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Sublethal effects of the pesticide Diazinon on olfactory function in mature male Atlantic salmon parr

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Diazinon, an organophosphate pesticide, had a sublethal effect on the olfactory system of mature male Atlantic salmon parr. The olfactory responses of the parr to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were studied after exposure of the epithelium to different concentrations of Diazinon in water. Electrophysiological recordings from the epithelium indicated that the responses to this prostaglandin were significantly reduced at nominal concentrations as low as 1·0 $\mu g\ l^{-1}$ and the threshold of detection was reduced 10-fold at 2·0 $\mu g\ l^{-1}$. Mature male salmon parr exposed for a period of 120 h to Diazinon (nominal concentrations 0·3, 0·8, 1·7, 2·7, 5·6, 13, 28 and 45 $\mu g\ l^{-1}$) also had significantly reduced levels of the reproductive steroids, 17,20β-dihydroxy-4-pregnen-3-one, testosterone and gonadotrophin II in the blood plasma after priming with ovulated female salmon urine. Both prostaglandin $F_{2\alpha}$ and ovulated female urine are known to have important roles in synchronizing reproductive physiology and behaviour in salmonids as well as other fish species. The results are therefore discussed in relation to the possible sublethal effects of Diazinon on reproduction in the Atlantic salmon and possible effects on populations of salmonids.

Key words: *Salmo salar*; Diazinon; toxicity; pesticide; olfaction; pheromones.

INTRODUCTION

In the U.K. there is increasing concern over the agricultural use of organophosphate pesticides and their subsequent fate within the aquatic environment. Of particular concern are the effects upon the biota of inland waters and the potential effects upon fish populations and the fisheries dependent upon them. One organophosphate pesticide that is widely used in the U.K. is Diazinon (*O*,*O*-diethyl *O*-2-isopropyl-6-methylpyrimidin-4-yl phosphorothiate; IUPAC), which is considered to be toxic to fish. Diazinon is one of the active ingredients used in dips to prevent and treat ticks, lice and scab on sheep. The main sheep dipping areas occur in upland regions and dipping often occurs close to rivers and tributaries supporting populations of Atlantic salmon *Salmo salar* L. and sea trout *S. trutta* L. In certain areas Diazinon often persists at low levels (3–68 ng l^{-1}), but high transient peaks (3·98–200 $\mu g\ l^{-1}$), have been recorded in certain watercourses (Virtue & Church, 1993).

Diazinon is an inhibitor of acetylcholinesterase (AChE), the enzyme that hydrolyses the neurotransmitter acetylcholine. The effects on fish populations may either be acute, resulting in death, or chronic where the effects may be longer term and more difficult to quantify. Sublethal effects of Diazinon on

fish have been shown to include vertebral malformation (Nishiuchi, 1971), alterations of blood constituents (Anees, 1978), impaired reproduction (Goodman *et al.*, 1979), inhibition of AChE activity (Goodman *et al.*, 1979; Keizer *et al.*, 1991), reduced larval growth (Seikai, 1982), reduced adult growth (Chatterjee & Konar, 1984), reduction of liver DNA, RNA and protein content (Ansari & Kumar, 1988), impaired swimming and changes in pigment levels (Alam & Maughan, 1992), ultrastructural changes in muscle (Sakr & Gabr, 1992) and structural changes to gills (Dutta *et al.*, 1993).

One aspect that has not been studied previously is the possible sublethal effects of Diazinon on the teleost olfactory system. As a result of the geographical and seasonal use of Diazinon and its occurrence in salmonid rivers, there is concern that the pesticide may constitute a potential hazard to the Atlantic salmon. Recently, it has been demonstrated that low pH water has a deleterious effect on the Atlantic salmon's ability to detect important odorants and pheromones which may be important mediators of reproductive physiology and behaviour (Moore, 1994).

This study looked at two different aspects relating to the possible sublethal effect of Diazinon on the Atlantic salmon olfactory system. First, an electrophysiological study was carried out to investigate its effects on the olfactory responses of mature male Atlantic salmon parr to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). $PGF_{2\alpha}$ is a potent odorant in mature male parr (Moore & Scott, 1992), as well as other teleosts, where it has a role in synchronizing spawning physiology and behaviour between males and females (Sorensen, 1992; Sorensen & Goetz, 1993). More recently $PGF_{2\alpha}$ has also been shown to have a priming effect on plasma steroid and gonadotrophin levels in mature male salmon parr (Moore & Waring, 1995). Second, an endocrinological study was carried out to investigate the effects of Diazinon on the levels of plasma reproductive steroids and gonadotrophin in mature male parr after exposure to the urine of ovulated female Atlantic salmon. It has been shown recently that urine, detected via the olfactory system, has an important role in salmonid reproduction. For instance, the urine of reproductively mature female rainbow trout *Oncorhynchus mykiss* (Walbaum) contains a priming pheromone which increased the levels of 17,20β-dihydroxy-4-pregnen-3-one (17,20βP), testosterone and gonadotrophin II (GtH II) in the blood plasma of reproductively mature male rainbow trout (Scott *et al.*, 1994). Ovulated female salmon urine is also a potent attractant to mature male parr (Moore & Scott, 1992), eliciting strong rheotactic behaviour in fish similar to that described for testosterone (Moore, 1991). In addition, electrophysiological studies on mature male Atlantic salmon parr have demonstrated that to detect 17,20β-dihydroxy-4-pregnen-3-one 20-sulphate (17,20β-P-sulphate; a conjugate of the oocyte-maturation-inducing steriod in salmonids), the olfactory receptors of the male must be pre-exposed first to the urine of ovulated female Atlantic salmon (Moore & Scott, 1992).

This paper reports the results of a study to ascertain the possible sublethal effects on olfactory mediated aspects of reproduction in mature male Atlantic salmon parr exposed to Diazinon. Although the concentrations of Diazinon which are toxic to Atlantic salmon are not known, data from other salmonid species suggest that the 96 h LC_{50} varies from 100–1700 $\mu g\ l^{-1}$ depending upon species and water chemistry (Eisler, 1986). The choice of Diazinon

concentration during this study (0·3–45 μg l^{-1}) was based on a limited number of sampling studies in river catchments containing salmonids and reflects environmental levels of the pesticide present in water courses (National Rivers Authority, National Centre for Toxic and Persistent Substances, Peterborough, U.K.). The results are discussed in relation to reproduction in Atlantic salmon and the longer term effects on salmonid stocks and the fisheries dependent upon them.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Atlantic salmon parr (98–176 mm in length, 1+ in age) 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 fish were maintained under natural light conditions in 1000-l tanks with a constant flow of aerated, dechlorinated water (85 l min^{-1}). Water temperatures during the period ranged between 5·5 and 15·6° C; pH 7·5; alkalinity 156 mg HCO_3 l^{-1}; total calcium 166 mg l^{-1}; total hardness 405 mg l^{-1} as $CaCO_3$; aluminium <10–32 μg l^{-1}; sodium 37·3 mg l^{-1}; magnesium 12 mg l^{-1}; NO_3 0·2–49·1 mg l^{-1}; SO_4 10 μg l^{-1}. Fish were fed to satiation with commercial salmon pellets.

Mature male parr were studied between October and December 1994 (the water temperature during this period ranged between 7·5–10·7° C). After each experiment (4–5 h in duration) the fish was killed, sexed and its gonadosomatic index (GSI) calculated. Most of the parr tested were spermiating (i.e. they had free running milt) and had GSI of 7·57 ± 0·37% (mean ± S.E.M.; n=14).

ELECTROPHYSIOLOGICAL STUDIES

The study used the same electrophysiological technique (electro-olfactogram: EOG) as that in previous studies on mature male Atlantic salmon parr (Moore & Scott, 1991, 1992; Moore, 1994; Moore *et al.*, 1994). EOG recording measures trans-epithelial voltage gradients from the surface of the olfactory epithelium and is considered to reflect multi-unit cell activity (Evans & Hara, 1985). The fish were anaesthetized with 2-phenoxyethanol (0·4 ml l^{-1}) and the skin and the cartilage removed to expose the olfactory rosettes. The fish were then immobilized with an intramuscular injection of gallamine triethiodide (0·3 mg kg^{-1} of 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^{-1}). Paired silver electrodes were attached subcutaneously to the fish to monitor the heart rate and level of anaesthesia during each experiment. The output was displayed continuously on an oscilloscope (Textronic 465 B). This also provided an indication of the stability and health of the preparation. Electrophysiological recordings were made by using glass pipettes filled with saline–agar (2%) bridged to an Ag–AgCl electrode (Type EH-3MS, Clark Electromedical Instruments) filled with 3 M KCl. The tip of the electrode (90–100 μm) was placed close to the olfactory epithelium at the base of the largest posterior lamella. This was where the maximum response to 10^{-5} M L-serine (mean 0·95 ± 0·07 mV), and minimum responses to dechlorinated water (mean 0·08 ± 0·01 mV) were obtained. 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 DC preamplifier (Digitimer Ltd) and either displayed directly on a pen recorder (Lectromed MX 212) or digitized and stored for later analysis on an Apricot XEN-PC computer by using Asystant+scientific software (Asyst Inc.). Previous studies using the same electrophysiological technique have indicated that there were no changes in responsiveness to odorants by the parr during the duration of the experiments (5–6 h) (Moore & Scott, 1991, 1992; Moore, 1994; Moore *et al.*, 1994).

TESTING PROCEDURE

Serial dilutions of $PGF_{2\alpha}$ (Sigma Chemicals), ranging from 10^{-6}–10^{-9} M were prepared from a stock solution consisting of 500 μg ml^{-1} of 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 until required (room temperature 7·5–10·7° C). Control dilutions of ethanol were also prepared and tested. A stock solution of 10^{-5} M L-serine in dechlorinated water was also prepared weekly. Diazinon (Greyhound Chromatography and Allied Chemicals) was prepared from a stock solution of 200 mg l^{-1} of absolute ethanol, and stored in amber glass bottles. The stock solution was prepared fresh prior to each experiment.

The responses of the olfactory epithelium of mature male salmon parr to a 10^{-9} M concentration of $PGF_{2\alpha}$ ($n=6$) were recorded after perfusion of the olfactory rosette with different concentrations of the pesticide Diazinon. At the start and end of each EOG experiment the responses to 10^{-5} M L-serine, ethanol and water control were tested. The olfactory epithelium was perfused with control water (no Diazinon) for 30 min. A constant volume of 10^{-9} M $PGF_{2\alpha}$ (50 μl) was then injected, via a remote-control switch, into the second inlet of a three-way solenoid valve (Lee Company) carrying a constant flow of the water (3 ml min^{-1}) over the olfactory epithelium, and the EOG response recorded. The stimulus lasted 2·5 s and the flow rate was unaltered by the addition of the test substance. Without significantly altering the flow rate, the control water was switched to water containing Diazinon at a concentration of 0·1 μg l^{-1}, and the olfactory epithelium perfused for a further 30 min. At the end of this period another constant volume of 10^{-9} M $PGF_{2\alpha}$ was injected and the EOG response again recorded. Similar recordings were made after perfusion of the epithelium with Diazinon at concentrations of 1·0, 2·0, 5·0, 10·0 and 20·0 μg l^{-1}. Water samples were collected at the end of the experiment and stored in 1-l amber glass bottles at 5° C in the dark until analysed for Diazinon content (7 days later).

Dose–response studies were carried out using serial dilutions of $PGF_{2\alpha}$ and water containing a range of Diazinon concentrations (0·1, 1·0, 2·0, 5·0 and 10·0 μg l^{-1}). At the start of each EOG experiment the $PGF_{2\alpha}$ was presented to the olfactory epithelium in order of increasing concentration with a 2-min recovery interval between each stimulus. The responses to 10^{-5} M L-serine, ethanol and water control were tested at the beginning and end of each dilution. Initially the olfactory rosettes were perfused with control water for 30 min before serial dilutions of $PGF_{2\alpha}$ (10^{-12}–10^{-7} M) were presented to the olfactory epithelium and the EOG responses recorded. The olfactory epithelium was then perfused with a solution of Diazinon (0·1 μg l^{-1}) for 30 min and serial dilutions of $PGF_{2\alpha}$ were again presented. This procedure was repeated at Diazinon concentrations of 1·0, 2·0, 5·0 and 10·0 μg l^{-1}.

The recovery time of the olfactory epithelium of three salmon parr after perfusion with water containing Diazinon (concentration 1·0 μg l^{-1}) was studied. The response of the olfactory epithelium to a 10^{-9} M concentration of $PGF_{2\alpha}$, was recorded after perfusion with control water for 30 min. The olfactory epithelium was then perfused with water containing Diazinon (concentration 1·0 μg l^{-1}) for 30 min. The response of the epithelium to the $PGF_{2\alpha}$ was then recorded every 10 min thereafter for a period of 270 min. The responses to 10^{-5} M L-serine, ethanol and water control were tested at the beginning and end of each experiment. The recorded responses were expressed as a percentage of the initial response recorded to the $PGF_{2\alpha}$ after perfusion with the control water.

Control experiments were carried our prior to the dose–response studies to examine the effects of repeated exposure of the olfactory epithelia to $PGF_{2\alpha}$. Three mature male parr were exposed to a 10^{-8} M concentration of $PGF_{2\alpha}$ every 2 min for 120 min. The responses to 10^{-5} M L-serine, ethanol and water control were tested at the beginning and end of the period. The amplitude of each EOG response was expressed as a percentage response of the initial L-serine standard.

DATA ANALYSIS

The amplitude of each EOG response was measured from the baseline to the peak of each phasic displacement and expressed in mV. Any replicates were averaged and the

TABLE I. Nominal and measured concentrations of diazinon in the electrophysiological and endocrinological experiments

	Nominal ($\mu g\ l^{-1}$)	Measured ($\mu g\ l^{-1}$)	% of nominal
Electrophysiology experiment			
Control	0	0	0
	0·1	0·22	220
	1	0·4	40
	2	0·83	41·5
	5	1·35	27
	10	3·45	34·5
	20	2·61	13·05
Endocrine experiment			
Control	0	0·01	0
	0·3	0·06	20
	0·8	0·26	32·5
	1·7	0·49	28·8
	2·7	1·42	52·6
	5·6	3·12	55·7
	13	4·82	37
	28	7·52	26·8
	45	15·22	33·8

values 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 epithelium to prostaglandins (Moore & Scott, 1992; Bjerselius & Olsen, 1993). In all experiments the EOG responses recorded at each concentration of Diazinon were compared to the responses recorded to the relevant control water taken from the inlet of the salmon tank, using a paired two sample for means *t*-test. The threshold value of $PGF_{2\alpha}$ at each concentration of Diazinon was estimated as the lowest dose whose 95% confidence interval did not include 0. All values in the text and figures are expressed as the arithmetic means ± S.E.M. EOG responses to $PGF_{2\alpha}$ were similar in shape to those described in other studies (Moore & Scott, 1991, 1992; Bjerselius & Olsen, 1993).

EFFECT OF DIAZINON ON RESPONSIVENESS TO URINE FROM OVULATED FEMALES

At the end of October 1994, spermiating male parr [length 129·9 ± 1·5 mm, weight 23·5 ± 0·8 g, GSI 7·4 ± 0·2% (mean ± S.E.M.)] were removed from the stock tanks and transferred into 63-l glass tanks in a separate aquarium system. Each tank had a constant flow of aerated dechlorinated tap water ($1\ l\ min^{-1}$) with no recirculation. A natural photoperiod was followed and the water temperature was 13 ± 1° C. Males were stripped of milt gently (see below) and groups of five were placed into each tank and left to recover for 72 h without feeding.

Stock solutions of Diazinon were produced by dissolving in industrial methylated spirit (IMS) and stored in amber glass bottles. The nominal concentrations aimed for during the study are shown in Table I. Diazinon was added to the tanks via a multichannel peristaltic pump (Technicon) and silicon tubing (Altec) and vigorously mixed by aeration. Stock solutions were renewed every 12 h. In addition to the treated tanks there were three additional treatment groups: (1) untreated salmon parr (no urine); (2) salmon parr exposed to female urine (urine) and (3) salmon parr exposed to female urine+IMS (IMS).

After 120 h, 630 µl of freshly-thawed urine pooled from several ovulated female Atlantic salmon from the National Rivers Authority, Kielder hatchery (see Moore & Scott, 1992 for details) was squirted into each tank to give a final dilution of one part in 10^5. An equivalent volume of water was squirted into the tank containing the non-stimulated control fish. To minimize disturbance to the fish, squirting was carried out using a 1-ml plastic syringe with a 10-cm long plastic straw fitted so that the operators' hands would not come into contact with the water and the mesh lids would not have to be removed from the tanks.

After 3-h exposure to ovulated female salmon urine, the males were anaesthetized in 0·4 ml l^{-1} 2-phenoxyethanol and blotted lightly with tissue paper to remove water from the urinogenital area. The males were held belly down and the abdomen was stroked, applying slight pressure, in an anterior to posterior direction towards the gonadal papilla. The milt expressed was collected in tared weigh boats. The males were then pithed, measured, weighed, and blood sampled from the caudal vessels using heparinized syringes fitted with 23-G needles. Blood was centrifuged for 10 min at 2000 ***g*** and the plasma was stored at −20° C. Water samples were collected at the end of the experiment and stored in 1-l amber glass bottles at 5° C in the dark until analysed for Diazinon content (14 days later).

Radioimmunoassays for 17,20βP, testosterone, and 11-ketotestosterone (11-KT) were carried out by the procedures of Scott *et al.* (1982) and Scott *et al.* (1984) on diethyl ether extracted plasma. The radioimmunoassay for GtH II was carried out on 50 µl plasma samples as described by Suzuki *et al.* (1988) and Swanson *et al.* (1989). Water Diazinon concentrations were measured by gas chromatography–mass spectrometry as described below.

DATA ANALYSIS

The levels of expressible milt and plasma levels of 17,20βP, testosterone, 11-KT and GtH-II (expressed as ng ml^{-1} of plasma) were analysed using a 1-way ANOVA. Where a significant difference was found to have occurred ($P<0·05$), individual groups were compared to the no urine control group (no urine) using a Student–Neuman–Keuls test.

Water Diazinon concentrations were measured by gas chromatography–mass spectrometry (GC/MS) at the MAFF, Burnham-on-Crouch Laboratory. Water samples (1 l) were extracted twice with 50 ml dichloromethane and the pooled solvent layer was dried over anhydrous sodium sulphate and reduced in volume under oxygen-free nitrogen using a Turbovap evaporator to approximately 5 ml. Prior to final analysis the dichloromethane was further reduced in volume and the solvent exchanged for iso-octane. The GC/MS pressure was 25 psi. Samples (1 µl) were injected into the GC in the splitless mode at an oven temperature of 90° C, the injection port temperature was 270° C, the transfer line was held at 275° C and the ion trap at 220° C. After injection the oven temperature was held at 90° C during the 2 min splitless period, then programmed to increase at 15° C min^{-1} to 165° C and then further at 5° C min^{-1} to a final temperature of 250° C. Electron impact spectra data were acquired across the mass range 50–350 amu. A three point external standard calibration curve was constructed and the ions 137, 179 and 304 summed and used for quantification.

RESULTS

WATER DIAZINON CONCENTRATIONS

The measured water Diazinon concentrations from the electrophysiological study were in the range 13–42% of the nominal concentrations with one anomalous reading of 225% possibly due to contamination during analysis (Table I). The measured concentrations in the water sampled from the urine priming study tanks ranged from 20–55% of the nominal concentrations aimed for (Table I). However, since these were terminal measurements and we have no

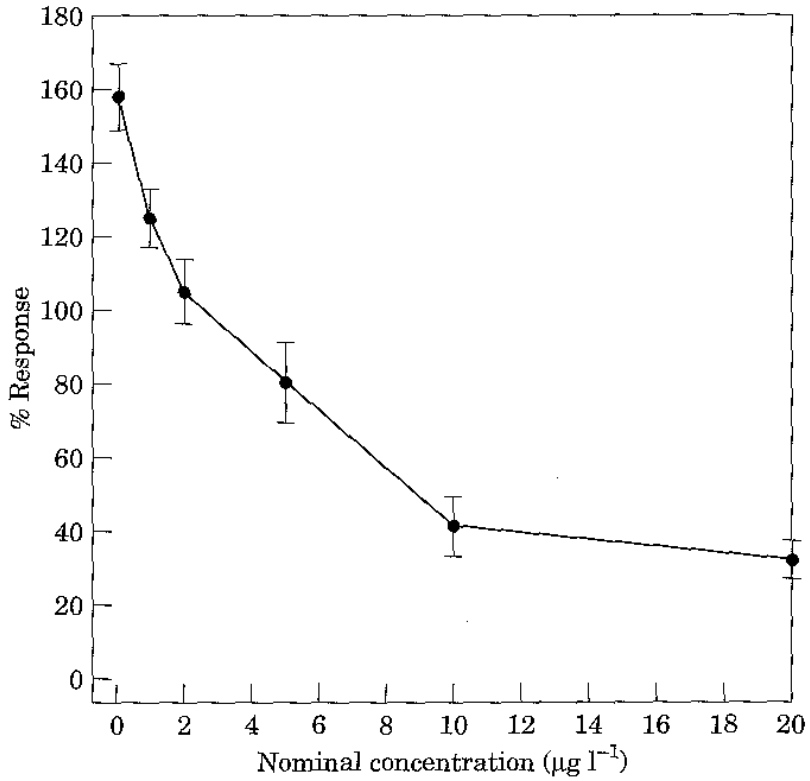

FIG. 1. EOG recordings from the olfactory epithelia of mature male Atlantic salmon parr to $PGF_{2\alpha}$ (concentrations 10^{-9} M; $n=6$), after perfusion of the olfactory epithelium with Diazinon (nominal concentrations 0·1, 1·0, 2·0, 5·0, 10·0 and 20 μg l^{-1}). The amplitude of each EOG response was measured from the baseline to the peak of each phasic displacement and expressed as a percentage of the response to a 10^{-5} M concentration of L-serine. Vertical bars represent standard deviations.

data regarding the rate of Diazinon degradation over the full 120-h period, the fish may have been exposed to water concentrations nearer those of the nominal levels earlier in the time-course. The water samples from both experiments suffered an unavoidable delay in being analysed, and although stored in the dark at a low temperature our data suggests a significant degree of degradation occurred before the GC/MS analysis was performed. Therefore we refer to the nominal concentrations in the rest of the text.

ELECTROPHYSIOLOGICAL STUDIES

Electrophysiological responses recorded from the olfactory epithelium of mature male Atlantic salmon parr to 10^{-9} M $PGF_{2\alpha}$ were significantly reduced after perfusion of the epithelium with nominal concentrations of Diazinon ranging from 1·0–20 μg l^{-1} (Fig. 1). Although at the highest concentration tested (20·0 μg l^{-1}), responses were still recorded from the epithelium the mean amplitudes were only 20·9 ± 3·9% compared to the control. Mean EOG recordings from the olfactory epithelium of the parr to each concentration of Diazinon were: control − 1·7 ± 0·88 mV; 0·1 μg l^{-1} − 1·7 ± 0·87 mV; 1·0 μg l^{-1} − 1·32 ± 0·72 mV; 2·0 μg l^{-1} − 1·11 ± 0·6 mV; 5·0 μg l^{-1} − 0·86 ± 0·46 mV; 10·0 μg l^{-1} − 0·45 ± 0·24 mV; 20·0 μg l^{-1} − 0·35 ± 0·18 mV. Diazinon did not produce a recordable EOG response from the olfactory epithelium of the parr or effect the shape of the recorded response to $PGF_{2\alpha}$ at the Diazinon concentrations tested.

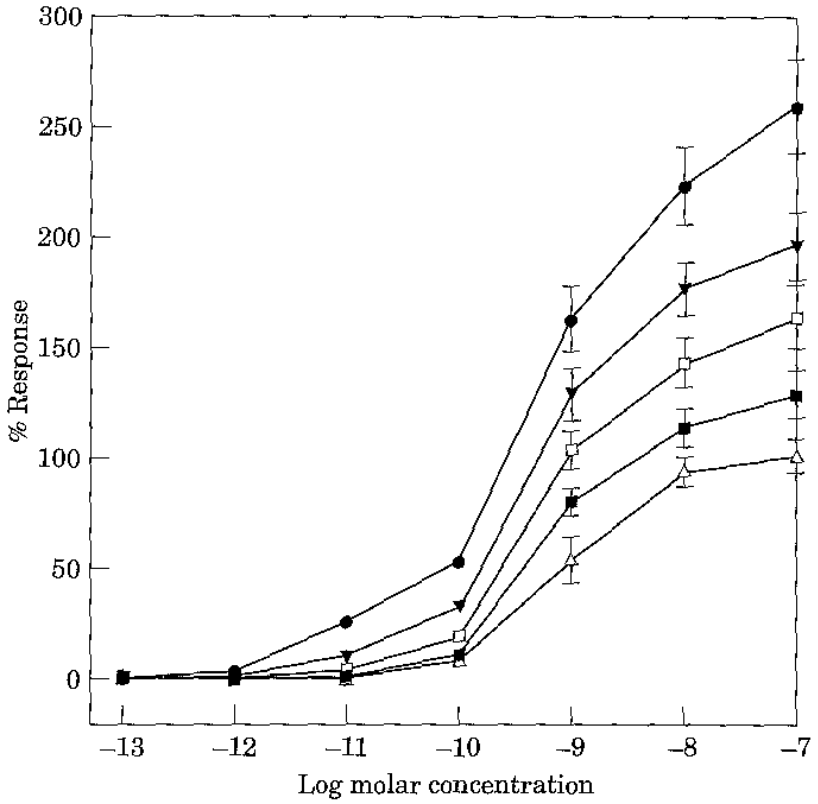

FIG. 2. Semi-logarithmic plots of the concentration response relationship in mature male Atlantic salmon parr to $PGF_{2\alpha}$ at control water (●), 1·0 $\mu g\,l^{-1}$ (▼), 2·0 $\mu g\,l^{-1}$ (□), 5·0 $\mu g\,l^{-1}$ (■) and 10 $\mu g\,l^{-1}$ (△) Diazinon. The responses to 0·1 $\mu g\,l^{-1}$ Diazinon were the same as the control and have not been included. The amplitude of each EOG response was measured from the baseline to the peak of each phasic displacement and expressed as a percentage of the initial response to a 10^{-5} M concentration of L-serine. Vertical bars represent standard deviations.

There was a similar significant decrease in the recorded response to the standard 10^{-5} M L-serine after exposure to Diazinon (range 1·0–20 $\mu g\,l^{-1}$). Mean EOG recordings from the olfactory epithelium of the parr before and after exposure to Diazinon were $0{\cdot}90 \pm 0{\cdot}07$ mV and $0{\cdot}26 \pm 0{\cdot}04$ mV respectively ($P<0{\cdot}01$; Student's t-test).

Concentration response studies also indicated that there was a significant reduction in the EOG recordings from the olfactory epithelium to all concentrations of $PGF_{2\alpha}$ after perfusion with Diazinon concentration of 1·0 $\mu g\,l^{-1}$ ($P<0{\cdot}01$); 2·0 $\mu g\,l^{-1}$ ($P<0{\cdot}01$); 5·0 $\mu g\,l^{-1}$ ($P<0{\cdot}001$) and 10·0 $\mu g\,l^{-1}$ ($P<0{\cdot}001$) (Fig. 2). There was a significant change in threshold concentration of $PGF_{2\alpha}$ in mature male parr at higher concentrations of Diazinon. After perfusion with concentrations >2·0 $\mu g\,l^{-1}$ there was a corresponding 10-fold decrease in the sensitivity of the olfactory epithelium to $PGF_{2\alpha}$.

Repeated exposure over a 120-min period of the olfactory epithelium to a 10^{-8} M concentration of $PGF_{2\alpha}$ did not significantly affect the amplitude of the recorded response. Recorded responses at the beginning and end of the study were $214 \pm 23{\cdot}4\%$ (mean ± S.E.M.) and $222 \pm 21{\cdot}6\%$ (mean ± S.E.M.) respectively of the response to a 10^{-5} M L-serine standard ($P>0{\cdot}05$; Student's t-test).

The olfactory epithelium showed only slight recovery after exposure to a 1·0 $\mu g\,l^{-1}$ nominal concentration of Diazinon. After Diazinon exposure the

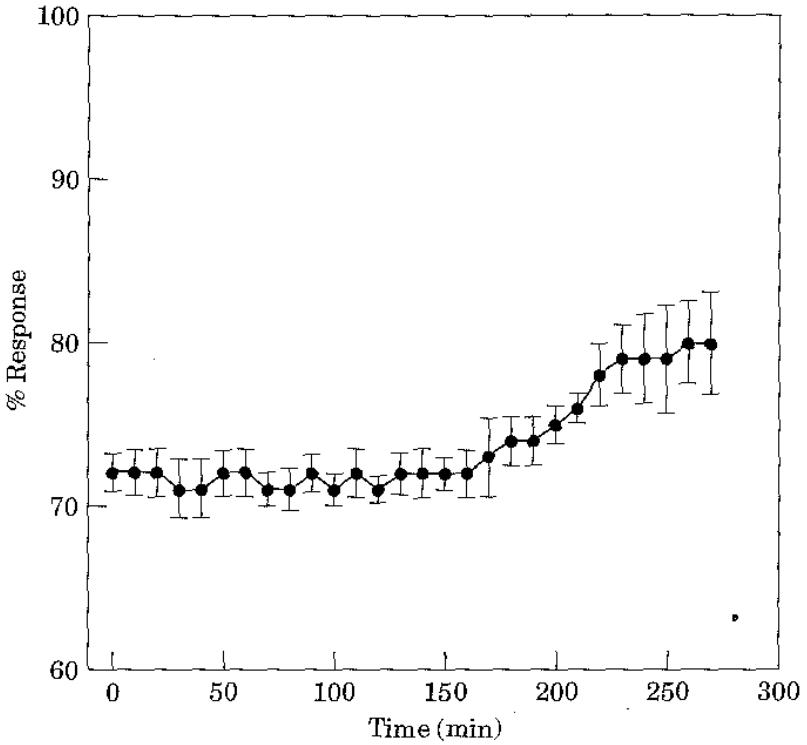

FIG. 3. Recovery time of the olfactory epithelium of mature male Atlantic salmon parr in response to $PGF_{2\alpha}$ (concentration 10^{-9} M; $n=3$), after perfusion of the olfactory epithelium for 30 min with 1·0 μg l^{-1} Diazinon. The responses are presented as a percentage response to the initial stimulus of $PGF_{2\alpha}$ after perfusion with control water (no Diazinon). During recovery the epithelium were stimulated at 10-min intervals.

recorded response to 10^{-9} M $PGF_{2\alpha}$ was still 80 ± 3·1% (mean ± S.E.M.) after 270 min in control water (Fig. 3).

ENDOCRINOLOGICAL STUDIES

17,20βP and GtH II

Exposure of mature male salmon parr to the urine from ovulated female salmon, elevated plasma levels of 17,20βP ($P<0·05$) significantly [Fig. 4(a)]. However, exposure of the male parr to nominal concentrations of Diazinon ranging from 0·3–45 μg l^{-1}, abolished this priming effect and there was no significant difference in the plasma level of 17,20βP when compared to fish not exposed to urine [Fig. 4(a)]. Similar results were obtained for plasma levels of GtH II although slightly higher nominal concentrations of Diazinon were required (0·8–45 μg l^{-1}) to abolish the priming effect of female salmon urine on plasma levels of GtH II [Fig. 4(b)].

Testosterone and 11-KT

Exposure of mature male salmon parr to the urine from ovulated female salmon, also elevated plasma levels of testosterone ($P<0·05$) [Fig. 5(a)] and 11-KT ($P<0·05$) significantly [Fig. 5(b)]. However, exposure to Diazinon did not reduce significantly the priming effect of female urine on plasma levels of testosterone and 11-KT at certain concentration. Exposure to nominal concentrations of 0·3, 0·7, 2·7, 5·6 and 13 μg l^{-1} had no significant effect on the

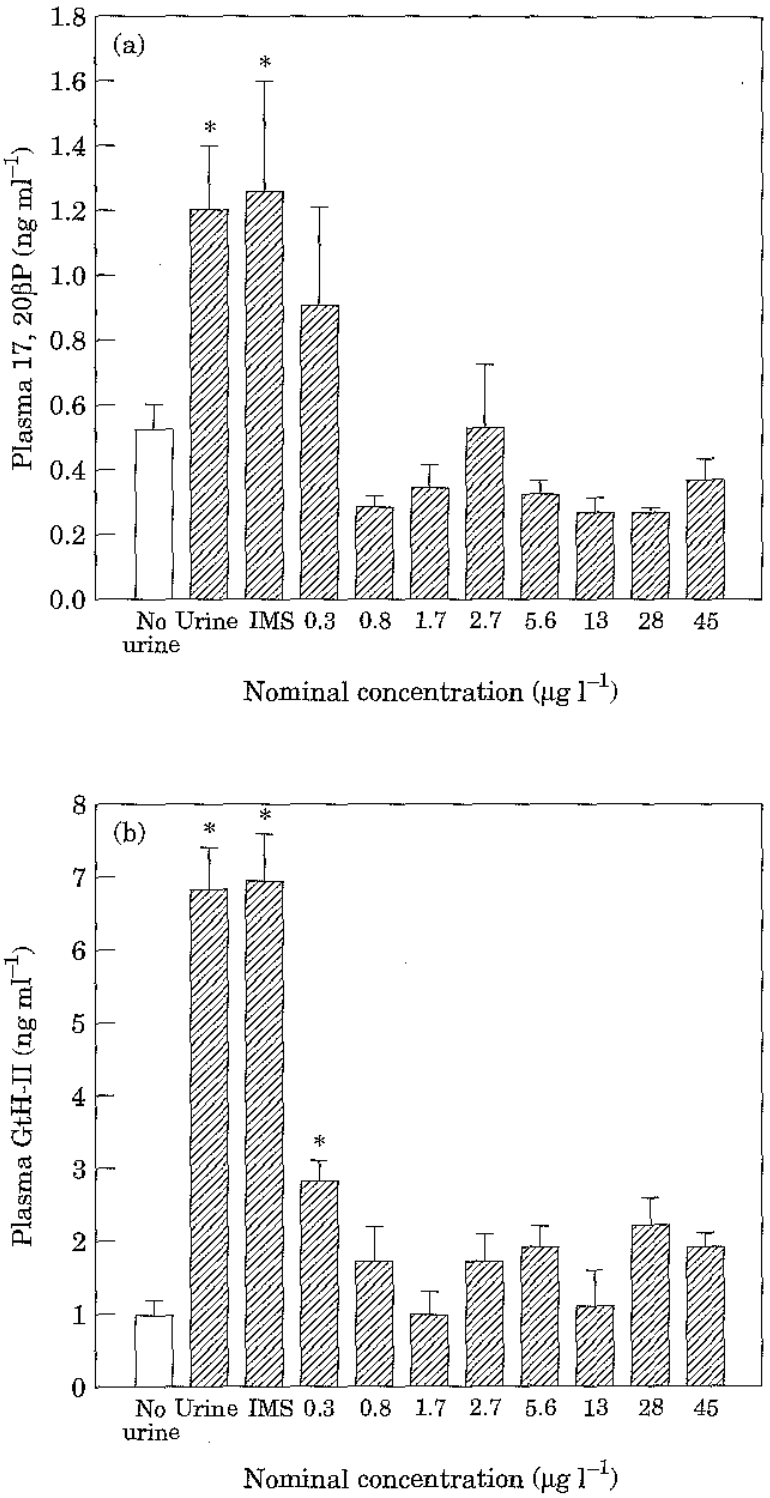

FIG. 4. Mean level (± S.E.M.) of (a) plasma 17,20βP (ng ml^{-1}) and (b) GtH II (ng ml^{-1}) from groups of mature male parr exposed to Diazinon (nominal concentration 0·3–45 μg l^{-1}; n=5), after priming with ovulated female salmon urine. □, Control group that was not stimulated with ovulated female urine. For statistical analysis see text. *A significant difference from the control group not stimulated with ovulated female urine at the 5% level.

priming effect of female salmon urine on plasma testosterone levels (P>0·05) [Fig. 5(a)], and exposure to nominal concentrations of 0·3, 5·6 and 13 μg l^{-1} similarly had no significant effect on the priming effect of female salmon urine on plasma 11-KT levels in mature male parr (P>0·05) [Fig. 5(b)].

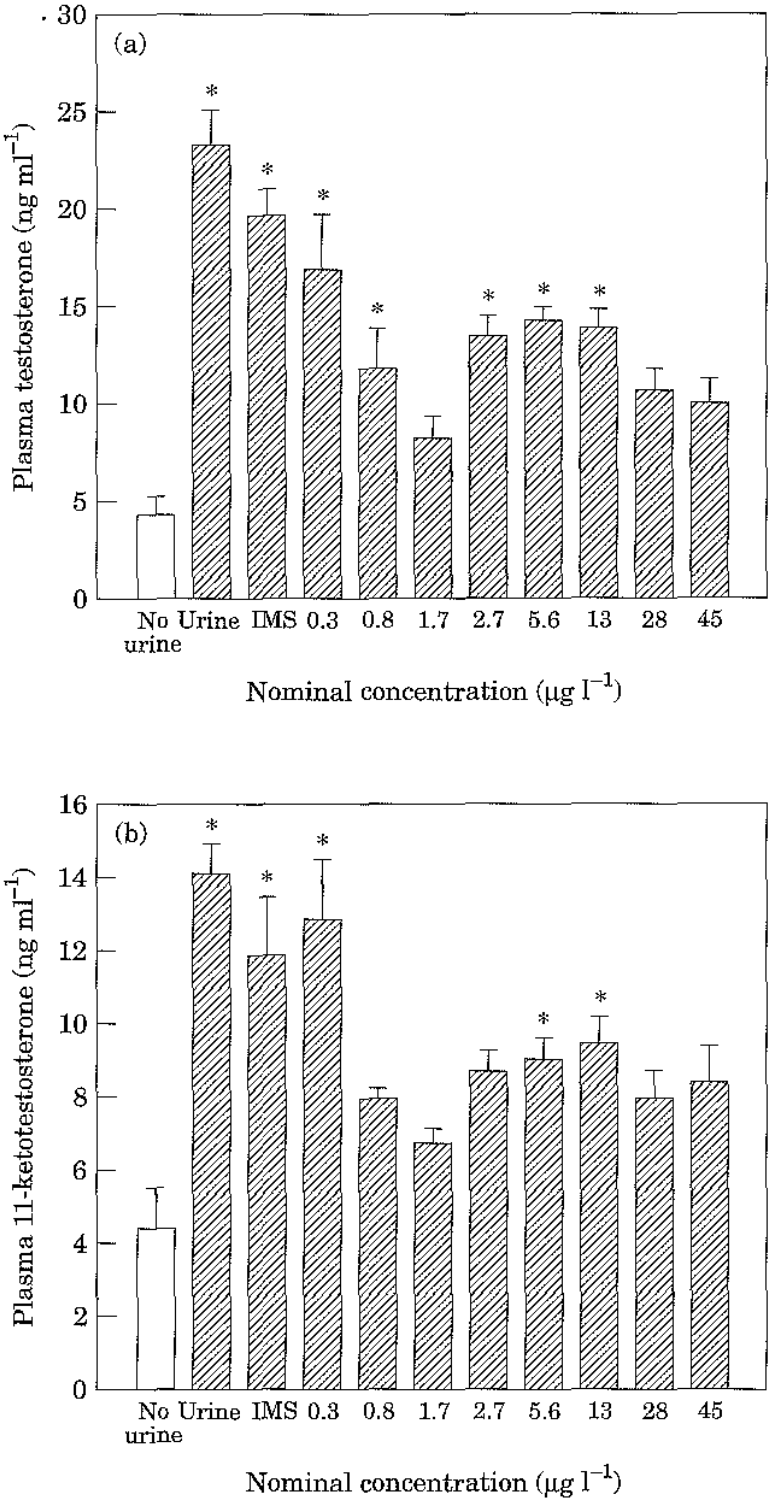

FIG. 5. Mean level (± S.E.M.) of (a) testosterone (ng ml^{-1}) and (b) 11-ketotestosterone (ng ml^{-1}) from groups of mature male parr exposed to Diazinon (nominal concentration 0·3–45 μg l^{-1}; n=5), after priming with ovulated female salmon urine. Key as for Fig. 4.

Expressible milt

The levels of expressible milt were elevated significantly in male parr 3 h after exposure to the urine from an ovulated female salmon (P<0·05) (Fig. 6). However, exposure of mature male parr to all concentrations of Diazinon (0·3–45 μg l^{-1}) depressed this priming effect significantly and there was no

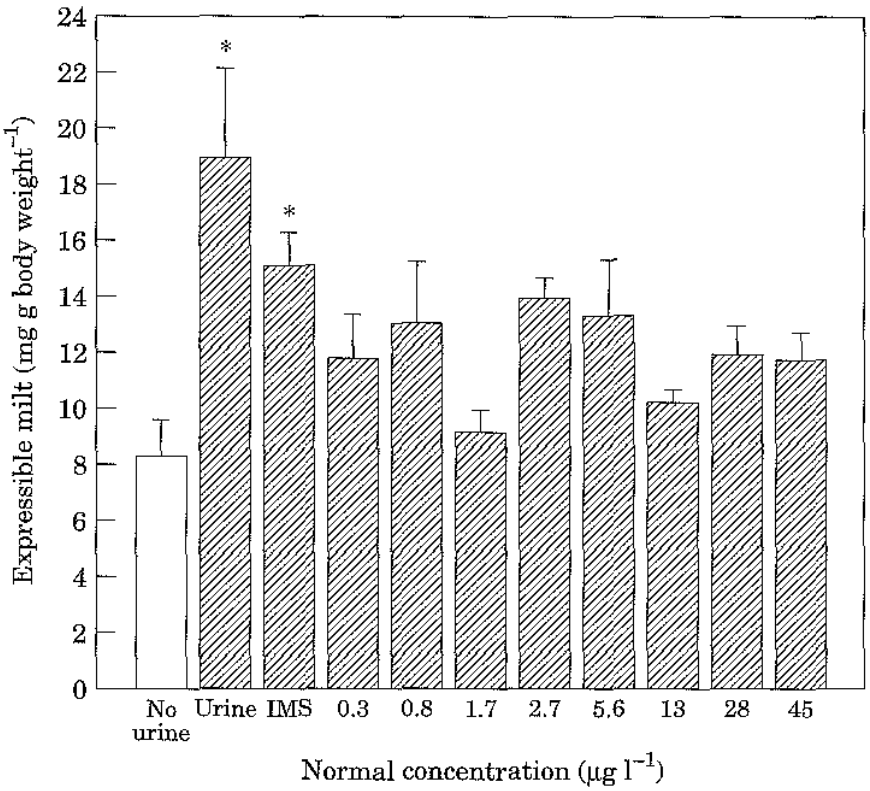

FIG. 6. Mean level (± S.E.M.) of expressible milt (mg g body weight^{-1}), from groups of mature male parr exposed to Diazinon (nominal concentration 0·3–45 μg l^{-1}; n=5), after priming with ovulated female salmon urine. Key as for Fig. 4.

significant difference in the level of expressible milt when compared to fish not exposed to female urine (P<0·05) (Fig. 6).

DISCUSSION

The results of this study demonstrate clearly that when mature male Atlantic salmon parr are exposed to the organophosphate pesticide Diazinon, there is a significant sublethal effect on the olfactory system. After perfusion of the olfactory epithelium with Diazinon, the recorded responses to $PGF_{2\alpha}$ were diminished significantly, and after exposure to levels between 2·0 and 10 μg l^{-1}, the sensitivity to $PGF_{2\alpha}$ was decreased by a factor of 10. The suppression of plasma steroids and GtH-II after priming with ovulated female urine may also indicate that exposure to Diazinon has affected the salmon's olfactory system. Elevation of plasma steroids as a result of a priming pheromone has been shown to be mediated via the olfactory system (Olsen & Liley, 1993).

A significant reduction in the ability of Atlantic salmon to detect and respond to odorants and pheromones involved in reproduction may have long term implications for individuals and populations. F-type prostaglandins are released by mature male charr *Salvelinus alpinus* (L.) to attract females and elicit their spawning behaviour (Sveinsson & Hara, 1995). More recently $PGF_{2\alpha}$ has been shown to have a priming effect on plasma levels of 17,20βP, GtH-II, testosterone, 11-ketotestosterone and milt levels in mature male Atlantic salmon parr (Moore & Waring, 1995). Studies on other species of teleosts have demonstrated also that prostaglandins and urine involved in synchronizing reproductive physiology

and behaviour between male and female (see Sorensen, 1992; Olsen & Liley, 1993; Scott *et al.*, 1994). Suppression of plasma steroids and GtH-II may also have significant deleterious effects on salmon reproduction. Chemical stimuli from ovulated (nesting) female salmonids are believed to be essential to synchronize the spawning readiness of the male with that of the female (Sorensen, 1992). The elevations in plasma GtH II and gonadal steroids in stimulated males are also believed to be necessary to maintain full reproductive behaviour (Liley *et al.*, 1993; Liley & Stacey, 1983). Elevated plasma 17,20βP levels also play a role in restoring courtship behaviour in castrated male rainbow trout (Mayer *et al.*, 1994). Moreover, 17,20βP is believed to be important in male salmonids for the acquisition of sperm motility (Nagahama, 1994). Although expressible milt volume was not affected as markedly as plasma 17,20βP levels in the Diazinon-exposed male salmon in response to female urine, we cannot discount the possibility of a deleterious effect on sperm quality. Since 17,20βP is thought to be important in the final maturation of the milt stored in the spermatic duct (Ueda *et al.*, 1985; Miura *et al.*, 1992), there remains the possibility that the attenuated plasma GtH II and 17,20βP responses of males to female urine may have affected adversely the quality rather than the quantity of the milt expressed.

To what extent the sublethal effects of Diazinon on the olfactory detection of prostaglandin and the suppression of milt, plasma steroids and GtH-II would have on reproductive success in wild populations of Atlantic salmon is a matter of conjecture. However, a life cycle study on fathead minnow *Pimephales promelas* Rafinesque demonstrated that at 60·3 $\mu g\ l^{-1}$ Diazinon, no spawning occurred, with only limited spawning at 6·9–28 $\mu g\ l^{-1}$ (Allison & Hermanutz, 1977). At even lower concentrations (3·5 $\mu g\ l^{-1}$) only 65% of the spawned eggs hatched. Growth and survival of the progeny at 3·5 $\mu g\ l^{-1}$ Diazinon were not affected, indicating that the above effects related to parental exposure. In a similar study on brook trout *Salvelinus fontinalis* Mitchill it was demonstrated that additional mortality occurred during spawning at 4·8 and 9·6 $\mu g\ l^{-1}$ Diazinon (Allison & Hermanutz, 1977). Similar results were obtained by Goodman *et al.* (1979) on the estuarine sheepshead minnow *Cyprinodon variegatus* Lacépède. The authors also found impaired reproduction and after longer exposure to sublethal levels of Diazinon reproduction remained impaired for at least 3–4 weeks after fish were returned to clean water. It is possible that in these fish a reduction in spawning was due to impaired olfactory ability to detect relevant reproductive odorants and subsequent low levels of plasma steroids as a result of exposure to Diazinon.

During the present study 30 min exposure to 1·0 $\mu g\ l^{-1}$ Diazinon was sufficient to affect significantly the olfactory mediated detection of $PGF_{2\alpha}$, whilst longer term exposure suppressed plasma levels of steroids and GtH-II in response to female urine. There was also no rapid recovery of the olfactory epithelium which remained affected for up to 4·5 h after exposure for a similar period. The effects on and recovery time of the epithelium to longer term exposure to sublethal levels of Diazinon is not known.

It is not understood how Diazinon affects the olfactory ability of the mature male parr to detect $PGF_{2\alpha}$. Diazinon is considered to be selectively toxic to fish species (Vittozzi & De Angelis, 1991) and is a known inhibitor of AChE activity

(Goodman *et al.* 1979; Keizer *et al.*, 1991). The additional effect of Diazinon on the olfactory response of the parr to the amino acid L-serine may indicate that the pesticide acts upon the olfactory receptor system modulating general cell function or sensitivity. Previous studies on the toxicity of heavy metals on the electro-olfactogram of the Atlantic salmon have suggested that the toxic effect operates on the transduction mechanisms of the olfactory receptor cells (Winberg *et al.*, 1992; Bjerselius *et al.*, 1993). However, during the present study Diazinon did not affect the shape or form of the EOG response as previously reported for heavy metals (Winberg *et al.*, 1992; Bjerselius *et al.*, 1993). It is unlikely that the observed deleterious effects on the recorded response to $PGF_{2\alpha}$ during the present study was due to fatigue or adaptation of the olfactory receptors. Repeated exposure of the olfactory epithelium to $PGF_{2\alpha}$ in the absence of Diazinon did not effect the shape or amplitude of the recorded response.

It is also not fully understood how Diazinon affects the priming effect of the urine on the plasma levels of steroids and GtH II. It is possible that if $PGF_{2\alpha}$ is the priming pheromone in female Atlantic salmon urine as suggested by Moore & Waring (1995) then a significant reduction in the ability to detect this pheromone will in turn effect priming in the male. However, the possibility that exposure to Diazinon may have suppressed plasma levels of steroids and GtH II in the fish by a means other than a direct effect on olfactory mediated priming by the female urine cannot be discounted. A number of organophosphate pesticides have been shown to have a deleterious impact on testicular steroidogenesis (Kime, 1995). However, because the 17,20βP and androgen levels in the plasma of Diazinon exposed fish were not significantly lower than the group not exposed to female urine, this would suggest that there was no direct effect of Diazinon on testes steroidogenesis during this study. There was also a differential effect of Diazinon exposure on the plasma levels of the androgens and 17,20βP in mature male parr. The reasons for this are unknown but it may suggest differential regulation of C19 and C21 steroids after priming with female urine. This requires further study.

Diazinon is also known to be concentrated by tissues and organs of a number of freshwater species (Goodman, 1979; Seguchi & Asaka, 1981; Tsuda *et al.*, 1989, 1990; Keizer *et al.*, 1991; Sancho *et al.*, 1992, 1993). Excretion rates from the fish are often rapid but do vary between tissues and organs (Tsuda *et al.*, 1989, 1990; Sancho *et al.*, 1992). In a study on two species of fish Keizer *et al.* (1993) considered that the mechanism of toxicity was species selective. In guppy *Poecilia reticulata* Peters toxicity of Diazinon was due its metabolism to a highly toxic metabolite, possibly Diazoxon, whilst in the zebra fish *Brachydanio rerio* Hamilton-Buchanan, toxicity was due to the accumulation of the parent compound.

For Diazinon to have a significant sublethal impact on reproduction in wild populations of Atlantic salmon, it must satisfy a number of criteria. The pesticide should be present within the aquatic environment in biologically significant amounts, remain biologically active and occur in spawning tributaries during the main salmon spawning season. Although Diazinon is not intentionally applied directly to water courses, it does occur as the result of inadequate storage/disposal of waste, or run-off from agricultural land due to rainfall.

During a study on sheep dipping practices in the R. Tweed catchment, Virtue & Church (1993) detected short-term spikes of Diazinon ranging between 0·3 and 200 $\mu g\ l^{-1}$ in adjacent watercourses during the months October and November. At the majority of the sites sampled, the streams were known to contain spawning populations of Atlantic salmon (W. A. Virtue pers. comm.). Diazinon has also been detected in a number of rivers throughout England and Wales during routine monitoring by the National Rivers Authority (NRA National Centre for Toxic and Persistent Substances, Peterborough). Little is known of the fate and accumulation of Diazinon in the aquatic environment. The biological activity and toxicity of Diazinon in water is influenced by a number of physical parameters, such as pH (Chatterjee & Konar, 1984) and soil type (see Allison & Hermanutz, 1977). The pesticide has a half-life of around 70·5 h in water (Ferrando *et al.*, 1991). Diazinon may therefore occur in rivers in significant amounts and in locations where it may have potential effects on spawning Atlantic salmon and subsequent long term effects on populations.

Diazinon is only one of the many organophosphate pesticides that are used regularly in agriculture and that occur in varying concentrations in inland waters. Its occurrence throughout the year may also have significant effects on other olfactory mediated behaviour during the Atlantic salmon life cycle. Possible effects on olfactory imprinting during the smolt stage and homing of adults are of concern. Deleterious effects of long term exposure on reproduction are not understood, but there may be implications for adult salmon that spend more extended periods in fresh water, such as the early spring-run Atlantic populations. Further work on the sublethal effects of organophosphate pesticides on Atlantic salmon are therefore of great importance.

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References

Alam, M. K. & Maughan O. E. (1992). The effect of malathion, Diazinon and various concentrations of zinc, copper, nickel, lead, iron and mercury on fish. *Biological Trace Element Research* **34,** 225–236.

Allison, D. T. & Hermanutz, R. O. (1977). Toxicity of Diazinon to brook trout and fathead minnows. *United States Environmental Research Center Ecological Research Series* EPA-600/3-77-060.

Anees, M. A. (1978). Haematological abnormalities in a freshwater teleost, *Channa punctatus* (Bloch), exposed to sub lethal and chronic levels of three organophosphorus insecticides. *International Journal on Ecological and Environmental Sciences* **4,** 53–60.

Ansari, B. A. & Kumar, K (1988). Diazinon toxicity: Effect on protein and nucleic acid metabolism in the liver of zebrafish, *Brachydanio rerio* (Cyprinidae). *The Science of the Total Environment* **76,** 63–68.

Bjerselius, R. & Olsén, K. H. (1993). A study of the olfactory sensitivity of crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*) to 17*α*,20*β*-dihydroxy-4-pregnen-3-one and prostaglandin $F_{2\alpha}$. *Chemical Senses* **18,** 427–436.

Bjerselius, R., Winberg, S., Winberg, Y. & Zeipel, K. (1993). Ca super (2+) protects olfactory receptor function against acute Cu (II) toxicity in Atlantic salmon. *Aquatic Toxicology* **25,** 125–138.

Chatterjee, K. & Konar, S. K. (1984). Effects of the pesticide Diazinon at various pH and turbidity on fish and aquatic ecosystems. *Environmental Ecology* **2,** 49–53.

Dutta, H. M., Richmonds, C. R. & Zeno, T. (1993). Effects of Diazinon on the gills of bluegill sunfish *Lepomis macrochirus. Journal of Environmental Pathology, Toxicology and Oncology* **12,** 219–227.

Eisler, R. (1986). Diazinon hazards to fish, wildlife, and invertebrates: A synoptic review. *U.S. Department of the Interior, Fish and Wildlife Service, Contaminant Hazards Reviews, Report No. 9, Biological Report* **85** (1.9).

Evans, R. E. & Hara, T. J. (1985). The characteristics of the electro-olfactogram (EOG): its loss and recovery following olfactory nerve section in the rainbow trout (*Oncorhynchus mykiss*). *Brain Research* **330,** 65–75.

Ferrando, M. D., Alarcon, V., Fernandez, A., Gamon, M. & Andreu-Moliner, E. (1991). Persistence of some pesticides in the aquatic environment. *Bulletin of Environmental Contamination and Toxicology* **48,** 747–755.

Goodman, L. R., Hansen, D. J., Coppage, D. L., Moore, J. C. & Matthews, E. (1979). Diazinon: Chronic toxicity to, and brain acetylcholinesterase inhibition in, the sheepshead minnow, *Cyprinodon variegatus. Transactions of the American Fisheries Society* **108,** 479–488.

Keizer, J., Giuseppina, D'A. & Vitozzi, L. (1991). The importance of biotransformation in the toxicity of xenobiotics to fish. I. Toxicity and bioaccumulation of Diazinon in guppy (*Poecilia reticulata*) and zebra fish (*Brachydanio rerio*). *Aquatic Toxicology* **21,** 239–254.

Keizer, J., Giuseppina, D'A., Nagel, R., Gramenzi, F. & Vitozzi, L. (1993). Comparative Diazinon toxicity in guppy and zebra fish: Different role of oxidative metabolism. *Environmental Toxicology and Chemistry* **12,** 1243–1250.

Kime, D. E. (1995). The effects of pollution on reproduction in fish. *Reviews in Fish Biology and Fisheries* **5,** 52–96.

Liley, N. R. & Stacey, N. E. (1983). Hormones, pheromones and reproductive behavior in fish. In *Fish Physiology*, Vol. IXB (Hoar, W. S., Randall, D. J. & Donaldson, E. M., eds), pp. 1–63. London: Academic Press.

Liley, N. R., Olsen, K. H., Foote, C. J. & Van Der Kraak, G. J. (1993). Endocrine changes associated with spawning behavior in male Kokanee salmon (*Oncorhynchus nerka*) and the effects of anosmia. *Hormones and Behavior* **27,** 470–487.

Mayer, I., Liley, N. R. & Borg, B. (1994). Stimulation of spawning behaviour in castrated rainbow trout (*Oncorhynchus mykiss*) by 17α,20β-dihydroxy-4-pregnen-3-one, but not by 11-ketoandrostenedione. *Hormones and Behaviour* **28,** 181–190.

Miura, T., Yamauchi, K., Takahashi, H. & Nagahama, Y. (1992). The role of hormones in the acquisition of sperm motility in salmonid fish. *Journal of Experimental Zoology* **261,** 359–363.

Moore, A. (1991). Behavioural and physiological responses of precocious male Atlantic salmon (*Salmo salar* L.) parr to testosterone. In *Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish* (Scott, A. P., Sumpter, J. P., Kime, D. E. & Rolfe, M. S., eds), pp. 194–196. Sheffield: FishSymp91.

Moore, A. & Scott, A. P. (1991). Testosterone is a potent odorant in precocious male Atlantic salmon (*Salmo salar* L.) parr. *Philosophical Transactions of the Royal Society of London Series* **B 332,** 241–244.

Moore, A. & Scott, A. P. (1992). 17α,20β-dihydroxy-4-pregnen-3-one 20-sulphate is a potent odorant in precocious male Atlantic salmon (*Salmo salar* L.) parr which have been pre-exposed to the urine of ovulated females. *Proceedings of the Royal Society of London Series* **B 249,** 205–209.

Moore, A. (1994). An electrophysiological study on the effects of pH on olfaction in mature male Atlantic salmon (*Salmo salar*) parr. *Journal of Fish Biology* **45,** 493–502.

Moore, A., Ives, M. J. & Kell, L. T. (1994). The role of urine in sibling recognition in Atlantic salmon (*Salmo salar* L.) parr. *Proceedings of the Royal Society of London Series* **B 255,** 173–180.

Moore, A. & Waring, C. P. (1995). The effects of water quality on olfaction in the Atlantic salmon (*Salmo salar* L.). In *Proceedings of the Workshop "Fish Pheromones: Origins and Mechanisms of Action"* (Canario, A. V. M. & Power, D. M., eds) University of Algarve, Portugal.

Nagahama, Y. (1994). Endocrine regulation of gametogenesis in fish. *International Journal of Developmental Biology* **38,** 217–229.

Nishiuchi, Y. (1971). Toxicity of pesticides to several freshwater organisms. *Aquaculture* **19,** 151–157.

Olsen, K. H. & Liley, N. R. (1993). The significance of olfaction and social cues in milt availability, sexual hormone status and spawning behaviour of male rainbow trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology* **89,** 107–118.

Sakr, S. A. & Gabr, S. A. (1992). Ultrastructural changes induced by Diazinon and neopybuthrin in skeletal muscles of *Tilapia nilotica*. *Bulletin of Environmental Contamination and Toxicology* **48,** 467–473.

Sancho, E., Ferrando, M. D., Gamon, M. & Andreu-Moliner, E. (1992). Organophosphorus Diazinon induced toxicity in the fish *Anguilla anguilla* L. *Comparative Biochemistry and Physiology* **103C,** 351–356.

Sancho, E., Ferrando, M. D., Gamon, M. & Andreu-Moliner, E. (1993). Bioconcentration and excretion of Diazinon by eel. *Bulletin of Environmental Contamination and Toxicology* **50,** 578–585.

Scott, A. P., Sheldrick, E. L. & Flint, A. P. F. (1982). Measurement of 17α,20β-dihydroxy-4-pregnen-3-one in plasma of trout (*Salmo gairdneri* Richardson): seasonal changes and response to salmon pituitary extract. *General and Comparative Endocrinology* **46,** 444–451.

Scott, A. P., MacKenzie, D. S. & Stacey, N. E. (1984). Endocrine changes during natural spawning in the white sucker, *Catostomus commersoni*. II. Steroid hormones. *General and Comparative Endocrinology* **56,** 349–359.

Scott, A. P., Liley, N. R. & Vermeirssen, E. L. M. (1994). Urine of reproductively mature female rainbow trout, *Oncorhynchus mykiss* (Walbaum), contains a priming pheromone which enhances plasma levels of sex steroids and gonadotrophin II in males. *Journal of Fish Biology* **44,** 131–147.

Seguchi, K. & Asaka, S. (1981). Intake and excretion of Diazinon in freshwater fishes. *Bulletin of Environmental Contamination and Toxicology* **27,** 244–249.

Seikai, T. (1982). Acute toxicity of organophosphorus insecticides on the developmental stages of eggs, larvae and juveniles of Japanese striped knifejaw *Oplegnathus fasciatus*. *Bulletin of the Japanese Society of Science and Fisheries* **48(5),** 599–603.

Sorensen, P. W. (1992). Hormones, pheromones and chemoreception. In *Fish Chemoreception* (Hara, T. J., ed), pp. 199–228. London: Chapman & Hall.

Sorensen, P. W. & Goetz, F. W. (1993). Pheromonal and reproductive function of F prostaglandins and their metabolites in teleost fish. *Journal of Lipid Mediators* **6,** 385–393.

Suzuki, K., Kanamori, A., Nagahama, Y. & Kawauchi, H. (1988). Development of salmon GTH I and GTH II radioimmunoassays. *General and Comparative Endocrinology* **71,** 459–467.

Sveinsson, T. & Hara, T. J. (1995). Mature male of Arctic charr, *Salvelinus alpinus*, release F-type prostaglandins to attract conspecific mature females and stimulate their spawning behaviour. *Environmental Biology of Fishes* **42,** 253–266.

Swanson, P., Bernard, M., Nozaki, M., Suzuki, K., Kawauchi, H. & Dickhoff, W. W. (1989). Gonadotropins I and II in juvenile coho salmon. *Fish Physiology and Biochemistry* **7,** 169–176.

Tsuda, T., Aoki, S., Kojima, M. & Harada, H. (1989). Bioconcentration and excretion of Diazinon, IBP, malathion and fenitrothion by willow shiner. *Toxicological and Environmental Chemistry* **24,** 185–190.

Tsuda, T., Aoki, S., Kojima, M. & Harada, H. (1990). Bioconcentration and excretion of Diazinon, IBP, malathion and fenitrothion by carp. *Comparative Biochemistry and Physiology* **96C,** 23–26.

Ueda, H., Kambegawa, A. & Nagahama, Y. (1985). Involvement of gonadotrophin and steroid hormones in spermiation in the Amago salmon, *Oncorhynchus rhodurus*, and goldfish, *Carassius auratus. General and Comparative Endocrinology* **59,** 24–30.

Virtue, W. A. & Church, A. M. (1993). A survey of sheep dipping practices in the River Tweed Catchment. *Journal of Institute of Water and Environmental Management* **7,** 395–403.

Vittozzi, L. & De Angelis, G. (1991). A critical review of comparative acute toxicity data on freshwater fish. *Aquatic Toxicology* **19,** 167–204.

Winberg, S., Bjerselius, R., Baatrup, E. & Døving, K. B. (1992). The effect of Cu (II) on the electro-olfactogram (EOG) of the Atlantic salmon (*Salmo salar* L.) in artificial freshwater of varying inorganic carbon concentrations. *Ecotoxicology and Environmental Safety* **24,** 167–178.