracted ovulated females and ripe males. It also stimulated digging behaviour in mature females. It is concluded that F-type PGs or their derivatives are released by ripe males on the spawning grounds to attract females and elicit their spawning behaviour.

ACKNOWLEDGEMENTS

I am very grateful to my supervisor, Dr. Toshiaki J. Hara, for his guidance, patience and enthusiasm for this project. I am also grateful to the other members of my advisory committee, Drs. R.M. Evans, J.D. Geiger, J.I. Nagy, and T.J. Wiens, for their constructive advice and encouragement throughout this project. Especial thanks to Dr. T.J. Wiens for critical evaluation of an earlier draft of this thesis.

I also like to thank members of the FIGS (Freshwater Institute Graduate Students) group for stimulating and entertaining discussions. Thanks to several staff members of the Freshwater Institute, DFO, and the Department of Zoology, University of Manitoba, which provided help and support. My sincere thanks to my spouse, Kristjana Gunnarsdóttir, and my son, Sveinn Torarinsson, for their support and understanding throughout the study.

I wish to express my appreciation to the Department of Fisheries and Oceans for financial support and for access to their research facilities at the Freshwater Institute, Winnipeg. This project was also supported by NSERC grant to Dr. T.J. Hara.

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LIST OF ABBREVIATIONS

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

%AA: percent of 10 μM L-serine phasic response

%PG: percent of 10 nM $PGF_{2\alpha}$ phasic response

EIA: enzyme immunoassay

EOG: electroolfactogram

F-IM: immature female

F-OV: ovulated female

F-OO: "old-ovulated" female

F-PO: pre-ovulatory female

GtH: gonadotrophic hormone

IM: immature fish

M-IM: immature male

M-RI: ripe male

NTR: nerve-twig recording

PG: prostaglandin

CHAPTER I

General Introduction

Pheromones are substances excreted by a living organism and received by conspecific individuals in which they induce specific reactions. This definition for the term pheromone was introduced by Karlson and Lüscher (1959). Their definition has been criticized for being too vague and general by mammalian and fish biologists (Liley, 1982; Drickamer, 1989). However, recent findings on chemical signals in fish, including the present study, provide compelling reason to use the original definition (Sorensen, 1992). Furthermore, the definition should include chemicals which have hormonal or other physiological functions within the body as well as metabolic by-products, if they also serve as conspecific signals. Two types of pheromones can be distinguished, primer and releaser pheromones (Wilson and Bossert, 1963). A primer pheromone induces physiological changes without any immediate change in behaviour, while releaser pheromones trigger behavioural responses.

Kittredge *et al.* (1971) argued that aquatic organisms are likely to use hormones and their metabolites as sex pheromones; this would only require evolution of excretion of the relevant compounds and external expression of receptors which already exist as internal receptors. This argument can be turned around. Unicellular organisms are known to use sex pheromones, e.g. *Blepharisma* (Van Houten and Preston, 1987) and *Saccharomyces cerevisiae* (Cole *et al.*, 1990). Hormones, in general, may have evolved from pre-existing pheromones; this may not have involved

any immediate changes regarding expression of receptors on cell surfaces nor excretion of relevant compounds. Both arguments may be valid, i.e. hormones may originally have evolved from pheromones, but at some later time, pheromones may again have evolved from these hormones. However, a lack of information on pheromones in aquatic invertebrates makes any further speculations impossible. Recent findings on pheromones in fish, including the present study, establish that fish sex pheromones and sex hormones are at least in some instances identical or very similar (for review see Sorensen *et al.*, 1991a; Sorensen, 1992). At present there is no information on the internal and external receptors for these chemical signals. Also, with the exception of the African catfish, *Clarias gariepinus* (Schoonen *et al.*, 1988), no information on the biosynthesis and excretion mechanisms of pheromones are available. Again, without better knowledge on the above issues, any evolutionary speculation at this time would be disputable.

Tavolga (1956) was the first to demonstrate that substances emanating from a mature fish can influence the behaviour of the opposite sex. His study on the estuarine gobiid *Bathygobius soporator* showed that small quantities of water in which gravid females had been held rapidly elicited male courtship behaviour. Ovarian fluid from gravid females and egg washings were both effective. By destroying the male's olfactory sense, he established that the behavioural effect was mediated by olfaction. Unfortunately, the chemical nature of the substance involved was not established, and further research on pheromones in this species has not yet been conducted.

The chemical identity of a possible reproductive pheromone was first established in black gobies, *Gobius jozo* (Colombo *et al.*, 1980; Colombo *et*

al., 1982). Their study showed that etiocholanolone glucuronide, which is found in the testes-associated mesorchial gland, attracted ovulated females and induced oviposition. A pheromonal signal that is released by ovulated female zebrafish, *Brachydanio rerio*, and stimulates male reproductive behaviour has been suggested to be a mixture of estradiol glucuronide and testosterone glucuronide produced by the ovaries (van den Hurk and Lambert, 1983; Lambert *et al.*, 1986). 5β-Pregnan-3α,17α-diol-20-one-3α-glucuronide has been hypothesized to act as a reproductive pheromone in the African catfish *Clarias gariepinus*; it is released by the male to attract and stimulate female spawning (Resink *et al.*,1987a,b; Resink *et al.*,1989a,b,c,d). Conjugated and reduced steroids have also been hypothesized to act as pheromones in plaice, *Pleuronectes platessa*, and dab, *Limanda limanda* (Canario and Scott, 1989a,b).

The most complete study on reproductive pheromones in fish has, however, been conducted on goldfish (Partridge *et al.*, 1976; Stacey and Sorensen, 1986; Sorensen *et al.*, 1988,1989; Stacey *et al.*, 1989). Although many questions remain to be answered regarding goldfish spawning pheromones, it is apparent that goldfish have evolved a very sophisticated pheromonal system. It comprises both pre-ovulatory primer pheromone and post-ovulatory releaser pheromone. The pre-ovulatory primer pheromone in goldfish is 17α,20β-dihydroxy-4-pregnen-3-one (17,20P) and it stimulates gonadotrophic hormone (GtH) secretion from the pituitary in mature male goldfish (Stacey and Sorensen, 1986; Dulka *et al.*, 1987). The GtH surge then increases milt production presumably in anticipation of spawning. The releaser pheromone signal in the goldfish consists of prostaglandins ($PGF_{2\alpha}$ and 15-keto-$PGF_{2\alpha}$ or related compounds) and stimulates the sexual behaviour of the goldfish (Sorensen *et al.*, 1988;

Sorensen *et al.*, 1991a). In spite of a very rapid growth in knowledge on fish pheromones recently, we are still just scratching the surface. A lot more studies need to be done before we can begin to understand the function of these pheromones.

The apparent relationship between pheromones and reproductive hormones in goldfish is intriguing. It immediately brings up the question of how widespread this type of pheromone is among fish and among other vertebrates. The steroid androstenol is known to function as a pheromone in pigs (Melrose *et al.*, 1971) and humans (Cowley and Brooksbank, 1991). If it is commonplace that hormones and their metabolites are used as pheromones, then how species-specific are these pheromones (Sorensen, 1992)? Both steroids and prostaglandins (PGs) provide basic structures which are known to exist in great diversity. These chemicals may exist in many forms: reduced; combined with hydroxy or keto groups in several positions; conjugated with glucuronic acid, taurine or sulfate; and perhaps others. Furthermore, different mixtures of the same chemicals could provide species-specificity, as is known for pheromones in insects (Linn and Roelofs, 1989).

Of the animal sensory systems, the chemosensory systems are the least understood in terms of reception mechanisms. Whether specific ligand-binding molecules (receptors) exist, which reversibly bind to the stimuli and initiate specific chemical cascades within the receptor cells, has long been debated (Lancet, 1986). If receptors exist, then the number of different types of these structures and their individual specificities are not known. Such information on the logic of peripheral receptor mechanisms has been a key in providing insight into the processing of other sensory modalities, such as vision (Nathans *et al.*, 1986).

Several recent studies show similarities between the olfactory transduction mechanisms and those for hormones, neurotransmitters, and light. Most recently several genes, encoding seven-transmembrane-segment (7-TMS) proteins in the olfactory epithelium, were identified in rat (Buck and Axel, 1991). Members of this family of 7-TMS genes are the visual pigments and receptors for several different hormones and neurotransmitters (Dohlman *et al.*, 1991). Buck and Axel (1991) suggested that the products of the 7-TMS genes they found and which were only expressed in the olfactory epithelium were the much searched for olfactory receptors. Evidence for that speculation has, however, not yet been brought forth. Odorants have also been shown to activate second messenger systems such as adenylyl cyclase/cAMP (Pace *et al.*, 1985), and phospholipase C/IP_3 (Huque and Bruch, 1986; Breer *et al.*, 1990; Boekhoff *et al.*, 1990a,b). Furthermore, the odorants affect these systems through GTP-dependent proteins (G proteins; Pace and Lancet, 1986; Jones and Reed, 1989; Anholt, 1989), as do agonists of many other receptors. These similarities provide strong evidence for the existence of olfactory receptors. However, classical ligand-binding studies have not proven applicable for olfactory receptors in mammals despite extensive efforts by many laboratories (for review see Anholt, 1989; Snyder *et al.*, 1989).

Mammals possess an olfactory system of enormous discriminatory power and can distinguish among thousands of distinct odorants (see Lancet, 1986; Reed *et al.*, 1990). Although many potent odorants in human and other mammals are known, their biological significance is not understood. Amino acids have been shown to stimulate feeding behaviour in some species of fish (Adron and Mackie, 1978). However, their role in the biology of fish is not clear. Besides amino acids, bile salts are known

stimulants (Døving *et al.*, 1980; Hara *et al.*, 1984), but the biological significance of these stimuli is also poorly comprehended.

Heterogeneity of olfactory receptors for amino acids in fish has been established (Caprio and Byrd, 1984; Sveinsson and Hara, 1990a,b). These studies have shown that amino acid receptors are not very specific for their agonists. Furthermore, low affinity of the receptors for their ligands and apparent accumulation into membrane vesicles in the binding assay (Brown and Hara, 1981,1982) have hindered further progress in purification of these receptors. Chemical stimuli with known biological significance and high affinity for their receptors would be of great value as a tool to identify, characterize and delineate transduction mechanisms in olfaction. I anticipated that pheromones in aquatic species should be suitable candidates for such signals. Recently, Lancet (1991) suggested the same.

At the start of the project which is presented in this thesis, I found by using EOG recordings that Arctic char and lake trout respond to low concentrations of $PGF_{2\alpha}$. However, rainbow trout and brook trout did not respond. Several studies on olfaction have established Arctic char as a good model for studying both behavioural and electrophysiological aspects of olfaction (Døving *et al.*, 1973,1974,1980; Døving and Belghaug, 1977; Belghaug and Døving, 1977; Jones *et al.*, 1985; Olsén, 1985; Sveinsson, 1985; Sveinsson and Hara, 1990a,b). Arctic char was also easily available for the present project and was therefore used to answer the following questions: (1) What are the electrophysiological characteristics of the reception and transduction mechanisms of $PGF_{2\alpha}$? (2) Can the receptors for $PGF_{2\alpha}$ be characterized and isolated by biochemical methods? (3) What are the behavioural and biological functions of $PGF_{2\alpha}$ as a chemosensory signal? Chapters II, III, and IV address these three questions, respectively.

CHAPTER II

Electrophysiological studies of the olfactory receptor for prostaglandins in Arctic char, *Salvelinus alpinus*

Introduction

Recent studies have shown that prostaglandins (PGs), steroid hormones, and their metabolites may function as pheromones in fish (Colombo *et al.*, 1980; Colombo *et al.*, 1982; van der Hurk and Lambert, 1983; Stacey and Sorensen, 1986; Sorensen *et al.*, 1988; see Chapter I for more information). It is also clear that these pheromones are at least to some extent species-specific (Sorensen *et al.*, 1991a).

Although precocious male parr of Atlantic salmon (*Salmo salar*) are very sensitive to testosterone (detection threshold of 0.01 pM), immature parr are unresponsive (Moore and Scott, 1991). Of different developmental stages in goldfish, prostaglandin (PG) sensitivity has only been examined in mature males (Sorensen *et al.*, 1988). However, goldfish of different maturational stages show similar sensitivity to 17α,20β-dihydroxy-4-pregnen-3-one (17,20P; Sorensen *et al.*, 1987). Information on changes in olfactory sensitivity at different developmental stages could give important clues for the biological function of the compounds involved.

The electroolfactogram (EOG) is a transient potential change recorded from the surface of the olfactory epithelium in response to odour stimulation (Ottoson, 1956). It is believed to represent summated receptor potentials in the olfactory neurons (Ottoson, 1956,1971), and has proved

useful in the study of olfactory sensitivity to chemical stimuli in fish (Silver *et al.*, 1976; Caprio, 1977,1978; Silver, 1982; Evans and Hara, 1985; Sorensen *et al.*, 1987,1988,1990; Caprio *et al.*, 1989). Furthermore, EOG activity has been correlated with electrophysiological recordings from the olfactory nerve and tract (Silver, 1982; Sveinsson, 1985; Sorensen *et al.*, 1991b), endocrine responses (Stacey *et al.*, 1989), and behavioural responses (Sorensen *et al.*, 1989). EOG recordings can be performed with only minor surgical procedure, and thus represent a reliable, fast, and convenient method for screening a large number of chemicals for potential pheromonal functions.

Cross-adaptation experiments have been used to identify separate and independent receptors or transduction mechanisms for olfactory stimuli (Caprio and Byrd, 1984; Sveinsson and Hara, 1990b). In these studies, the olfactory epithelium is continuously perfused with an adapting stimulus. If the concentration of the adapting stimulus is high enough to saturate the receptor sites, it will render the receptors reacting with that stimulus unable to respond to an additional stimulus (the test stimulus). Other receptors, not reacting with the adapting stimulus and not sharing transduction mechanisms with the receptor reacting with the adapting stimulus, are presumed to have unaffected capability to respond to the appropriate test stimuli. Thus, any response to the test stimulus during cross-adaptation indicates that separate receptors and transduction mechanisms exist for the two stimuli, the test stimulus and the adapting stimulus.

The objective of this phase of the study was threefold: (1) to test electrophysiologically the effectiveness of several different PGs in stimulating the olfactory system of Arctic char, (2) to identify the number

and specificity of receptors involved in PG detection, and (3) to examine whether the responsiveness is dependent on the maturational stage of the char. In this chapter, I show that Arctic char have an olfactory receptor which is very sensitive and highly specific for $PGF_{2\alpha}$. This receptor is functionally separate from receptors for amino acids and bile salts. I further show that the electrophysiological response of this receptor is independent of sex or stage of sexual maturation with the possible exception of post-ovulatory females.

Materials and Methods

Arctic char (*Salvelinus alpinus*) hatched from native breeding stock taken from Nauyuk Lake (Northwest Territories, Canada) were obtained from the Rockwood Aquaculture Research Centre of the Freshwater Institute, DFO. Fish were held in laboratory tanks supplied with a continuous flow of dechlorinated water (temp. 10.5-11.5°C), and fed commercial fish pellets *ad lib*. Maturation was induced by a long photoperiod (18L:6D) presented to groups of fish in which each fish had reached at least 500 g in weight (age: 3+ to 4+). In 4-6 months, 50-80% of the fish became ripe. After each EOG experiment, the sex and stage of sexual maturation of the char were determined and the fish sacrificed. The following classification was used:

M-IM: Immature male or male in early stage of maturation (gonads not fully developed).

M-RI: Ripe male; milt could be stripped by applying gentle pressure on the abdomen.

F-IM: Immature female or female in early stage of maturation.

F-PO: Preovulatory female; eggs could be felt inside the abdominal cavity but could not be easily stripped.

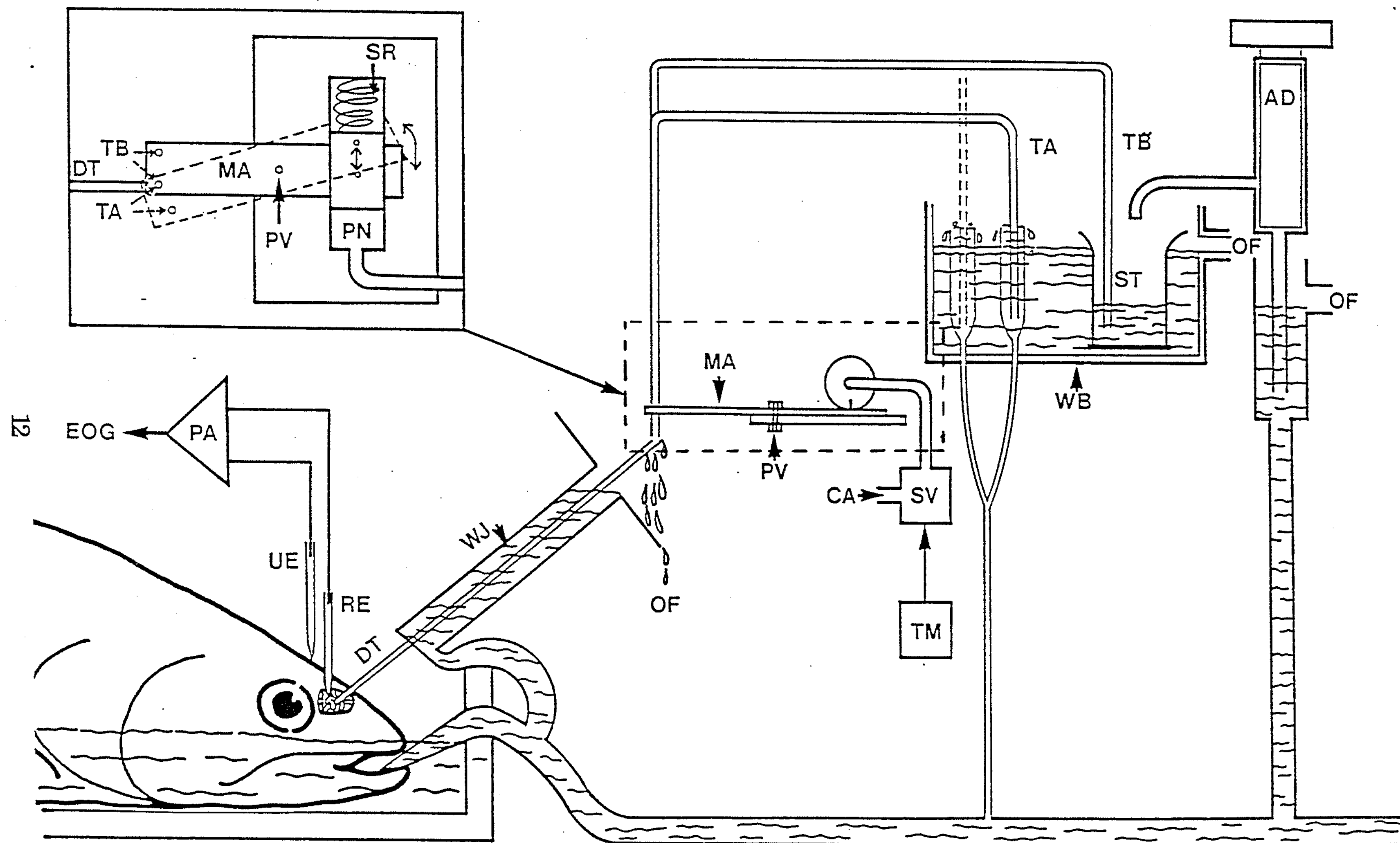
F-OV: Ovulated female; clear eggs could be stripped by applying gentle pressure on the abdomen.

F-OO: "Old-ovulated" female; opaque eggs or eggs with white spots could be easily stripped.

For EOG recording, a fish immobilized with an intramuscular injection of Flaxedil (gallamine triethiodide, 3-4 mg/kg body wt.) was secured in a holding apparatus and placed in a Plexiglas trough. A tube placed in the mouth of the fish perfused the gills with water throughout the experiments. As it has been shown that even low tranquilizing doses of the fish anaesthetic agent MS-222 affects the olfactory epithelium (Lewis *et al.*, 1985), it was not used.

The apparatus used to deliver stimulus solutions is shown in Figure 1. It applies a stimulus abruptly to the olfactory epithelium for a preset duration with no dilution of a given stimulus solution. The apparatus consists of a delivery system and an activating system. The activating system consists of a movable arm, a pneumatic actuator (Chromatronix type PA-875), spring return actuator (Chromatronix type SR-1), solenoid valve (Chromatronix Model SOL-3-24 V DC; Hara *et al.*, 1973), and a electronic time switch (Omron HC3A). The solenoid valve is a three-way valve which pressurizes the actuator when the electronic timer is turned on, and then vents off automatically after an adjustable period of time. When the actuator is pressurized it moves the arm; when it vents, the arm returns to its original position. The delivery system has a glass

Fig. 2.1 The stimulus delivery apparatus and the setup for the EOG recording. The same stimulus delivery system was used for the nerve-twig recording (NTR). AD= adjustable dispenser; CA= compressed air; DT= delivery tube (glass); MA= movable arm; OF= overflow; PA= preamplifier; PN=pneumatic actuator; PV= pivot; RE=recording electrode; SR= spring return actuator; ST= stimulus solution; SV= solenoid valve; TA=tubing A (polyethylene); TB= tubing B (ployethylene); TM= timer; UE= reference electrode; WB= waterbath; WJ= waterjacket. Broken lines within the insert show the arm and holes in switched position. See text for explanation.


Top view of enclosed region
SR
TB
DT
MA
TA
PV
PN
TA
TB
AD
OF
ST
OF
MA
WB
EOG
PA
PV
CA
SV
WJ
UE
RE
OF
TM
DT

capillary going through a water jacket, which provides constant temperature. The glass capillary then supplies either nostril with water or stimulus solution. At the upper end of the glass capillary is the arm of the activating system, which holds one end of each of two PE connecting tubes (50 cm PE 240 with 5 cm PE 190 inserted at the end). The other ends of these PE tubes are placed respectively in plain water and in the stimulus solution of choice. Depending on the position of the arm, one of the tubes delivers fluid to the glass capillary while the fluid from the other is drained. Prior to stimulation, one tube is placed into a freshly made stimulus solution in a siliconized (Sigmacote) beaker which is located in the water bath to provide constant temperature. Once this solution fills the tubing, the timer may be activated, which results in movement of the arm and the tube supplying the glass capillary with stimulus solution. The water is driven by gravity through the tube and the glass capillary. The vertical distance from the water level of the water bath to the upper end of the glass capillary is 18 cm; the length of the glass capillary is 18 cm, tilting ca. 45°. All these values are adjustable. The flow of water or solutions through the tubing is more than through the glass capillaries thus creating overflow which is drained. There is no apparent interruption or disturbance of the flow (6.7 ml/min) to the nostril when the exchange of tubes takes place. A delay of about 1 s elapses from the time of switching until the new solution reaches the nostril. Because the stimulus can be applied for a prolonged time if desired, both phasic and tonic components of the response can be studied.

For the EOG, the overlying skin, cartilage, and dorsal aspect of the olfactory sac were removed. EOG responses were recorded differentially with two Ag-AgCl electrodes (WP Instruments Type EH-1S) filled with 3 M KCl via saline gelatin-filled (8%) capillary pipettes (tip diameter ca. 100

μm). A recording electrode was placed in the water flow, perpendicular to and slightly above the mucosal surface of the central ridge of the olfactory rosette. This location was found to give the largest responses to both standard (10 μM L-serine) and 10 nM $PGF_{2\alpha}$ stimulation. The reference electrode was placed on the skin adjacent to the olfactory cavity. The electrical signal was amplified with a DC preamplifier (Grass 7P1).

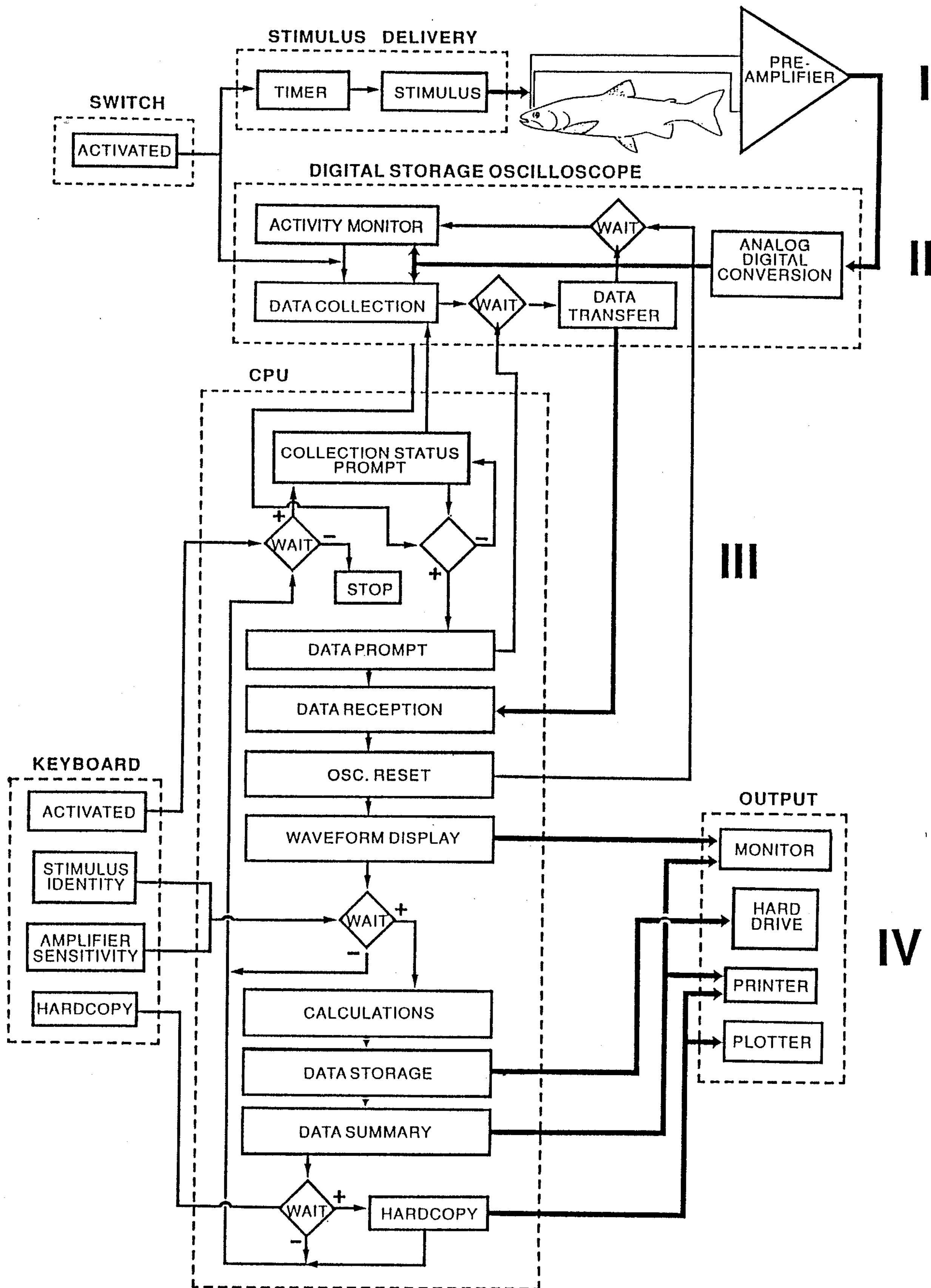
Olfactory nerve-twig recordings (NTR) were carried out as previously described (Sveinsson and Hara, 1990a,b). The olfactory bulbs and the central part of the olfactory nerves were exposed by removing overlying cartilage and skin. A paralene coated tungsten microelectrode (1-2 MΩ, DC; tip diameter = 15 μm; tip length = 20 μm) was penetrated into one of the olfactory nerves. Another electrode of the same kind was placed in the tissue fluid between the nerves. The electrical activity was recorded differentially with the two electrodes and amplified by an AC-preamplifier (Grass P511) with a bandpass of 30-10,000 Hz and integrated (Grass 7P3B) with a time constant of 0.5 s. Only preparations which gave responses to standard (10 μM L-serine) stimulation were used to collect data.

An automated system for data collection and analysis was developed using digital storage oscilloscope (Tektronix 2220) and microcomputer (Mind 386SX) with hardware/software utilities (GURU II, Tektronix). The functional operation of the system can be divided into four steps (Fig. 2.2, I-IV):

Step I: Delivery of the stimulus, recording of the electrical signal (EOG, NTR), and amplification (see also Fig. 2.1).

Step II: Analog-digital conversion of the signal and temporary storage by the oscilloscope (data collection). A switch simultaneously turns on the timer of the stimulus delivery system in Step I and triggers

Fig. 2.2 Flow chart showing the automated data collection and analysis system. CPU= central processing unit. See text for explanation.



STIMULUS DELIVERY
TIMER
STIMULUS
PRE-AMPLIFIER
I
SWITCH
ACTIVATED
DIGITAL STORAGE OSCILLOSCOPE
ACTIVITY MONITOR
WAIT
ANALOG DIGITAL CONVERSION
II
DATA COLLECTION
WAIT
DATA TRANSFER
CPU
COLLECTION STATUS PROMPT
+
WAIT
−
−
+
STOP
III
DATA PROMPT
DATA RECEPTION
OSC. RESET
KEYBOARD
ACTIVATED
STIMULUS IDENTITY
AMPLIFIER SENSITIVITY
HARDCOPY
WAVEFORM DISPLAY
WAIT
+
−
CALCULATIONS
DATA STORAGE
DATA SUMMARY
WAIT
+
−
HARDCOPY
OUTPUT
MONITOR
HARD DRIVE
PRINTER
PLOTTER
IV

the oscilloscope for the data collection in Step II.

Step III: Transmission of the digitized data from the oscilloscope to the computer. When the computer is activated it will prompt the oscilloscope for data collection status every second until data collection is completed. Then the computer prompts the oscilloscope for the data and the oscilloscope sends the data to the computer.

Step IV: Receiving stimulus identity from the computer keyboard, calculation of parameters (response latency, phasic and tonic response magnitudes, durations, etc.), data storage onto a hard drive, printing a data summary and plotting a hardcopy if required. The phasic response was measured as the maximum response level reached during the first 5 s after activation of the stimulus delivery system. The tonic response was measured as the response level after 10 s of stimulation (see Fig. 2.3).

The stored information was then retrieved from the hard drive to a spreadsheet or other programs for a final analysis and graphic display. The curve-fitting procedure of Sigmaplot^{TM} (Jandel Scientific) was used to fit the following equation to the concentration-response curves:

$$R = C + Q \cdot S / (K + S) \qquad \text{Eq. 1}$$

where R is response magnitude, and S is stimulus concentration. The parameter C represents response to control stimulus (plain water), C + Q corresponds to maximum response level, and K corresponds to the concentration where response is half-way between the C + Q and C levels. This equation represents a single component model (Sveinsson and Hara,

1990a,b). Multicomponent models were found to indicate multiple types of receptors (Sveinsson and Hara, 1990a,b). However, they were unable in the present study to improve the fit significantly over that of the single component model and were therefore rejected.

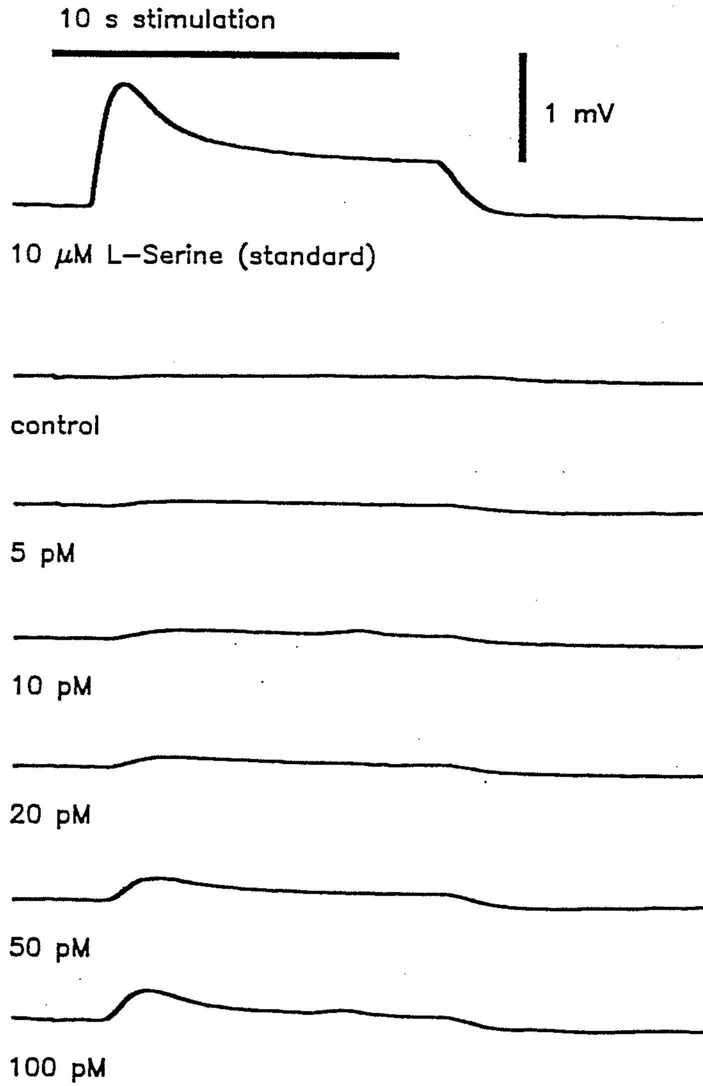
Prostaglandins were dissolved in HPLC grade methanol (1 mg/ml) from which 10 μM stock solutions were made in distilled water. A stock solution of 1 mM L-serine in distilled water wasmade. The test solutions were made from these stock solutions (just a few seconds before application) in the same water as was perfusing the naris. When concentration series were used they were always tested in order of ascending concentration. Each concentration of each chemical was only tested once on each fish. Responses were either expressed in mV, or as percent of response to 10 μM L-serine (%AA), or as percent of response to 10 nM $PGF_{2\alpha}$ (%PG). All prostaglandins and analogs were obtained from Cayman Chemical (Ann Arbor, MI). Other chemicals were from Sigma Chemical (St. Louis, MO).

Results

The EOG to $PGF_{2\alpha}$ application showed a rapid, phasic response, followed by a tonic response that was maintained throughout the stimulation period (Fig. 2.3). The shape of the EOG waveform in response to PG stimulation was similar to that of amino acids (e.g. 10 μM L-serine) except that the tonic/phasic response ratio for PGs was considerably smaller. In addition to $PGF_{2\alpha}$, seventeen synthetic prostaglandins and analogs were tested at 10 nM (Fig. 2.4). Responses were expressed in %PG (percent of 10 nM $PGF_{2\alpha}$ phasic response) in Fig. 2.4, because this gave less variance than if the responses were expressed in %AA (percent of 10 μM

Fig. 2.3 Samples of EOG traces to a concentration series of $PGF_{2\alpha}$ and 10 µM L-serine (all from the same fish). Each trace shows 20 seconds of recording. The duration of the stimulus delivery activation (10 s) and 1 mV deflection are indicated.

Prostaglandin $F_2\alpha$ EOG responses



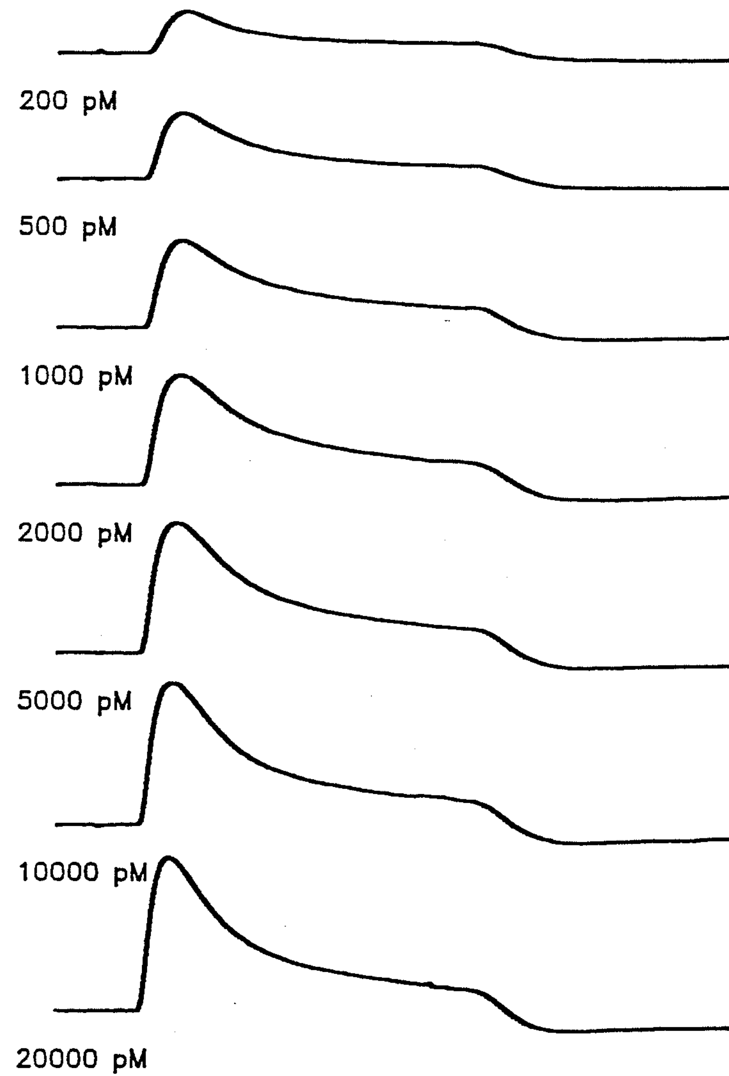
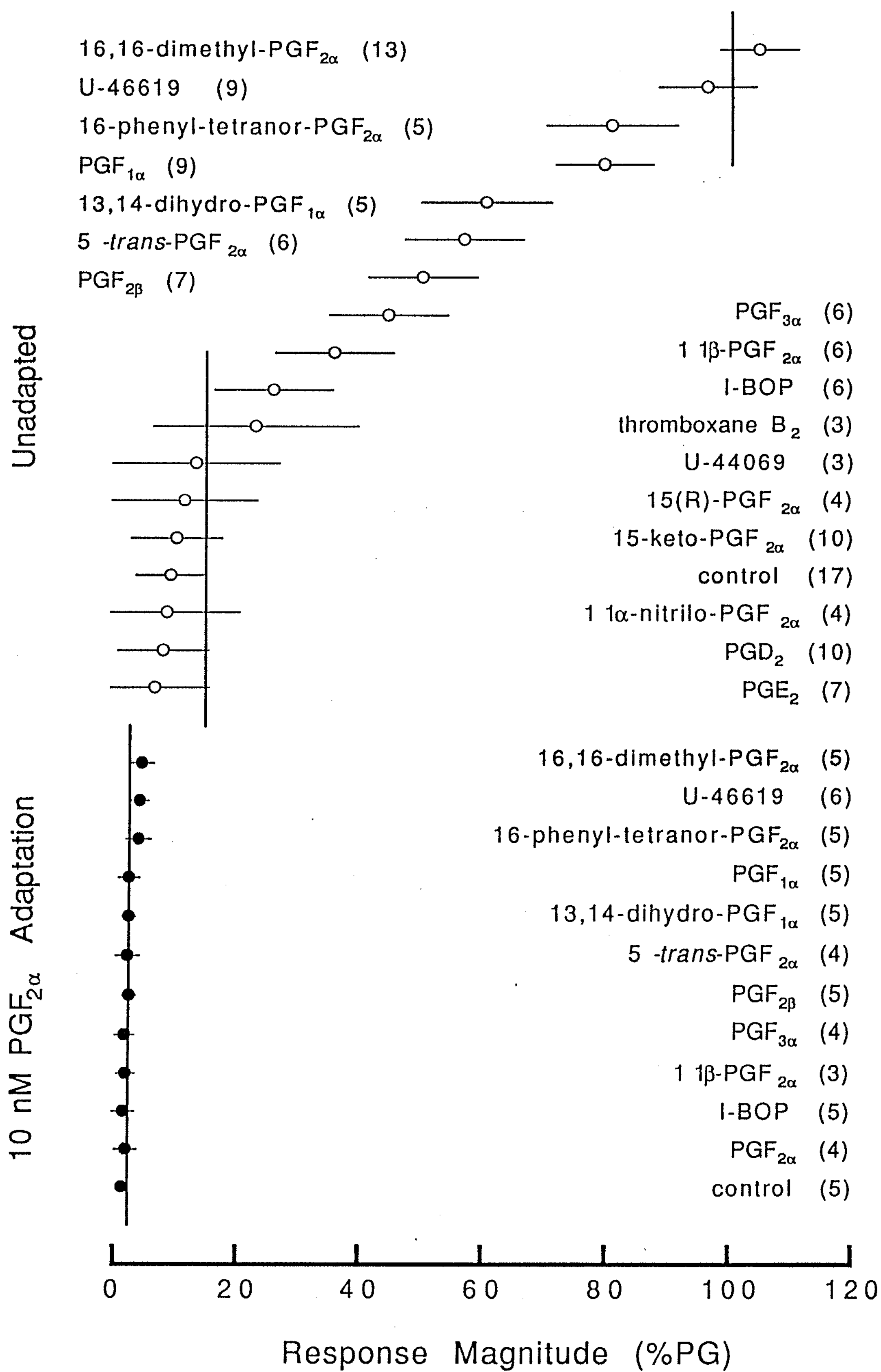


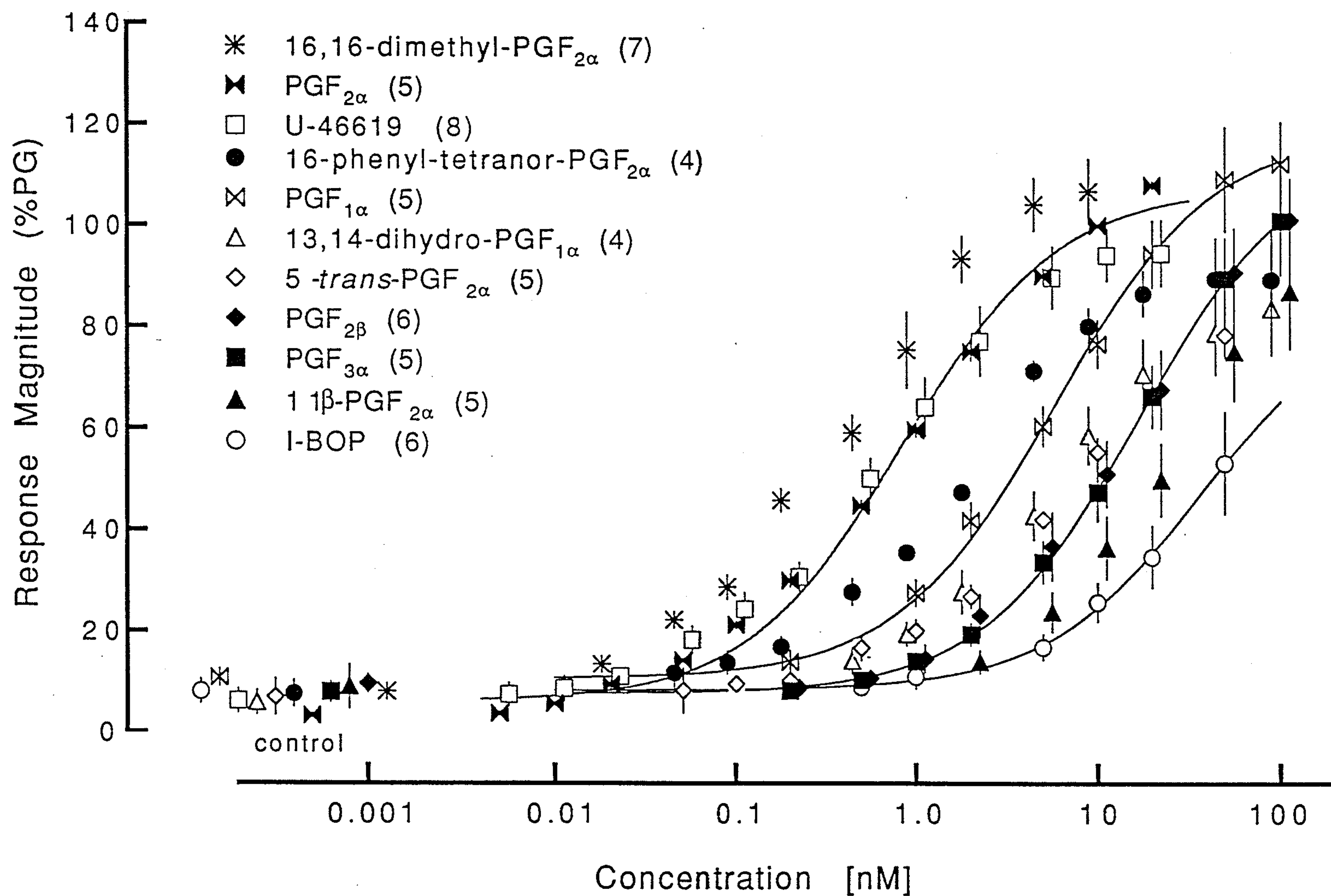
Fig. 2.4 The amplitudes of the phasic EOG responses to various synthetic prostaglandins and analogs tested at 10 nM, without (open symbols) and during 10 nM $PGF_{2\alpha}$ adaptation (filled symbols). Responses are expressed as percentage of response to 10 nM $PGF_{2\alpha}$ (%PG) Means (symbols) and T'-values (error bars) are indicated. Where error bars overlap, means are not significantly different (P<0.05). The two vertical lines show the response to 10 nM $PGF_{2\alpha}$ (100%PG) and the upper T'-value for the control response (15%PG). Number of fish tested in parentheses. All fish tested were immature or in early stage of sexual maturation (age: 1+ to 2+).


Unadapted
16,16-dimethyl-PGF2α (13)
U-46619 (9)
16-phenyl-tetranor-PGF2α (5)
PGF1α (9)
13,14-dihydro-PGF1α (5)
5 -trans-PGF 2α (6)
PGF2β (7)
PGF3α (6)
1 1β-PGF 2α (6)
I-BOP (6)
thromboxane B 2 (3)
U-44069 (3)
15(R)-PGF 2α (4)
15-keto-PGF 2α (10)
control (17)
1 1α-nitrilo-PGF 2α (4)
PGD2 (10)
PGE2 (7)
10 nM PGF2α Adaptation
16,16-dimethyl-PGF2α (5)
U-46619 (6)
16-phenyl-tetranor-PGF2α (5)
PGF1α (5)
13,14-dihydro-PGF1α (5)
5 -trans-PGF 2α (4)
PGF2β (5)
PGF3α (4)
1 1β-PGF 2α (3)
I-BOP (5)
PGF2α (4)
control (5)
0
20
40
60
80
100
120
Response Magnitude (%PG)

L-serine phasic response). The pooled variance was 88 for the %PG responses and 948 for the %AA data ($F[112,112]=10.77$; $P<0.01$). Only two analogs (16,16-dimethyl-$PGF_{2\alpha}$ and U-46619) gave responses as large as $PGF_{2\alpha}$ did. At 10 nM, seven of the substances tested gave responses less or not significantly larger than those to control (plain water) stimulation. Concentration-response curves for the chemicals that gave significant responses at 10 nM and lower concentrations are shown in Figure 2.5. The concentration-response curve for $PGF_{2\alpha}$ has a sigmoid shape with threshold of detection around 5-10 pM and reaches a plateau at 5-10 nM. The curves for the other compounds have similar shapes, but are shifted to the right to various degrees. Equation 1 was fitted to the curves and resulting parameters are shown in Table II.1. The maximum response level obtained by the fitting procedure (the Q parameter) was similar for all the curves (78-109%). The K parameter is therefore negatively correlated with the responses at 10 nM, namely the PGs which had the largest responses at 10 nM had the lowest K values. The chemicals which had the lowest Q value differed structurally from $PGF_{2\alpha}$ by changes on the side chain rather than on the penta-ring.

The concentration-response relationship of the integrated NTR response to $PGF_{2\alpha}$ had detection threshold lower than 100 pM and reached plateau at 1 nM (Fig. 2.6). The plateau value was lower than that for the EOG responses when expressed as %AA (EOG plateau for $PGF_{2\alpha}$ was 109 %AA, but the integrated NTR plateau was 42 %AA). The NTR recordings indicate that the EOG to $PGF_{2\alpha}$ is indeed transduced into nerve activity and transmitted to the brain. The integrated NTR responses to 10 nM $PGF_{1\alpha}$ were about 80% of the response to $PGF_{2\alpha}$ which is about the same as that observed in EOG. Although the $PGF_{2\alpha}$ responses were significantly larger

Fig. 2.5 Concentration-response relationship for eleven prostaglandins and analogs. Curves fitted according to the model (eq. 1) are shown for four compounds ($PGF_{2\alpha}$, $PGF_{1\alpha}$, $PGF_{3\alpha}$, and I-BOP; parameters in Table II.1). Number of fish tested in parentheses. All fish tested were immature or in early stage of sexual maturation (age: 1+ to 2+).

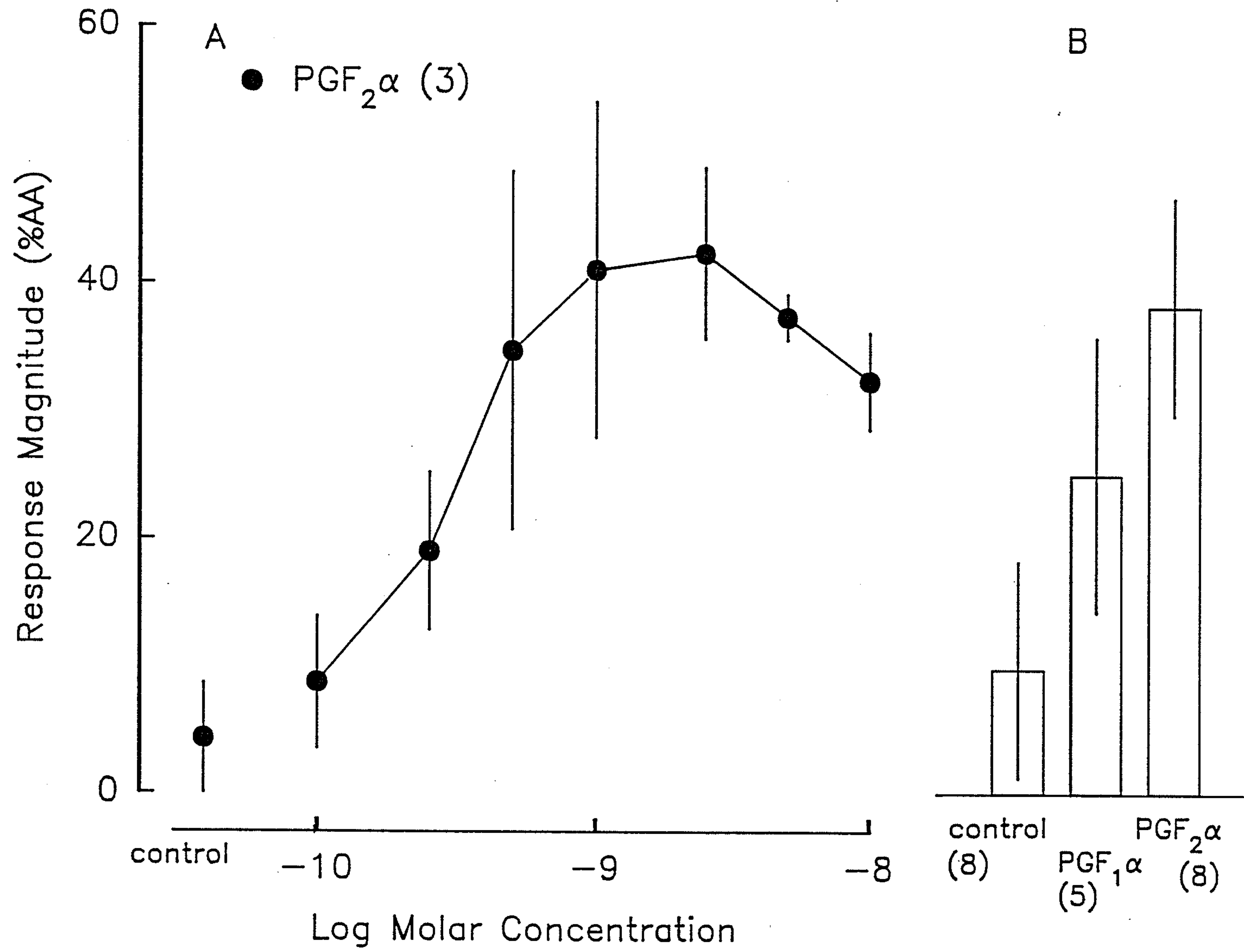


16,16-dimethyl-PGF2α (7)
PGF2α (5)
U-46619 (8)
16-phenyl-tetranor-PGF2α (4)
PGF1α (5)
13,14-dihydro-PGF1α (4)
5 -trans-PGF 2α (5)
PGF2β (6)
PGF3α (5)
1 1β-PGF 2α (5)
I-BOP (6)
Response Magnitude (%PG)
140
120
100
80
60
40
20
0
control
0.001
0.01
0.1
1.0
10
100
Concentration [nM]

Table II.1 Estimates of the parameters obtained from the fitting of Eq. 1 to the responses shown in Fig. 2.5. Standard deviation as calculated by Sigmaplot in parentheses

Name	K [nM]	Q [%PG]	C [%PG]
16,16-dimethyl-$PGF_{2\alpha}$	0.5 (1.2)	101 (8)	11 (2)
U-46619	0.6 (0.5)	90 (3)	8 (1)
$PGF_{2\alpha}$	0.9 (0.9)	102 (5)	6 (1)
16-phenyl-tetr.-$PGF_{2\alpha}$	2.0 (0.6)	84 (2)	9 (1)
13,14-dihydroxy-$PGF_{1\alpha}$	5.2 (1.3)	83 (3)	7 (2)
$PGF_{1\alpha}$	5.8 (0.9)	108 (2)	11 (1)
5-trans-$PGF_{2\alpha}$	6.6 (1.1)	78 (2)	9 (1)
$PGF_{2\beta}$	14.9 (1.4)	106 (2)	9 (1)
$PGF_{3\alpha}$	17.1 (0.8)	109 (1)	7 (1)
11-$PGF_{2\alpha}$	27.1 (3.2)	101 (3)	8 (1)
I-BOP	38.2 (6.3)	79 (6)	8 (1)

Fig. 2.6 Integrated nerve-twig recording (NTR) responses to (A) concentration series of $PGF_{2\alpha}$ [average (symbols) and SE (error bars) indicated], and (B) 10 nM $PGF_{2\alpha}$ and $PGF_{1\alpha}$, and control [average (bars) and T'- values (error bars) indicated; averages are not significantly different where error bars overlap]. Number of fish tested in parentheses. All fish tested were immature or in early stage of sexual maturation (age: 1+ to 2+).

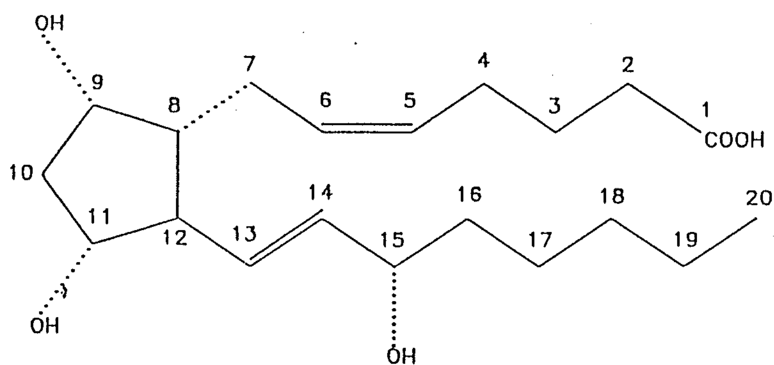

A
PGF2α (3)
B
Response Magnitude (%AA)
60
40
20
0
control
−10
−9
−8
Log Molar Concentration
control (8)
PGF1α (5)
PGF2α (8)

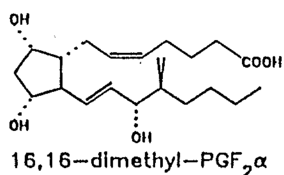
than control responses, the $PGF_{1\alpha}$ responses were not significantly different from control nor $PGF_{2\alpha}$ responses (Fig. 2.6B). Because NTR proved unstable and very tedious to perform, only EOG was used to further characterize the PG responses.

The chemical structures of the substances tested are shown in Figure 2.7. $PGF_{2\alpha}$ and the more stimulatory compounds are at the top and those that were nonstimulatory at 10 nM are in the two rows at the bottom. The structural requirements for PGs to elicit EOG response can be summarized from these experiments as follows. The hydroxy groups attached to positions 11 and 15 seem important for effective stimulation. The ability of the compound to elicit response was eliminated by changing the direction of either of the bonds in these positions from downward to upward (11β-$PGF_{2\alpha}$, 15(R)-$PGF_{2\alpha}$). Any other changes to these groups had the same effect (PGD_2, 11α-nitrilo-$PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$, U- 44069) with one exception; when oxygen, covalently bound to a carbon atom, was attached to position 11α (U-46619) instead of the hydroxy group, the compound was as stimulatory as before. Changing the direction of the hydroxy group in position 9 ($PGF_{2\beta}$) reduced the response at 10 nM to 50% and keto group (PGE_2) eliminated the response. However, when carbon was attached to this position (U-46619), no reduction in stimulatory effectiveness was observed. Reduction of the two double bonds on the side chain ($PGF_{1\alpha}$, 13,14-dihydro-$PGF_{1\alpha}$) or adding a third double bond ($PGF_{3\alpha}$) decreased the stimulatory effectiveness by 10-55%PG at 10 nM. Also changing the double bond in position 5,6 from *cis* to *trans* had a similar effect. Other changes on the side chain caused no (16,16-dimethyl-$PGF_{2\alpha}$) or some (16-phenyl-tetranor-$PGF_{2\alpha}$) reduction in stimulatory effectiveness of the compounds.

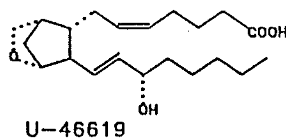
Fig. 2.7 Chemical structures of the prostaglandins and analogs tested.

$PGF_2\alpha$

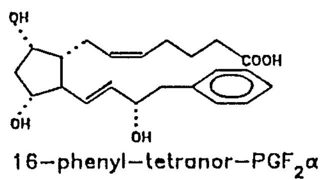




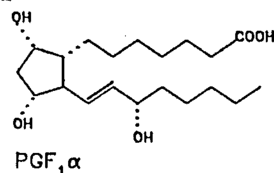
16,16–dimethyl–$PGF_2\alpha$



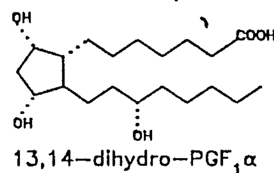
U–46619



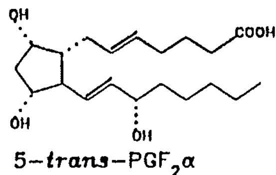
16–phenyl–tetranor–$PGF_2\alpha$



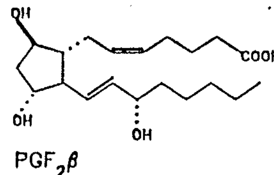
$PGF_1\alpha$



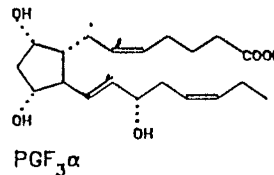
13,14–dihydro–$PGF_1\alpha$



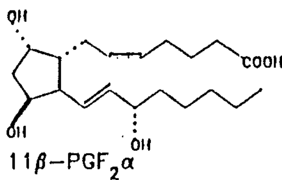
5–*trans*–$PGF_2\alpha$



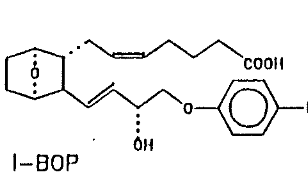
$PGF_2\beta$



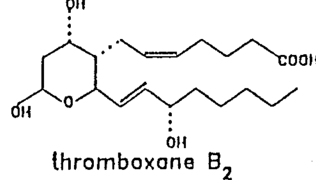
$PGF_3\alpha$



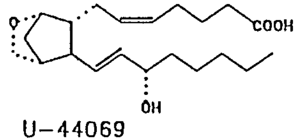
11β–$PGF_2\alpha$



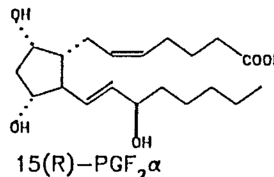
I–BOP



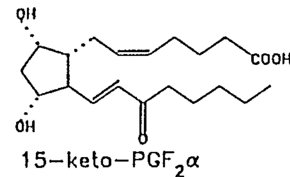
thromboxane B_2



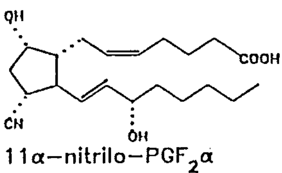
U–44069



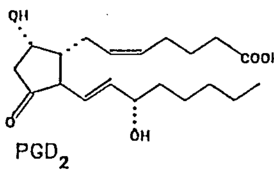
15(R)–$PGF_2\alpha$



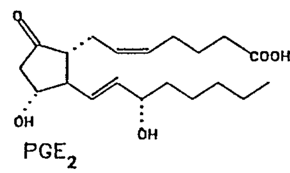
15–keto–$PGF_2\alpha$



11α–nitrilo–$PGF_2\alpha$



PGD_2



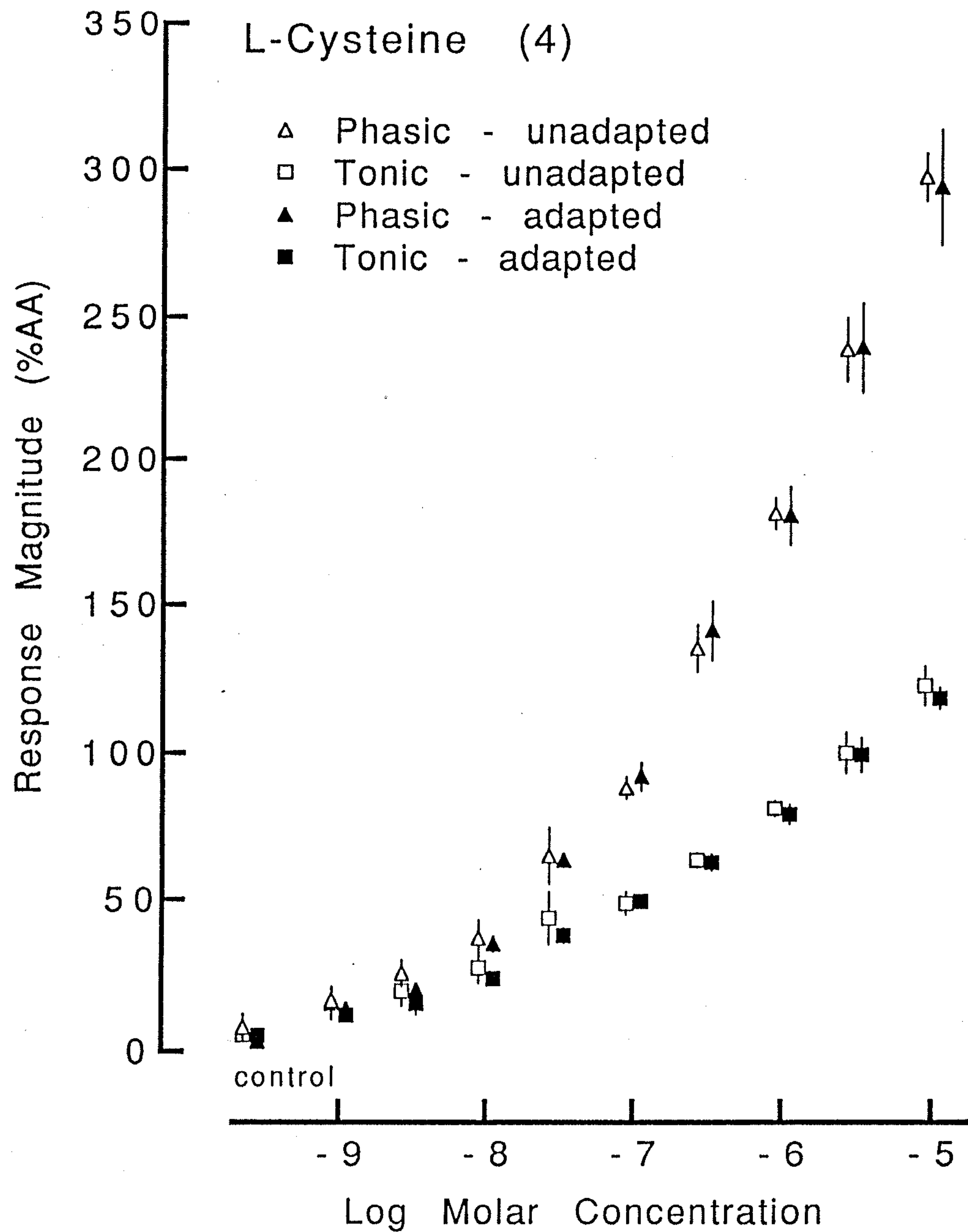
PGE_2

Two other analogs, both of which had structural changes on the penta-ring (thromboxane B_2, I-BOP), were only slightly stimulatory at 10 nM.

The responses to 10 nM solutions of the PGs and analogs were totally eliminated during 10 nM $PGF_{2\alpha}$ cross-adaptation (Fig. 2.4). Although the responses to 10 nM $PGF_{2\alpha}$, 16,16-dimethyl-$PGF_{2\alpha}$, and U-46619 were larger than the response to plain water, the difference was not statistically significant (Fig. 2.4). This suggests that all these chemicals bind to the same receptors or share the same transduction mechanisms. Concentration series of L-cysteine, L-arginine, and taurocholate were also tested just before and while 10 nM $PGF_{2\alpha}$ was used to adapt its receptors (Fig. 2.8, 2.9, and 2.10). No change was observed in either the phasic or tonic response to any of these chemicals. This indicates that these chemicals do not bind to the same receptors as $PGF_{2\alpha}$ does. Larger variance of the PG responses when expressed as %AA than when expressed as %PG (see above) further suggests functional independence of the PG receptor from the amino acid receptors.

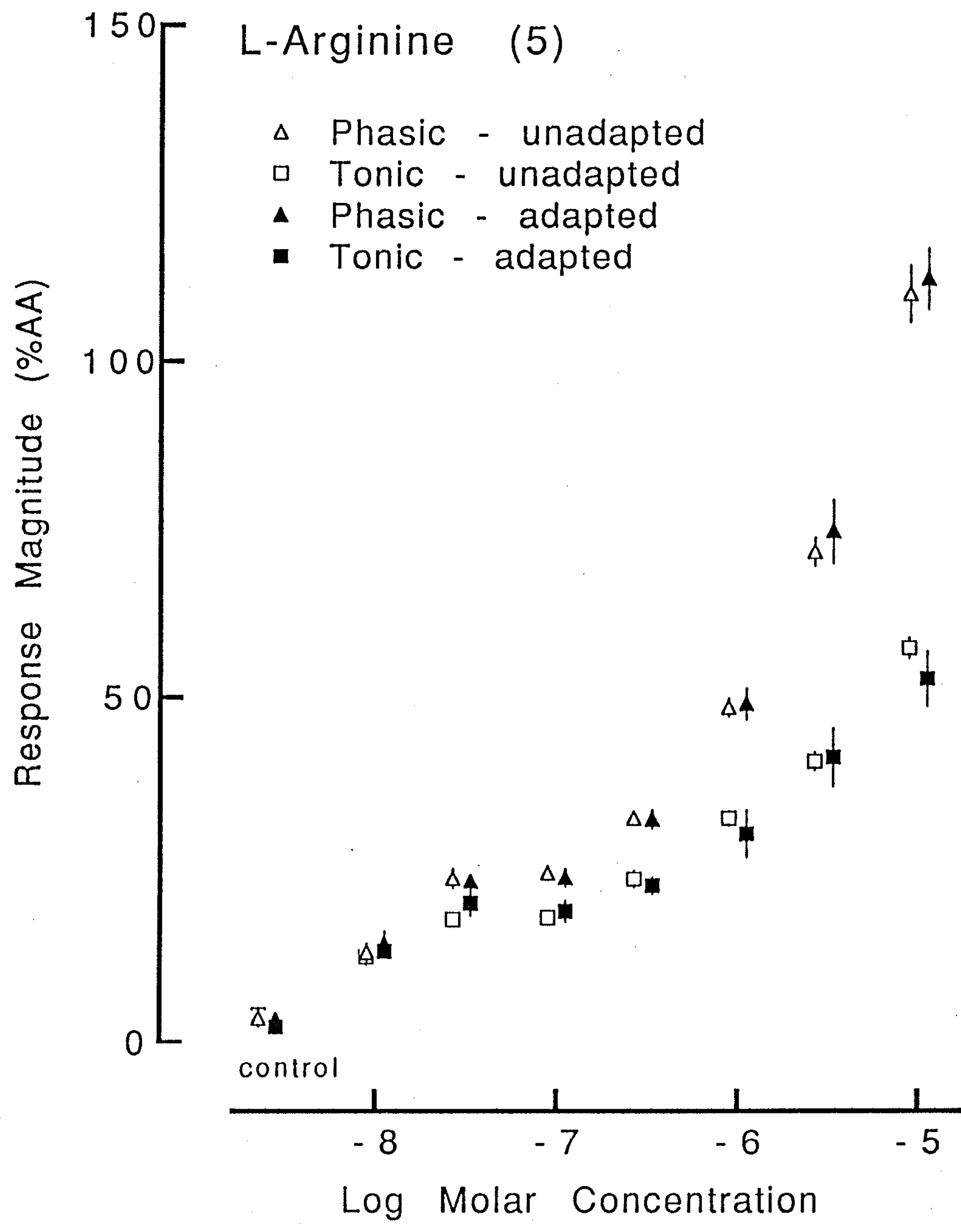
The EOG responses to 10 nM $PGF_{2\alpha}$ are plotted against the EOG responses to 10 µM L-serine in Fig. 2.11 for char at different stages of sexual maturation. The average responses to 10 nM $PGF_{2\alpha}$ and 10 µM L-serine were 0.86 mV and 0.96 mV, respectively. The variances were 273697 and 266960, respectively. The variability of the PG response is thus not different from the variability of the L-serine response. Product-moment correlation coefficient (r) for the pooled data was 0.598 ($P<0.05$). Thus, only 36% ($r^2=0.36$) of the variability in the responses to 10 nM $PGF_{2\alpha}$ can be accounted for by variability in the responses to 10 µM L-serine. When the correlation coefficients were calculated for each maturational stage and compared, none was significantly different from any of the other ones. The correlation

Fig. 2.8 EOG responses to L-cysteine before and during adaptation to 10 nM $PGF_{2\alpha}$. Averages (symbols) and standard error of means (error bars) are indicated. Number of fish tested in parentheses. All fish tested were immature or in early stage of sexual maturation (age: 1+ to 2+).



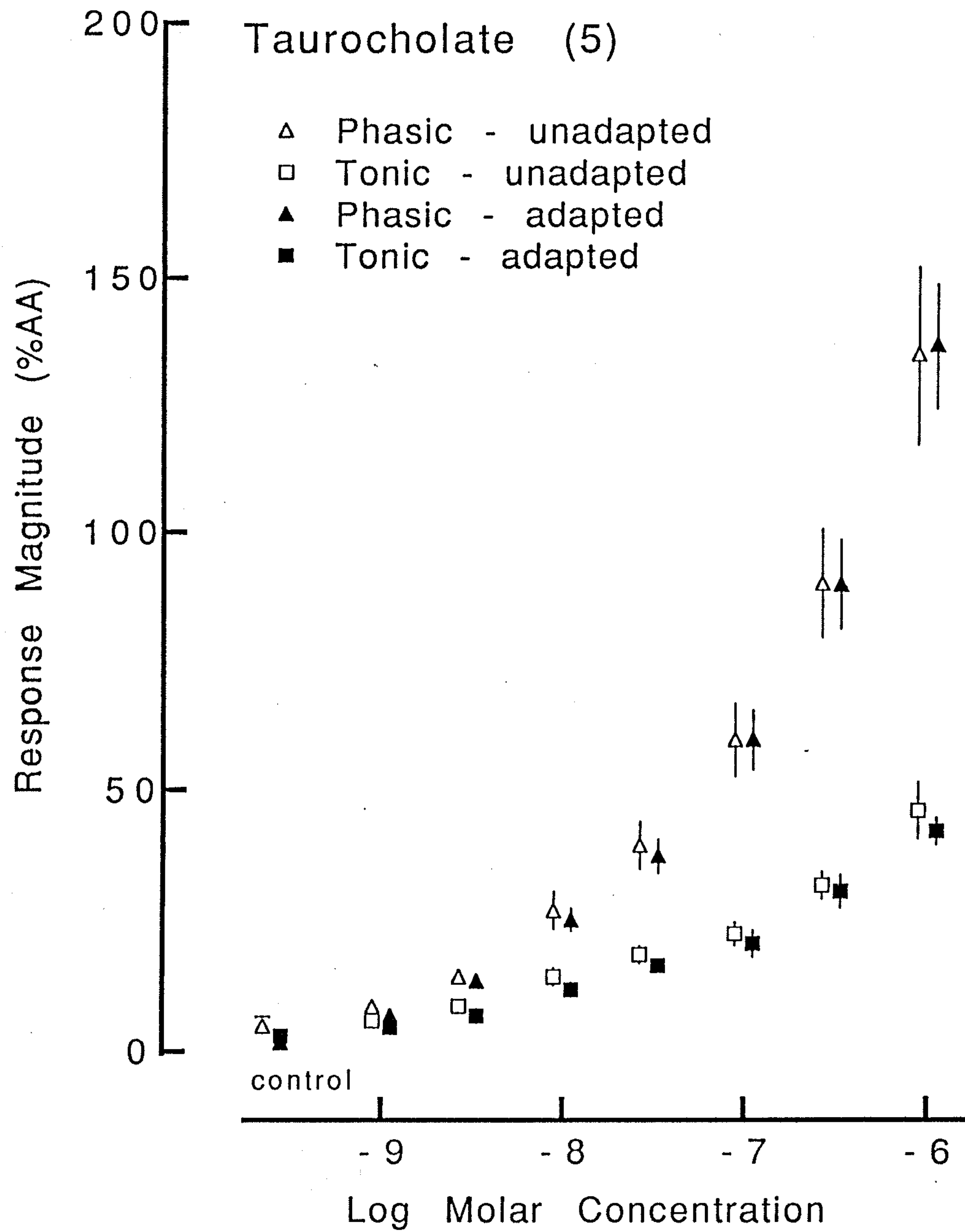
L-Cysteine (4)
Phasic - unadapted
Tonic - unadapted
Phasic - adapted
Tonic - adapted
350
300
250
200
150
100
50
0
Response Magnitude (%AA)
control
- 9
- 8
- 7
- 6
- 5
Log Molar Concentration

Fig. 2.9 EOG responses to L-arginine before and during adaptation to 10 nM $PGF_{2\alpha}$. Averages (symbols) and standard error of means (error bars) are indicated. Number of fish tested in parentheses. All fish tested were immature or in early stage of sexual maturation (age: 1+ to 2+).



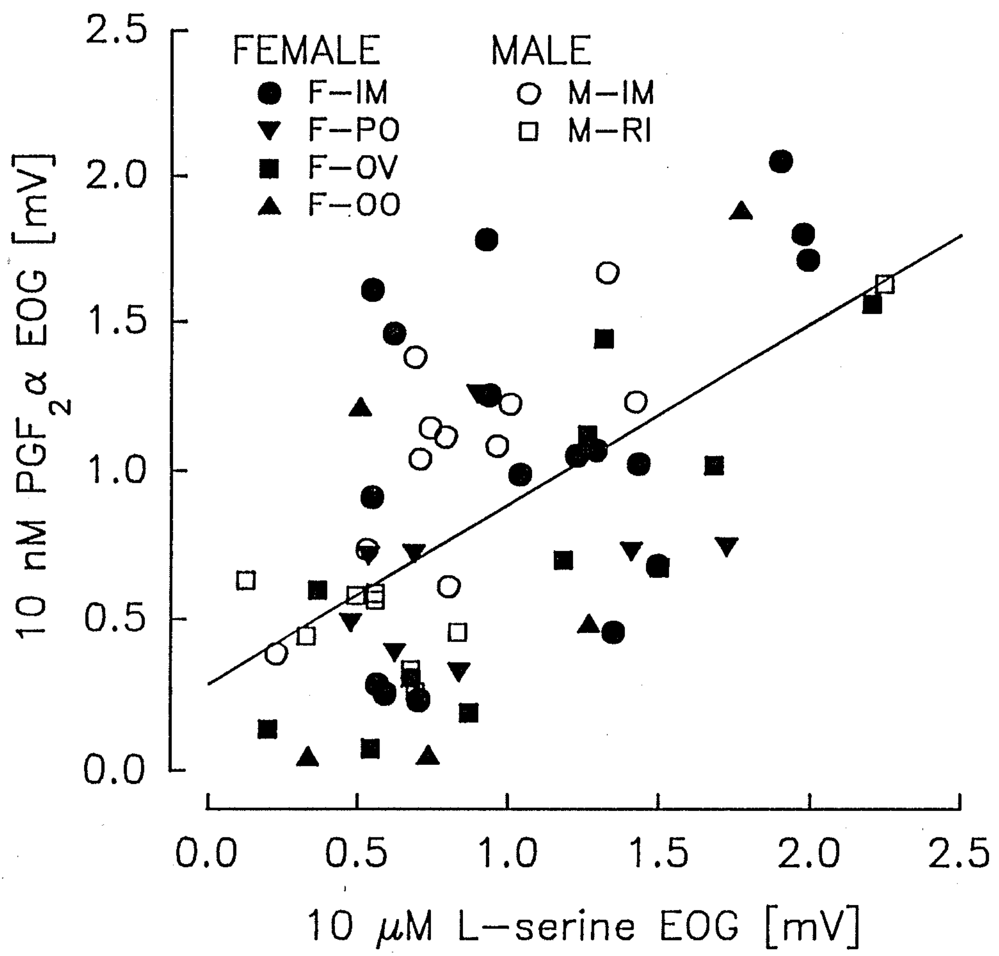
L-Arginine (5)
Phasic - unadapted
Tonic - unadapted
Phasic - adapted
Tonic - adapted
150
100
50
0
Response Magnitude (%AA)
control
- 8
- 7
- 6
- 5
Log Molar Concentration

Fig. 2.10 EOG responses to taurocholate before and during adaptation to 10 nM $PGF_{2\alpha}$. Averages (symbols) and standard error of means (error bars) are indicated. Number of fish tested in parentheses. All fish tested were immature or in early stage of sexual maturation (age: 1+ to 2+).



Taurocholate (5)
Phasic - unadapted
Tonic - unadapted
Phasic - adapted
Tonic - adapted
Response Magnitude (%AA)
200
150
100
50
0
control
-9
-8
-7
-6
Log Molar Concentration

Fig. 2.11 The amplitude of the phasic EOG response to 10 nM $PGF_{2\alpha}$ plotted against the amplitude af the phasic EOG response to 10 μM L-serine for each individual char. Different maturational stages are indicated (abbreviations defined in text, page 10).


FEMALE
F–IM
F–PO
F–OV
F–OO
MALE
M–IM
M–RI
10 nM PGF$_{2}\alpha$ EOG [mV]
10 μM L–serine EOG [mV]
0.0
0.5
1.0
1.5
2.0
2.5

coefficients for all stages were significant ($P<0.05$), exept for the F-PO and F-OO data. Three females (2 F-OO and 1 F-OV) gave EOG response to 10 nM $PGF_{2\alpha}$ less than 0.1 mV (0.046 mV, 0.071 mV, 0.051 mV; the three lowest points on Fig. 2.11) and were barely larger than the control responses (0.022 mV, 0.014 mV, and 0.037, respectively). Although two of the smallest responses came from F-OO, the second largest response came also from a F-OO female. When the variance of the responses to F-OO females was tested against the pooled variance of the other fish, it was found to be significantly larger (ANOVA, $F[4,55]=3.04$; $P<0.05$).

To further compare the $PGF_{2\alpha}$ responses for different maturational stages, attempts were made to fit equation 1 to the responses to a concentration series (5 pM - 20 nM) of $PGF_{2\alpha}$ for each individual char. The fitting procedure converged for all char except to those three that gave responses less than 0.1 mV to 10 nM $PGF_{2\alpha}$. Average values of the parameters for each maturational stage are given in Table II.2. No significant difference in parameters across maturational stages were observed (ANOVA on log transformed data, $F[5,94]=0.68$; $P>0.05$). Furthermore, there was no difference in responses to 10 nM PGD_2, PGE_2, $PGF_{1\alpha}$, 15-keto-$PGF_{2\alpha}$, and 16,16-dimethyl-$PGF_{2\alpha}$ across maturational stages (Table II.3; ANOVA, $P>0.05$ for each chemical).

Discussion

The EOG is considered to represent the summated receptor potential of olfactory receptor neurons (Ottoson, 1956,1971; Getchell, 1974). It has proven to be a reliable indicator of chemosensitivity to pheromonal cues in fish (Sorensen *et al.*, 1987,1988,1990; Resink *et al.*, 1989d). In goldfish,

Table II.2 Average of the estimates of parameters from the model fitting for EOG responses to $PGF_{2\alpha}$ for each maturational stage (Mat. stage). Standard error of mean is shown in parentheses.

Mat. stage	N	K [nM]	Q [%PG]	C [%PG]
M-IM	4	0.8 (0.1)	102 (2)	9 (2)
M-RI	8	1.2 (0.1)	103 (4)	9 (2)
F-IM	4	1.0 (0.1)	102 (1)	8 (1)
F-PO	8	1.4 (0.2)	104 (3)	11 (1)
F-OV[1]	10	1.2 (0.2)	99 (3)	12 (2)
F-OO[1]	3	1.1 (0.3)	96 (6)	12 (5)

[1] One F-OV and two F-OO char had too small responses for the fitting procedure to converge successfully and are thus not included.

Table II.3 Average EOG responses [%PG] to 10 nM PGD_2, PGE_2, $PGF_{1\alpha}$, 15-keto-$PGF_{2\alpha}$ (15K), and 16,16- dimethyl-$PGF_{2\alpha}$ (DM) for each maturational stage (Mat. stage). Standard error of mean is shown in parentheses.

Mat. stage	N	PGD_2	PGE_2	$PGF_{1\alpha}$	15K	DM
M-IM	2	9 (4)	5 (1)	78 (13)	9 (1)	105 (1)
M-RI	9	8 (2)	7 (3)	65 (4)	10 (3)	100 (4)
F-IM	4	10 (3)	7 (3)	78 (1)	9 (1)	102 (3)
F-PO	2	4 (3)	3 (1)	65 (6)	8 (4)	108 (2)
F-OV	7	12 (2)	9 (2)	73 (4)	11 (1)	101 (4)
F-OO	4	7 (2)	9 (3)	71 (6)	10 (3)	87 (4)

responses to pheromones are transmitted through the medial olfactory tracts (Sorensen *et al.*, 1991b). The terminal nerve, which has been proposed to mediate pheromonal responses in vertebrates (Demski and Northcutt, 1983), was unresponsive to pheromones in goldfish (Fujita *et al.*, 1991). The EOG in the present study was found, at least for $PGF_{2\alpha}$, to correlate with neural impulses activity transmitted to the brain through the olfactory nerve.

Several lines of evidence indicate independence of PG olfactory receptor and transduction mechanisms from those for other known stimuli. First, responses to L-cysteine, L-arginine and taurocholate were unaffected by adaptation of the olfactory epithelium to $PGF_{2\alpha}$. Secondly, there was a poor correlation between the responses to L-serine and $PGF_{2\alpha}$. This resulted in higher variance of the PG responses when expressed as percent of L-serine (%AA) rather than percent of $PGF_{2\alpha}$ (%PG). PG receptors in goldfish were also found to be independent of other olfactory receptors (Sorensen *et al.*, 1988). However, unlike goldfish which have two independent types of PG receptor, no evidence for more than one type of olfactory receptor for PGs was found in the present study in Arctic char. The concentration-response curves for the PGs were best fitted by a single component model, and this has been shown to indicate a single type of receptor (Sveinsson and Hara, 1990a,b). Furthermore, adaptation to one PG chemical ($PGF_{2\alpha}$), eliminated the responses to all the other PGs.

Multicomponent models, where each component was represented by equation 1, were successfully used to describe and analyze concentration-response relationship of olfactory receptors for amino acids (Sveinsson and Hara, 1990a,b). However, multicomponent models were unable in the present study to improve the fit significantly over that of the single

component model and were therefore rejected. These models, including the single component model, assume that the measured response is linearly related to the number of occupied receptor molecules. Therefore, the parameter K is equivalent to the dissociation constant (K_d) of the stimulus-receptor interaction. Of various types of model tested, these types of models were found to fit the integrated NTR responses best (Sveinsson and Hara, 1990a,b). The model fitting in the present study suggests that the K_d for the interaction of $PGF_{2\alpha}$ with its receptor is 0.9 nM. This is comparable to the receptor-binding affinities of various neurotransmitters that have been used in classical ligand-binding studies (Yamamura *et al.*, 1985). This matter will be further discussed in Chapter III.

In fish, F-type and E-type PGs have been found to be involved in ovulation, female sexual behaviour, and possibly in gonadotropin secretion (for review see Stacey and Goetz, 1982). No attempts have been made to further determine the specificity of the receptors responsible for these responses. However, five types of PG receptors have been described in mammalian tissues (various types of smooth muscles and blood platelets) and at least some of them can be further divided into subtypes (for review see Coleman, 1987; Gardiner, 1990). These types have been named DP, EP, FP, IP, and TP, where the naturally occurring compounds PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2 are the most potent agonists for each, respectively. In the present study $PGF_{2\alpha}$ was the most stimulatory of the naturally occurring compounds tested, suggesting that a receptor comparable to the FP type mediates the observed EOG responses. PGD_2 and PGE_2 were ineffective as agonists, implying that olfactory receptors of type DP and EP are not present in Arctic char. The results regarding the TP type of receptor are somewhat conflicting. Thromboxane A_2 is a very

unstable compound, making it unsuitable as a TP agonist in many experimental settings. Instead, U-46619 has been used as a TP agonist for several years (Coleman *et al.*, 1981; Coleman, 1987). Although U-46619 was as stimulatory as $PGF_{2\alpha}$ in the present study, I-BOP, a new more potent TP agonist (Morinelli *et al.*, 1990), was ineffective. Furthermore, cross-adaptation experiments did not reveal that $PGF_{2\alpha}$ and U-46619 were reacting with different receptors, refuting the possibility of a separate TP-type receptor. Studies with PG agonists have usually used mammalian tissue preparations which contain more than one type of PG receptor, thus making agonist characterization for each type more difficult (Coleman, 1987). However, it seems that the PG olfactory receptor in Arctic char is less specific, in regard to U-46619 at least, than the FP hormone receptor in mammals. Unfortunately, no further information is available on the number or specificity of internal eicosanoid receptor types in fish.

The present study revealed that the three hydroxy groups in positions 9, 11, and 15 are important for these agonists in eliciting responses. $PGF_{2\alpha}$ has all these hydroxy groups in the same plane, i.e. they are all in the 'down' position (Fig. 2.7). Changing the direction of any of these to the 'up' position renders the resulting chemical ineffective in eliciting responses. These groups are, however, not necessarily directly involved in binding to the receptor. These are polar groups on an otherwise rather hydrophobic molecule and directional changes of the bonds could have profound effects on the conformation of the molecule.

The only structural difference among the naturally occurring agonists for the FP, EP, and DP receptors is exchange of a keto group for a hydroxy group in either position 9 or 11 (Coleman, 1987). Thus, the conservatism of groups in these positions is in agreement with what is

expected for a PG receptor. Conservatism at position 15 is not unexpected either. This site is used to metabolise and presumably inactivate PGs by 15-hydroxy prostanoate dehydrogenase in mammals (Samuelsson *et al.*, 1975; Granström and Kumlin, 1987). Furthermore, $PGF_{2\alpha}$ with a keto group in this position (15-keto-$PGF_{2\alpha}$) was the most stimulatory PG tested in goldfish (Sorensen *et al.*, 1988); its threshold was around 1 pM, lower than that of the most stimulatory PG in the present study. However, the threshold for $PGF_{2\alpha}$ in goldfish was 100 pM which is about ten times higher than found here for the same chemical in Arctic char. In goldfish, two independent receptors were found to be responsible respectively for $PGF_{2\alpha}$ and 15-keto-$PGF_{2\alpha}$ responses (Sorensen *et al.*, 1988). Thus it seems that the receptor for the latter is not comparable to any other previously known eicosanoid receptor. As is known for other PG receptors, changes on the side chains of the PGs are more permissive than changes on the penta-ring in retaining their effectiveness as agonists (Coleman, 1987; Gardiner, 1990).

No differences were found in sensitivity or specificity of the PG olfactory receptor among different stages of sexual maturation in the present study (possibly with the exception of three females). If the PG pheromones have no biological significance for immature fish it appears a waste of energy to maintain high sensitivity receptors for PG through the juvenile life stage. Because very little is known about the receptors and transduction mechanisms for pheromones (further discussed in Chapter III), it is at present impossible to estimate the actual cost of maintaining functional receptors. Also, it cannot be excluded that the PG receptor described in the present study provides important information in early life in relation to learning, imprinting and migration (Hara *et al.*, 1984; Morin *et al.*, 1989a,b; Dodson, 1988; also see Chapter IV for further discussion).

Sorensen *et al.* (1987) found that, although only mature males showed endocrine and behavioural responses to 17,20P, both mature females and gonadally regressed males had functional olfactory receptors for it. However, precocious Atlantic salmon males have functional receptors for testosterone only temporarily (Moore and Scott, 1991). The biological function of testosterone as an external chemical cue in this species is, however, not known.

Down-regulation of hormonal and neurotransmitter receptor upon prolonged exposure to agonists is well known (Creese and Sibley, 1981; Sibley and Lefkowitz, 1985; Dohlman *et al.*, 1991). The present study indicates that post-ovulatory females may down-regulate or modulate their PG receptors. Three post-ovulatory females showed EOG responses less than 0.1 mV to 10 nM $PGF_{2\alpha}$, lower than any other group. Also, post-ovulatory females showed no significant correlation between PG and L-serine responses. Upon ovulation female brook trout (*Salvelinus fontinalis*) ovaries produce $PGF_{2\alpha}$ and elevated plasma level of $PGF_{2\alpha}$ can be detected (Cetta and Goetz, 1982; Goetz *et al.*, 1989). The char used in the present study had been kept in isolation for at least one week before testing. No immunoreactive F-type PGs were found in water from the tanks holding individual Artic char females (Chapter IV). It is thus unlikely that the unresponsiveness of the females is due to down-regulation by exposure to PGs released by the female itself. Sex hormones have been shown to modulate the electrical activity of the olfactory bulb in goldfish (Hara, 1967). However, the functioning of the olfactory receptors was not investigated in that study. Whether the observed reduction in olfactory responsiveness of post-ovulatory females to $PGF_{2\alpha}$ occurs generally should be investigated

further. Also, information on the mechanisms controlling such changes would be of great interest.

CHAPTER III

Biochemical and kinetic studies of the prostaglandin olfactory receptor in Arctic char, *Salvelinus alpinus*

Introduction

Ligand-binding experiments have been used successfully to identify and characterize several hormone and neurotransmitter receptors (Cuatrecasas, 1975; Snyder, 1984; Yamamura *et al.*, 1985,1990). This method has also been applied to taste and olfactory receptors for amino acids in fish (Krueger and Cagan, 1976; Cagan and Zeiger, 1978; Novoselov *et al.*, 1980; Brown and Hara, 1981,1982; Fesenko *et al.*, 1983). Amino acid olfactory receptors have relatively low affinities for their ligand compared to many neurotransmitter and hormone receptors. This has made further characterization and purification of the receptor difficult. Furthermore, some aspects of the apparent binding suggest that it represents accumulation of amino acids into vesicles rather than binding to a receptor (Brown and Hara, 1981,1982).

Binding studies are not always successful. Particularly, binding studies for odorant recognition sites in mammals have not been successful (Snyder *et al.*, 1988; Lancet *et al.*, 1988; Anholt, 1989). Several laboratories, some with long experience in binding studies on neurotransmitters, have failed to demonstrate binding of odorants to receptor sites. One of the drawbacks in these studies is the lack of biologically meaningful odorants. Pheromones with known physiological or behavioural function have been

suggested to be better suited for binding studies (Lancet, 1991). Pheromone receptors are known for their high sensitivity (Sorensen *et al.*, 1987; Sorensen *et al.*, 1988; Chapter II). They might, therefore, be expected to have higher affinity for their ligands than other olfactory receptors. In support of this idea is a recently identified specific binding of the pheromone 17,20P to an olfactory preparation in goldfish (Rosenblum *et al.*, 1991). The binding site has high affinity for the ligand (K_d=1 nM) and shows some correlation with physiological and behavioural data.

Ligand-binding experiments have demonstrated the existence of high affinity binding sites for $PGF_{2\alpha}$ in mammalian ovaries (Wright *et al.*, 1979; Bussmann, 1989; Orlicky, 1990). Radiolabelled $PGF_{2\alpha}$ and membrane preparations were used in these studies. Thus, the use of a radiolabelled $PGF_{2\alpha}$ in classical ligand-binding experiments has proven valid.

In Chapter II, I used electrophysiological techniques to show that the olfactory epithelium of Arctic char has very specific and highly sensitive EOG responses to $PGF_{2\alpha}$. The concentration-response curve shows half-maximum response at 0.8 nM, suggesting a high-affinity receptor for this ligand. This chapter describes binding experiments which I conducted in an attempt to further identify and characterize the olfactory receptor for $PGF_{2\alpha}$ in Arctic char. Several binding procedures that have proven successful for other receptors (e.g., the amino acid receptors in olfactory epithelium of fish and the $PGF_{2\alpha}$ receptors in ovaries of mammals), were tried. Contrary to expectation I was unable to demonstrate any specific binding of $PGF_{2\alpha}$ to tissue preparations of the olfactory epithelium of Arctic char. However, I was able to demonstrate accumulation of L-alanine by these preparations of Arctic char, as has already been shown for various

other species of fish (Cagan and Zeiger, 1978; Rhein and Cagan, 1983; Brown and Hara, 1981,1982; Bruch and Rulli, 1988).

To further investigate possible reasons for the failure of the ligand-binding experiments, I examined EOG in response to concentration series of $PGF_{2\alpha}$ during adaptation to different concentrations of the same chemical, $PGF_{2\alpha}$ (self-adaptation). Previously published kinetic analysis of olfactory responses to amino acids analysed only the phasic component of the response to the test stimulus (Sveinsson and Hara, 1990a,b). The theory which the model is based on, however, assumes that the ligand-receptor interaction has reached a steady state. It is more likely that the interaction has reached steady state once the response has levelled to a tonic level. Therefore, both the phasic and tonic components were used in the kinetic analysis in the present study. The results suggest that either the receptor has lower affinity than predicted from the concentration-response curve for unadapted responses alone, or that the receptor is switched to a low affinity state when exposed to a stimulus. In light of these latter results, possible reasons for the inability to demonstrate specific binding of $PGF_{2\alpha}$ to olfactory tissue preparations are discussed.

Materials and Methods

Arctic char from the same stock as used in Chapter II were used in these experiments. All fish used (500-1000 g) were immature or in an early stage of maturation.

Unless otherwise indicated, the procedure for the binding experiments was the same as those previously used by others for studies on amino acid binding or accumulation by olfactory tissue preparations in fish

(Cagan and Zeiger, 1978; Brown and Hara, 1981,1982). Briefly, ten to twelve fish were decapitated and the olfactory rosettes were excised with a scalpel. They were placed in a homogenizing buffer (1 mM $CaCl_2$, pH 7.5 w/$NaHCO_3$, 150 μM indomethacin) with 300 mM sucrose and were washed 3 times. The rosettes then were transferred to homogenizing buffer (without sucrose) and cut into small pieces with a razor blade. They were homogenized in a glass homogenizer with a teflon piston by using 10-15 strokes. The crude homogenate was centrifuged at 1100 x g for 15 min (pellet resuspended is defined as a P1 fraction) and the supernatant was again centrifuged at 7300 x g for 60 min. The supernatant was discarded and the pellet resuspended (defined as a P2 fraction) with binding buffer (10 mM 2-[(2-amino-2-oxoethyl)-amino]ethanesulfonic acid (ACES), with 10 mM NaCl, 150 μM indomethacin, 0.1 % bovine serum albumin, at pH 7.0). Incubations were performed in 150 μl polypropylene tubes in a final volume of 50 μl containing approximately 100 μg protein of the tissue suspension. Duplicate samples with 1-10 nM [5,6,8,9,11,12,14,15n-^{3}H]-$PGF_{2\alpha}$ (Amersham, 352 cpm/fmole) or 150 nM L-[2,3-^{3}H]alanine (Amersham, 95 cpm/fmole) were incubated for 60 min on ice. Non-specific binding was determined by adding 10 mM unlabelled $PGF_{2\alpha}$ (Cayman Chemical) or 10 mM unlabelled L-alanine to a duplicate set of reaction vessels in addition to the labelled ligand. The reaction was terminated and the bound radioligand was separated from the free radioligand by filtration under reduced pressure through pre-washed (with distilled water) glass fiber filters (HAWP, Millipore; on a 1225 Sampling Manifold, Millipore). The filters were then washed twice with 5 ml of ice-cold buffer and placed into plastic vials with 5 ml of scintillation fluid (ScintiVerse Bio-HP, Fisher). Radioactivity was measured with scintillation spectrometry (Bechman LS-

7500). Specific binding was defined as the difference in radioactive binding between the samples without unlabelled ligand (total bound) and the samples with unlabelled ligand (non-specific binding). Protein concentrations were determined by the method of Lowry *et al.* (1951), using human serum albumin as standard.

In an effort to obtain specific binding of $PGF_{2\alpha}$, several changes in procedure were made to the binding assay in different binding experiments.

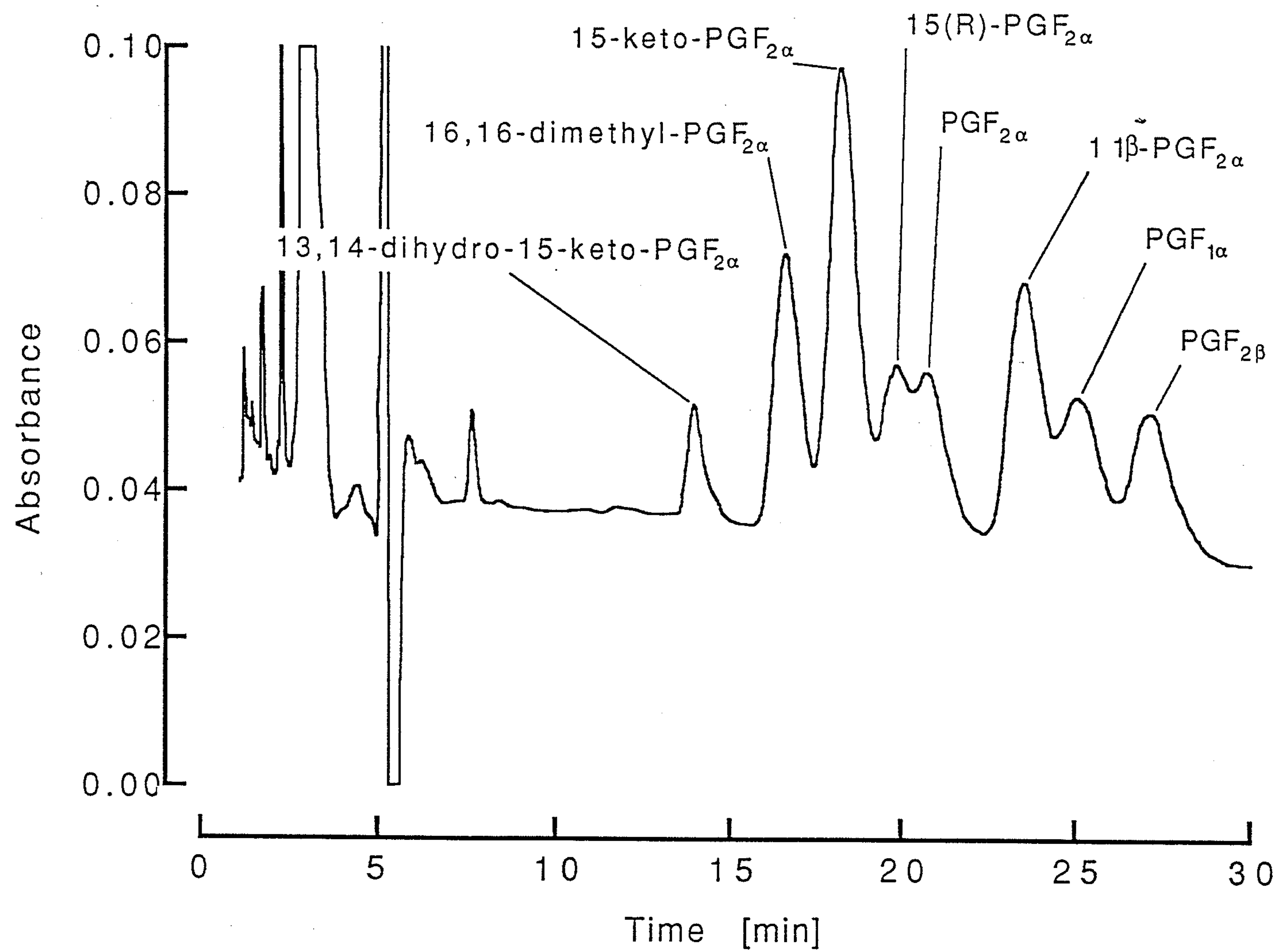
1. Tissue fractions: crude homogenates were tested and also different concentrations of tissue preparation (50-1400 mg protein/ml incubation medium).
2. Buffers: binding buffers without bovine serum albumin or indomethacin were tested, as was replacement of the ACES with 10 mM Tris-HCl (pH 7.0).
3. Assay tubes: glass tubes instead of polypropylene tubes were used and final volumes of 100 μl and 500 μl were tried.
4. Incubation temperatures and time: temperature of 10°C in addition to 0°C was used and the incubation time was varied (0-120 min).
5. Filtration and filters: various treatments of the samples and the filters before and after filtration were tested. To reduce non-specific binding to the filters, they were pre-washed with 5 ml of either binding buffer alone or binding buffer containing 10 μM unlabelled $PGF_{2\alpha}$. Also the samples were diluted with 1 ml of binding buffer (with and without 10 μM unlabelled $PGF_{2\alpha}$) a few seconds before filtration. The filters were treated with 0.3% polyethylenimine (Snyder *et al.*, 1988) for 12 hours before filtration to trap soluble proteins (crude homogenate was used in this experiment). Hydroxyapatite was added to the

samples a few seconds before filtration to precipitate proteins (Orlicky, 1990). Various washing procedures of the filters after the filtration were also tested.

6. Chemical inhibitors: various inhibitors and antioxidants were included in the binding buffer to eliminate possible chemical modification of the receptor or the labelled ligand in the incubation medium. To protect SH groups, 1 mM dithiothreitol was included. Metyrapone (1 mM and 1 μM) was included to inhibit cytochrome P-450 monooxygenase. Antioxidants (hydroquinone at 1 mM and 1 μM, and Trolox (Aldrich) at 15 μM) were used to inhibit oxidizing reactions in the incubation medium. EDTA at 1 mM was used to inhibit kinases (Orlicky, 1990).

7. Endogenous ligand and tracer stability: purity of the tracer was checked by using a normal-phase HPLC procedure which separates the most common F-type PGs (Fig. 3.1). A two pump gradient system was used (Gilson) with a flow rate of 2 ml/min and u.v detection at 208 nm wavelength. The column (5m- Zorbax Silica Analytical Cartridge, 46 x 250 mm) was equilibrated to a 15:85 mixture of solvent A (50% methanol, 5% isopropanol, 45% hexane, 0.1% acetic acid) and solvent B (99.9% hexane, 0.1% acetic acid). After an extraction with C_{18}-extraction column a sample was evaporated to dryness and the residue was dissolved in solvent B. Ten μl of this sample was then injected onto the column and after a 33 min run the solvent system was gradually changed over the next 2 min to 100% solvent A.
A fraction collector (Gilson) was used to collect 2 ml (1 min) fractions from the column. Radioactivity in aliquots from the fractions was measured by scintillation spectrometry as before. When pure tracer

Fig. 3.1 HPLC chromatogram of prostaglandin standards. See text for the chromatographic procedure.



Absorbance
0.10
0.08
0.06
0.04
0.02
0.00
0
5
10
15
20
25
30
Time [min]
13,14-dihydro-15-keto-PGF$_{2\alpha}$
16,16-dimethyl-PGF$_{2\alpha}$
15-keto-PGF$_{2\alpha}$
15(R)-PGF$_{2\alpha}$
PGF$_{2\alpha}$
11β-PGF$_{2\alpha}$
PGF$_{1\alpha}$
PGF$_{2\beta}$

was injected onto the HPLC column, the fraction collected during the 20th min of the HPLC run contained >90% of the injected radioactivity. After 60 min incubation with tissue as used in the ligand-binding assay, samples were diluted with 10 ml distilled water and extracted with C_{18}-extraction columns and checked on the HPLC. No difference was found between these runs and runs with a pure tracer. Tissue samples were also measured for $PGF_{2\alpha}$ content by extracting the samples as just described and assaying with EIA (enzyme immunoassay; see Chapter IV for EIA procedure, page 88).

In the self-adaptation experiments, EOG was recorded in response to concentration series of $PGF_{2\alpha}$, before and during adaptation to $PGF_{2\alpha}$. The EOG was recorded as described in Chapter II, and the same curve-fitting procedure was also used. If it is assumed that the measured response is linearly related to the number of occupied receptors, then the K parameter estimated under the unadapted condition is the dissociation constant (K_d) of the ligand-stimulus interaction (Sveinsson and Hara, 1990a). Furthermore, if no change in the affinity of the receptor for the ligand occurs, the value of the K parameter can be predicted (Sveinsson and Hara, 1990b; eqs 1b and 1c):

$$K = K_d \cdot (1 + S'/K'_d) \qquad \text{Eq. 2}$$

where S' and K'_d are the concentration and the dissociation constant of the adapting stimulus, respectively. In self-adaptation experiments the adapting stimulus and the test stimulus are the same compound, thus $K_d = K'_d$ if no change in affinity occurs in the presence of the adapting stimulus.

All chemicals except those indicated above were purchased from Sigma Chemical (St. Louis).

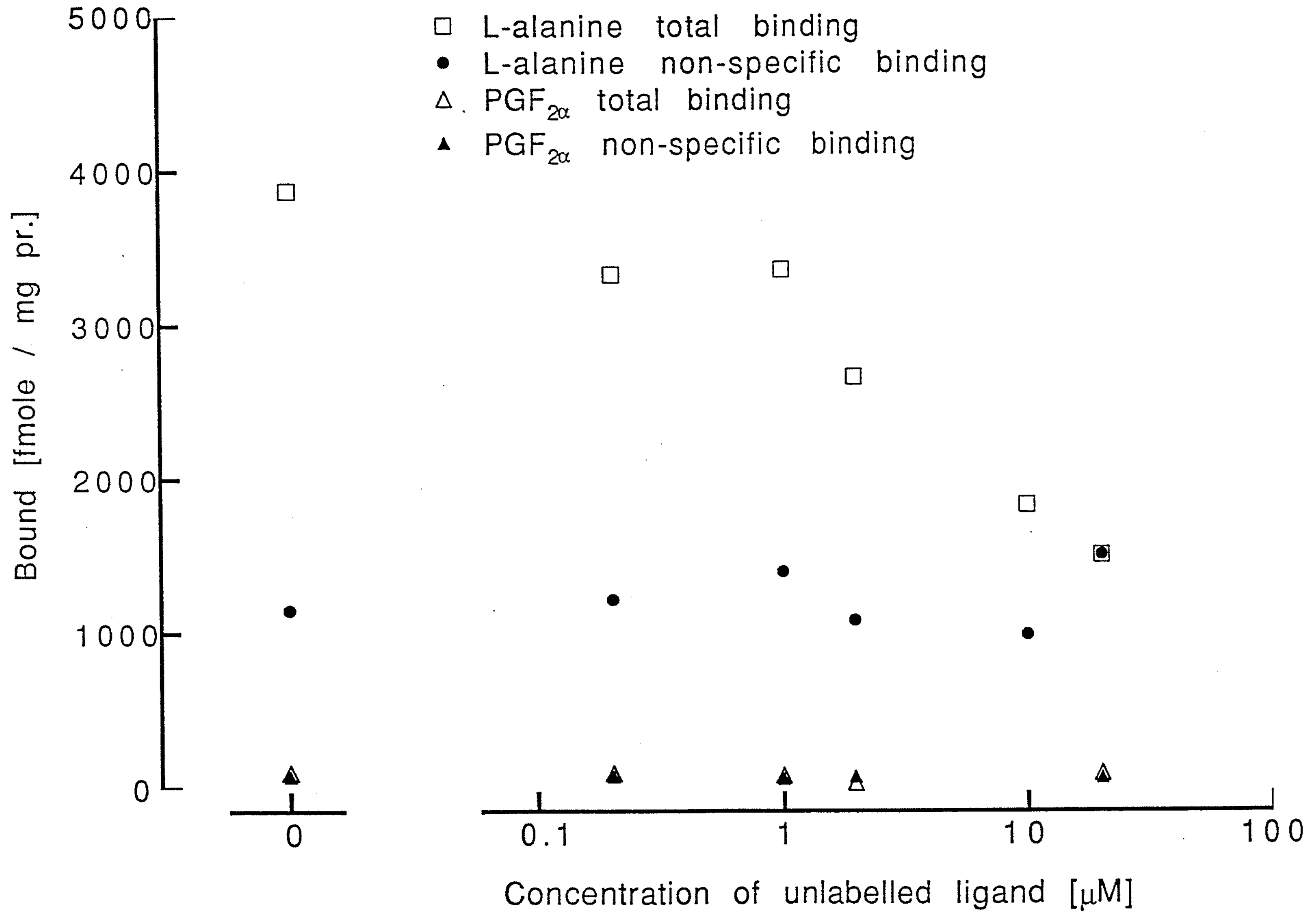
Results

No specific binding was observed of tritiated $PGF_{2\alpha}$ to the P2 fraction of olfactory tissue from Arctic char (Fig. 3.2). Different fractions, the crude homogenate, and the supernatant after the first and second centrifugation, were tested with no detection of specific binding. With decreases in the number or the volume of the washing steps, both the non-specific binding and the variability of binding between samples increased (increasing the noise level). However, no significant increase was observed in specific binding, which might be partially due to increased noise level. Increased washing lowered the non-specific binding without revealing specific binding. Contrary to PG's failure to bind, amino acid binding to the P2 fraction of the olfactory epithelium was demonstrated (see Fig. 3.2). The specific binding of labelled L-alanine was 2700 fmole per mg protein and was gradually displaced by increasing concentration (0.5-20 μM) of unlabelled L-alanine in the incubation medium.

The HPLC analysis confirmed the purity of the tracer and further showed that it was not metabolised in the incubation medium. The EIA analysis of the tissue preparation revealed concentrations of immunoreactive PGFs which correspond to 0.01-0.1 nM of $PGF_{2\alpha}$ in the final incubation suspension of the binding assay. This is less than 10% of the amount of tritiated $PGF_{2\alpha}$ added to the incubation vials (1-10 nM final).

The concentration-response relationship of the EOG response to $PGF_{2\alpha}$ was examined during self-adaptation to 0.1, 1.0 and 10.0 nM $PGF_{2\alpha}$.

Fig. 3.2 Binding of 1.3 nM [5,6,8,9,11,12,14,15n-^{3}H]-$PGF_{2\alpha}$ and 153 nM L-[2,3-^{3}H]alanine to the olfactory tissue preparation from Arctic char with various concentrations of unlabelled $PGF_{2\alpha}$ and L-alanine, respectively, in the incubation medium. Average values of duplicate samples are shown.



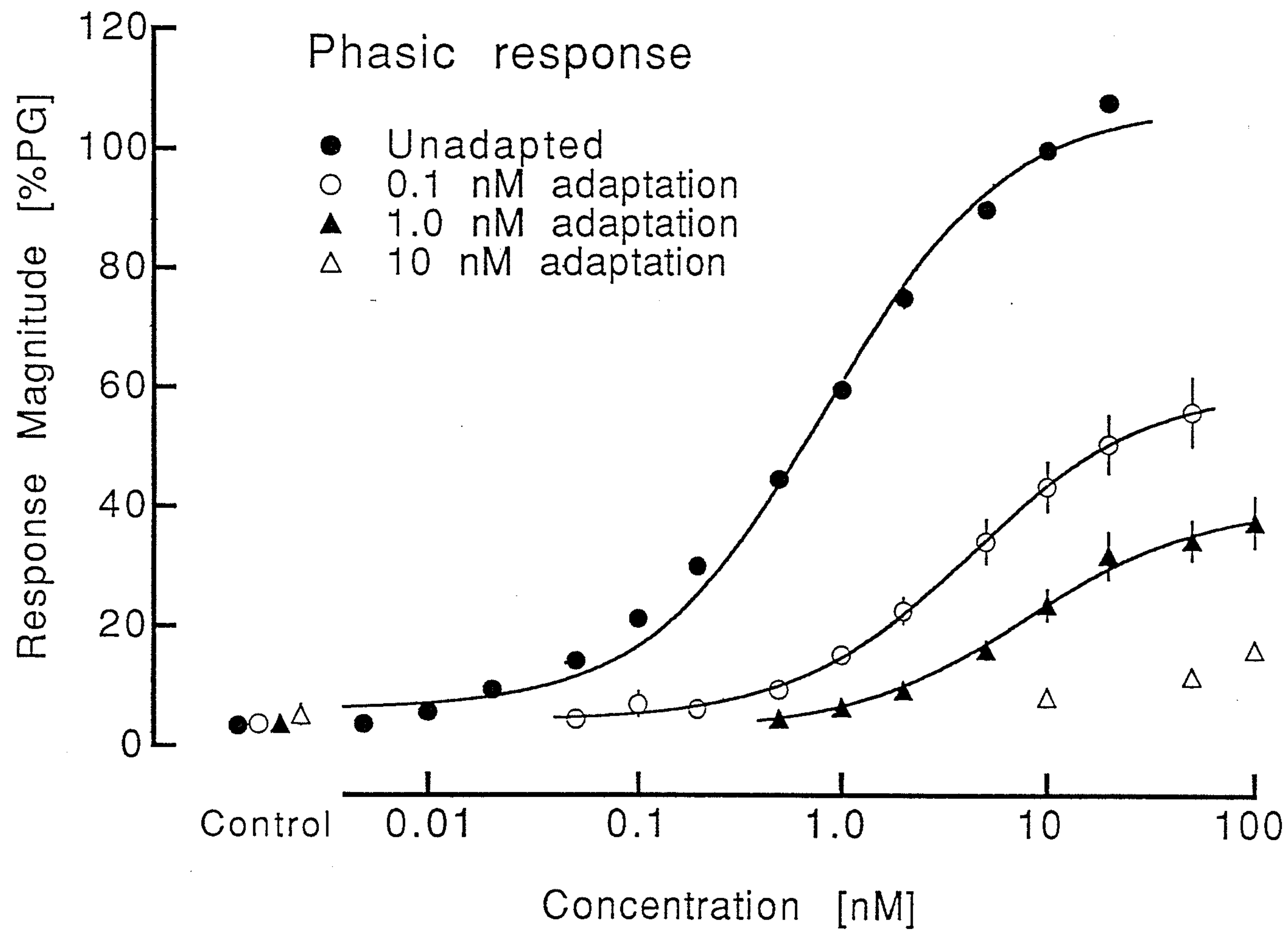
L-alanine total binding
L-alanine non-specific binding
PGF2α total binding
PGF2α non-specific binding
5000
4000
3000
2000
1000
0
Bound [fmole / mg pr.]
0
0.1
1
10
100
Concentration of unlabelled ligand [μM]

Both the phasic response magnitudes (Fig. 3.3) and the tonic response magnitudes (Fig. 3.4) were examined. Equation 1 (Chapter II) was fit to the data and the estimates of the parameters are shown in Table III.1. Predicted values of K as calculated by Eq. 2 are lower than the values found by the fitting procedure by a factor of 4 for the phasic response and 3 for the tonic response. This suggests either: (1) that the $PGF_{2\alpha}$ receptor has lower affinity for its ligand than predicted from kinetic analyses of the unadapted responses, or (2) that the receptor is switched to a low affinity state when exposed to a stimulus.

Discussion

Although no specific binding of tritiated $PGF_{2\alpha}$ was observed, I could demonstrate specific binding of tritiated L-alanine to the P2 fraction obtained from the olfactory tissue preparation. At the concentrations used in the binding assay, $PGF_{2\alpha}$ elicits an EOG response of about the same magnitude as a response to 10 μM L-serine (Chapter II), but L-alanine elicits an EOG response which is only about 30% of the respone to 10 μM L-serine (Sveinsson, 1985). If the magnitude of the response is proportional to the number of receptors bound to the ligand, then binding of 1 nM $PGF_{2\alpha}$ to an olfactory tissue preparation should be expected to be more than binding of 150 nM L-alanine to the same tissue preparation. Even if binding sites exist in a tissue preparation, several factors can make them unobservable in a classical ligand-binding assay. These factors are: (1) a high level of non-specific binding resulting in high noise level; (2) a high endogenous level of a ligand in the incubation medium that out-competes the radiolabelled ligand for binding sites; (3) ligand metabolization or

Fig. 3.3 Concentration-response relationship of the phasic EOG response to $PGF_{2\alpha}$ during adaptation to various concentrations of the same compound. Means (symbols) and standard error of means (error bars) of recordings from 5 fish are indicated. %PG: percent of response to 10 nM $PGF_{2\alpha}$.



Phasic response
Unadapted
0.1 nM adaptation
1.0 nM adaptation
10 nM adaptation
Response Magnitude [%PG]
120
100
80
60
40
20
0
Control
0.01
0.1
1.0
10
100
Concentration [nM]

Fig. 3.4 Concentration-response relationship of the tonic EOG response to $PGF_{2\alpha}$ during adaptation to various concentrations of the same compound. Means (symbols) and standard error of means (error bars) of recordings from 5 fish are indicated. %PG: percent of response to 10 nM $PGF_{2\alpha}$.

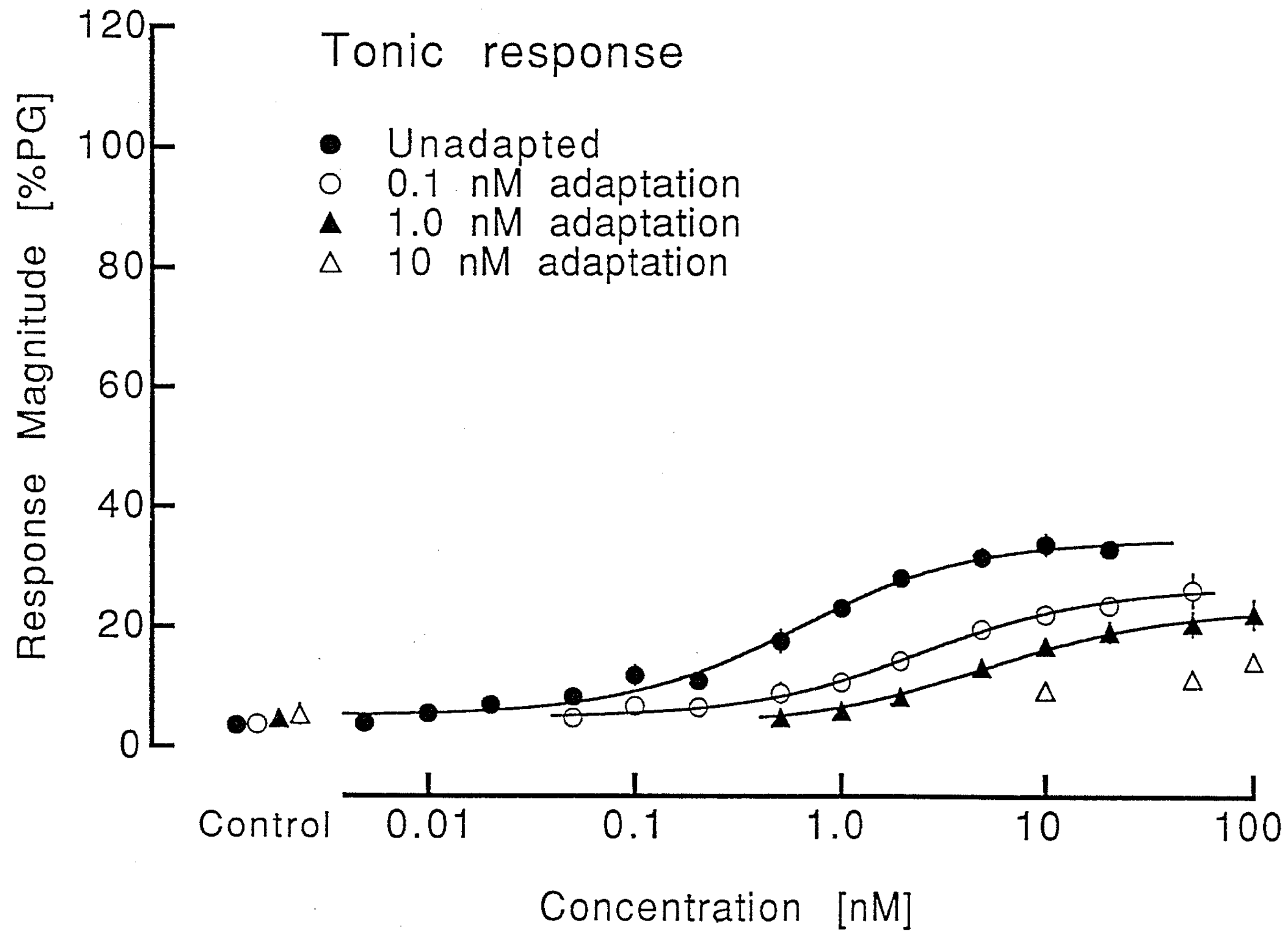

Tonic response
Unadapted
0.1 nM adaptation
1.0 nM adaptation
10 nM adaptation
Response Magnitude [%PG]
120
100
80
60
40
20
0
Control
0.01
0.1
1.0
10
100
Concentration [nM]

Table III.1 Estimates of the parameters obtained from fitting of Eq. 1 (Chapter II) to the responses shown in Fig. 3.3 and 3.4. Standard Error as calculated by Sigmaplot^{TM} is in parentheses. Predicted K values are calculated according to Eq. 2.

Adaptation	K [nM]	Q [%PG]	C [%PG]
	Phasic response		
None	0.9 (0.9)	102 (5)	6 (1)
0.1 nM	4.3 (0.6)	56 (1)	4 (1)
- predicted	1.0		
1.0 nM	8.0 (2.0)	38 (2)	3 (1)
- predicted	1.9		
	Tonic response		
None	0.6 (1.2)	30 (2)	5 (1)
0.1 nM	2.5 (1.1)	22 (1)	3 (1)
- predicted	0.7		
1.0 nM	5.3 (1.6)	19 (1)	4 (1)
- predicted	1.6		

inactivation in the incubation medium; (4) the absence of functional perireceptor enzymes which must modify the stimulus before it can bind to the receptor; (5) low density of the receptor; (6) low affinity of the receptor for the ligand (fast dissociation rate of the ligand from the receptor); (7) the receptor's desensitization or switching to a low affinity state. As discussed below factors (6) and (7) are considered to be the most probable factors contributing to the lack of specific binding in the present study and factors (4) and (5) may also be contributing.

Compared to L-alanine's non-specific binding, $PGF_{2\alpha}$ showed little non-specific binding. This is at least partly due to the lower concentration of radioactive $PGF_{2\alpha}$ used in the binding assay. However, a high level of non-specific binding cannot be considered the major problem in the present study.

The ability of various tissues to produce eicosanoids during trauma is well known (Samuelsson *et al.*, 1975; Granström and Kumlin, 1987). Indomethacin, a very potent inhibitor of PG synthesis (Benedetto and Slater, 1987), was included throughout the tissue handling and the binding assay in the present study. Furthermore, EIA revealed only insignificant amounts of immunoreactive F-type PG in the incubation medium when indomethacin was used. It is thus unlikely that endogenous $PGF_{2\alpha}$ was competing with the radioactive ligand for binding sites. Although the presence of other endogenous ligands in the tissue preparation - undetectable by the immunoassay - seems rather unlikely, it cannot be ruled out.

Studies on perireceptor events have just begun and have shown that they play an important role in the function of the olfactory system (for review see Carr *et al.*, 1990a,b). Binding studies on the external

chemoreceptors in slime mold are complicated by the presence of potent enzymes which degrade and inactivate the stimuli (Van Houten and Preston, 1987). A cytochrome P-450 gene was found to be uniquely expressed at high levels in the olfactory epithelium of rat (Nef *et al.*, 1989). The activation of this gene coincided temporally with a postnatal increase in olfactory sensitivity to odorants, suggesting a role for this enzyme in olfactory function. In liver, cytochrome P-450-dependent oxidative metabolism transforms hydrophobic compounds into more polar derivatives which frequently conjugate with glucuronic acid, glutathione, or sulfate (for review see Nebert and Gonzalez, 1987). Interestingly, expression of an olfactory-specific UDP-glucuronosyl-transferase (UGT), which conjugates odorants with glucuronic acid, was recently identified in bovine olfactory epithelium (Lazard *et al.*, 1991). This enzyme showed preference for odorants over standard UGT substrates, and the conjugation abolished the stimulatory effectiveness of the odorants. Despite the use of several different inhibitors in the present study, an increase in specific binding was not observed. Furthermore, HPLC analysis of the ligand indicated that the ligand was not metabolized in the incubation medium.

The possibility that metabolism or modification of the $PGF_{2\alpha}$ molecule is necessary for it to bind to a receptor can not be discounted. This is required for the mating pheromone in the yeast, *Rhodosporidium toruloides* (Miyakawa *et al.*, 1982,1987). Two types of haploid cells are identified in this species, type A and type a. Type A haploid cells release a peptide which is a biologically inactive pheromone. This peptide is hydrolysed by a peptidase present on the surface of type a cells before it can bind to its receptor on the surface of the same type a cells.

It is also possible that $PGF_{2\alpha}$ is not the compound which in nature is released and binds to the olfactory receptor. The compound naturally released may for example be a metabolite of $PGF_{2\alpha}$. If $PGF_{2\alpha}$ does not bind to the receptor – only a certain metabolite binds – the use of $PGF_{2\alpha}$ as a ligand in the binding assay is not appropriate. However, the fact that very low concentration of $PGF_{2\alpha}$ elicits EOG responses argues against that possibility or suggests that perireceptor enzymes modify $PGF_{2\alpha}$ before it reacts with the olfactory receptor responsible for the EOG responses.

In the African catfish, 5β-pregnane-3α,17α-diol-20-one-3α-glucuronide is an effective stimulant while the non-conjugated compound is not (Resink *et al.*, 1989). The sulfate conjugate of 17,20P is stimulatory in goldfish, while 17,20P-glucuronide is not (Sorensen *et al.*, 1991a). The possibility that $PGF_{2\alpha}$ requires conjugation with glucuronic acid, glutathione or sulfate before it binds to its olfactory receptor in Arctic char cannot be ruled out. If cytochrome P-450 and conjugation enzymes are present in the olfactory epithelium of Arctic char, they may be lost in the supernatant when the P2 fraction is used in the binding assay. Although they should be present in the crude homogenate, they may not be functional because of a lack of oxidizing agents. Only reducing agents were tested in the present experiments. Also, oxidizing and reducing agents may respectively up- and down-regulate the density of the olfactory receptors as is known for the thromboxane A_2/PGH_2 receptor in human platelet membranes (Dorn, 1990).

Binding proteins in the mucus of mammalian olfactory epithelium (olfactory binding protein, OBP; Pelosi, *et al.*, 1982; Bignetti *et al.*, 1985; Pevsner *et al.*, 1985) and in the sensillar lymph of arthropod antennae (pheromone binding protein, PBP; Lerner *et al.*, 1990) have been identified.

These proteins non-discriminatorily bind hydrophobic odorant molecules and increase their water solubility (Pelosi and Maida, 1990). Although they are likely to play a role in olfaction their function remains unknown. Whether aquatic organisms have similar binding proteins remains to be seen. However, if the primary function of these binding protein is to make airborne molecules more soluble in the aqueous layer between air and the receptor, they may not be present in aquatic organisms.

Because of the very small size of olfactory receptor cells, opening of a few ion channels may be sufficient to depolarize the cell membrane and initiate impulses (see Lancet, 1986). Furthermore, with the help of an efficient transduction cascade system (e.g. the adenylyl cyclase system discussed below), occupancy of only one or a very few receptor sites would be needed to elicit physiological responses. Therefore, the density of olfactory-receptor sites may be too low to be detectable in a classical ligand-binding experiment.

If the magnitude of electrophysiological responses is any indication of the relative number of receptor binding sites, then the number of $PGF_{2\alpha}$ binding sites should not be less than those for L-alanine at the concentrations of ligands used in the binding assay in the present study. However, the transduction mechanisms involved in the detection of these two different groups of chemicals could be quite different and thus not comparable. Furthermore, amino acid binding to fish olfactory preparations has some characteristics of ligand accumulation into membrane vesicles (Brown and Hara, 1981,1982). Although such accumulation may serve as part of the amino acid reception mechanism, measured binding would then not reflect the actual number of binding or

transport sites; ligand-binding assays would profoundly overestimate the number of binding sites.

Olfactory receptors have been predicted to be of low affinity (Lancet, 1986). High olfactory sensitivity would be accomplished by a large number of receptor sites and efficient transduction cascades. High concentrations of adenylyl cyclase and kinases in the olfactory epithelium are consistent with this hypothesis. Electrophysiological responses to odorant stimulation return to the pre-stimulus baseline within 1 or 2 seconds (Fig 2.3). This implies either that the stimulus molecule dissociates from the receptor site very rapidly or that the receptor-ligand complex changes at high rate to an inactive state (Kamo *et al.*, 1980). Although the latter possibility was rejected for amino acid receptors (Sveinsson and Hara, 1990a,b), it still could apply to PG receptors. Treatment with hydroxyapatite has been used to precipitate the ligand-receptor complex of $PGF_{2\alpha}$ with luteal receptors and prevent dissociation of the ligand from the receptor (Orlicky, 1990). Hydroxyapatite did not increase specific binding in the present experiments, indicating that $PGF_{2\alpha}$ was not "washed off" the receptor during the filtration.

By use of models which were fitted by non-linear regression to the integrated NTR phasic responses for amino acids, changes in the K parameter during self- and cross-adaptations were successfully predicted in earlier work (Sveinsson and Hara, 1990a,b). In the present study the estimated values of the K parameter during self-adaptation were three to four times higher than the values predicted from the estimated values for the unadapted state. This is either because the K_d value in the unadapted state is actually higher than estimated by the fitting process or because K_d increases in the presence of the adapting stimulus. Unlike the integrated

NTR responses to amino acids, the maximum EOG response to $PGF_{2\alpha}$ decreased during self-adaptation. The maximum level for the phasic response decreased by 46%PG (from 102%PG to 56%PG, Table III.1) when adapted to 0.1 nM concentration. However, the unadapted phasic and tonic magnitudes in response to 0.1 nM $PGF_{2\alpha}$ are only 21%PG (Fig. 3.3) and 12%PG (Fig. 3.4), respectively. The decrease in the maximum level of the phasic response is thus more than the response to the adapting stimulus when tested alone. This again may indicate that the receptors are desensitized or down regulated upon exposure to adapting stimulus.

Many G protein-coupled receptors are desensitized when continuously stimulated by an agonist (Dohlman *et al.*, 1991). Phosphorylation appears to be a pivotal step in this process. Most studies have focused on the coupling of the receptor to the G protein and have found that phosphorylation of the receptor interferes with this coupling and inhibits transduction. Bruch and Kalinoski (1987) found that GTP lowered the affinity of amino acid olfactory receptors in catfish, implying that the receptors interact with G proteins which subsequently modulate their affinity. Whether this modulation is due to direct interaction or to effects on phosphorylation or methylation of the receptors is not known. The PGE receptor on frog erythrocytes is shifted by guanine nucleotides from a high (K_d=77 nM) to a low (K_d=56 μM) affinity state (Lefkowitz *et al.*, 1977). Adenylyl cyclase is only stimulated by the low affinity form of the receptor.

A few studies have looked at the effect of phosphorylation on the affinity of the receptors for their ligands. The slime mold (*Dictyostelium discoideum*) has cell surface receptors for cAMP which mediate its chemotactic response (Van Houten and Preston, 1987). Kinetic analysis of cAMP binding to intact cells revealed three types of receptors, S, H, and L

(K_ds= 12.5, 60, and 450 nM, respectively; Van Haastert and De Wit, 1984). While the S receptor is stable, the H and L forms are interconvertible. In the presence of a ligand, 90% of the receptor population is in the L state after 45-60 sec. The dissociation rate of the ligand from this receptor is fast, with a half-life of about 0.7 sec. This fast dissociation leaves the filtration assay incapable of detecting binding because during the washing step all the ligand will dissociate from the receptor. The folate receptor in slime mold has also been shown either to interconvert between high- and low-affinity forms or to change in number in response to ligand binding and guanine nucleotides (De Wit and Van Haastert, 1985; De Wit and Bulgakov, 1985). In yeast, *Saccharomyces cerevisiae*, phosphorylation of the pheromone receptor obstructed transduction of the mating signal. However, no change in the affinity of the receptor for its ligand, the α-mating factor, was observed (Jenness and Spatrick, 1986).

Methylation, in addition to phosphorylation, is involved with sensory transduction of chemical signals in prokaryotes (Ordal, 1985; Stock *et al.*, 1990; Bourret *et al.*, 1991). While phosphorylation is involved with activating chemotactic responses, methylation or demethylation of the transducer is involved in adaptation of the chemotaxis. In humans, formylmethionyl-leucyl-phenylalanine (FMLP) binds to a highly specific receptor on the leucocyte membrane (Williams *et al.*, 1977). To maintain the FMLP receptor in a functional, high-affinity state it has to be methylated (Pike and Snyderman, 1982). The receptor exists in two interconvertible affinity states (K_d = 26 nM and K_d = 5-6 nM; Yuli *et al.*, 1982). The higher affinity state of the receptor (methylated) mediates the chemotactic response while the lower affinity state (demethylated) mediates the secretory response. Thus, methylation/demethylation of chemotactic

receptors occurs in two life-forms which are evolutionarily very far apart (bacteria and humans). Whether olfactory receptor cells have a similar methylation/demethylation system which affects the affinity of their receptors for odorants has not yet been investigated.

The high-affinity β-adrenergic receptor on intact astrocytoma and glioma cells converts to a low affinity state in the presence of an agonist (Toews *et al.*, 1983). However, in membrane preparations it remains in the high-affinity state, presumably because it is uncoupled from the GTP-dependent protein (G-protein); thus, classical binding studies are made possible. In olfactory cilia preparations, odorants stimulate second messenger systems through G-proteins (Pace *et al.*, 1985; Sklar *et al.*, 1986; Bruch and Teeter, 1990), thus indicating that the transduction cascade is functional. High basal activity of the adenylyl cyclase in olfactory cilia preparations suggests that the transduction cascade is partly activated in the absence of exogenous odorant (Bakalyar and Reed, 1990). Also, high activities of protein kinases were observed in an olfactory cilia preparation from frog (Chen *et al.*, 1986). From the above observations, it is logical to suspect that desensitization and down-regulation processes are also active in the tissue or cilia preparations used in biochemical studies on olfactory receptors. It is thus possible that membrane preparations of olfactory receptors differ from those of neurotransmitters, in that the receptors exist in low-affinity rather than high-affinity state. Furthermore, this may explain why classical ligand-binding studies are generally not applicable to olfactory receptors.

Recently, binding of the steroidal pheromone 17,20P to olfactory membrane preparations in goldfish was reported (Rosenblum *et al.*, 1991). The measured dissociation constant (1.02 nM) is in good agreement with

EOG responses (Sorensen *et al.*, 1987,1990). However, the ability of androstenedione to displace the binding is not consistent with cross-adaptation experiments where EOG responses to each of the two steroids were not affected by adaptation to the other (Sorensen *et al.*, 1990). Binding experiments have to be interpreted cautiously and any inconsistency casts doubt on the biological significance of the data (Burt, 1985). However, because of the high affinity of 17,20P's binding to olfactory tissue, it would be of great interest to further study and characterize this site.

Molecular cloning techniques have recently entered the arena of olfactory research. Buck and Axel (1991) cloned and characterized 18 different members of a multigene family that are explicitly expressed in the olfactory epithelium of rat. Other members of this family, the seven transmembrane proteins, include several different types of receptors, e.g. light receptor, cholinergic muscarine receptors, α- and β-adrenergic receptors etc. (for review see Dohlman *et al.*, 1991). Molecular cloning was used to isolate the receptor genes of the T-lymphocyte (Hedrick *et al.*, 1984) and the light receptor of the cone cells (Nathans *et al.*, 1986). Getchell *et al.* (1990) isolated poly (A+)RNA from catfish barbels and injected it into *Xenopus* oocytes. The oocytes expressed amino acid receptors that exhibited characteristics similar to those of catfish taste receptors. Ligand binding studies have been used in olfaction for over a decade (Zelson and Cagan, 1978; Brown and Hara, 1981,1982) and despite intensive efforts by several laboratories (Brown and Hara, 1981,1982; Snyder *et al.*, 1988; Lancet *et al.*, 1988; Anholt, 1989) have not been able to positively identify odorant recognition sites. Future advances in our understanding of transduction in olfaction is likely to come from the use of molecular cloning techniques.

Above, I have discussed several possibilities which could account for the failure to observe specific binding of $PGF_{2\alpha}$ to the olfactory tissue preparation in Arctic char. This discussion can be summarized as follows. The study shows that the ligand is not metabolically modified in the incubation medium. Furthermore, the results do not support the idea that a high concentration of endogenous ligand out-competes the radiolabelled ligand for binding sites. The following possibilities remain: (1) metabolic modification is necessary in order for the ligand to bind to its receptor; (2) the number of receptors (relative to non-specific binding) is too low to be detected in the assay used (comparison to amino acid binding possibly being misleading because the latter may represent accumulation of ligand into membrane vesicles but not true binding to binding sites); (3) the affinity of the receptor(s) for the ligand is too low or it is switched to a low state when exposed to the ligand. Although the EOG self-adaptation experiment presented in this chapter supports the last possibility, all three possibilities may contribute to the failure of the ligand-binding experiments in the present study to demonstrate $PGF_{2\alpha}$ binding sites.

CHAPTER IV

Release of F-type prostaglandins by ripe males and behavioural reactions to $PGF_{2\alpha}$ of both sexes of Arctic char, *Salvelinus alpinus*

Introduction

Several studies have shown that ripe fish are attracted to water scented with substances originating from the opposite sex (see Liley, 1982 for review). Among the salmonids, attraction of males by chemical cues from ripe females has been demonstrated in rainbow trout (Newcomb and Hartman, 1973; Emanuel and Dodson, 1979; Honda, 1980) and two species of Pacific salmon (Honda, 1982). Attraction of rainbow trout females to chemical cues from ripe males has also been documented (Newcomb and Hartman, 1973). Furthermore, Foster (1985) found that adult lake trout (*Salvelinus namaycush*, Walbaum), searching for a spawning site, were attracted to substances originating from the faeces of juveniles. In these experiments, it is not clear whether the fish were responding to specific chemicals (pheromones) emitted specifically for the purpose of attracting conspecifics or whether the fish were responding to metabolites inevitably released to the environment (Liley, 1982).

Recently, the nature of the chemical stimuli involved in conspecific attraction and behavioural responses was discovered in a few species. Glucuronided sex steroids (probably a mixture of testosterone glucuronide and estradiol glucuronide) were shown to mediate attraction of zebrafish males (*Brachydanio rerio*) to ovulated females (van den Hurk and Lambert, 1983). Ethiocholanolone glucuronide, which is produced in the mesorchial

gland of *Gobius jozo* males, attracts and induces oviposition in females (Colombo *et al.*, 1980; Colombo *et al.*, 1982). F-type PGs, assumed to be released by female goldfish, induce sexual behaviour in males (Sorensen *et al.*, 1988; Sorensen *et al.*, 1989). In the African catfish (*Clarias gariepinus*), glucuronided steroids (most likely 5β-pregnane-3α,17α-diol-20-one-3α-glucuronide), which are produced in the seminal vesicle of the male, attract the female and stimulate ovulation (Resink *et al.*, 1989a,b,c). However, 17α,20β-dihydroxy-progesterone, which induces an gonadotrophic surge (Dulka *et al.*, 1987; Stacey *et al.*, 1989) and milt production in goldfish males (Stacey and Sorensen, 1986; Stacey *et al.*, 1989), has only minor effects on behaviour (Sorensen *et al.*, 1989).

Reproductive behaviour has been described for many salmonids, including Arctic char (Fabricius and Gustafson, 1954; Sigurjónsdóttir and Gunnarsson, 1989), Dolly Varden (Needham and Vaughan, 1952), Miyabe char (Maekawa, 1983), lake trout (Foster, 1985), eastern brook trout (Smith, 1941), steelhead trout (Needham and Taft, 1934), rainbow trout (Tautz and Groot, 1975; Honda, 1980), Atlantic salmon (Belding, 1934; Jarvi, 1990), cutthroat trout (Smith, 1941), as well as many of the Pacific salmons (Hanson and Smith, 1967; Tautz and Groot, 1975; Honda, 1982; Kwain, 1982; Keenleyside and Dupuis, 1988). The same general pattern can be identified for all salmonids. Males are usually more numerous than the females and defend territory and/or establish dynamic hierarchy dominance among themselves. The female selects a spawning site and performs stereotyped digging behaviour: turning on her side and flapping her tail down on the substratum a few times as she moves forward. Males court the females by quivering at their side which eventually results in simultaneous release of gametes from both sexes (spawning).

I demonstrated in Chapter II that the Arctic char has a very specific and sensitive olfactory receptor for $PGF_{2\alpha}$. Because F-type PGs are known to function as reproductive pheromones in goldfish (Sorensen *et al.*, 1988), and also because they are involved in ovulation in salmonids (Cetta and Goetz, 1982; Stacey and Goetz, 1982; Goetz *et al.*, 1989), I anticipated F-type PGs to be reproductive pheromones in Arctic char. If they are, they would be expected to: (1) be released into the water by mature char at a specific time prior to or during spawning; (2) be released by only one of the sexes in response to a specific stimuli; and (3) elicit specific behavioural effects in one or both of the sexes. In the present study I conducted a series of experiments to test these three predictions. Experiments 1, 2, and 3 were designed to test predictions 1, 2, and 3, respectively. In these experiments, I showed that immunoreactive F-type PGs are released by actively spawning fish. Then I found not only that ripe males are more odorous to other char than females or immature fish, but also that they increase their odour production and release immunoreactive F-type PGs when they are exposed to water from ovulated females. Finally, I examined the behavioural response of Arctic char to waterborne $PGF_{2\alpha}$. I found that water containing a low concentration of $PGF_{2\alpha}$ attracted ripe males and females, but was avoided by non-ripe females. Furthermore, it induced "digging" behaviour in some of the ripe females. From these experiments I conclude that ripe Arctic char males release $PGF_{2\alpha}$ or its metabolites to attract females to the spawning grounds and elicit their spawning behaviour.

Materials and Methods

Arctic char from the same stock as used in Chapter II was used in these experiments, and same procedure was used to induce sexual maturation. ANOVA and the T′-method for comparison of means (T′-value) were used for statistical analyses (Sokal and Rohlf, 1981).

Experiment 1

This experiment was conducted to test whether F-type PGs are released to the water by Arctic char during spawning. Ripe char, two females and three males, were put into a 450 l observation tank (100 x 150 x 50 cm, with 25-30 cm deep water) with a gravel-covered bottom (5-7 cm depth, stones were 2-5 cm in diameter). Water was recirculated to a 250 l head tank by a pump (ca. 10 l/min). The water was chilled in the head tank by a cooling unit before it entered the observation tank again. Water temperature in the observation tank was 4-7°C. A flow of fresh water into the head tank and overflow from the observation tank provided continuous renewal (1 l/min) of the recirculating water. Every second day duplicate water samples were taken from the overflow of the observation tank and from the fresh replacement water to the head tank. The water samples were extracted as described below and later analysed by enzyme immunoassay (EIA) for $PGF_{2\alpha}$. Spawning activity was monitored at least three times a day (half an hour each time). Spawning activity was recorded for males if they were aggressive and courted the females. Spawning activity was recorded for the females if they displayed "digging" behaviour.

Experiment 2A

This experiment was conducted to test whether mature fish with no contact with other fish release F-type PGs to the water. How odorous fish of different maturational stages are, as revealed by EOG recording, was also tested in this experiment. Fish were placed individually in 45 l glass aquaria. Inflow of freshwater was adjusted to approximately 1 l/min for each aquarium. Outflow water was collected for extraction and EIA analysis (described below). The effectiveness of the outflow water in eliciting EOG responses was also tested. Immature Arctic char (age: 1+ to 2+) were used in the EOG recordings which were conducted as previously described (Chapter II, page 10).

Experiment 2B

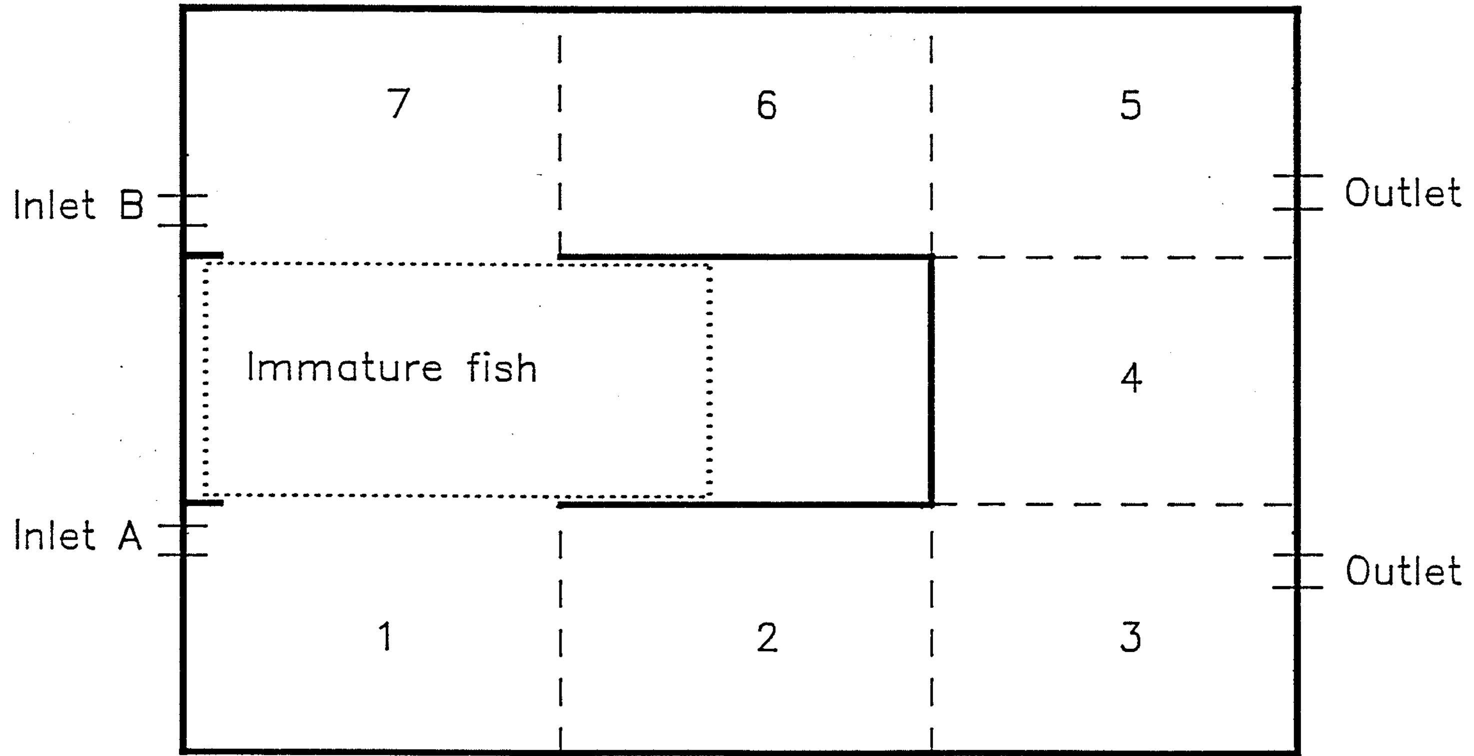
This experiment was conducted to examine whether mature fish will, upon receiving chemosensory signals from other fish: (1) change their odour production; (2) release F-type PGs to the water. The same aquaria as used in experiment 2A were arranged in two sets on a rack, where one set (donor tanks) was on a shelf 80 cm above the other set (recipient tanks). At time zero, the outlet water from each of the donor tanks was fed into one of the recipient tanks, one outlet to each recipient tank. Because the original freshwater inflows were kept intact, each recipient tank now received two water inlets (the primary water inlet and outlet water from one of the donor tanks). After 30, 60, 90, and 150 min, water samples were taken from the recipient tank's outflow and tested for EOG effectiveness as before. The EOG response elicited by each sample was compared to an “expected” EOG response to the outflow water from the recipient tank water before any interaction took place. The "expected" EOG response was found by taking

water samples before the start of the experiment (from both donor and recipient tanks) and mixing them in the same proportion as the respective flows through the tanks, and then recording the EOG in response to this mixture. Assuming that there is complete mixing inside the tanks and no change in odour production of either the donor or the recipient fish, this is the EOG expected response to the outlet water from the recipient tank when a steady state has been reached. Any difference in the response from the expected value was concluded to be due to a change in odour production by the recipient char under the influence of chemosensory signals in the donor water. Water samples for extraction and EIA analysis were also collected after 150 min from the start of the experiment. At the end of each trial the maturational stage of both the donor and recipient fish were determined. The same classification of maturational stages as before was used (Chapter II, page 9). Each fish was only used for one trial.

Experiment 3

This experiment was conducted to examine the behavioural reactions of fish of different maturational stages to a low concentration of $PGF_{2\alpha}$. A schematic top view of the trough used in this experiment is shown in Fig. 4.1. The trough was U-shaped with one inlet into each arm and two outlets on the opposite side. The U-shaped trough was 150 cm long and 100 cm wide with the width of the arms 33 cm. For the behavioural analysis the trough was divided into seven equal-sized sectors (numbered 1 to 7; Fig. 4.1) with imaginary boundaries (shown by broken lines in Fig. 4.1; see next paragraph for further explanations). One pipe fed water at 3 l/min (10-11°C) into each arm. A glass aquarium was placed between the arms of the U (shown by dotted lines in Fig. 4.1), and during experiments it held

Fig. 4.1. Top view of the trough used to study the behavioural responses of Arctic char to $PGF_{2\alpha}$. The glass aquarium in the middle (dotted line) contained immature fish to provide visual cues. The inflow was 3 l/min into each arm. The dimensions of the trough are 100x150 cm and the water depth was 20 cm. The bottom was covered with gravel, 2-5 cm in diameter. Solid lines represent opaque walls. Broken lines show imaginary boundaries between the sectors which were inserted afterwards when the recorded behaviour was analysed from video tapes.


7
6
5
Inlet B
Outlet
Immature fish
4
Inlet A
Outlet
1
2
3

immature fish that could only be seen from the ends of the arms (from sectors 1 and 7). However, no water-borne chemical signals were received by the char in the U-shaped trough from the char in the glass aquarium as its drain was separate from that for the U-trough. The behaviour of the char was video taped in top view, for a period of 30 min before $PGF_{2\alpha}$ water was introduced (a control period) and another 30 min period after the infusion started (a test period). Two test solutions were made; one contained 100 nM $PGF_{2\alpha}$ in distilled water and the other (a control solution) contained only distilled water. For stimulus infusion, these two solutions were pumped (3 ml/min for 30 min) into each inlet pipe, 50 cm from where they discharged into the arms of the trough. Turbulent flow in the pipes ensured mixing of the solutions with the water. Thus, water containing 0.1 nM $PGF_{2\alpha}$ entered one of the arms of the trough. The infused stimuli ($PGF_{2\alpha}$ and control stimuli) spread evenly into the arms as observed with a dye solution (ruthenium red) and their front passed through the first two sectors in each arm (i.e., sectors 1,2 and 7,6) in about 6 min. About 33 l of water filled each sector so the average concentration at that time in the arms would be ca. 0.027 nM. A few fish showed strong preference for one of the arms during the control period and seldom entered the other. If the fish had been stationary in one of the arms throughout the last 2 min before introduction of the stimulus solutions, $PGF_{2\alpha}$ was infused into that arm and control solution into the other. This was to ensure that the fish would be exposed to the $PGF_{2\alpha}$ stimulus during the test period. Otherwise the solutions were randomly allocated to the arms.

The behaviour of the fish was analysed by playing back at a double speed the previously recorded video tapes onto a video screen (the boundaries as shown by broken line in Fig. 4.1 were drawn onto the video

screen). The activity of the fish on the video screen was monitored visually and recorded manually via a computer keyboard whenever the tip of the nose moved across boundaries between sectors. A GWBASIC program was written to tabulate the entries and afterwards calculate a mean position and activity of the fish in the trough for each period (the control period and the test period). The mean position was calculated as the weighted average of the sections' values, where the time spent in each section was used as the weighting factor. The values ranged from +1 for the section closest to the inlet used for $PGF_{2\alpha}$ infusion to –1 for the section closest to the other inlet; the intervening sections had values + 2/3, + 1/3, 0, –1/3, and –2/3, respectively (section #4 always had 0 value, while values for the others depended on which inlet was chosen for $PGF_{2\alpha}$ infusion). Avoidance-preference (A-P) was then calculated as the difference in mean position between the control period and the test period (+ value indicates preference, – indicates avoidance). Activity was calculated as the total number of times the fish's nose crossed a boundary between two adjacent sections over each of the 30 min period. Change in activity (CA) was calculated as the percent change from the control period to the test period (+ value indicates increase in activity, – value indicates decrease in activity):

$$CA=100\cdot(\text{test period activity} - \text{control period activity})/(\text{control period activity}) \quad \text{Eq. 3}$$

Extractions

Each water sample was filtered through a 500 mg C_{18} extraction column which was pre-washed with 10 ml of methanol and equilibrated with 20 ml of distilled water. After the extraction the columns were washed

with 20 ml of distilled water and then eluted with 2 ml of methanol which was collected and kept at -17°C until EIA analysis. To one half of the control water samples, 20 picomoles of $PGF_{2\alpha}$ was added; these samples were used to monitor extraction and EIA analysis efficiencies. These efficiencies ranged from 70% to 95% (with the loss assumed to be due to a chemical break-down of the PGs before and during analyses), but all presented values were corrected for this. Tritium-labelled $PGF_{2\alpha}$ (10,000 cpm/sample, ca. 0.03 nM) was added to all samples to monitor extraction recovery which ranged from 90% to 100%.

Enzyme Immunoassay (EIA)

The ACE^{TM} enzyme immunoassay kit for $PGF_{2\alpha}$ was bought from Cayman Chemical (Ann Arbor, MI). Although the assay was for $PGF_{2\alpha}$ it had 100% cross-reactivity for $PGF_{1\alpha}$, and 7% for PGD_2 but less than 0.1% for PGE_2. Duplicate aliquots of the extraction eluents were evaporated to dryness under vacuum in a Savant SpeedVac Concentrator. The residue was dissolved in EIA buffer (0.1 M potassium phosphate, 0.4 M NaCl; 1 mM Na_4EDTA, 0.1% bovine serum albumin, 0.01% NaN_3, at pH 7.4) and assayed according to kit instructions. Briefly, a microtiter plate (NUNC certified) was coated with mouse monoclonal anti-rabbit IgG and then treated with saturation buffer (EIA buffer with 0.3% bovine serum albumin and 0.03% NaN_3). After the plate had been washed 3 times with wash buffer (0.01 M potassium phosphate, pH 7.4; 0.05% Tween 20), each well of the plate received: 1) 50 µl of either sample, $PGF_{2\alpha}$ standard (for standard curves), or EIA buffer (for determining maximal binding, B_0); 2) 50 µl of $PGF_{2\alpha}$ acetylcholinesterase tracer; 3) 50 µl of $PGF_{2\alpha}$ antiserum except the wells that were used to determine non-specific binding (they received 50 µl of EIA

buffer instead). The plate was incubated for 18 hours at room temperature. Then, the plate was washed 6 times with wash buffer and 200 μl of Ellmann's reagent (acetylthiocholine iodide and 5,5′-dithiobis[2-nitro benzoic acid]) was added to each well. The optical density (at 412 nm) of the solution in each well was measured with a spectrophotometer. The density of the colour was proportional to the amount of tracer bound to the well which was inversely proportional to the amount of immuno-reactive F-type PGs in the well. The detection limit of the assay corresponded to about 0.5 pM $PGF_{2\alpha}$ in the water samples. Parallel binding was demonstrated to extracted water samples.

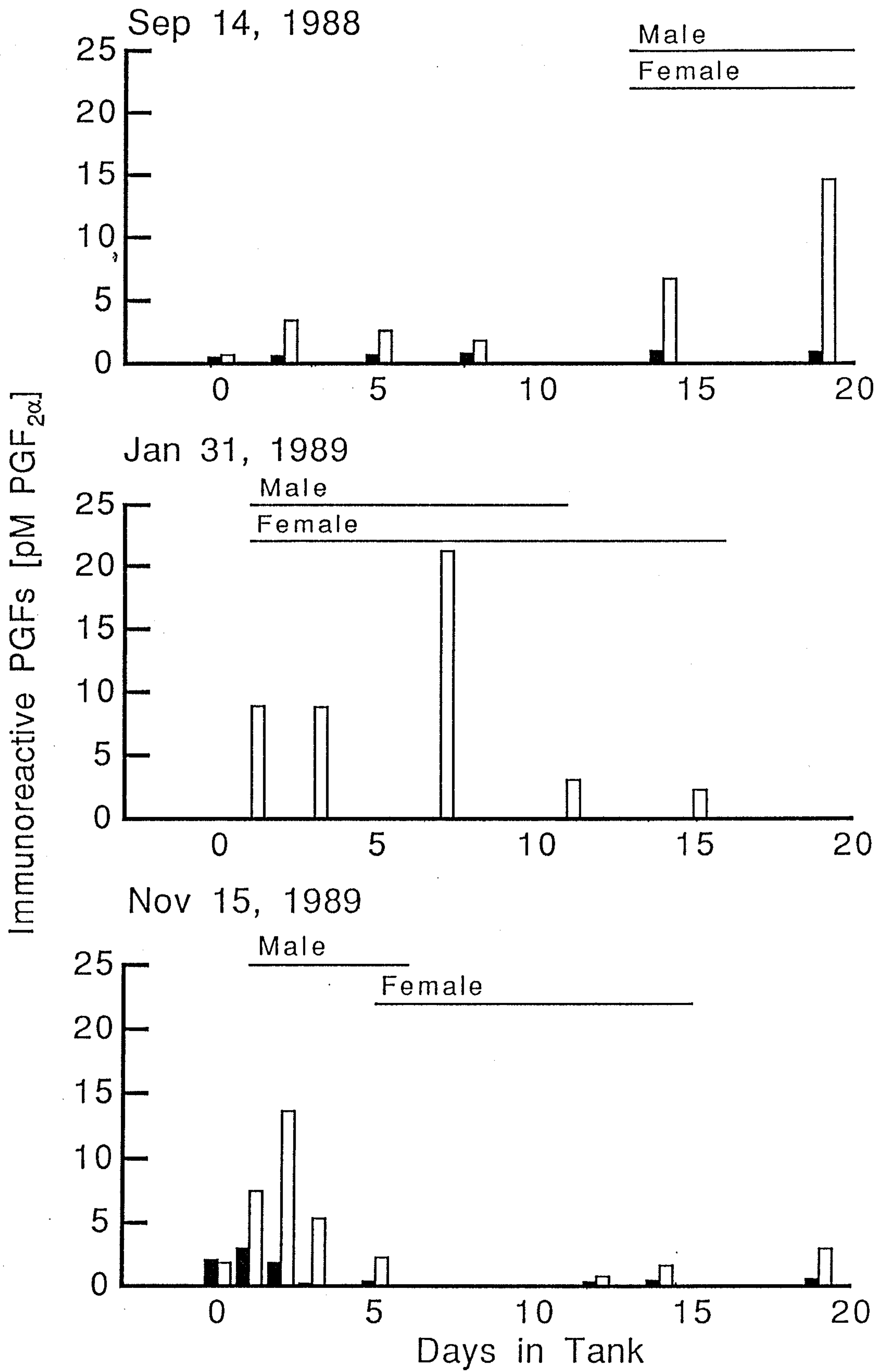
Results

Experiment 1

Spawning activity of both sexes appeared to be an "on or off" behaviour. Once spawning behaviour started it continued for several days, with occasional breaks for only few minutes at each time. Males which showed spawning behaviour were aggressive and territorial. They attacked males and inactive females, but courted females which showed spawning behaviour. This was done by gliding along the female's side and quivering at the same time. Females which showed spawning behaviour stayed close to the bottom with the anal fin usually touching the gravel. Occasionally they turned on their side and flapped their tail down on the gravel a few times. Then they circled and took the same position again, close to the bottom.

Actively spawning char released F-type PGs into the water as revealed by the immunoassay (Fig. 4.2). Increase in the amount of

Fig. 4.2. Concentration of immunoreactive F-type prostaglandins in the inlet (filled bars) and outlet (open bars) water of the spawning tank of Experiment 1, holding 2 ovulated females and 3 ripe males of Arctic char (flow 1 l/min; 4-7°C). Lines above the bars indicate the period in which spawning behaviour was observed in each sex (see text for details).



Sep 14, 1988
Male
Female
25
20
15
10
5
0
0
5
10
15
20
Jan 31, 1989
Male
Female
Nov 15, 1989
Male
Female
Immunoreactive PGFs [pM PGF2α]
Days in Tank

immunoreactive PGs in the outlet water coincided more consistently with the spawning activity of the males rather than the females (Fig. 4.2). Only three trials of Experiment 1 were successful, i.e., spawning activity of both sexes was observed during a trial. Several factors may have been contributing. They are: 1) ripe fish of both sexes were only available about twice a year and only for a few weeks each time; 2) the ripeness of the sexes was not always synchronized; 3) ripe males were very susceptible to infection and usually died within 10-14 days after being transferred from the stock tank to the observation tank (all the above experiments were terminated because of death or bad health of the males); if the females did not ovulate during that time, no spawning activity was observed.

Experiment 2A

Water samples from tanks holding ripe males produced significantly larger EOG responses in immature char than samples from tanks holding ripe females or immature fish (Fig. 4.3). However, the immunoassay was unable to detect F-type PGs in the water from any of the tanks. Therefore, no fish was found to release F-type PGs when kept separated from other fish. However, ripe males were found to be more odorous than other fish.

Experiment 2B

This experiment consisted of 19 trials. They were grouped according to the maturational stages of both the donor and recipient fish (Fig. 4.4). At the start of a trial the recorded response to the outflow water usually deviated considerably from the "expected" response (Fig. 4.4). This is because the samples at time 0 were taken before any amount of water from the donor tanks had entered the recipient tanks, and thus before any

Fig. 4.3. EOG responses of immature char to water from the tanks in Experiment 2A. Each tank was holding one char, of which the maturational stage was determined and classified as indicated. Means (symbols) and T′-values (bars) are indicated. Number of water samples in parentheses. Only one water sample was taken from each fish in the tanks and only tested once in an EOG recoding. On each EOG preparation, 1-8 water samples were tested. Where bars do not overlap, the means are significantly different from each other ($P<0.05$). IM: immature fish; F-PO: pre-ovulatory female; F-OV: ovulated female; F-OO: old-ovulated female (over-ripe); M-RI: ripe male; %AA: percent of response to 10 μM L-serine.

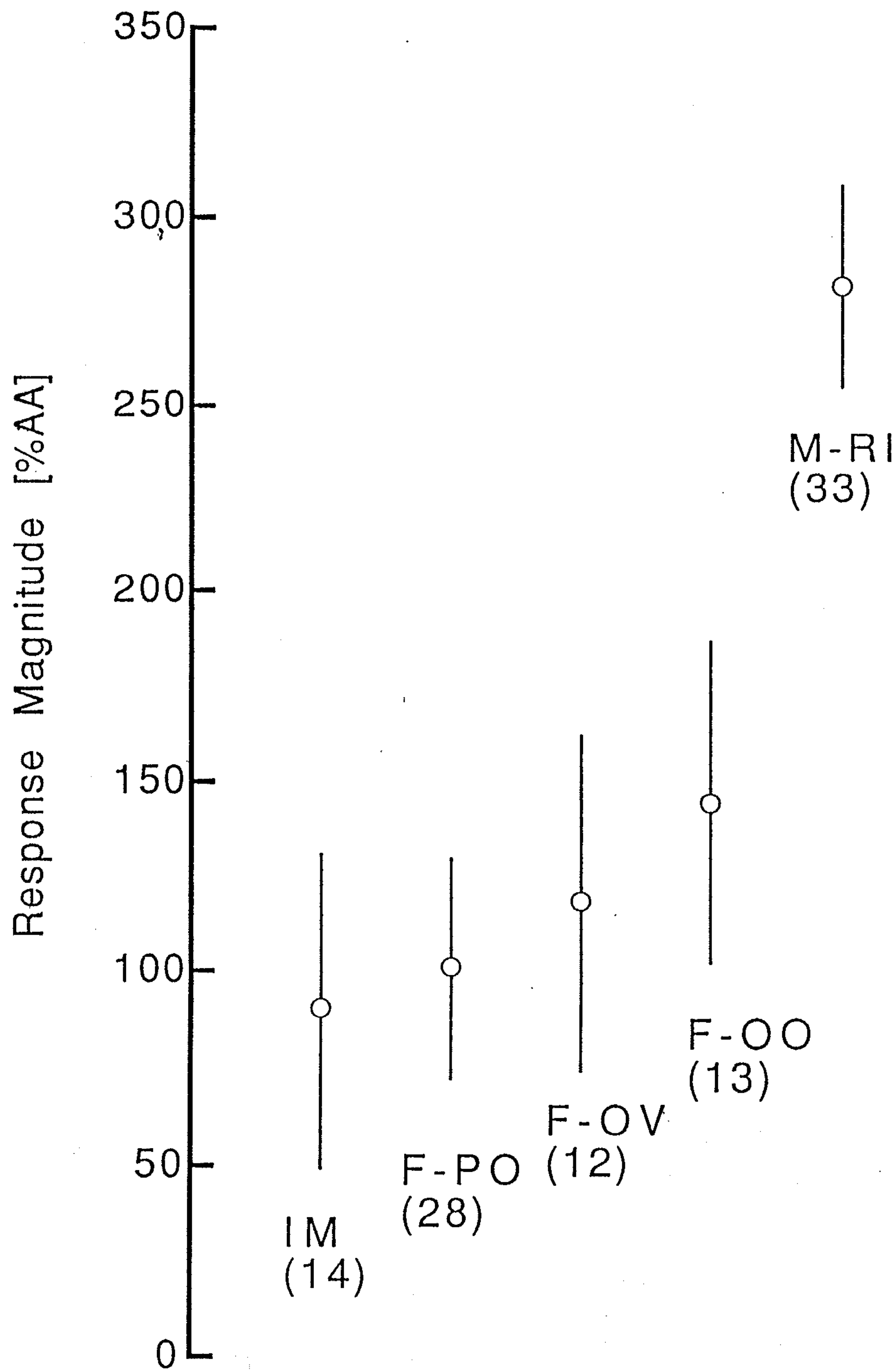
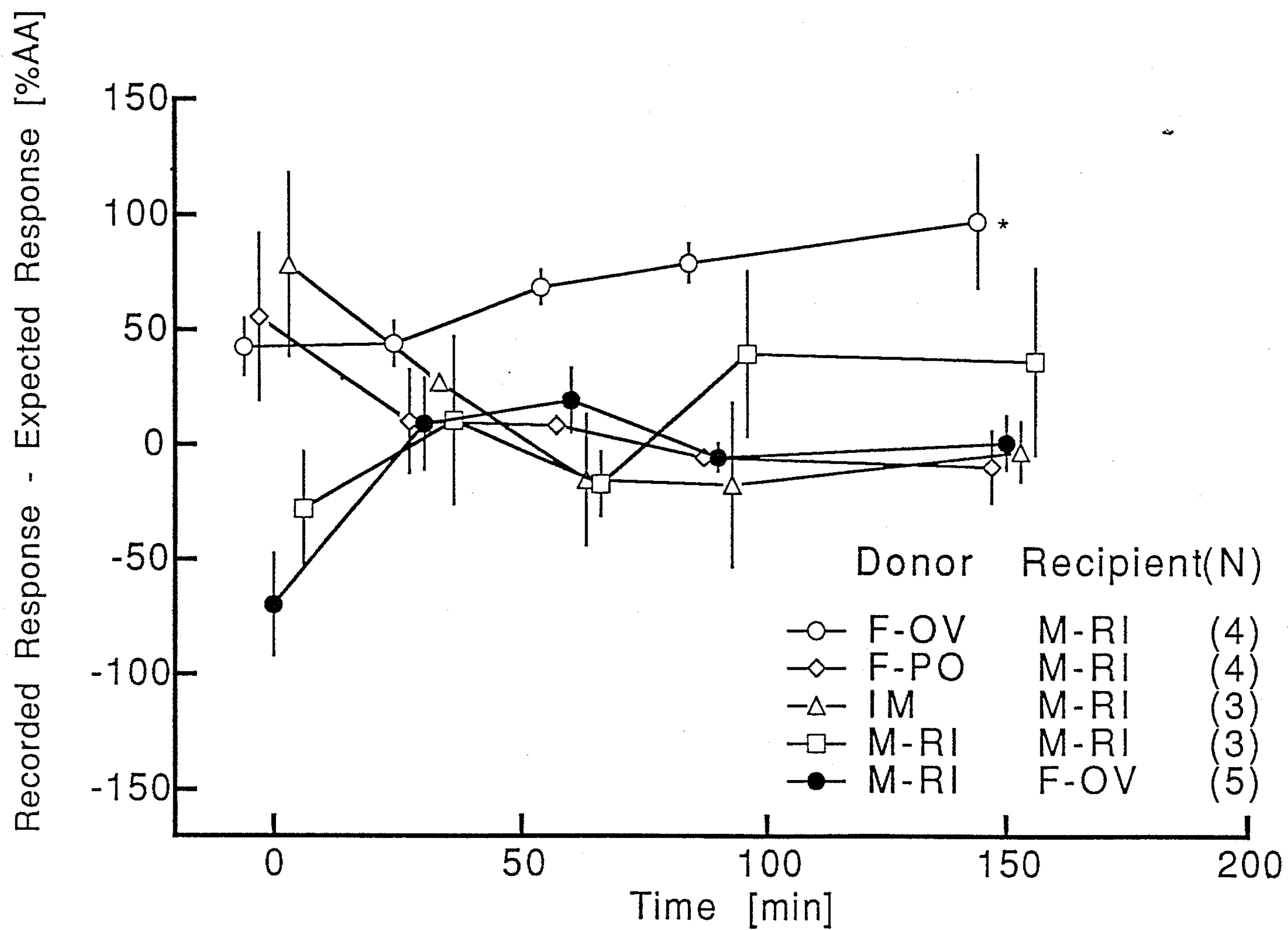

350
300
250
200
150
100
50
0
Response Magnitude [%AA]
IM
(14)
F-PO
(28)
F-OV
(12)
F-OO
(13)
M-RI
(33)

Fig. 4.4. Differences between recorded and expected EOG response to water drained from recipient tanks in Experiment 2B. At time 0 each tank (holding one fish) received the outlet water from a donor tank (holding another fish). Expected EOG responses were found by recording responses to a mixture of water from both recipient and donor tank in proportion to the outlet flow from each tank at time 0. Trials (one donor tank and one recipient tank) were grouped according to the maturational stages of both the donor fish and the recipient fish as shown (see Fig. 4.3 for abbreviations). Number of trials for each group is shown in parentheses. Means (symbols) and standard error of means (error bars) of each group are indicated.

* This mean response was significantly different from the three lowest means at this time point (time 150 min; ANOVA and T′-method). In the samples of this mean, 9.1 pM (average, SE = 3.8 pM) of immunoreactive F-type prostaglandins were measured by the EIA assay, but none in the other water samples at this time point or in water samples taken at time 0.


Recorded Response - Expected Response [%AA]
150
100
50
0
-50
-100
-150
0
50
100
150
200
Time [min]
*
Donor Recipient(N)
F-OV M-RI (4)
F-PO M-RI (4)
IM M-RI (3)
M-RI M-RI (3)
M-RI F-OV (5)

mixing had occurred. A response to water from a recipient tank, where the recipient fish was a odorous ripe male (M-RI), was more than expected because the less odorous water from the donor tank (except when the donor fish also was a ripe male, M-RI), had not yet "diluted" the water in the recipient tank. When the donor fish was a ripe male (M-RI), the response at time 0 was less than expected because its odour had not yet entered and mixed with the water inside the recipient tank. After about 60-90 min from the start of a trial, complete mixing seemed to have taken place and the response to the outlet water had reached a steady state level close to the expected value. There was an exception, however, when the donor fish was an ovulated female (F-OV) and the recipient fish was a ripe male (M-RI). The response to the outlet water from the recipient tank did not decline to the expected value, but instead continued to increase throughout the experiment. The responses measured after 150 min from the start of the trials showed a significant difference among groups (Fig. 4.4; ANOVA: $F[4,14] = 3.35$, $P<0.05$); the responses to the water from the recipient tanks that held ripe males and received water from donor tanks holding ovulated females were larger than expected, assumed to be because of increased odour production by the recipient males. Furthermore, the immunoassay revealed that only water samples taken from the recipient tanks where the males showed this increased odour production contained immunoreactive F-type PGs (average: 9.1 pM, SE: 3.8 pM), but none of the others. No PGs were detected in any of the tanks at the start of the trials (at time 0).

<u>Experiment 3</u>

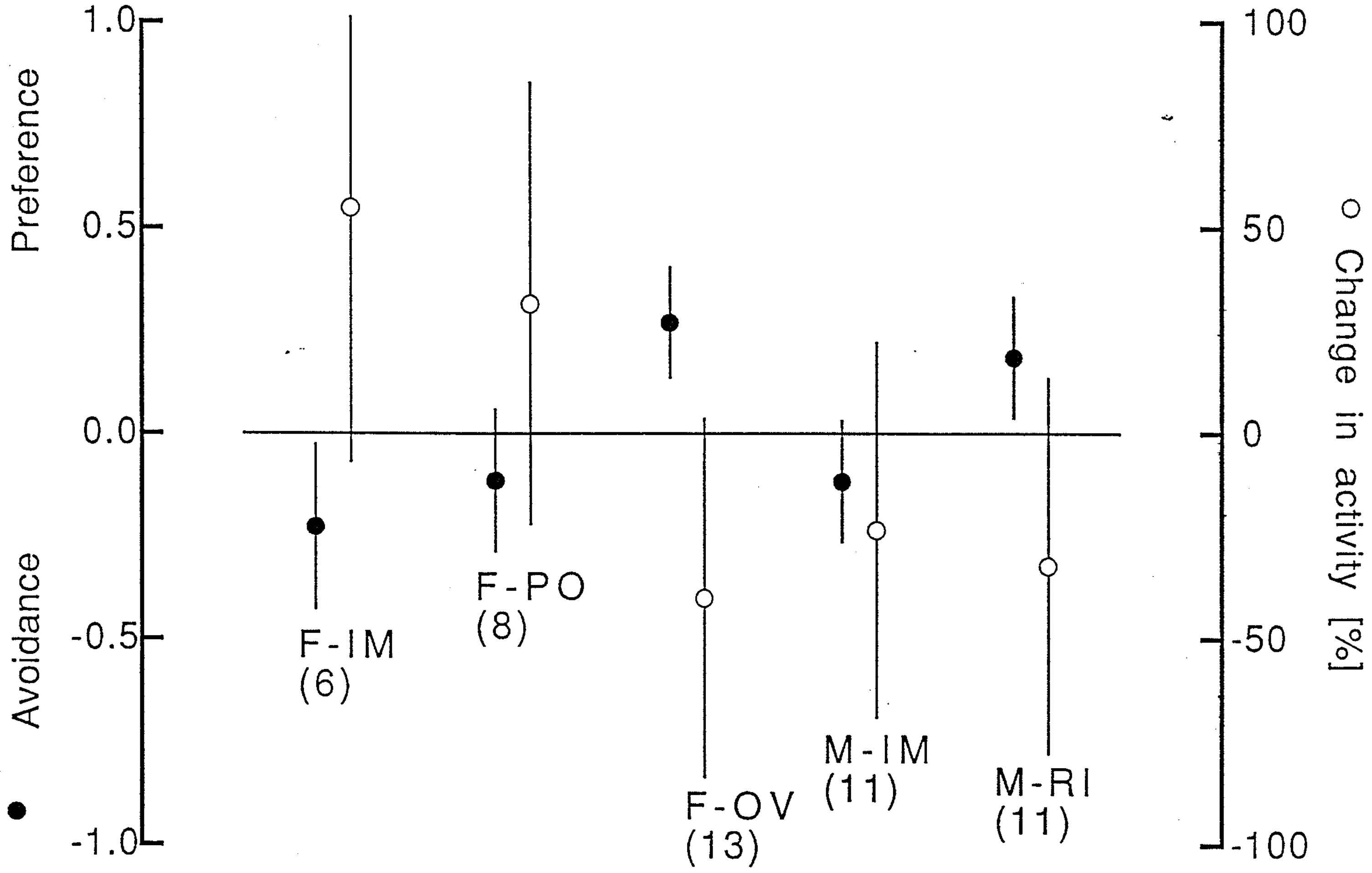
Forty-nine fish were tested in this experiment. During the control period, the behaviour of the fish inside the trough was very variable. Some

fish moved around and showed no preference for location inside the trough during the control period, whereas others showed preference for certain locations. However, no location inside the trough was preferred significantly more often than any other. For example the arm with inlet A was preferred by 7 fish and 9 fish preferred the arm with inlet B. The activity also varied considerably: one fish moved from one section to another only 4 times during the 30 min control period, while another fish crossed a boundary 886 times during this period (almost once every 2 seconds).

The means and T′-values of both the A-P and the CA scores for each maturational stage are shown in Fig. 4.5. Administration of $PGF_{2\alpha}$ altered mean position significantly, as reflected in non-zero A-P scores (Fig. 4.5; ANOVA: $F(4,44)=7.55$, $P<0.001$). Only immature females (F-IM) showed significant avoidance in response to $PGF_{2\alpha}$ (mean A-P score is negative and the error bar does not cross the zero), while both ovulated females (F-OV) and ripe males (M-RI) showed significant preference for $PGF_{2\alpha}$. However, $PGF_{2\alpha}$ administration had no significant effect on the activity among groups: the differences were not significant for the CA scores. One of the ovulated females increased her activity from 5 during the control period to 242 during the test period or by +4740%. Because this value was much larger than any other value and created enormous error bars, it was not included in Fig. 4.2.

During the experiment some of the F-OV and F-PO females showed digging behaviour, with the female turning on her side and flapping her tail down. Only one female (F-OV) showed this behaviour during the control period and did so nearly continuously throughout both control and test periods. Six females that showed no such behaviour during the control

Fig. 4.5. Change in mean distance from odour source (A-P score; solid symbol) and change in activity (CA score; open symbol) between two 30 min periods, one before and one during $PGF_{2\alpha}$ stimulation (Experiment 3). See text for further explanation of variables and their calculation. Top view of the behavioural trough which was used in this experiment is shown in Fig. 4.1. Means (symbols) and T′-values (error bars) are indicated. Number of fish tested in parentheses. Where bars do not overlap, the means are significantly different from each other ($P<0.05$). Abbreviation for maturational stages is in the legend for Fig. 4.3.


1.0
0.5
0.0
-0.5
-1.0
Preference
Avoidance
●
100
50
0
-50
-100
○ Change in activity [%]
F-IM
(6)
F-PO
(8)
F-OV
(13)
M-IM
(11)
M-RI
(11)

period performed this act during the $PGF_{2\alpha}$ exposure. One of these did so nearly continuously throughout the test period, commencing as soon as she entered the PGF-odour plume. The other females displayed this behaviour only 1-4 times during the 30 min test period. The probability of showing the digging behaviour only during the test period purely by chance by these six females is 0.016 (0.5^6) and is thus below significance level.

Discussion

A few studies including the present one have found that water inhabited by ripe fish contains immunoreactive F-type PGs. F-type PGs were detected in the water inhabited by spawning goldfish and were found to stimulate the spawning behaviour of males (Sorensen *et al.*, 1988; Sorensen *et al.*, 1989). Holding water of ovulated cobitid loach females contained a large amount of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ for which the males have a high olfactory sensitivity (EOG threshold 10^{-13} M; Kitamura and Ogata, 1991). The detection of immunoreactive F-type PGs in the water of the spawning tank in the present study only when the males show active spawning behaviour (aggression and courting) was the first indication that the PGs serve as chemosignals in connection with the sexual behaviour of Arctic char males. The presence of PGs during male sexual behaviour in experiment 1 in the present study suggests that either (1) the PGs stimulate the spawning behaviour of the males, or (2) the males release PG into the water during their sexual behaviour. The results from experiments 2 and 3 support the latter possibility. In experiment 2, immunoreactive PGs of F-type were detected in the water from isolated males when they received chemosensory cues from ovulatory females. In experiment 3, low concentrations of $PGF_{2\alpha}$ induced more specific behavioural response in the

females than the males. Both ovulated females and ripe males were attracted to $PGF_{2\alpha}$. Furthermore, $PGF_{2\alpha}$ induced digging behaviour in some of the ripe females, a stereotyped behaviour and a part of the female's spawning behaviour in salmonids (see Introduction). These results are consistent with the idea that $PGF_{2\alpha}$ or compounds closely resembling it are released by males during their spawning activity, and act primarily upon females to elicit or enhance their spawning behaviour. Secondly, these compounds may attract other males.

Very limited studies have been conducted on PGs and PG metabolisms in male fish (for review see Stacey and Goetz, 1982). It could be argued that the measured PGs in the water in experiment 1 are due to some immune reaction in the males related to their imminent death. However, this cannot explain the males' production of PGs in experiment 2 only when they smell ovulated females. Furthermore, the behavioural response of the fish to $PGF_{2\alpha}$ supports the previous explanation.

Several previous studies on ripe salmonids have indicated that chemosensory cues are involved in communication between the sexes around the time of spawning. In experiments with rainbow trout, the strongest response observed was the attraction of ripe females to water from ripe males (Newcombe and Hartman, 1973). No attempts were made to identify the chemical nature of the stimuli involved. However, this observation is consistent with the results of the present study, namely, release of PGFs from males and attraction of females to $PGF_{2\alpha}$.

Rainbow trout males were weakly attracted or showed positive rheotaxis to water from ripe females (Newcombe and Hartman, 1973; Emanuel and Dodson, 1978; Honda, 1980). A similar phenomenon was reported in *Oncorhynchus rhodurus* and *O. masou* and the responses were

species-specific (Honda, 1982). Honda (1980) found decreased courting behaviour in anosmic rainbow trout males and suggested that pheromones are present in ovaries of ovulated females. Liley *et al.* (1991) reported that anosmic males of rainbow trout and kokanee salmon (*O. nerka*) spawned as readily as intact males when paired with nesting females in laboratory stream tanks. However, at a natural spawning area, anosmic males of kokanee salmon were less active and less persistent in response to nesting females than intact males. Also, anosmic rainbow trout and kokanee salmon males had lower concentrations of plasma steroids and less "strippable" milt volume than intact males (Liley *et al.*, 1991). All of these experiments suggest that although chemical cues from females are not always essential to the performance of sexual behaviour of salmonid males, they might play a role in maintaining arousal and milt production. Although PG stimuli can not be excluded from involvement in mediating the response of males to female odour. However, the "goldfish model" could be invoked and it proposed that some derivatives of sex-steroids released by the females are responsible for the males' responses. In the goldfish, 17,20P has profound effects on the male endocrine system (gonadotrophin hormone surge; Dulka *et al.*, 1987; Stacey *et al.*, 1989) but only minor immediate behavioural effects (Sorensen *et al.*, 1989), while PG chemosignals stimulate sexual behaviour but exert no known direct endocrine effects (Sorensen *et al.*, 1989).

Reproductive behaviour of Arctic char on a natural spawning ground was recently described (Sigurjónsdóttir and Gunnarsson, 1989). The behaviour is similar to that described previously for this species in an experimental setting (Fabricius and Gustafson, 1954). Males congregate on the spawning grounds and are usually more numerous than the females

(Sigurjónsdóttir and Gunnarsson, 1989). There they seem to display and establish a dominance hierarchy wherein size is an important factor; colour and other cues may also contribute (see below for further discussion on this point). When females arrive, they search for a spawning site and, once this has been selected, they use digging behaviour to clean the spawning site or to form a nest, depending on the substratum. Males – usually more than one – take position around the female and the one highest in the dominance hierarchy takes a guarding position. The guarding male stays behind and above the female. He attacks the other males and periodically courts the female. The other males, called satellite males, try to steal fertilization either: (1) by sneaking in and spawning with the female while the guarding male is chasing other satellite males; or (2) by rushing in and releasing milt while the guarding male is spawning with the female.

The males of lake trout arrive at the spawning area before females do (Miller and Kennedy, 1948). Under artificial conditions, Arctic char males were observed to establish territories before females moved in to dig redds (Fabricius and Gustafson, 1954). In the field the Arctic char males were always more numerous than the females, especially early in the season, and were likely to arrive there before the females (Sigurjónsdóttir and Gunnarsson, 1989). Both studies (Fabricius and Gustafson, 1954; Sigurjónsdóttir and Gunnarsson, 1989) reported rather unstable territories. Foster (1985) studied lake trout (*Salvelinus namaycush*) and concluded that the mating strategy fits the definition of the lek. However, according to the definition by Vehrencamp and Bradbury (1984), the males neither defend a resource nor their mates in lek-mating systems. This latter definition also states that no paternal care of the offspring is undertaken; males establish

display territories where they advertise themselves to females. As Arctic char males (and most salmonids; see reference above) defend (guard) the females rather than a display territory, they do not fit this definition of the lek.

Size was found to be correlated with success in guarding position (dominance hierarchy) among Arctic char males (Sigurjónsdóttir and Gunnarsson, 1989). The same was found for pink salmon, *O. gorbuscha* (Keenleyside and Dupuis, 1988). Yet, it cannot be excluded that other cues such as colour and chemical cues are also important, for example among evenly sized males. In the present study, I demonstrated that ripe males are more odorous than other char, as reflected in EOG responses. Although it is impossible to form a conclusions about the purpose of this odour production by ripe males, it is a part of their identity and makes them distinct from females. Such sexual dimorphism is widely known for visual (colour, antlers) and auditory cues (songs) among vertebrate groups (Morse, 1980). These sensory cues are usually involved in advertising sexuality and in mate-getting activities. It is thus possible that ripe Arctic char males use chemical displays on the spawning ground when establishing the dominance hierarchy and attracting females.

As mentioned above all the salmonids show the same general behavioural pattern during spawning. However, it cannot be concluded that they use the same chemical cues or pheromones. Honda (1982) found species-specificity of behavioural responses to ovarian fluid in Yamame (*Oncorhynchus masou*) and Amago (*O. rhodurus*) salmons. EOG recordings showed that while lake trout are as sensitive to $PGF_{2\alpha}$ as Arctic char, rainbow trout and brook trout did not respond at all (Sveinsson and Hara, unpublished). This is interesting from an evolutionary point of view

because genetic analysis has indicated that lake trout and Arctic char are more closely related than either one is with brook trout or rainbow trout (Grewe *et al.*, 1990). Although the same behavioural pattern is used by salmonids, the importance and utilization of different sensory cues could be quite variable among the salmonid species.

The importance of synchronous release of gametes in externally fertilizing species like salmonids is obvious when it is considered that the sperm are only viable for a brief time after release. The behavioural trajectory in salmonids, which has the ultimate goal of reproduction, consists of two phases: (1) migration from feeding area to selected spawning grounds, and (2) spawning behaviour involving mate selection and gamete release (Hasler, 1971; Quinn and Dittman, 1990). The synchronization achieved at each phase is on two different time scales; synchronization of the sexual maturation and arrival of both sexes at the spawning grounds (on the level of days/weeks) and synchronization of the release of gametes (on the level of seconds/subseconds). The former phase depends on the ability of the individuals to receive environmental signals by which to decide when to start the migration, and to find the route to the spawning grounds. The photoperiod has been shown to be important in timing reproductive maturation in several salmonids, and is thus also likely to be involved in timing the migration (Bromage *et al.*, 1984; MacQuarrie *et al.*, 1979). However, the mechanism by which the salmonids find their way to the spawning grounds has been a matter of debate for years (Quinn, 1990; Quinn and Dittman, 1990; Brannon and Quinn, 1990). It is generally accepted that they use olfaction, at least for the final stage of migration (Wisby and Hasler, 1954; Hasler, 1971). They are noted for their tendency to spawn at their natal site or site of previous spawning (for

review see Stabell, 1984; Quinn, 1990). The "imprinting" hypothesis, proposed by Hasler and Wisby (1951), states that the juvenile fish learn the odour of their freshwater habitat before they migrate to the ocean. When they return years later for spawning they are attracted to these odours. Another hypothesis, proposed by Nordeng (1971,1977), states that pheromones, released by young on the spawning grounds, attract the migrating adults. Foster (1985) concluded that juvenile lake trout release chemical cues in their faeces which attract the adults when they return to spawn. The chemical nature of these proposed pheromones is not known, but the possibility that they are PGs or PG derivatives should be considered in future experiments on this subject. Although a recent report has provided evidence which supports the imprinting hypothesis (Brannon and Quinn, 1990), it can not be discounted that pheromones (or PGs) contribute to the odour the juvenile salmonids learn before they migrate to the ocean. It should also be considered that, if the males who first arrive on the spawning grounds release F-type PGs, they would attract and guide the later arriving males to the spawning beds. Furthermore, it would attract ripe females to the spawning ground and stimulate their digging behaviour. In fact, the present report (Experiment 3) supports these last two ideas.

The chain of stimulus-reaction events, which ensures the synchronous release of gametes from both sexes, has recently been studied in himé salmon, *Oncorhynchus nerka* (Satou *et al.*, 1991). Visual cues (colour, shape, and posture; Takeuchi *et al.*, 1987) and vibrational cues (Satou *et al.*, 1987) from the female elicit quivering behaviour (courting) of the male. The quivering creates a vibration signal which elicits vibration in the female which in turn elicits spawning (gamete release) by the male

(Satou *et al.*, 1991). Subsequently, the vibrational signal created by the male spawning seems to be requisite for gamete release by the female. Although olfactory signals were not excluded in the above studies they seem unlikely to be used for such rapid and "private" communication. Olfactory cues could, however, provide an ongoing motivating stimulus and prime the endocrine system for readiness for spawning as already discussed.

In conclusion, the present study shows that: (1) immunoreactive F-type PGs are released into the water by actively spawning Arctic char; (2) ripe males are very "odorous" to other char; (3) ripe male increase their odour production and release immunoreactive F-type PGs when they smell ovulated females; and (4) $PGF_{2\alpha}$ attracts ovulated females and incite their "digging" behaviour, and also attracts ripe males.

CHAPTER V

General Conclusions

This study was initiated to answer the following questions: (1) What are the electrophysiological characteristics of the reception and transduction mechanisms of $PGF_{2\alpha}$ in Arctic char? (2) Can the olfactory receptors for $PGF_{2\alpha}$ be characterized and isolated by biochemical methods? (3) What are the behavioural and biological functions of F-type PGs as chemosensory signals in Arctic char?

In Chapter II, I used electro-olfactogram (EOG) to demonstrate that Arctic char have olfactory receptors with high sensitivity and specificity for $PGF_{2\alpha}$. Responses from the olfactory nerve provide evidence that these responses to $PGF_{2\alpha}$ are transduced and transmitted to the brain. In cross-adaptation experiments it was shown that the receptor for this PG response is separate and independent from receptors for amino acids and bile salts. This was further supported by poor correlation between the magnitudes of the responses to L-serine and $PGF_{2\alpha}$, indicating that the response to each is independent from the other. Analysis of PG responses of char at different maturational stages revealed no significant differences in specificity or sensitivity. However, post-ovulatory females showed more variability in response magnitude to PGs than other stages, with some individuals responding very poorly.

The high sensitivity and the shape of the concentration-response curve suggested that the affinity of the receptor involved might be high enough to be used in ligand-binding studies and biochemical

characterization and isolation of the receptor protein. The binding studies described in Chapter III were, however, unable to achieve this objective. Several reasons could account for this: a lower affinity of the receptor for the ligand than expected from electrophysiological data; a switch from high- to low-affinity state of the receptor when exposed to ligand; a need for the ligand to be enzymatically modified before it can bind to the receptor; or a low density of receptor molecules.

In Chapter IV, I showed that immunoreactive F-type PGs are released during spawning of Arctic char by ripe males. I found that ripe males are very odorous to other char and that they possibly use this odour production as a chemical display in mate-getting activities. Chemical signals emanating from ovulated females stimulated the males to release the immunoreactive F-type PGs and to increase their odour production. Furthermore, I found that $PGF_{2\alpha}$ attracts ovulated females and stimulates their digging behaviour. It also attracts ripe males. The possibility exist that PGs or steroid-derived pheromones are used by salmonids to locate their natal spawning grounds.

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