

## ELECTROPHYSIOLOGICAL AND ENDOCRINOLOGICAL EVIDENCE THAT F-SERIES PROSTAGLANDINS FUNCTION AS PRIMING PHEROMONES IN MATURE MALE ATLANTIC SALMON (*SALMO SALAR*) PARR

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### Summary

Previous work has demonstrated that the urine from mature female salmonids contains a priming pheromone that significantly enhances the reproductive physiology of receiving males. The chemical identity of the pheromone(s) has not yet been identified. The present study investigated the possibility that F-type prostaglandins (PGFs) may have a pheromonal role in Atlantic salmon by measuring the olfactory sensitivity of male salmon to PGFs, the effect of waterborne PGFs on male reproductive physiology and the PGF content of mature female urine. Electrophysiological studies demonstrated that the olfactory epithelium of mature male salmon parr was acutely sensitive to PGF<sub>1α</sub> and PGF<sub>2α</sub>, with detection threshold concentrations of 10<sup>-11</sup> mol l<sup>-1</sup>. The olfactory epithelium was also sensitive to the PGF metabolite 15-ketoPGF<sub>2α</sub> (threshold 10<sup>-8</sup> mol l<sup>-1</sup>), but did not detect a further metabolite, 13,14-dihydro-15-ketoPGF<sub>2α</sub>. Sensitivity to both PGF<sub>1α</sub> and PGF<sub>2α</sub> increased

as the reproductive season progressed. Exposure of mature male parr to waterborne PGF<sub>1α</sub> and PGF<sub>2α</sub> (10<sup>-8</sup> mol l<sup>-1</sup>) resulted in significant increases in levels of expressible milt and in the plasma concentrations of 17,20β-dihydroxy-4-pregnen-3-one, testosterone and 11-ketotestosterone. Urine from ovulated female salmon also contained large quantities (18 ng ml<sup>-1</sup>) of immunoreactive PGFs, whereas urine from mature males and non-ovulated mature females contained significantly smaller amounts (<1 ng ml<sup>-1</sup>). The results support the theory that PGFs function as priming pheromones in Atlantic salmon and are released in the urine of ovulated female salmon. The involvement of these pheromones in synchronising male–female salmon spawning is discussed.

Key words: Atlantic salmon, *Salmo salar*, pheromone, prostaglandins, reproduction, physiology, urine, olfaction.

### Introduction

Pheromonal-mediated synchronisation of spawning is now known to occur in a wide variety of teleosts species (reviewed by Stacey *et al.* 1994). The best understood example of this is the goldfish *Carassius auratus*. Female goldfish release a variety of pheromones during the peri-ovulatory period which are detected by the olfactory system of males and lead to subsequent alterations in the male reproductive physiology (priming activity) and/or behaviour (releaser activity). Pre-ovulatory females release 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) and 17,20β-dihydroxy-4-pregnen-3-one 20-sulphate (17,20βP-S) into the water (Stacey and Sorensen, 1991; Sorensen *et al.* 1995; Scott and Sorensen, 1995), which elevate plasma gonadotrophin II (GtH II) concentrations in males and increase the amount of expressible milt (Stacey *et al.* 1989). Post-ovulatory female goldfish release prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) and its metabolites into the water, which induce behavioural changes in males (Sorensen *et al.* 1988, 1989).

In Atlantic salmon (*Salmo salar* L.), the urine from an ovulated female salmon is both a potent odorant to mature male salmon parr (Moore and Scott, 1992) and also contains at least one priming pheromone which elevates plasma GtH II, testosterone and 17,20βP concentrations in mature males (Moore and Waring, 1996; Waring *et al.* 1996). Waring *et al.* (1996) demonstrated that two of the steroids that are detected by the olfactory system of mature male parr, testosterone (Moore and Scott, 1991) and 17,20βP-S (the sulphated form of 17,20βP; Moore and Scott, 1992), have no significant priming effect (in the case of testosterone) or a weak and inconsistent (in the case of 17,20βP-S) priming effect on the reproductive system of mature male salmon parr.

The aims of the present study were therefore to determine the identity of the priming pheromone released by the female Atlantic salmon and which, once detected, enhances the physiological status of mature male parr. The present study concentrated on the role of F-series prostaglandins (PGFs),

which have also previously been shown to be potent odorants in mature male parr (Moore and Scott, 1992).

The study examined three different aspects relating to the possible role of PGFs as priming pheromones synchronising reproductive physiology in male salmon. First, an electrophysiological study (electro-olfactogram; EOG) was carried out during the reproductive season (September–December) to investigate the olfactory responses of immature male parr and mature male parr to PGFs. Second, during the same period, an endocrinological study was carried out to investigate the possible priming effect of PGFs on plasma levels of reproductive steroids and levels of expressible milt in mature male parr. Third, an investigation of the levels of immunoreactive PGFs (irPGF) in salmon urine was carried out to demonstrate the possible mode of release of the putative priming pheromones to the environment.

The results of this study are discussed in relation to the role of pheromones in priming males and in synchronising reproductive physiology in the Atlantic salmon. Parts of these data have been published previously in abstract form (Moore and Waring, 1995; Waring and Moore, 1995).

## Materials and methods

### Experimental animals

Atlantic salmon parr (*Salmo salar* L.) were collected from the National Rivers Authority, Cynrig Hatchery, Wales (water pH 7.6), in September, October and November 1994 and transported to the Lowestoft Fisheries Laboratory. The parr were derived from salmon collected from the River Usk and were maintained at the Cynrig hatchery under natural light conditions. The stock tanks were fed by water from the River Cynrig, which was extracted from a site inaccessible to Atlantic salmon. The parr were therefore not exposed in any way to adult salmon. At Lowestoft, the fish were maintained under natural light conditions in 1000 l stock tanks with a constant flow of aerated, dechlorinated water (rate 85 l min<sup>-1</sup>). Water temperatures were between 5.5 and 15.6 °C; pH 7.5; alkalinity 156 mg HCO<sub>3</sub><sup>-</sup> l<sup>-1</sup>; total calcium 166 mg l<sup>-1</sup>; total hardness 405 mg l<sup>-1</sup> as CaCO<sub>3</sub>; aluminium <10–32 µg l<sup>-1</sup>; Na<sup>+</sup> 37.3 mg l<sup>-1</sup>; Mg<sup>2+</sup> 12 mg l<sup>-1</sup>; NO<sub>3</sub><sup>-</sup> 0.2–49.1 mg l<sup>-1</sup>; SO<sub>4</sub><sup>2-</sup> 10 µg l<sup>-1</sup>. Fish were fed to satiation with commercial salmon pellets.

### Experiment 1: electrophysiological recordings from the olfactory epithelium

Immature male parr (length 124±11 mm) and mature male salmon parr (length 135±15 mm) were studied between September and December 1994 (water temperature 6.4–13.8 °C). After each experiment (4–5 h in duration), the fish were killed, sexed and the gonadosomatic index (GSI) calculated (testes weight/body weight × 100%). Most mature parr tested were spermiating (i.e. they had free-running milt) and had a GSI of 8.4±1.4%. Immature parr had a GSI of 0.04±0.002%.

The study used the same electrophysiological technique

(electro-olfactogram, EOG) as that in previous studies on mature male Atlantic salmon parr (Moore and Scott, 1991, 1992; Moore, 1994; Moore *et al.* 1994). EOG recording measures transepithelial voltage gradients from the surface of the olfactory epithelium and is considered to reflect multi-unit cell activity (Evans and Hara, 1985).

The fish were anaesthetised with 2-phenoxyethanol (0.4 ml l<sup>-1</sup>), and the skin and the cartilage were removed to expose the olfactory rosettes. The fish were then immobilised with an intramuscular injection of gallamine triethiodide (0.3 mg kg<sup>-1</sup> body mass) and placed in a V-shaped clamp in a Perspex flow-through chamber. The gills were constantly perfused with water containing 2-phenoxyethanol (0.2 ml l<sup>-1</sup>). Paired silver electrodes were attached subcutaneously to the fish to monitor the heart rate and level of anaesthesia during each experiment. The output was continuously displayed on an oscilloscope (Textronic 465 B). This also provided an indication of the stability and health of the preparation. Electrophysiological recordings were made using glass pipettes filled with saline–agar (2%) bridged to a Ag–AgCl electrode (type EH-3MS, Clark Electromedical Instruments) filled with 3 mol l<sup>-1</sup> KCl (Fig. 1). The tip of the electrode (100–150 µm in diameter) was placed close to the olfactory epithelium at the base of the largest posterior lamella. This was where the maximum response to 10<sup>-5</sup> mol l<sup>-1</sup> L-serine (mean 0.93±0.08 mV) and minimum responses to dechlorinated water (mean 0.07±0.02 mV) were obtained. This was also the area of the epithelia where the maximum responses to F-series prostaglandins were recorded. A reference electrode of the same type was grounded and placed lightly on the skin of the nares of the fish. The signal was amplified using a Neurolog Systems d.c. preamplifier (Digitimer Ltd) and either displayed directly on a pen recorder (Lectromed MX 212) or digitised and stored for later analysis on an Apricot XEN-PC computer using Asystant+ scientific software (Asyst Inc.).

A constant volume (100 µl) of the test substance was injected, *via* a remote-control switch, into the second inlet of a three-way solenoid valve (Lee Company) carrying a constant

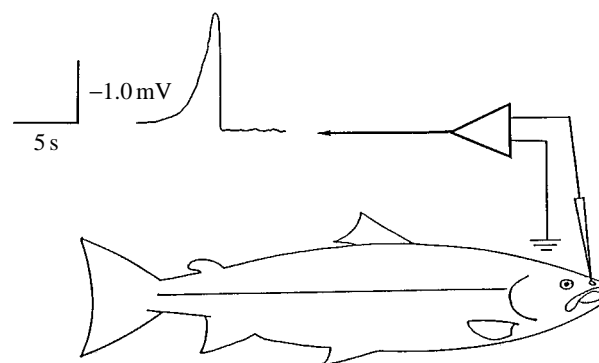


Fig. 1. Experimental arrangement for recording electro-olfactograms (EOGs) and a typical response recorded from the olfactory epithelium of spermiating mature male Atlantic salmon parr to 10<sup>-8</sup> mol l<sup>-1</sup> PGF<sub>2α</sub>. The rising edge of the EOG response has been retouched for clarity.

flow (3 ml min<sup>-1</sup>) of dechlorinated water over the olfactory epithelium. The stimulus lasted for 1.5 s, and the flow rate was unaltered by the addition of the test substance. Little, if any, mechanical response was therefore associated with the EOG recording.

Four PGFs (PGF<sub>1α</sub>, PGF<sub>2α</sub>, 15-ketoPGF<sub>2α</sub> and 13,14-dihydro-15-ketoPGF<sub>2α</sub>) were obtained from Sigma Chemicals. PGF<sub>1α</sub> and PGF<sub>2α</sub> were studied during September, October, November and December whereas 15-ketoPGF<sub>2α</sub> and 13,14-dihydro-15-ketoPGF<sub>2α</sub> were studied during November and December 1994. Serial dilutions of each prostaglandin ranging from 10<sup>-6</sup> to 10<sup>-13</sup> mol l<sup>-1</sup> were prepared from stock solutions consisting of 10 µg ml<sup>-1</sup> in absolute ethanol. The dilutions were prepared fresh before each experiment with water taken from the inlet pipe of the salmon tank and allowed to stand at room temperature (7.5–10.7 °C) until required. At the end of October, midway through the study, new stocks of PGF<sub>1α</sub> and PGF<sub>2α</sub> were purchased and fresh stock solutions were prepared. Control dilutions of ethanol were also prepared and tested. A stock solution of 10<sup>-5</sup> mol l<sup>-1</sup> L-serine in dechlorinated water was also prepared weekly.

At the start of each EOG experiment, the prostaglandins were presented to the olfactory epithelium in order of increasing concentration with a 2 min recovery interval between each stimulus. The order in which the prostaglandins were tested each time was chosen at random. Each dilution was tested twice, and the responses to 10<sup>-5</sup> mol l<sup>-1</sup> L-serine and a water control were tested at the beginning and end of each dilution.

#### *Experiment 2: cross-adaptation studies*

In order to determine whether the EOG responses elicited by both PGF<sub>1α</sub> and PGF<sub>2α</sub> were transduced by a similar mechanism, a series of cross-adaptation experiments was performed. The methodology was similar to that previously described (Caprio and Byrd, 1984; Sorensen *et al.* 1987). Cross-adaptation studies involved first the measurement of the responses to a test substance and then perfusing the olfactory epithelium with an adapting odorant into which pulses of the test odorant (made up in the adapting odorant) were added. The experiments were carried out in November 1995 during a period when the responses of the olfactory epithelium to both PGF<sub>1α</sub> and PGF<sub>2α</sub> were similar. Initially, PGF<sub>2α</sub> was used as the adapting stimulus at a concentration of 10<sup>-7</sup> mol l<sup>-1</sup>, which produces a near-maximal EOG response and indicates possible receptor site saturation. The adapting stimulus was then switched to 10<sup>-7</sup> mol l<sup>-1</sup> PGF<sub>1α</sub>, which again produces a near-maximal EOG response. Adaptation was first verified by testing the responses of the olfactory epithelia to an equimolar concentration of the adapting substance, and then the responses to 10<sup>-5</sup> mol l<sup>-1</sup> L-serine and blank water were tested as controls.

The amplitude of each EOG response was measured from the baseline to the peak of each phasic displacement and expressed in millivolts (mV). All duplicates were averaged and then expressed as a percentage response of the initial L-serine

standard. All recordings in response to the dechlorinated water controls were subtracted from the EOG responses. L-Serine was chosen as a standard as it has been used as such in previous studies measuring the EOG responses of fish olfactory epithelia to prostaglandins (Moore and Scott, 1992; Bjerselius and Olsén, 1993). The threshold of response of each prostaglandin was estimated as the lowest concentration whose 95 % confidence interval did not include the value zero (see Cardwell *et al.* 1995).

The EOG recordings in response to all the prostaglandins were similar in waveform and polarity (negative) to those described and illustrated in other studies (Moore and Scott, 1991, 1992; Bjerselius and Olsén, 1993). A sample recording of the EOG response to 10<sup>-8</sup> mol l<sup>-1</sup> PGF<sub>2α</sub> is shown in Fig. 1.

#### *Experiment 3: The effect of waterborne PGFs on male reproductive physiology*

Sixty spermiating male parr (length 128±2 mm; mass 25±2 g; GSI 6.7±0.2 %) were used in this experiment. On various dates from October to December 1994, groups of five males were stripped of milt, moved from the stock tanks and placed in one of a series of 631 glass tanks supplied with flow-through dechlorinated tapwater. The water flow rate to each tank was 11 min<sup>-1</sup> (temperature 13±1 °C) and a natural photoperiod was followed. Males were allowed to recover for 72 h.

PGF<sub>2α</sub> and PGF<sub>1α</sub> (Sigma Chemicals) were dissolved in ethanol and diluted to 2 ml with dechlorinated water to give final concentrations of 10<sup>-8</sup> mol l<sup>-1</sup>. For controls, an equivalent volume of ethanol was diluted to 2 ml with dechlorinated water. The test solutions were added to the tanks using 2 ml syringes fitted with 10 cm lengths of plastic straw. The solutions were added in the vicinity of the inflowing water, which was subsequently dispersed into the tanks *via* vigorous aeration. The water flow rate to each tank was unaltered and the males were left undisturbed. After 3 h of exposure, the fish were anaesthetised with 2-phenoxyethanol (0.4 ml l<sup>-1</sup>) and lightly blotted dry with tissue paper to remove excess water from the urinogenital area. The fish were held belly down, and expressible milt was collected and quantified using the method of Moore and Waring (1996). The fish were then killed, their length measured, weighed and blood was collected from the caudal vasculature using heparinised syringes fitted with 23 gauge needles. Blood was centrifuged for 10 min at 2000 g and the plasma was stored at -20 °C.

Plasma steroids were extracted with diethyl ether, which was then evaporated to dryness at 37 °C. Steroids were resuspended in 1 ml of 0.1 mol l<sup>-1</sup> phosphosaline buffer (pH 7.6) and stored at -20 °C before analysis. Details of the radioimmunoassays (RIAs) used to measure 17,20βP, testosterone and 11-ketotestosterone are as described by Scott *et al.* (1982, 1984).

#### *Experiment 4: the steroid and immunoreactive PGF content of female and male salmon urine*

In October 1994, five pre-ovulatory females (mass 587–1107 g; GSI 10–19 %) and five spermiating males (mass 284–874 g; GSI 3–8 %) were anaesthetised in 0.3 ml l<sup>-1</sup> 2-

phenoxyethanol and a cannula was implanted in the urinary bladder using the procedure of Eddy and Talbot (1985). In November 1994, five ovulated females (860–1360 g; GSI not measured) were cannulated in the urinary bladder using identical procedures. The exact timing of when the females ovulated was not determined, but females were checked at least once a day for ovulatory state and were cannulated immediately if found to have ovulated. Fish were allowed to recover for 24 h in individual rectangular hatchery tanks (1.9 m × 0.5 m × 0.25 m) containing 180 l of dechlorinated tapwater (temperature 8–10 °C) with a water flow rate of 2.5 l min<sup>-1</sup>. The following day, urine was collected, on ice, for 8 h, pooled and then split into samples and stored at -20 °C. The female salmon were taken from a stock maintained at the Lowestoft Fisheries Laboratory and had been reared from eggs collected from the National Rivers Authority, Kielder Hatchery (Moore *et al.* 1994).

Free steroids were extracted from urine using diethyl ether. Glucuronidated and sulphated steroids were isolated using the method of Scott and Liley (1994) and then extracted with diethyl ether. All the diethyl ether extracts were evaporated to dryness at 37 °C in borosilicate glass tubes and the steroids were redissolved in 1 ml of 0.1 mol l<sup>-1</sup> phosphosaline buffer (pH 7.6) and kept at -20 °C until analysed. Details of the RIAs for 17,20βP, testosterone and androstenedione were as described by Scott *et al.* (1982, 1984). Sulphated and glucuronidated steroids have been expressed as nanograms of free steroid and values have not been adjusted to take into account the mass of the conjugating moiety. Immunoreactive PGFs were extracted from urine using Sep-Pak C<sub>18</sub> cartridges (Waters) and the method outlined by Powell (1982). Briefly, Sep-Paks were washed with 10 ml of ethanol followed by 10 ml of distilled water. Urine (1 ml), which was acidified to pH 3.0 using 1 mol l<sup>-1</sup> HCl, was then drawn through the column by vacuum. The columns were washed with 2 ml of distilled water, 2 ml of 15% ethanol and 2 ml of petroleum ether. The prostaglandins were eluted by two 2 ml washes with methyl formate. The methyl formate was then dried down at 32 °C under a stream of nitrogen, and the PGFs were redissolved in 1 ml of 0.1 mol l<sup>-1</sup> phosphosaline buffer (containing 0.1% gelatine) (pH 7.6).

Immunoreactive PGFs were measured using an RIA with anti-PGF<sub>2α</sub> serum (S. Klinger Ltd, St Albans, Hertfordshire, UK; cross-reactivities being 100% PGF<sub>2α</sub>; 38% PGF<sub>1α</sub>; <0.1% 13,14 dihydro-15-ketoPGF<sub>2α</sub>; <0.1% 15-ketoPGF<sub>2α</sub>; <0.2% E-series prostaglandins) at a final dilution of 1:2000. [5,6,8,9,11,12,14,15(*n*)-<sup>3</sup>H]PGF<sub>2α</sub> was purchased from Amersham International Ltd. The assay used approximately 5000 disintegrations min<sup>-1</sup> of radiolabel per tube and separation of bound and free PGFs was achieved using dextran-coated charcoal (0.5% charcoal, 0.05% dextran T<sub>70</sub> in RIA buffer). The assay displayed parallelism of diluted urines (100% to 5%) with the standard curve and the minimum detectable limit of the RIA was 8 pg tube<sup>-1</sup>. Extraction efficiencies, measured by adding [<sup>3</sup>H]PGF<sub>2α</sub> to urines, were greater than 90% and the measured values were not corrected.

### Statistical analyses

Data from experiment 3 were log-transformed where necessary to ensure homogeneity of variance and analysed using a two-way analysis of variance (ANOVA) with date and treatment as factors. Where significance was indicated, multiple comparisons were carried out using Student–Newman–Keuls (SNK) multiple-range tests. Urine data from experiment 4 were log-transformed and analysed using a one-way ANOVA followed by SNK tests for comparing the urine concentrations of the different groups. All values in the text, figures and tables are expressed as the untransformed arithmetic means ± S.E.M.

## Results

### Experiment 1: electrophysiological recordings from the olfactory epithelium

The olfactory epithelium of spermiating mature male parr was acutely sensitive to PGF<sub>1α</sub> and PGF<sub>2α</sub>, less sensitive to 15-ketoPGF<sub>2α</sub> and did not respond at all to 13,14-dihydro-15-ketoPGF<sub>2α</sub> (Fig. 2). In spermiating mature male parr studied during November, the response threshold for both PGF<sub>1α</sub> and PGF<sub>2α</sub> was 10<sup>-11</sup> mol l<sup>-1</sup> and for 15-ketoPGF<sub>2α</sub> the threshold was 10<sup>-8</sup> mol l<sup>-1</sup>.

There was a significant increase in sensitivity of male parr to PGF<sub>2α</sub> with increasing sexual maturity of the fish and as the reproductive season progressed (Fig. 3A). The threshold of detection of PGF<sub>2α</sub> increased 100-fold from 10<sup>-9</sup> mol l<sup>-1</sup> in immature fish in September to 10<sup>-11</sup> mol l<sup>-1</sup> in non-spermiating and spermiating mature male parr in October. Although the sensitivities of non-spermiating and spermiating male parr were similar, the amplitude of the recorded response was

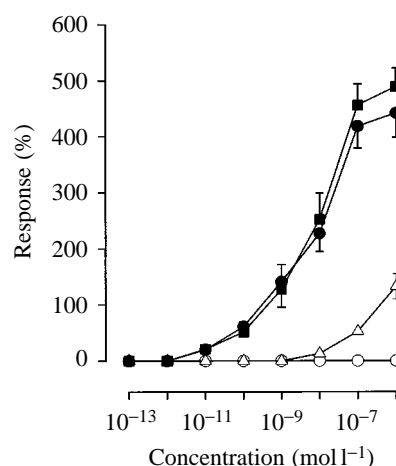


Fig. 2. Plots of the concentration–response relationship recorded from the olfactory epithelium of mature male Atlantic salmon parr during November in response to four F-series prostaglandins, PGF<sub>1α</sub> (■), PGF<sub>2α</sub> (●), 15-ketoPGF<sub>2α</sub> (△) and 13,14-dihydro-15-ketoPGF<sub>2α</sub> (○) (*N*=12). The amplitude of each EOG response was measured from the baseline to the peak of each phasic displacement and is expressed as a percentage of the initial response to 10<sup>-5</sup> mol l<sup>-1</sup> L-serine. Vertical bars represent S.E.M.

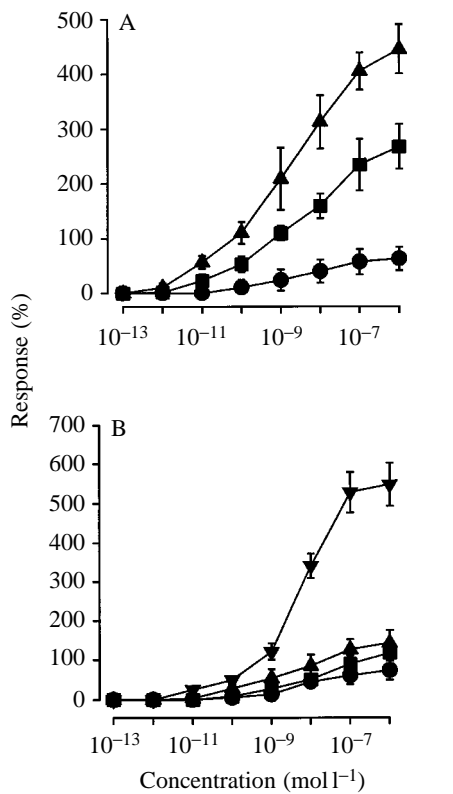


Fig. 3. Plots of the concentration–response relationship recorded from the olfactory epithelium of immature (●, September), non-spermiating (■, October) and spermiating (▲, October) mature male Atlantic salmon parr in response to (A) PGF<sub>2α</sub> and (B) PGF<sub>1α</sub> (N=32). The symbol ▼ denotes recordings from spermiating male parr during November in response to PGF<sub>1α</sub>. The amplitude of each EOG response was measured from the baseline to the peak of each phasic displacement and is expressed as a percentage of the initial response to 10<sup>-5</sup> mol l<sup>-1</sup> L-serine. Vertical bars represent S.E.M.

significantly greater in spermiating parr at concentrations between 10<sup>-11</sup> and 10<sup>-6</sup> mol l<sup>-1</sup> (Fig. 3A). Typical EOG responses to 10<sup>-8</sup> mol l<sup>-1</sup> PGF<sub>2α</sub> recorded from the olfactory epithelium during November were 290–320 % greater than the response to 10<sup>-5</sup> mol l<sup>-1</sup> L-serine (Fig. 2).

Recordings from the olfactory epithelium of male salmon parr indicated that there was a more marked change in seasonal sensitivity to PGF<sub>1α</sub> than to PGF<sub>2α</sub> (Fig. 3B). During

September and October, the response threshold for PGF<sub>1α</sub> of immature male, non-spermiating mature male and spermiating mature male parr was 10<sup>-10</sup> mol l<sup>-1</sup>. However, towards the end of November there was a 10-fold increase in sensitivity to PGF<sub>1α</sub> with a response threshold of 10<sup>-11</sup> mol l<sup>-1</sup>. Sensitivity to PGF<sub>1α</sub> remained equally high during December (not shown). There was also a corresponding increase in the amplitude of the response recorded from the olfactory epithelium. The response to 10<sup>-8</sup> mol l<sup>-1</sup> PGF<sub>1α</sub> in November increased from 92 to 295 % of the response recorded to 10<sup>-5</sup> mol l<sup>-1</sup> L-serine (Fig. 3B).

There was, however, no corresponding seasonal increase in the amplitude of the recorded EOG response to the standard 10<sup>-5</sup> mol l<sup>-1</sup> L-serine. Mean EOG responses recorded from the olfactory epithelium were: immature parr (September) 0.62±0.08 mV; non-spermiating parr (October) 0.67±0.076 mV; spermiating parr (October) 0.70±0.09 mV; spermiating parr (November) 0.67±0.07 mV.

Experiment 2: cross-adaptation studies

When the olfactory epithelium was adapted to 10<sup>-7</sup> mol l<sup>-1</sup> PGF<sub>2α</sub>, the EOG responses to 10<sup>-7</sup> mol l<sup>-1</sup> PGF<sub>1α</sub> were completely eliminated (Table 1). However, responses to L-serine were unaffected by adaptation. Conversely, when the olfactory epithelium was adapted to 10<sup>-7</sup> mol l<sup>-1</sup> PGF<sub>1α</sub>, the EOG responses to 10<sup>-7</sup> mol l<sup>-1</sup> PGF<sub>2α</sub> were also completely eliminated. Again, responses to L-serine were unaffected by adaptation (Table 1).

Experiment 3: the effects of waterborne PGFs on male reproductive physiology

There was a seasonal increase in the levels of expressible milt in males exposed to waterborne PGF<sub>1α</sub> and PGF<sub>2α</sub> (Fig. 4A). During October, PGFs had no significant effect on the levels of expressible milt in male fish. However, during November and December, exposure to waterborne PGF<sub>1α</sub> and PGF<sub>2α</sub> increasingly elevated the levels of expressible milt in the male parr (Fig. 4A). PGF<sub>1α</sub> and PGF<sub>2α</sub> had similar effects on the quantities of expressible milt produced by the male parr throughout the study period.

During the first sampling date in October, plasma 17,20βP concentrations in male salmon parr were unaffected by exposure to either PGF (Fig. 4B). Thereafter, there was an increase in the effect of PGF<sub>2α</sub> on the levels of plasma 17,20βP

Table 1. Cross-adaptation experiment

PGF <sub>1α</sub>				PGF <sub>2α</sub>			
PGF <sub>2α</sub>		10 <sup>-5</sup> mol l <sup>-1</sup> L-serine		PGF <sub>1α</sub>		10 <sup>-5</sup> mol l <sup>-1</sup> L-serine	
Before	During	Before	During	Before	During	Before	During
1.9±0.08	0*	0.93±0.06	0.90±0.07	1.96±0.065	0*	0.85±0.12	0.89±0.09

Electro-olfactogram responses (mV ± S.E.M.; N=?) elicited from the olfactory epithelium before and during adaptation to 10<sup>-7</sup> mol l<sup>-1</sup> PGF<sub>1α</sub> or PGF<sub>2α</sub>.

Before and during responses were compared using paired t-tests; \*P<0.01.

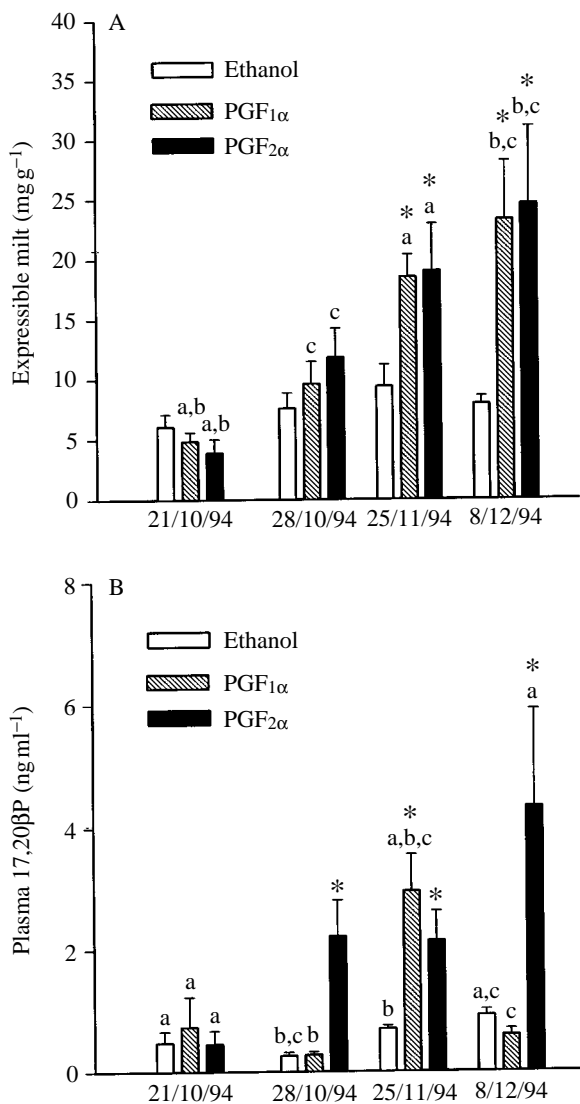


Fig. 4. Levels of expressible milt (A) and plasma 17,20βP (B) concentrations in mature male salmon parr 3 h after exposure to various test solutions on various dates in the reproductive season.  $N=5$  for each observation. Asterisks represent statistically significant differences ( $P<0.05$ ) between PGF-exposed males and ethanol-treated control males on each sampling date. For within-treatment comparisons, values on various sampling dates with the same letter are significantly different ( $P<0.05$ ) from each other. Values are means + S.E.M.

in fish with time. During the latter part of October and during November and December, PGF<sub>2α</sub> significantly elevated levels of plasma 17,20βP. However, there was a significant differential response in fish exposed to PGF<sub>1α</sub> compared with PGF<sub>2α</sub> during the study period. Only on the November sampling date did exposure to PGF<sub>1α</sub> significantly elevate the levels of plasma 17,20βP in mature male salmon parr (Fig. 4B).

The levels of plasma testosterone and 11-ketotestosterone in male parr after exposure to waterborne PGFs are shown in Fig. 5A,B. There was a significant seasonal increase in the

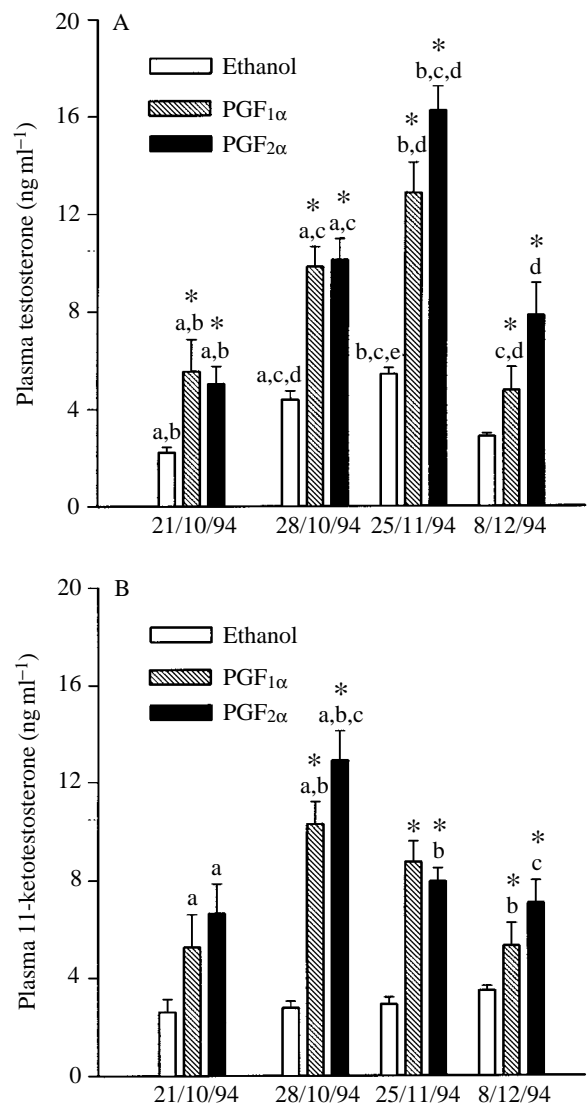


Fig. 5. Plasma testosterone (A) and 11-ketotestosterone (B) concentrations in mature male salmon parr 3 h after exposure to various test solutions on various dates in the reproductive season.  $N=5$  for each observation. Asterisks represent statistically significant differences ( $P<0.05$ ) between PGF-exposed males and ethanol-treated control males on each sampling date. For within-treatment comparisons, values on various sampling dates with the same letter are significantly different ( $P<0.05$ ) from each other. Values are means + S.E.M.

levels of plasma testosterone in fish after exposure to both PGFs when compared with the control groups (Fig. 5A). Plasma testosterone was maximally stimulated in the male fish during November, although levels were still significantly elevated during December. Plasma testosterone concentrations in control fish showed a similar seasonal trend. Both PGFs tested were equally stimulatory to male salmon parr throughout the sampling period. Plasma 11-ketotestosterone concentrations were also elevated by both PGFs throughout the study period except on the initial sampling date in October (Fig. 5B). Unlike plasma testosterone, there was no seasonal

Table 2. The urinary concentrations of testosterone, 17,20 $\beta$ P, androstenedione and immunoreactive PGFs and their sulphated (-S) and glucuronidated (-G) conjugates

	Male urine	Non-ovulated female urine	Ovulated female urine
Testosterone	3.6 $\pm$ 0.2 <sup>a</sup>	3.6 $\pm$ 0.2 <sup>a</sup>	3.7 $\pm$ 0.2 <sup>a</sup>
Testosterone-S	5.9 $\pm$ 0.2 <sup>a</sup>	6.0 $\pm$ 0.4 <sup>a</sup>	8.3 $\pm$ 2.0 <sup>a</sup>
Testosterone-G	12.8 $\pm$ 2.8 <sup>a</sup>	1.8 $\pm$ 0.5 <sup>b</sup>	136.6 $\pm$ 15.4 <sup>c</sup>
17,20 $\beta$ P	1.8 $\pm$ 0.1 <sup>a</sup>	1.8 $\pm$ 0.04 <sup>a</sup>	3.4 $\pm$ 0.6 <sup>b</sup>
17,20 $\beta$ P-S	9.7 $\pm$ 1.3 <sup>a</sup>	1.0 $\pm$ 0.6 <sup>b</sup>	119.9 $\pm$ 1.8 <sup>c</sup>
17,20 $\beta$ P-G	1.7 $\pm$ 0.6 <sup>a</sup>	0.2 $\pm$ 0.04 <sup>b</sup>	8.5 $\pm$ 1.5 <sup>c</sup>
Immunoreactive PGFs	0.7 $\pm$ 0.1 <sup>a</sup>	0.8 $\pm$ 0.2 <sup>a</sup>	18.2 $\pm$ 2.2 <sup>b</sup>
Androstenedione	ND	ND	ND

Concentrations in urine are expressed as ng ml<sup>-1</sup> urine.

Data represent the mean  $\pm$  S.E.M. of five fish per group; ND, not detectable.

For each compound, urine concentrations with the same letter are not significantly different from each other.

increase in plasma 11-ketotestosterone concentrations in control fish (Fig. 5B). The responses to both PGFs were similar throughout the study, again indicating that there was no differences in the priming effect on male parr between PGF<sub>1 $\alpha$</sub>  and PGF<sub>2 $\alpha$</sub> . The responses were significantly larger in the later October sampling point compared with earlier in October and in December (Fig. 5B).

#### Experiment 4: the steroid and immunoreactive PGF content of female and male salmon urine

Table 2 shows the steroidal and irPGF contents of female and male salmon urines. There was no marked difference in the urinary steroid and irPGF contents of male and non-ovulated females for most of the compounds measured. Urine of ovulated females, however, was characterised as having markedly increased concentrations of testosterone glucuronide, 17,20 $\beta$ P sulphate and irPGFs (greater than 10-fold increases for all three compounds) and of 17,20 $\beta$ P glucuronide.

## Discussion

### PGFs as priming pheromones in salmon

The results of the present study support the hypothesis that not only are the PGFs (PGF<sub>1 $\alpha$</sub>  and PGF<sub>2 $\alpha$</sub> ) potent odorants in mature male salmon parr but they also have a priming effect on these fish, elevating levels of plasma 11-ketotestosterone, testosterone and 17,20 $\beta$ P as well as the levels of expressible milt. Moreover, significant quantities of irPGFs, probably PGF<sub>1 $\alpha$</sub>  and PGF<sub>2 $\alpha$</sub>  judging from the antibody specificities, are excreted to the environment in the urine of ovulated female salmon. Therefore, on the basis of the present results, we suggest that PGFs function as priming pheromones in male Atlantic salmon, increasing their physiological readiness prior to spawning. The priming effect of PGF<sub>1 $\alpha$</sub>  and PGF<sub>2 $\alpha$</sub>  on the plasma levels of reproductive steroids and milt in male fish is similar to the phenomena previously reported in rainbow trout

*Oncorhynchus mykiss* (Scott *et al.* 1994) and Atlantic salmon (Moore and Waring, 1996; Waring *et al.* 1996) after exposure of male fish to ovulated female urine. In both instances, the authors suggested that the effect on the male hormone system was in response to one or more priming pheromones present in the urine of ovulated females. Therefore, we suggest that in the case of the Atlantic salmon, on the basis of the present data, the priming pheromones present in the female urine are likely to be PGF-like compounds. Although ovulated female salmon also release significant quantities of testosterone glucuronide, 17,20 $\beta$ P glucuronide and 17,20 $\beta$ P-S, these compounds are either not detected by male parr olfactory epithelium (testosterone glucuronide and 17,20 $\beta$ P glucuronide: Moore and Scott, 1991, 1992) or have a weak and inconsistent priming-like effect on male parr physiology (17,20 $\beta$ P-S; Waring *et al.* 1996).

Scott *et al.* (1994) also demonstrated that female rainbow trout release at least one priming pheromone in their urine which elevates plasma GtH II, testosterone and 17,20 $\beta$ P concentrations in mature males, and chemical cues emitted by mature females have been shown to be attractive to conspecific mature males (Honda, 1980, 1982) and may also be important mediators of male spawning readiness (Olsén and Liley, 1993; Liley *et al.* 1993). However, the identity of the priming pheromones in female rainbow trout urine has yet to be ascertained, although a previous study suggests that the pheromones are unlikely to be PGFs. E. L. M. Vermeirssen and A. P. Scott (unpublished observations) were unable to demonstrate a priming effect of prostaglandins on male reproductive physiology.

Whilst a number of other species of fish have been shown to have acute olfactory sensitivity to PGFs (Cardwell *et al.* 1991, 1992, 1995; Sorensen *et al.* 1991a,b), in only two other species, the goldfish (*Carassius auratus*; Sorensen *et al.* 1988) and the loach (*Misgurnus anguillicaudatus*; Kitamura *et al.* 1994), have PGFs been shown to function as sex pheromones. On the basis of the results from the present study, it is reasonable to conclude that PGF<sub>1 $\alpha$</sub>  and PGF<sub>2 $\alpha$</sub>  are also both sex pheromones in that they meet the criteria for a pheromone as described by Karlson and Luscher (1959). Evidence indicating a possible pathway of release by the female to the environment, acute sensitivity of the olfactory epithelium and corresponding physiological responses in the male fish to PGFs indicate a pheromonal role.

In goldfish, the levels of plasma PGF<sub>2 $\alpha$</sub>  increase during ovulation, and PGF<sub>2 $\alpha$</sub>  acts endogenously on the brain to stimulate spawning behaviour (Stacey, 1987). An increase in PGF production also occurs at the time of ovulation (Sorensen and Goetz, 1993). Ovulated female goldfish also release a mixture of PGFs and their metabolites to the environment, where they function exogenously as pheromones releasing male courtship behaviour (for a review, see Sorensen and Goetz, 1993). The endogenous function of PGFs in Atlantic salmon is not clear, although in another salmonid, *Salvelinus fontinalis*, the production of PGF has also been related to ovulation (Goetz *et al.* 1989). The exogenous role of PGFs in

Atlantic salmon appears to differ from that in both goldfish (Stacey *et al.* 1986) and loach (Kitamura *et al.* 1994), where waterborne PGFs function as releaser pheromones, rapidly stimulating male courtship behaviour. The present study demonstrates that in the Atlantic salmon PGFs have a priming effect. Preliminary behavioural studies have not yet indicated a behavioural response of mature male salmon parr to waterborne PGFs (A. Moore and C. P. Waring, unpublished observations) or even the presence of a releaser pheromone in female urine capable of stimulating male sexual behaviour. Although the urine from an ovulated female is a potent attractant to the male salmon (Moore and Scott, 1992), in the absence of other relevant reproductive cues (visual, chemical and mechanosensory), urine alone may not be sufficient to release full male sexual behaviour. There are also differences between salmonid species in relation to the exogenous role of PGFs. Sveinsson and Hara (1995) demonstrated that during spawning in Arctic charr (*Salvelinus alpinus*) it is mature male fish that released irPGFs, which attract the females and stimulate their spawning behaviour. In Atlantic salmon, however, males primed with female urine do not significantly increase their urinary excretion of irPGFs (C. P. Waring and A. Moore, unpublished observations).

#### *Changes in sensitivity to PGFs with time*

Although there was a clear increase in sensitivity to PGFs as the reproductive season progressed in terms of both the levels of expressible milt and the EOG response of the olfactory epithelium to waterborne PGFs, this was less clear-cut for the plasma endocrine data. Plasma 17,20 $\beta$ P concentrations responded much more strongly in November and December, whereas the responses of the two androgen hormones measured were evident earlier in the season, and in the case of testosterone this occurred during the first sampling date in October, and showed a peak in response later in October and November; however, the response was more attenuated in December. Although the reason for these differential patterns in the gonadal steroid responses are not known, they do mirror the shift in endogenous steroid production by the testes from predominantly C-19 androgen production to C-21 progestogen production as the spawning season progresses (Sakai *et al.* 1989; Scott and Sumpter, 1989; Barry *et al.* 1990; Abdullah and Kime, 1994).

The temporal changes in olfactory sensitivity of male salmon parr to both PGF<sub>1 $\alpha$</sub>  and PGF<sub>2 $\alpha$</sub>  are similar to those previously reported for testosterone (Moore and Scott, 1991). This suggests that sensitivity to pheromones and other olfactory odorants may be strongly dependent upon the maturation stage of the fish and possibly the particular period in the spawning season. The mechanism controlling the changes in peripheral sensitivity to steroids and PGFs is unknown, although it is probably under the control of the endocrine system(s) of the fish. It may possibly involve the reorientation of previously 'hidden' receptor sites, so that they become available to bind ligands, or conformational changes in receptor proteins in the membrane (Hara, 1994).

Alternatively, it may involve the seasonal development of discrete populations of olfactory receptors specifically to bind relevant pheromones when they become available.

It appears that the responses to both PGF<sub>1 $\alpha$</sub>  and PGF<sub>2 $\alpha$</sub>  are mediated by the same olfactory receptor. This is similar to the situation found in the goldfish (Sorensen *et al.* 1988, 1991a,b) and in two species of sucker (Sorensen *et al.* 1991a,b), where responses to PGF<sub>2 $\alpha$</sub> , 15-ketoPGF<sub>2 $\alpha$</sub>  and 13,14-dihydro-15-ketoPGF<sub>2 $\alpha$</sub>  are also mediated by the same olfactory receptor. However, in the present study, it is not clear why, if the two F-series prostaglandins share a similar receptor, there should be seasonal differences in the sensitivity to these two compounds. Further cross-adaptation studies throughout the reproductive season would be necessary to begin to explain this anomaly.

A number of other studies have also demonstrated the effects of maturity on olfactory sensitivity to sex pheromones (Sorensen *et al.* 1987, 1990; Sorensen and Goetz, 1993; Bjerselius and Olsén, 1993; Irvine and Sorensen, 1993). Resink *et al.* (1989) demonstrated that the behavioural responses of female African catfish *Clarias gariepinus* to steroid glucuronides was dependent on ovulatory state, and Colombo *et al.* (1980) showed that only black gobies *Gobius jazo* containing ovulated eggs showed a behavioural response to a steroid glucuronide. More recently, Cardwell *et al.* (1995) demonstrated that the hormonal state of male *Puntius schwanenfeldi* can influence their olfactory response to a PGF.

The mechanism controlling the changes in peripheral sensitivity to sex pheromones in salmon may be similar to, or have parallels with, the mechanisms involved in imprinting during smoltification in juvenile salmonids. Nevitt *et al.* (1994) produced evidence for a peripheral olfactory memory in imprinted salmon. The authors suggested that thyroid hormones might directly alter olfactory receptor cell sensitivity to odors present in the home stream. A similar mechanism operating during maturation in Atlantic salmon and controlled by circulating reproductive hormones may explain the windows of sensitivity of this species to certain steroids and sex pheromones. Recently, Nevitt *et al.* (1995) also showed a gonadotrophin-releasing hormone-immunoreactive cluster of neurones in the peripheral olfactory system of Atlantic salmon that may play a role in modulating receptor cell activity during reproduction.

There is increasing evidence that the Atlantic salmon pheromone system may differ significantly from the dual pheromone system proposed in the goldfish (Sorensen and Stacey, 1990). For instance, the pre-ovulatory pheromone in goldfish, 17,20 $\beta$ P (Sorensen *et al.* 1987), is not detected by the olfactory epithelium of mature male salmon (Moore and Scott, 1991) and the post-ovulatory pheromone in goldfish, PGFs and their metabolites (for a review, see Sorensen, 1992), function as releaser pheromones not as a priming pheromone as they do in Atlantic salmon. The Atlantic salmon may, therefore, offer a suitable comparative model to goldfish which may increase our understanding of the evolution of the hormonal pheromone system in diverse ecological habitats.



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