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Sublethal effects of a carbamate pesticide on pheromonal mediated endocrine function in mature male Atlantic salmon (*Salmo salar* L.) parr

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Abstract

The sublethal effect of the carbamate pesticide carbofuran on the priming pheromonal system of Atlantic salmon (*Salmo salar* L.) was investigated. Previous studies have demonstrated that ovulated female salmon release a priming pheromone in their urine (considered to be an F-type prostaglandin) which is subsequently detected by mature male salmon and results in increased levels of plasma sex steroids and expressible milt. In the present study, electrophysiological recordings from the olfactory epithelium of mature male salmon parr indicated that the responses to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were significantly reduced at nominal concentrations of carbofuran as low as 1.0 μg l^{-1}, and the threshold of detection was reduced 10-fold. A 5 day exposure to carbofuran significantly reduced the ability of male parr to respond to $PGF_{2\alpha}$ stimulation. The priming effect of $PGF_{2\alpha}$ on milt and plasma 17,20ß-dihydroxy-4-pregnen-3-one levels were abolished at water concentrations at and above 2.7 μg l^{-1}. In addition, the priming effect of $PGF_{2\alpha}$ on plasma testosterone and 11-ketotestosterone concentrations was abolished at water carbofuran concentrations above 6.5 μg l^{-1}. Exposure to similar concentrations of carbofuran also resulted in a reduction in the levels of free and glucuronidated steroids in the bile of $PGF_{2\alpha}$ primed male parr. The effect of carbofuran on the priming response did not appear to be due to a direct effect on the testes, since the ability of testes to respond to pituitary extract stimulation *in vitro* was not impaired in carbofuran-exposed males. Carbofuran appeared to reduce significantly or abolish the priming pheromonal system in mature male parr by directly affecting the ability of the olfactory system to detect $PGF_{2\alpha}$, although the toxicological mechanism involved is as yet unknown. The results are therefore discussed in relation to the possible sublethal effects of carbofuran on reproduction in the Atlantic salmon.

Introduction

Ovulated Atlantic salmon (*Salmo salar* L.) female, release a priming pheromone within their urine which, when detected by mature male parr, increases their levels of expressible milt and plasma sex hormone concentrations (Waring and Moore 1995; Waring et al. 1996). Evidence to date suggests that this priming pheromone is an F-type prostaglandin (Waring and Moore 1995; Moore and Waring 1996b) whose detection is mediated by receptors on the olfactory epithelium of males (Moore and Scott 1992; Moore and Waring 1995, 1996b). Priming pheromones are thought to be involved with the synchronization of spawning between the sexes and, in Atlantic salmon, the priming pheromonal system has recently been shown to have acute sensitivity to exposure to an organophosphate pesticide, diazinon. Diazinon exposed males when exposed to the priming pheromone in female salmon urine did not show increased plasma sex steroid levels nor an express-

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ible milt response (Moore and Waring 1996a). However, during that study it was not clear whether the inhibitory effect of diazinon on the priming response of mature male parr was operating via impaired olfactory detection or due to a direct effect on the testes.

In the present study, we have again looked at the impact of exposure to sublethal levels of a pesticide on the priming pheromonal system of Atlantic salmon. This study is an extension of the previous work on diazinon and investigates the effects of the carbamate pesticide, carbofuran. Carbamates are currently widely used as alternatives to organochlorine and organophosphate pesticides which have higher environmental persistence and greater toxicity to mammals and fish (Verma et al. 1982). Carbamate pesticides are reported to be metabolized and excreted rapidly in fish (reviewed by Eisler 1985).

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methyl carbamate) is a water-soluble systemic insecticide used on winter crops and it has been reported to be a contaminant in streams and rivers in the UK during the autumn/winter. The water concentrations used in the present investigations (0–22.7 μg l^{-1}) were based on the observations of Matthiessen et al. (1995) who measured water concentrations in an English stream of up to 26 μg l^{-1} in December. Although the toxicity of this pesticide to Atlantic salmon is not known, the concentrations used in the present experiments are expected to be well below the lethal concentration to fish which is reported to be 380–560 μg l^{-1} (reviewed by Eisler 1985).

The present study looked at two different aspects relating to the effects of carbofuran exposure on the response of male salmon to a priming pheromone. First, an electrophysiological study was carried out to investigate the effects of the pesticide on the olfactory ability of mature male Atlantic salmon parr to detect prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Second, an endocrinological study was carried out to investigate the effects of carbofuran exposure on the levels of plasma sex steroids and expressible milt in mature male parr after exposure to water-borne $PGF_{2\alpha}$. In order to investigate possible direct effects of the pesticide on the ability of the testes to respond to pituitary stimulation, testes were removed from carbofuran-exposed males and incubated *in vitro* with a pituitary extract from mature salmonid fish. In addition, free and glucuronidated steroid levels in the bile were measured to ascertain whether the pesticide altered aspects of the metabolism and excretion of steroids in mature male salmon parr.

Materials and methods

In October 1995, mature male Atlantic salmon parr were obtained from the Environment Agency, Cynrig hatchery, Wales and transported to the Lowestoft Fisheries Laboratory. The fish were kept in 1000 l tanks, under natural light conditions, with a constant flow of aerated dechlorinated water (flow rate of 85 l min^{-1}). Water temperature ranged from 7.5–10 °C. The physico-chemical characteristics of the water have been reported previously (Moore and Waring 1996a). The fish were fed to satiation twice a day with commercial salmon pellets.

Effect of carbofuran on the olfactory detection of $PGF_{2\alpha}$

The study used the same electrophysiological technique (electro-olfactogram: EOG) as that in previous studies on mature male Atlantic salmon parr (Moore and Scott 1992; Moore 1994; Moore and Waring 1996a,b). EOG recording measures trans-epithelial voltage gradients from the surface of the olfactory epithelium and is considered to reflect multi-unit cell activity (Evans and Hara 1985).

The testing procedure was the same as that previously reported for the study on diazinon (Moore and Waring 1996a). To examine the acute effect of carbofuran exposure on olfactory function, the responses of the olfactory epithelium of mature male salmon parr to a 10^{-9} M concentration of $PGF_{2\alpha}$ were recorded after perfusion of the olfactory rosette for a period of 30 min with different concentrations of carbofuran (0.1, 1.0, 2.0, 5.0, 10.0, and 20.0 μg l^{-1}). Dose response studies were carried out using serial dilutions of $PGF_{2\alpha}$ and water containing a range of carbofuran concentrations (0.1, 1.0, 2.0, 5.0, and 10.0 μg l^{-1}). The olfactory epithelium was perfused with each concentration of the pesticide for a period of 30 min, after

which the response to the range of $PGF_{2\alpha}$ concentrations were recorded.

The amplitude of each EOG response was measured from the baseline to the peak of each phasic displacement and expressed in millivolts (mV). All recordings in response to the dechlorinated water controls were subtracted from the EOG responses. In all experiments, the EOG responses recorded at each concentration of carbofuran were compared to the responses recorded to the relevant control water taken from the inlet of the salmon tank, using a paired *t*-test. The threshold value of $PGF_{2\alpha}$ at each concentration of carbofuran was estimated as the lowest dose whose 95% confidence interval did not include 0.

Effect of carbofuran on the priming response of males to $PGF_{2\alpha}$

In November 1995, spermiating male parr (weight 27–44 g; length 130–160 mm; GSI 5.7–9.3%), were transferred to 63 l glass tanks. Each tank had a constant flow of dechlorinated tap water (1 l min^{-1}) with no recirculation. A natural photoperiod was followed and the water temperature was 11 ± 1 °C. Males were gently stripped of milt and groups of 7 were placed into each tank and left to recover for 96h without feeding.

Stock solutions of carbofuran (from Greyhound, Birkenhead, UK) were produced by dissolving in water and were stored in amber glass bottles. Carbofuran was added to the tanks via a multichannel peristaltic pump (Technicon) and silicon tubing and vigorously mixed by aeration. Stock solutions were renewed every 12h. At the end of the experiment water samples were taken and stored in the dark at 4 °C in amber glass bottles for 5 days before analysis of actual carbofuran contents. Water carbofuran concentrations were measured using gas chromatography/mass spectrometry using the method outlined by Matthiessen et al. (1995).

Males were exposed to various concentrations of carbofuran (0–22.7 µg l^{-1}) for 5 days. At the end of this period groups of males were then either given a 5h exposure to $PGF_{2\alpha}$ or to the ethanol carrier. $PGF_{2\alpha}$ (Sigma) was dissolved in ethanol and then further diluted with tank water to give a final stock solution of 10^{-8} M. One ml aliquots of this stock solution were mixed with 1 ml of tank water and then added to the tanks. An extra group of males were exposed to the highest carbofuran concentration but were not exposed to $PGF_{2\alpha}$. This group was used to investigate the direct effect of carbofuran on milt and plasma sex steroids in non pheromonally-primed male parr.

At the end of the PGF-exposure period, males were anaesthetised in 0.4 ml l^{-1} 2-phenoxyethanol and milt and blood was sampled as described previously (Moore and Waring 1996a). Gall bladders were removed from each fish and the contents were left to drain into pre-weighed 1.5 ml microcentrifuge tubes. Bile was then diluted 1:10 (w/v) with distilled water and stored at –20 °C.

Testes were removed from some of the males and were then washed, chopped into 50 mg fragments and incubated *in vitro* as described by Nagler et al. (1996). Acetone-dried chum salmon (*Oncorhynchus keta*) pituitary powder was obtained from Syndel Laboratories (Vancouver, British Columbia, Canada). The powder was ground in 30 vol. (w/v) of incubation medium, centrifuged and the pellet was discarded. The supernatant (PE) was then used immediately for the experiment at a dosage of 0.025 mg of original powder per well. Testes fragments (50 mg per well) from each male were incubated, in the presence or absence of PE, at 11 °C in a humidified atmosphere on a shaking platform. After 18h, the media were removed, centrifuged, and the supernatants were stored at –20 °C until analysis.

Steroids were extracted from plasma (50 µl), bile (50 µl), and testes incubation medium (100 µl) with 3 ml of diethyl ether. Testosterone (T), 11-ketotestosterone (11-KT) and 17,20ß-dihydroxy-4-pregnen-3-one (17,20ßP) were measured using the radioimmunoassays described previously (Scott et al. 1982, 1984). In addition, samples of bile (50 µl) were treated with 'snail juice' (Sigma; containing 2000 I.U. ß-glucuronidase per sample) as described by Scott and Liley (1994) to measure the glucuronide conjugates of the three steroids, which have been expressed as µg of free steroid equivalents and have not been adjusted to take into account the mass of the glucuronide moiety.

The milt and the bile and plasma steroid data were analysed using a one-way ANOVA followed, if significance was indicated, by Student-Neuman-Keuls (SNK) tests as the multiple range

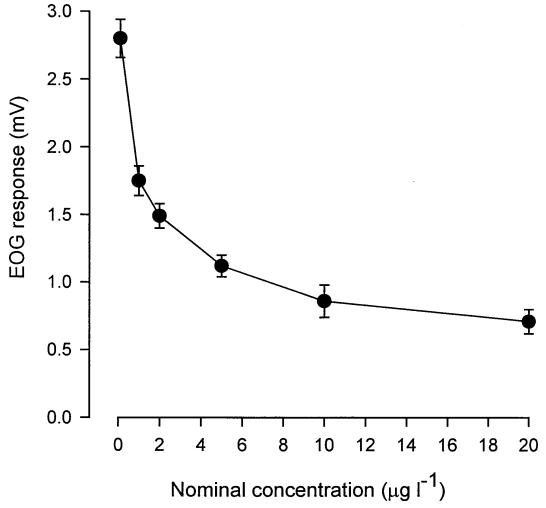

Fig. 1. EOG recordings from the olfactory epithelium of mature male Atlantic salmon parr to $PGF_{2\alpha}$ (concentrations 10^{-9} M; n = 6), after perfusion of the olfactory epithelium with carbofuran (nominal concentrations 0.1, 1.0, 2.0, 5.0, 10.0 and 20 µg l^{-1}). The amplitude of each EOG response was measured from the baseline to the peak of each phasic displacement and expressed as mV. The negative value of the EOG response has been changed and each recording expressed as positive mV. Vertical bars represent SEM.

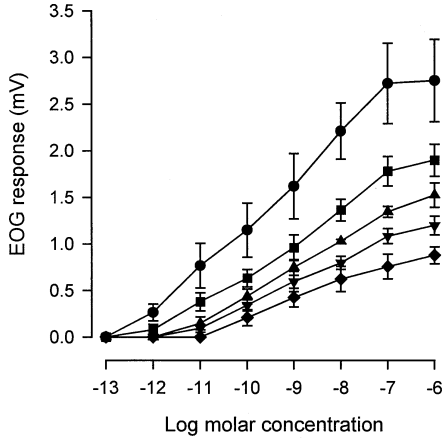

Fig. 2. Semi-logarithmic plots of the concentration response relationship in mature male Atlantic salmon parr to $PGF_{2\alpha}$ at control water (=), 1.0 µg l^{-1} (]), 2.0 µg l^{-1} (r) 5.0 µg l^{-1} (t) and 10 µg l^{-1} (}) carbofuran. The responses to 0.1 µg l^{-1} carbofuran were the same as the 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 in mV. The negative value of the EOG response has been changed and each recording expressed as positive mV. Vertical bars represent SEM.

test. The testes incubation data were analysed using a 2-way ANOVA with the prsence or absence of PE and pesticide treatment as factors. When significance was indicated, SNK was used as the multiple range test.

Results

Effect of carbofuran on the olfactory detection of $PGF_{2\alpha}$

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 carbofuran 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 amplitudes were only 21.5 ± 6.9% compared to the control.

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 carbofuran concentrations of 1.0 µg l^{-1} ($p < 0.01$); 2.0 µg l^{-1} ($p < 0.001$); 5.0 µg l^{-1} ($p < 0.001$) and 10.0 µg l^{-1} ($p < 0.001$) (Fig. 2). There was a significant change in the threshold concentration of $PGF_{2\alpha}$ in mature male parr at higher concentrations of carbofuran. After perfusion with > 1.0 µg l^{-1} there was a corresponding 10 fold decrease in the sensitivity of the olfactory epithelium to $PGF_{2\alpha}$.

There was also a significant reduction in the recorded response to 10^{-5} M L-serine. The mean EOG recording prior to carbofuran exposure was 0.86 ± 0.12 mV. After exposure to the highest concentration of the pesticide (20.0 µg l^{-1}), the EOG recording was reduced to 0.13 ± 0.03 mV, which was 15% of the initial response.

Carbofuran did not elicit a recordable response from the olfactory epithelium at any of the concentrations used in these experiments.

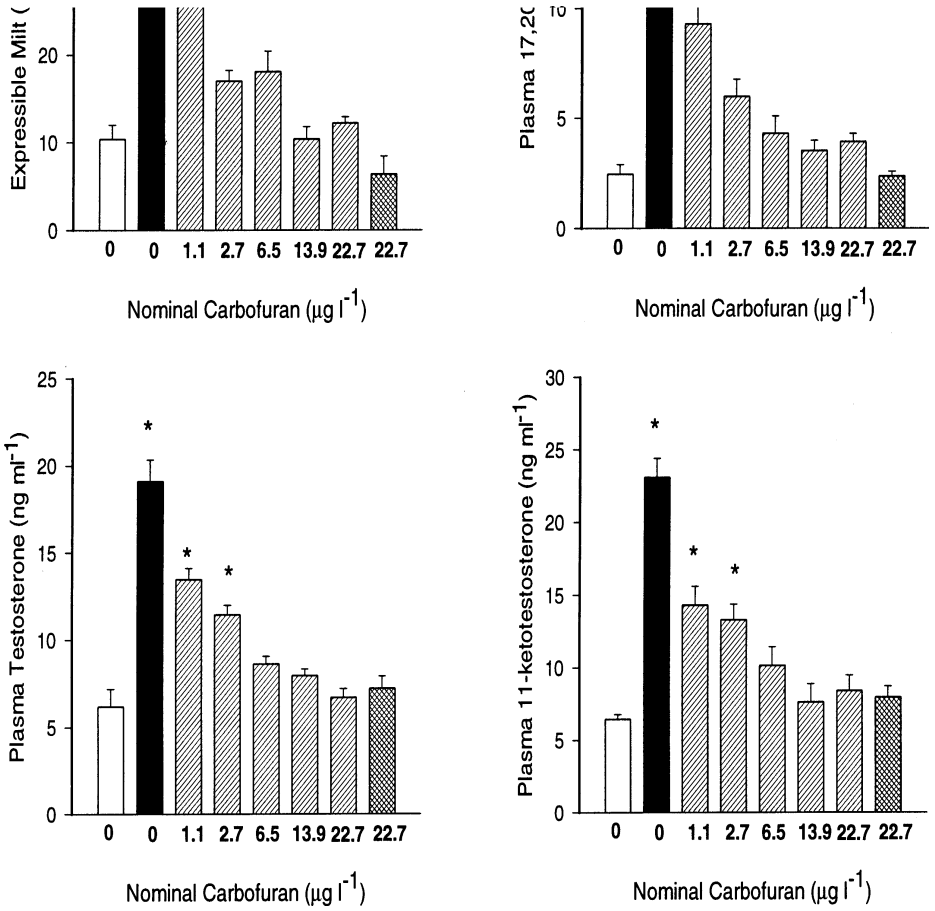

Fig. 3. The effect of carbofuran on expressible milt, plasma 17,20ßP, testosterone and 11-ketotestosterone concentrations in various groups of mature male salmon parr. Open column = controls not exposed to $PGF_{2\alpha}$; solid column = controls exposed to $PGF_{2\alpha}$; slashed column = exposed to carbofuran and $PGF_{2\alpha}$; cross hatched column = exposed to carbofuran but not $PGF_{2\alpha}$. Data represents the mean + SEM of 7 fish per group. * = $p < 0.05$ compared to control fish not exposed to $PGF_{2\alpha}$.

Effect of carbofuran on the priming response of males to $PGF_{2\alpha}$

Exposure to $PGF_{2\alpha}$ for 5h significantly increased levels of expressible milt and plasma 17,20ßP concentrations (Fig. 3) in male parr compared to controls and these responses were abolished when water carbofuran concentrations were at and above 2.7 µg l^{-1}. There was no significant difference in levels of expressible milt and plasma 17,20ßP concentrations between PGF- and non-PGF-exposed fish exposed to the highest water carbofuran concentration. Exposure to $PGF_{2\alpha}$ also increased plasma T and 11-KT concentrations in male parr (Fig. 3). Carbofuran present in the water also impacted on the plasma androgen response which was abolished at water concentrations at and above 6.5 µg l^{-1}. The plasma concentrations of both androgens did not significantly differ in both groups of males (i.e. non-PGF and PGF-primed) exposed to the highest carbofuran concentration.

Bile free and glucuronidated 17,20ßP levels were significantly elevated in the pheromonally-primed males compared to controls, but this response was abolished when water carbofuran concentrations were at and above 2.7 µg l^{-1} (Table 1). The increases in bile free and glucuronidated T concentrations shown by the PGF-exposed males, in the absence of carbofuran, were abolished when the pesticide was present at and above 6.5 µg l^{-1}.

Table 1. Bile free (in ng ml^{-1} bile) and glucuronidated (–G: in µg ml^{-1} bile) steroids in male salmon parr exposed to various concentrations of carbofuran

Carbofuran (µg l^{-1})	Steroid					
	17,20ßP	17,20ßP-G	T.	T.–G	11-KT	11-KT-G
0 (–PGF)	373 ± 30	8.5 ± 0.8	203 ± 13	50.6 ± 5.9	384 ± 24	31.1 ± 5.1
0 (+PGF)	620 ± 89*	58.7 ± 5.2*	348 ± 16*	143.6 ± 5.8*	329 ± 22	68.8 ± 4.8*
1.1	666 ± 33*	48.1 ± 8.3*	264 ± 21*	99.2 ± 10.8*	350 ± 33	49.9 ± 2.7*
2.7	428 ± 461	21.6 ± 5.4	290 ± 13*	83.4 ± 10.2*	292 ± 16	47.1 ± 6.3
6.5	461 ± 27	8.8 ± 0.8	206 ± 11	51.4 ± 4.6	355 ± 28	30.9 ± 3.8
13.9	394 ± 13	8.1 ± 1.2	200 ± 9	53.5 ± 2.6	351 ± 11	34.1 ± 3.6
22.7 (+PGF)	467 ± 31	8.7 ± 0.9	232 ± 4	52.3 ± 1.4	319 ± 11	34.6 ± 2.6
22.7 (–PGF)	399 ± 15	6.9 ± 0.9	237 ± 16	53.0 ± 5.1	288 ± 19	34.1 ± 3.4

Data represent means ± SE of 7 males per group. See text for explanation of steroid abbreviations. For the controls (0) and highest water carbofuran concentration (22.7) males were either exposed to $PGF_{2\alpha}$ (+PGF) or not (–PGF). * = $p < 0.05$ compared to control group [0 (–PGF)].

Bile free 11-KT levels were similar in all the groups, whereas the bile concentrations of the glucuronide conjugate of this steroid increased in pheromonally-primed males. However the presence of carbofuran at and above 2.7 µg l^{-1} abolished this increase (Table 1).

When exposed to PE *in vitro*, the release of 17,20ßP, T, and 11-KT from testes was significantly higher than those of corresponding controls (Fig. 4). In addition, there was no significant difference in the degree of PE stimulation for any of the three steroids between testes from carbofuran and non-carbofuran exposed males. Both the basal secretion and the degree of responsiveness to PE-stimulation were not influenced by whether the testes came from $PGF_{2\alpha}$-primed males or not.

Discussion

The present study clearly demonstrates that when mature male Atlantic salmon parr were exposed to carbofuran, there was a significant effect on the parrs' ability to detect and respond to the priming pheromone $PGF_{2\alpha}$. Both the recorded responses from the olfactory epithelium and the elevation of plasma levels of sex steroids and expressible milt after stimulation with the priming pheromone were significantly reduced or abolished as a result of exposure to the pesticide. Electrophysiological recordings from the epithelium indicated that the responses to the pheromone were significantly reduced at nominal concentrations of carbofuran as low as 1.0 µg l^{-1}, and the threshold of detection was reduced 10 fold. Exposure of salmon parr to nominal concentrations of the pesticide above 1.1 µg l^{-1} resulted in a significant impact on the priming response to the pheromone, and between 2.7 and 6.5 µg l^{-1} the response was completely abolished.

It is not yet evident what the toxicological mechanism is by which carbofuran disrupts the priming response in the male parr. However, it is probable that the pesticide is operating directly on the olfactory system of the fish. The fact that carbofuran significantly reduced the EOG response to $PGF_{2\alpha}$ strongly suggests the parr did not detect the pheromone, and this subsequently inhibited the priming of the males reproductive system. Elevation of plasma steroids as a result of a priming pheromone has also been shown to be mediated via the olfactory system in another salmonid (Olsén and Liley 1993). In addition, the data suggest that the disruption of the priming response is not the result of carbofuran directly affecting the testis or altering steroid excretion. Firstly, there was no significant difference in the circulating steroid concentrations nor in the level of expressible milt between PGF- and non-PGF stimulated males exposed to the highest carbofuran concentration. Secondly, testes from carbofuran-exposed males responded as well as the control fish testes to PE-stimulation. Both these lines of evidence indicate that testicular secretion of steroids was not impaired by carbofuran exposure. And thirdly, bile concentrations of free and glucuronidated

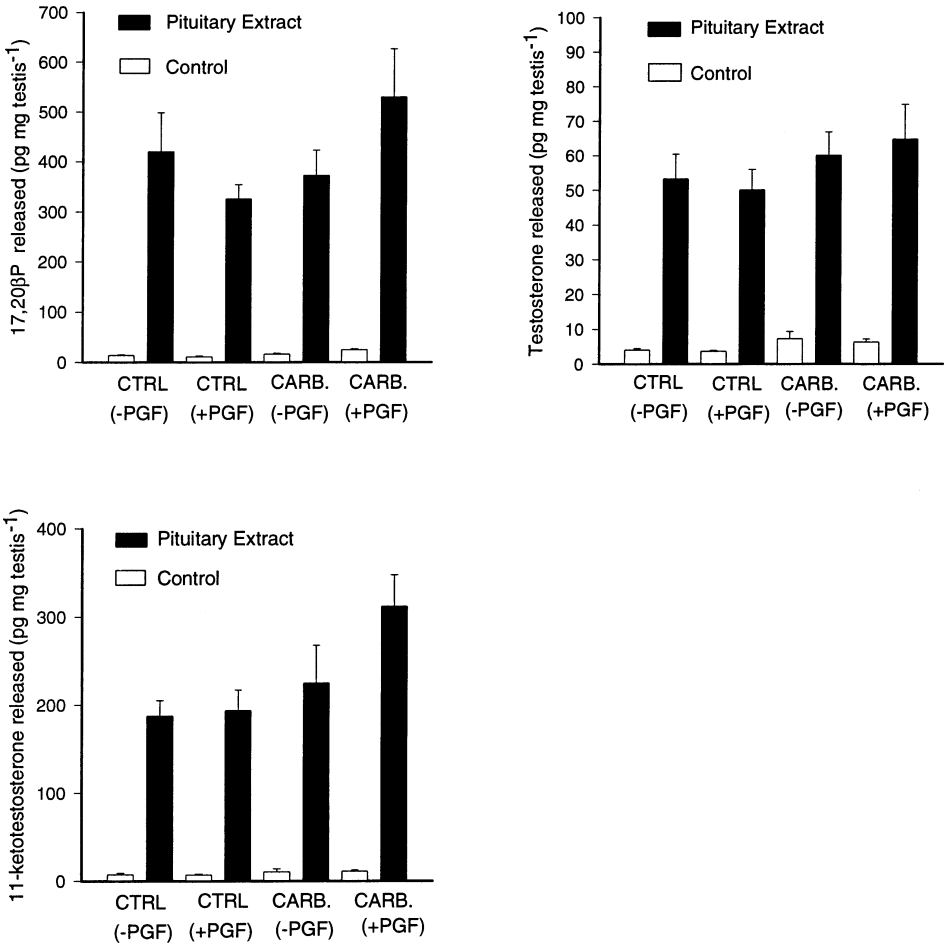

Fig. 4. In vitro release of free steroids from testes from different groups of salmon. CTRL = testes from control males; CARB = testes from males exposed to 22.7 μg l^{-1} carbofuran; –PGF = testes from fish not exposed to $PGF_{2\alpha}$; +PGF = testes from fish exposed to $PGF_{2\alpha}$. Data represent means ± SEM of 7 testes per group.

steroids were not increased in males unresponsive to pheromonal priming, which suggests that the unchanged plasma steroid concentrations were not a result of an increased plasma clearance rate.

During the priming experiments it was evident that the inhibition of the plasma androgen responses required exposure to higher water carbofuran concentrations compared to the inhibition of the plasma 17,20ßP response. An identical pattern was apparent in pheromonally-primed male salmon parr exposed to diazinon, an organophosphate pesticide (Moore and Waring 1996a). We had previously hypothesised that this reflected a differential regulation of C19 and C21 gonadal steroids in pheromonally-primed male salmon and although the present data supports this hypothess, the reason for it is unknown.

Mature male salmon parr appeared to be rendered anosmic, or at least severely hyposmic, to the priming pheromone after exposure of the olfactory epithelium to carbofuran. What effect this would have on the spawning potential and success of male salmon is not clear. Recent evidence from goldfish suggests that males detecting the priming pheromone released by females have not only greater quantities of milt but also increased numbers of sperm with greater motility (Defraipont and Sorensen 1993). Anosmic male rainbow trout

exhibited reduced levels of expressible milt and plasma sex hormones when paired with ovulated females compared to intact males (Olsén and Liley 1993). Despite this, however, Olsén and Liley (1993) noted that anosmic males spawned successfully with females, although Honda (1980) found that anosmic male rainbow trout had a dramatically reduced urge to court ovulated females when compared to intact males. Anosmic male kokanee salmon also showed reduced levels of milt and plasma sex hormones and were also much less vigorous and persistent in their courtship of females (Liley et al. 1993). Therefore, the evidence to date suggest that the ability to detect chemical cues emitted by ovulated and nesting female salmonids is important for male spawning readiness and success. The importance of chemical cues is likely to increase dramatically at night when the role of visual cues involved in reproduction are reduced. It is therefore probable that the inability of male salmon to respond to the priming pheromone in carbofuran-contaminated waters will affect their spawning potential and reproductive success and may have long term implications for individuals and populations.

The toxicological effect of carbofuran on the olfactory system was not only restricted to priming pheromones and reproduction. The pesticide also significantly reduced the ability of the olfactory epithelium to respond to the amino acid L-serine. The occurrence of carbofuran in the aquatic environment may therefore have significant effects on other olfactory mediated behaviour and physiology during the Atlantic salmon life cycle. The possible effects on olfactory imprinting during the smolt stage and the homing of the adults are of concern. In addition the effects of long term exposure of salmon to carbofuran are not known, but there may be implications for adult Atlantic salmon that spend more extended periods in fresh water, such as early spring-run populations.

The environmental impact of many pesticides on fish may be reduced by the individuals ability to detect the substances and avoid the contaminated area. A number of pesticides have been reported to be detected by the olfactory system of fish and these pesticide-contaminated waters can be avoided (Hidaka and Tatsukawa 1989; Ishida and Kobayashi 1995). However, the fact that carbofuran itself did not elicit an EOG response from the olfactory epithelium indicates that the pesticide is not an odorant to mature male parr at the concentrations tested. The inability to detect and avoid carbofuran-contaminated waters may have an important compounding effect on the environmental impact of carbofuran on salmon spawning success.

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