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Testosterone is a Potent Odorant in Precocious Male Atlantic Salmon (Salmo salar L.) Parr

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Testosterone is a potent odorant in precocious male Atlantic salmon (*Salmo salar* L.) parr

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SUMMARY

Electrophysiological recordings from the olfactory epithelia have shown that testosterone is a potent odorant in precocious male Atlantic salmon (*Salmo salar* L.) parr. However, the olfactory epithelia of these fish only appeared to be responsive for a limited period during the year (in October). Immature fish did not respond at any time. The precocious male parr were unresponsive to a large range of other steroids, including testosterone glucuronide. Free testosterone was not found in the urine of ovulating female Atlantic salmon. The results are discussed in relation to the role of testosterone in the physiology of the salmon and its possible role as a behavioural pheromone.

1. INTRODUCTION

In recent years it has been shown that teleost gonadal steroids can act as potent reproductive pheromones, releasing specific physiological and behavioural reactions in conspecifics. Steroids studied include 3α-hydroxy-5β-androstan-17-one-3α-glucuronide in the black goby, *Gobius jozo* (Colombo *et al.* 1980), 17α,20β-dihydroxy-4-pregnen-3-one in the goldfish, *Carassius auratus* (Stacey & Sorensen 1986; Dulka *et al.* 1987) and 3α,17α-dihydroxy-5β-pregnan-20-one-3α-glucuronide in the African catfish, *Clarias gariepinus* (Resink *et al.* 1989*a*).

Electrophysiological studies in the goldfish (Sorensen *et al.* 1987) and African catfish (Resink *et al.* 1989*b*) have shown that the olfactory epithelia of these fish are extremely sensitive to waterborne steroids, responding to concentrations as low as 10^{-13} M and 10^{-11} M, respectively. In the present study we examined the sensitivity of precocious male Atlantic salmon parr (*Salmo salar* L.) to a range of steroids, using the same electrophysiological technique (electro-olfactogram: EOG) as in the previous studies. EOG recording measures transepithelial voltage gradients from the surface of the olfactory epithelium and is thought to reflect multiunit receptor cell activity (Evans & Hara 1985).

The experiments were done in two successive years during the period of spermiation in precocious male parr. In the first year, a range of synthetic steroids was tested including steroids known to be present in the blood plasma of mature male and female Atlantic salmon. These steroids were testosterone, testosterone glucuronide, 17β-oestradiol and 17α,20β-dihydroxy-4-pregnen-3-one (see So *et al.* 1985). In the second year responsiveness to testosterone was studied in greater detail as this was the only steroid that had evoked a response from the olfactory epithelia the previous year.

An investigation was also made of the steroid content of urogenital fluids collected from ovulated female Atlantic salmon, to determine whether ovulated females might release testosterone.

2. MATERIALS AND METHODS

(*a*) *Experimental animals*

Atlantic salmon (*Salmo salar* L.) parr (147–158 mm in length, 1+ in age) were collected from the Welsh National Rivers Authority Cynrig hatchery in August 1988, September 1989 and May 1990 and transported to the Lowestoft Fisheries Laboratory. The fish were maintained under natural light conditions in plastic troughs with a constant flow of dechlorinated water (flow 40 l min^{-1}; temperature 8.9–14.0 °C).

Fish were studied during October 1988 and between October and December in 1989. Several immature fish were also tested in June 1990. After each experiment (2–4 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). Mature males had a GSI of 7.8% (s.e.m. 1.7% $n = 13$) and the immature males had a GSI of 0.02% (s.e.m. 0.004% $n = 5$). Immature females had a GSI of 0.019% (s.e.m. 0.003% $n = 5$).

(*b*) *Recording procedure*

The procedure was similar to that used by Sorensen *et al.* (1987) in the goldfish. The fish were anaesthetized with 2-phenoxy ethanol (0.4 ml l^{-1}) and the skin and cartilage removed to expose the olfactory rosettes. The fish were then immobilized with an intramuscular injection of gallamine triethiodide (0.3 mg kg^{-1} body mass) and placed in a V-shaped clamp in a Perspex flow-through chamber. The gills were constantly

perfused with water containing 2-phenoxy ethanol (0.2 ml l^{-1} concentration). Electrophysiological recordings were made by using glass pipettes filled with a saline-agar solution (4%), bridged to an Ag–AgCl electrode (Type EH-3MS, Clark Electromedical Instruments) filled with 3 M KCl. The tip of the glass pipette was placed close to the olfactory epithelium at the base of the largest posterior lamella. This was where maximum responses to 10^{-5} M L-serine and minimum responses to dechlorinated water controls were normally obtained. A reference electrode, of the same type, was grounded and placed lightly on the skin close to the nares of the fish. The signal was amplified by 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-i 386/100 computer by using Asystant+ scientific software (Asyst Inc.)

A constant volume of the test substance (dissolved in 200 µl of dechlorinated water) was injected via a remote control switch, into the second inlet of a three-way solenoid valve (Lee Company), carrying a constant flow of dechlorinated water over the olfactory epithelium (20 ml min^{-1}). The stimulus lasted 2 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.

(*c*) *Testing procedure*

The following steroids (Sigma Chemical Co. or Steraloids Ltd) were tested: 17α-hydroxy-4-pregnene-3,20-dione; 17α,20β-dihydroxy-4-pregnen-3-one; 17α,20α-dihydroxy-4-pregnen-3-one; 17α,21-dihydroxy-4-pregnene-3,20-dione (11-deoxycortisol); 3α,17α,20β-trihydroxy-5β-pregnane; 3α,17α-dihydroxy-5β-pregnan-20-one; 3α,17α,21-trihydroxy-5β-pregnan-20-one; 17α,21-dihydroxy-5β-pregnane-3,20-dione; testosterone; testosterone glucuronide (sodium salt); testosterone glucuronide (free acid); testosterone sulphate (sodium salt); androstenedione; 11-ketotestosterone; 3α,17β-dihydroxy-5β-androstane; 17β-oestradiol.

Serial dilutions of the steroids ranging from 10^{-14}–10^{-4} M concentration were prepared from stock solutions consisting of 500 µg steroid per millilitre of absolute ethanol. The dilutions were prepared fresh before each experiment and allowed to stand at room temperature until required. Similar concentrations of ethanol were prepared as controls and also tested. Serial dilutions of the amino acids L-cysteine and L-alanine and the bile acid taurocholic acid, ranging from 10^{-10} to 10^{-4} M, were also prepared fresh before each experiment.

The steroids and other compounds were presented to the olfactory epithelium in order of increasing concentration with a 2 min recovery interval between each stimulus. Each dilution was tested twice, and the responses to 10^{-5} M L-serine and a water control were tested at the beginning and end of each series of dilutions. The amplitude of each EOG response was measured from the baseline to the peak and expressed in mV. Replicates were averaged and then the values expressed as a percentage response of the most recently applied 10^{-5} M L-serine standard. L-serine was chosen as a standard as it has also been used as such in a previous study measuring the EOG responses of fish olfactory epithelia to steroids (Sorensen *et al.* 1987) and therefore permitted a comparison between studies of the potency of various steroids.

(*d*) *Radioimmunoassay of testosterone in urogenital fluids of ovulated female Atlantic salmon*

Samples of urine and ovarian fluid were collected, during the stripping of eggs for hatchery purposes, from 10 ovulated female Atlantic salmon during December 1989. Although an attempt was made to collect the urine and ovarian fluids separately, mixing occurred in all cases resulting only in samples of mixed urogenital fluids. They were frozen and stored until required for assay. 50 µl portions were then extracted, hydrolysed with β-glucuronidase and assayed as described previously (Scott & Canario 1990).

3. RESULTS

(*a*) *Electrophysiological recordings*

Of the steroids tested in 1988, testosterone was the only one that elicited an electrical response recordable from the olfactory epithelia of the precocious male parr ($n = 7$). On the basis of this finding the 1989 studies concentrated on characterizing the olfactory response to testosterone. Typical EOG responses to testosterone are shown in figure 1. The olfactory sensitivity to this steroid was extreme, with a threshold concentration of 10^{-14} M (figure 2). At concentrations of 10^{-10} to 10^{-8} M the measured EOG response was 134–168% greater than the response to 10^{-5} M L-serine. Responses to testosterone were obtained only from spermiating males. Non-spermiating precocious male parr, non-precocious male parr, and immature female parr were unresponsive to either testosterone or any of the other steroids during the study. The amplitude of the EOG response did not increase exponentially with increasing testosterone concentration, but appeared to level off above 10^{-7} M suggesting possible receptor site saturation.

EOG responses to testosterone were only obtained from spermiating male parr tested during the month of October after which there was a total loss of sensitivity (figure 2). The recorded response to testosterone was

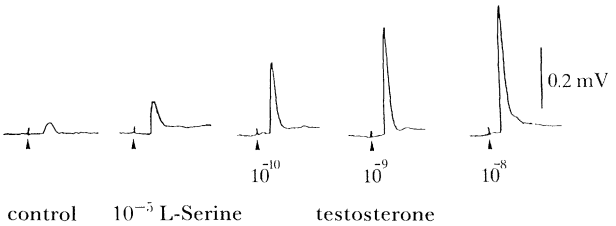

Figure 1. Typical EOG responses to testosterone recorded from the olfactory epithelia of spermiating precocious male salmon parr. The control is a solution of 10^{-8} M ethanol. Arrows indicate the addition of the odorant.

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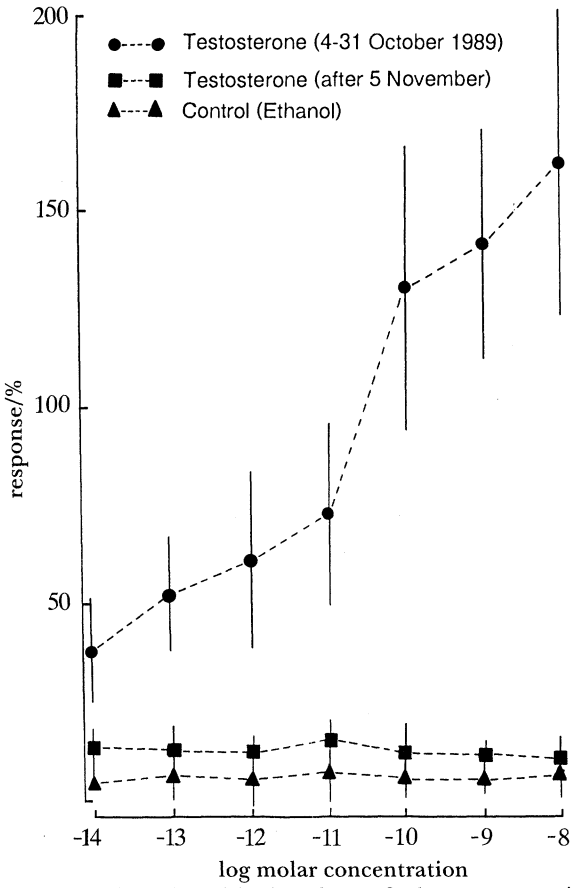

Figure 2. Semi-logarithmic plots of the concentration response relationship to testosterone in spermiating male precocious parr between 4–31 October 1989 ($n = 8$) and after 5 November 1989 ($n = 6$). The third plot is the response to control concentrations of ethanol. The responses are represented as a percentage of the response to a 10^{-5} M concentration of L-serine. Vertical bars represent standard errors.

then similar in amplitude to the controls although the precocious parr at this time still appeared to be spermiating.

The olfactory thresholds of precocious male parr to the L-amino acids and taurocholic acid were the same for non-precocious and female parr and similar to other teleost species (Sorensen *et al.* 1987; Hara 1982). Thresholds were 10^{-8} M for L-serine and 10^{-9} M for L-cysteine and taurocholic acid.

(*b*) *Testosterone content of urogenital fluids of female Atlantic salmon*

Levels of free testosterone in the ten samples were < 1 ng ml^{-1}. The mean level of testosterone glucuronide was 78.1 ng ml^{-1} (s.e.m. $\pm$20.9).

4. DISCUSSION

The present study shows that testosterone is a potent odorant in precocious male salmon parr, although the physiological and behavioural significance of this finding has yet to be fully established. A preliminary behavioural study on the Atlantic salmon has indicated that testosterone, at levels as low as 10^{-10} M concentration, is a strong attractant to spermiating precocious male salmon parr resulting in very positive rheotactic and searching behaviour (A. Moore, unpublished observations). Studies on at least two other species, also suggest that testosterone may act as an attractant or a releaser of reproductive behaviour. Spawning-run female sea lampreys, *Petromyzon marinus*, for example, have been shown to prefer water to which testosterone, at concentrations between 10^{-10} M and 10^{-12} M has been added (Adams *et al.* 1987). Testosterone, at a concentration of 10^{-11} M, has also been shown to induce female sexual behaviour in the Lake Baikal sculpin, *Cottocomephorus grewingki* Dyb. (Dmitrieva *et al.* 1988; Katsel *et al.* 1991). On the basis of the present results we suggest that the main role of testosterone, in female Atlantic salmon at least, may be as a pheromone, attracting the males to the females in the weeks leading up to spawning. The precocious male parr appear to lose their olfactory sensitivity to testosterone (end of October) some two weeks before spawning occurs in females from the same population. However, because mature female salmon were not tested in the present study we cannot preclude the possibility that the pheromone may also influence the females in some way.

To establish that waterborne testosterone has a behavioural or physiological role in Atlantic salmon parr, it will be necessary to show that it is synthesized and released into the water by conspecifics at detectable concentrations. It has already been well-established that blood plasmas from reproductively maturing male and female Atlantic salmon, in common with many other teleost species (see Scott & Sumpter 1983) have markedly elevated levels of testosterone (Idler *et al.* 1971; Stuart-Kregor *et al.* 1981; Hunt *et al.* 1982; So *et al.* 1985; Crim *et al.* 1986; Sangalang & Freeman 1988). Peak levels that have been reported in Atlantic salmon plasma range between 7 and 45 ng ml^{-1} in males and 22 and 120 ng ml^{-1} in females. Levels start to rise at an early stage in reproductive development, reach a peak some 1–5 weeks before the spawning and are significantly lower than peak levels at the time that spawning actually occurs. In both sexes most of the testosterone found in male and female Atlantic salmon is derived from the gonads (Idler *et al.* 1971 and Zhao & Wright 1985).

Our failure to detect free testosterone in the urogenital fluids of ovulated female salmon suggests either that: (*a*) testosterone is released from the females by some route other than the urine and/or ovarian fluid; (*b*) the females may not be the source of the testosterone (it is feasible that other male salmon may produce testosterone which may act as a male–male pheromone used to reduce milt production in conspecifics as part of a reproductive strategy); (*c*) the urines were sampled at the wrong time (see Discussion above), and (*d*) there is some other active substance in the urine, which although very similar to testosterone, cannot be detected by radioimmunoassay. Although there were fairly substantial amounts of glucuronidated testosterone, which is also found in the plasma (So *et al.* 1985), this did not evoke a response from the olfactory

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epithelium. Further research, including an investigations of the olfactory potency of male and female urines, is obviously required.

The limited period of responsiveness to testosterone suggests that receptiveness to some olfactory attractants or releasers may vary with the maturation stage. Resink *et al.* (1989*b*) showed that the behavioural response of the females was very dependent on ovulation. Similarly, Colombo *et al.* (1980) showed that only female black gobies containing ovulated eggs would show a behavioural response to 3α-hydroxy-5β-androstan-17-one-glucuronide. These findings contrast greatly, however, with those of Sorensen *et al.* (1987), who showed that neither the gender nor the reproductive condition of the goldfish affected the EOG response to 17,20β-P, and Resink *et al.* (1989*b*) who also showed that the EOG response to 3α,17-P-5β-glucuronide and other steroids was not influenced by the maturity stage of African catfish females.

The apparent saturation of the response to testosterone above a concentration of *ca.* 10^{-7} M is similar to that reported for 17,20β-P in the goldfish (Sorensen *et al.* 1987). In addition, behavioural results on the preference of landlocked sea lamprey for testosterone also show a reduced responsiveness to higher concentrations (Adams *et al.* 1987).

The references to proprietary products in this paper should not be construed as an official endorsement of these products, nor is any criticism implied of similar products that have not been mentioned.

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