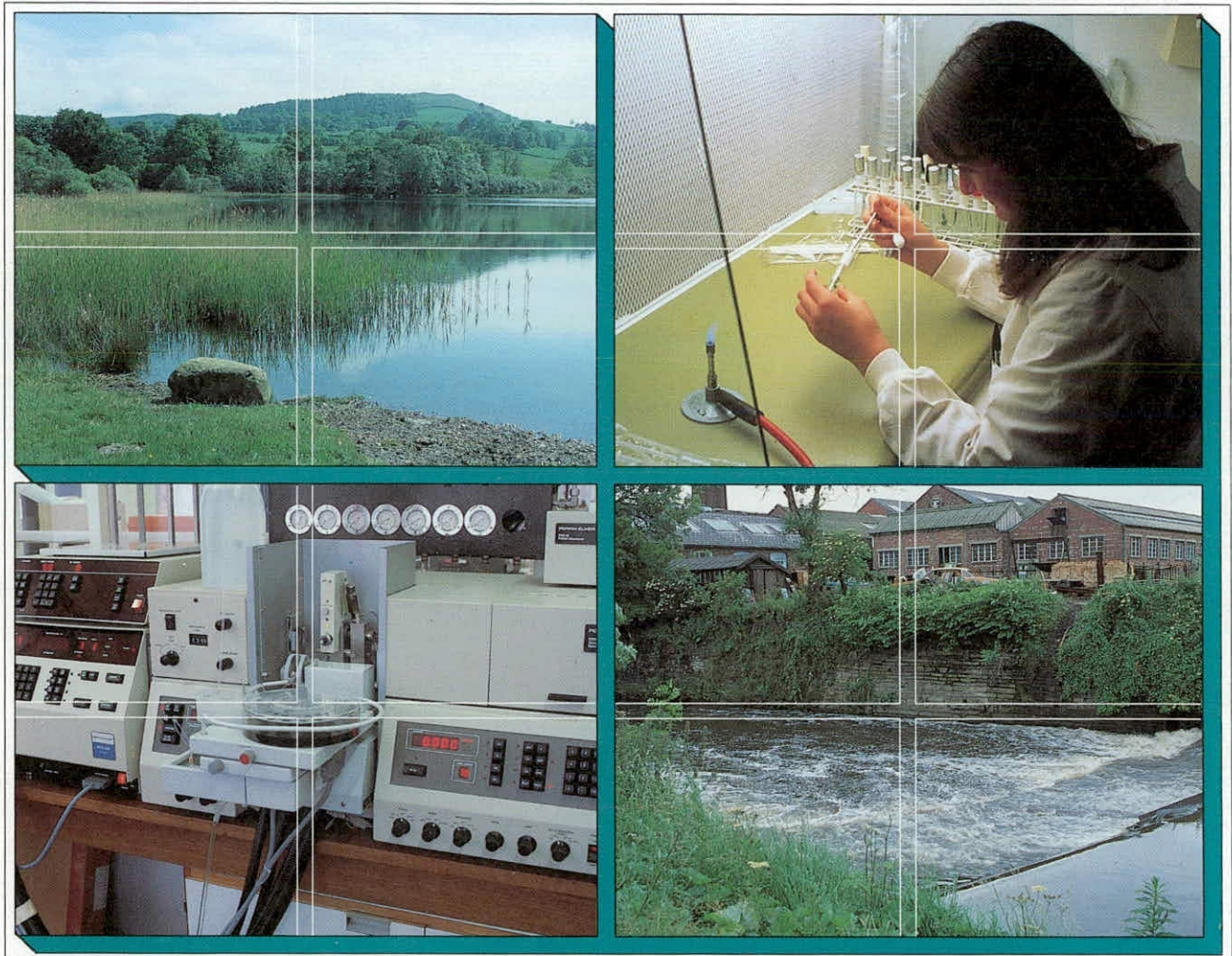


**PHEROMONE RECEPTION IN SALMONID FISH:  
IDENTIFICATION AND CHARACTERIZATION OF PUTATIVE  
STEROID RECEPTORS IN OLFACTORY TISSUE OF  
BROWN AND RAINBOW TROUT**

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A study commissioned by  
The Ministry of Agriculture Fisheries and Food,  
Chief Scientists Group (Fisheries),  
Nobel House, 17 Smith Square,  
London SW1P 3JR

April 1996

IFE Report No. WI/T11050V1/1

## SUMMARY

Specific binding sites for testosterone have been detected in three compartments of olfactory tissue from brown and rainbow trout. Binding of  $^3\text{H}$ -testosterone to the membrane fraction of olfactory tissue is of high affinity ( $K_d = 0.5 - 1.9 \text{ nM}$ ) and limited capacity ( $N_{\text{max}} = 30 - 60 \text{ fmol mg}^{-1} \text{ protein}$ ). Binding is reversible, and is eliminated by protease treatment. The binding site exhibits a high degree of ligand specificity,  $11\beta$ -hydroxytestosterone,  $11$ -ketotestosterone,  $17\alpha$ -hydroxyprogesterone,  $17\alpha$ ,  $20\beta$ -dihydroxy-4-pregnen-3-one, cortisol, and estradiol- $17\beta$  all fail to displace testosterone at 20-fold excess. These attributes are characteristic of specific steroid receptor proteins. Binding of testosterone within the cytosol fraction is of moderate affinity ( $K_d = 9.0 - 23.0 \text{ nM}$ ) and high capacity ( $N_{\text{max}} = 0.5 - 2.9 \text{ pmol mg}^{-1} \text{ protein}$ ) and binding of testosterone is more readily displaced by a number of steroid competitors than is the case for the membrane site. The rate of association and dissociation of testosterone from the cytosolic binding site is markedly more rapid than the equivalent processes in the membrane fraction. Binding of testosterone to the nuclear extract is of high affinity ( $K_d \sim 3.0 \text{ nM}$ ) and limited capacity ( $N_{\text{max}} \sim 50 \text{ fmol mg}^{-1} \text{ protein}$ ).

There are no marked differences between species or between sexes in the affinity or capacity of testosterone-binding sites in nuclear extract or membrane fraction. However, cytosolic testosterone-binding sites are three- to four-fold more abundant in rainbow trout than in brown trout, and female rainbow trout have more cytosolic binding sites than male rainbow trout, but a lower affinity for testosterone than male sites.

Preliminary evidence supports the involvement of the membrane-associated testosterone-binding site in olfactory processes. Rainbow trout display an EOG response to testosterone at a concentration ( $\geq 10^{-9} \text{ M}$ ) which is consistent with the equilibrium dissociation constant ( $K_d$ ) of the membrane-associated testosterone-binding site. The concentration of ligand at which half the binding sites are saturated ( $K_d$ ) is in the range  $0.5 - 2.0 \cdot 10^{-9} \text{ M}$  for membrane-associated testosterone binding. Binding of  $^3\text{H}$ -testosterone to the membrane-associated site shows a pH dependency which is comparable to the effects of pH on the EOG response to testosterone in intact fish.

The data concerning the attributes of the membrane associated testosterone-binding site are consistent with the olfactory tissue of salmonids possessing the capability to respond to testosterone present in the aquatic environment. The intracellular testosterone-binding sites show characteristics common to testosterone receptors in other fish tissues which are known androgen target tissues. This suggests that the development and/or function of salmonid olfactory tissue may be open to influence by endogenous testosterone. This is the first report of androgen binding to olfactory tissue in fish, and the first report of a membrane-associated androgen binding site in fish.

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# 1. INTRODUCTION

## 1.1 Background

It has long been appreciated that fish are extremely sensitive to chemical stimuli within their environment and that perception of chemical signals plays an important part in feeding, homing, kin recognition, schooling, and predator avoidance (Liley, 1982; Marui and Caprio, 1992; Olsen, 1992; Stabell, 1992; Hara, 1994). However, it is only relatively recently that the physiological mechanisms involved in chemosensory processes in fish have been studied in depth, and that the nature of links between olfaction, physiology and behaviour have become apparent. Understanding the crucial role of pheromones in the control and coordination of reproduction in fish is an area in which considerable progress has been made in recent years.

The definition and use of the term pheromone originally proposed by Karlson and Lüscher (1959) has remained unaltered to the present day. These authors suggested that pheromones are "*substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behaviour or a developmental process*". There has, however, been some debate over the applicability of this definition, as Sorensen (1992) discusses, focusing on the breadth of the definition, and whether metabolic by-products should be considered as pheromones. It is now clear that fish release many compounds into the water which conform to this definition of pheromones, among which are prostaglandins (Sorensen and Goetz, 1993; Sveinsson and Hara, 1995), steroids (Stacey *et al.*, 1989; Bjerselius *et al.*, 1995a) and steroid conjugates (Lambert and Resink, 1991; Scott and Vermeirsson, 1994).

The role of reproductive pheromones in fish is becoming increasingly well documented although the intricacies of the subject are far from being fully understood. Sorensen (1992) briefly describes the short history of research in this area. The goldfish (*Carrassius auratus*) is perhaps the fish in which the role, and identity, of pheromones involved in reproduction has been best described. Dulka (1993) summarises the reproductive pheromone system in goldfish as follows; preovulatory female goldfish release a pheromone, the primary component of which appears to be  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one ( $17\alpha,20\beta$ -P), which acts as a *primer* pheromone in



males. Gonadotropic hormone (GTH) secretion by the pituitary is stimulated in male goldfish resulting in increased milt production. A second pheromone is released by female goldfish following ovulation. The principal component of this pheromone appears to be a prostaglandin (PG). This compound(s) acts as a *releaser* pheromone and stimulates male sexual behaviour. Thus the pheromones released by the female optimise male gamete availability, trigger sexual behaviour, and synchronise the spawning act itself. Both Sorensen (1992) and Dulka (1993) extensively cite the original work which led to development of this model. Recent work suggests that  $17\alpha,20\beta$ -P also performs a similar function in the crucian carp (*Carrassius carassius*; Bjerselius *et al.*, 1995b) and common carp (*Cyprinus carpio*; Stacey *et al.*, 1994).

Not surprisingly, there are species differences in the pheromonal potency of specific compounds. A study which examined the olfactory sensitivity of eleven species of teleost fish to three prostaglandins ( $\text{PGF}_{2\alpha}$ , 15-keto- $\text{PGF}_{2\alpha}$ , 13,14-dihydro-15-keto- $\text{PGF}_{2\alpha}$ ), concluded that F-prostaglandins may be common as sex pheromones in cyprinids, but that non-cyprinids lack sufficient olfactory sensitivity to these compounds for them to perform this role (Kitamura *et al.*, 1994). Complicating matters further, *male* Arctic charr, *Salvelinus alpinus*, release large quantities of F-type prostaglandins into the water and this substance acts as an attractant for females (Sveinsson and Hara, 1995). Species variation in sensitivity to steroids as well as PG's is also apparent. Two species of catostomids (white sucker, *Catostomus commersoni* and longnose sucker, *C. catostomus*) show a high degree of sensitivity to F-prostaglandins but failed to show olfactory responses to free or conjugated gonadal steroids (Cardwell *et al.*, 1992).

There is considerable evidence that pheromones play a significant role in the reproduction of salmonid fish. It has been known for some time that female trout release a substance which attracts male fish (Newcombe and Hartmen, 1973). Anosmic kokanee salmon (*Oncorhynchus nerka*) are less vigorous and persistent in their courtship of females, and show reduced milt volume and plasma hormone levels compared to males with intact olfactory apparatus (Liley *et al.*, 1993). Similar evidence, supporting the importance of olfactory cues to reproductive processes, was obtained in rainbow trout (*O. mykiss*; Olsen and Liley, 1992). It has subsequently been demonstrated that urine of mature female rainbow trout contains a priming pheromone which elevates levels of  $17\alpha,20\beta$ -P, testosterone and GTH II in the blood of mature male

rainbow trout, although is without apparent effect on milt volume (Scott *et al.*, 1994). However, despite the fact that large quantities of steroidal compounds with potential to act as pheromones are released into the surrounding water by salmonids (Scott and Vermeirsson, 1994; Scott and Liley, 1994), the identity and role of specific pheromones related to these effects has yet to be established. Neither  $17\alpha,20\beta$ -P, or its conjugate  $17\alpha,20\beta$ -P-sulphate, are potent in eliciting physiological responses in trout (Scott *et al.*, 1994) despite the latter being abundant in the urine of rainbow trout and evoking an electrophysiological response in Atlantic salmon (*Salmo salar*; Moore and Scott, 1992). [Recent unpublished studies suggest that in fact salmonid fish may show a high degree of olfactory sensitivity to prostaglandins (A. Moore, pers. comm.). ]

## **1.2. Rationale for the study**

It is clear that pheromonal communication is intimately involved in assuring successful reproduction in fish. It is therefore important to understand the mechanisms by which pheromones exert their effects, and the identity of the compounds involved. Such knowledge is valuable, not only in furthering our understanding of the basic biology of an important animal group, but in assessing the potential impact on natural populations of fish of factors which might interfere with pheromonal communication. For example, water bodies are subject to pollution with a variety of organic and inorganic compounds - how might sublethal chemical pollution interfere with or otherwise affect pheromonal communication? Reproductive processes in fish are particularly sensitive to environmental stress - does acute or chronic stress interfere with the production and detection of pheromone signals?

Two approaches have traditionally been employed to identify pheromonal compounds and study their physiological and behavioural effects. One method is to expose fish to the putative pheromone and examine physiological and behavioural effects which result from such exposure. The second approach directly measures the ability of the fish to detect a specific compound by monitoring electrical activity in the olfactory tissue (electro-olfactogram, EOG). Both approaches have advantages. It is obviously ideal to obtain information on behavioural and physiological effects of a putative pheromone, as this exemplifies the role of the compound under natural conditions. However, such studies are complex and time-consuming. Initial screening of compounds likely to exert effects can be carried out employing electrophysiological techniques.

This approach is more easily set up, and provides exact data on the threshold of detection for the compound by the fish. Furthermore, the effect of other chemical factors on detection of the test compound by the olfactory tissue can be studied. However, only one individual may be screened at a time, and electrophysiological responses to a compound do not necessarily correspond to functional effects.

A further, complementary, approach is available. This is to employ biochemical methodology to identify specific receptor molecules for pheromonal compounds in the olfactory tissue. Characterisation of pheromone receptors would then permit the assessment of sensitivity to specific compounds in larger numbers of fish, and with better temporal resolution, than either of the alternative methodologies. In addition, screening of potentially active compounds could be readily accomplished by determining binding activities. In conjunction with existing techniques it would be feasible to address questions such as whether sensitivity to a given pheromone arises due solely to the appearance of the receptor, or whether responsiveness of the fish is dependent on other internal factors.

The study was carried out on rainbow trout and brown trout (*Salmo trutta*). The ready availability of rainbow trout, ease of maintenance and widespread experimental use as a "model" salmonid fish make it an ideal starting point for studies which will have relevance to both wild stocks of native salmonids and to aquaculture. The brown trout, in both its migratory and non-migratory forms, is a species of great economic significance in the UK.

### **1.3. Approach employed**

The only firm electrophysiological evidence for the detection of specific, putative pheromones in salmonid fish exists for the Atlantic salmon. Precociously mature male salmon display electrophysiological responses to very low concentrations (threshold for detection:  $10^{-14}$  M) of testosterone but this sensitivity is apparent for only a limited period of several weeks (Moore and Scott, 1991). No response was observed to  $17\beta$ -estradiol,  $17\alpha,20\beta$ -P or testosterone glucuronide. A substantial EOG response to  $17\alpha,20\beta$ -P-sulphate was also observed in precociously mature salmon parr but only after previous exposure of the olfactory tissue to urine from ovulated female salmon (Moore and Scott, 1992). As noted above, neither  $17\alpha,20\beta$ -P or  $17\alpha,20\beta$ -P-sulphate

elicit a physiological or behavioural response in rainbow trout (Scott *et al.*, 1994). The only previous study to examine the binding of a putative pheromone to the olfactory tissue of fish (goldfish; Rosenblum *et al.*, 1991) employed  $17\alpha,20\beta$ -P as a ligand, but these authors reported significant displacement of the binding of this ligand by testosterone. In the light of this limited evidence, it was therefore decided to initially seek specific binding sites for testosterone in the olfactory tissue of brown and rainbow trout.

In order to confirm the presence within a tissue of molecules which may function as receptors, a number of criteria must be satisfied. These can be summarised as follows (Orchinik and Murray, 1994; Hulme, 1990):

1. Binding of the ligand to the putative receptor should be saturable, reflecting a finite number of binding sites.
2. Specific binding should be abolished by conditions which are known to denature or degrade proteins.
3. Putative receptors should exhibit a ligand specificity which reflects their suggested physiological function.
4. The tissue distribution of the putative receptor should also reflect the physiological function(s) of the molecule.
5. Specific binding of the ligand to the putative receptor should be reversible.

The study was designed to address these factors with respect to the possible presence of testosterone-binding sites in the olfactory tissue of salmonid fish.

## 2. MATERIALS AND METHODS

### 2.1 Experimental fish

Three-year old brown and rainbow trout were maintained in the IFE experimental fish facility at Windermere. Both groups of fish were reared from eggs on site. Fish were held in 1500 l outdoor fibreglass tanks, each supplied with a constant flow of lake water ( $20 \text{ l min}^{-1}$ ) and fed once daily, five times per week, on commercial trout feed (BP Mainstream) at the manufacturers recommended rate.

### 2.2 Tissue preparation

Fish were netted from their holding tank into a trough containing anaesthetic (2-phenoxyethanol, 1:2000). When fully anaesthetized, the olfactory tissue was exposed and removed by dissection. The fish were then killed by a sharp blow to the head. Tissue was placed directly in homogenization buffer (0.2 M tris-HCl, pH 7.4, 12 mM monothioglycerol, 1.0 mM EDTA, 10.0 mM sodium molybdate, 20% glycerol) on ice. Within one hour of the commencement of sampling, the tissue was rinsed, wet weighed, and fresh buffer was added in the ratio 3 : 1 (volume : weight). The wet weight of individual olfactory rosettes from the fish sampled during these experiments was within the range 80 - 120 mg. The tissue was then homogenised, on ice, using an Ultra-Turrax TP 18/10. The homogenate was transferred to 13.5 ml polycarbonate centrifuge tubes and centrifuged at 1000 g for 15 mins at  $4^{\circ}\text{C}$  (3500 rpm in a Beckman J2-21 centrifuge with JA21 head). The pellet from this first spin, comprising nuclei and intact cells, was retained and the supernatant was transferred to clean tubes and centrifuged at 30,000 g for 60 mins at  $4^{\circ}\text{C}$  (19,000 rpm in J2-21). The resultant cytosol was dispensed in aliquots into capped polypropylene tubes and frozen at  $-70^{\circ}\text{C}$  until required. The pellet (membrane fraction) was resuspended by the addition of a similar volume of homogenization buffer and gentle homogenization. The resuspended membrane fraction was dispensed in aliquots and frozen at  $-70^{\circ}\text{C}$ . The nuclear pellet was washed three times by suspension in buffer and centrifugation at 1000 g for 15 mins. After the final wash, the pellet was resuspended in a similar volume of buffer containing 0.7 M KCl and incubated for 1 h at  $4^{\circ}\text{C}$ . The extract was then spun at 30,000 g for 60 mins at  $4^{\circ}\text{C}$  and the resulting supernatant (nuclear extract) was dispensed in aliquots and frozen at  $-70^{\circ}\text{C}$ .

### 2.3 Preliminary experiments

A first binding assay was carried out to determine whether there was any evidence for the specific binding of testosterone to either the membrane or cytosol fraction of trout olfactory tissue. Immature fish and mature female fish from two batches of 3+ rainbow trout (Stannan 1990 and Tasmanian 1990) were employed. Membrane and cytosol fractions were prepared from the olfactory tissue of these fish and 500  $\mu\text{l}$  (5-10 mg protein  $\text{ml}^{-1}$ ) of each was incubated together with 100  $\mu\text{l}$  of homogenization buffer containing 100,000 dpm (0.5 pmol) of [1,2,6,7- $^3\text{H}$ ]testosterone (3.33 Tbq  $\text{mmol}^{-1}$ , 11.3 Gbq  $\text{mg}^{-1}$ ; Amersham) with ( $B_{\text{NS}}$ ; 2 tubes) or without ( $B_{\text{T}}$ ; 2 tubes) a 1000-fold excess of inert testosterone (500 pmol, 144 ng). Incubation was carried out at 4°C for 1 h. In order to determine the extent to which  $^3\text{H}$ -T was bound to the membrane fraction, immediately following incubation, the tubes were vortex-mixed and 500  $\mu\text{l}$  of the contents were pipetted onto a glass microfibre filter (Whatman GF/B, retention 1.0  $\mu\text{m}$ , 2.5 cm) which had been pre-soaked in buffer overnight. A vacuum was applied to the filter and a 10.0 ml buffer wash was applied to the filter to remove unbound steroid and any steroid bound to soluble components of the preparation. The filter was then placed in a 5.0 ml scintillation vial, 4.0 ml of scintillation fluid (Ecoscint A, National Diagnostics) were added, and the samples were counted under standard  $^3\text{H}$  conditions in a liquid scintillation counter (Canberra-Packard 1900TR). Binding in the cytosol preparations was determined as follows. The tubes were placed on ice and 200  $\mu\text{l}$  of a dextran-charcoal suspension (DCC; 1.25% activated charcoal, 0.125% dextran, in homogenization buffer) were added to each tube. The tubes were vortex mixed, incubated on ice for 10 mins, then centrifuged to remove the DCC from suspension. A 300  $\mu\text{l}$  aliquot of supernatant from each tube was added to 4.0 ml of scintillation fluid in a 5.0 ml scintillation vial. Samples were counted under standard  $^3\text{H}$  conditions.

### 2.4 Effect of protein concentration on specific binding of $^3\text{H}$ -T to olfactory tissue membrane preparations

The aim of this experiment was to determine whether specific binding of T to olfactory tissue membrane preparation is directly proportional to protein content. Aliquots (400, 300, 200, 100 and 50  $\mu\text{l}$ ) of olfactory tissue membrane preparation from mature female rainbow trout were pipetted into groups of six assay tubes, and made up to a total volume of 400  $\mu\text{l}$  with buffer. Three tubes of each batch had previously received 20  $\mu\text{l}$  of ethyl acetate containing 0.5 nmol

inert T which was evaporated off under vacuum. A 100  $\mu$ l aliquot of buffer containing 100,000 dpm (0.5 pmol) of  $^3\text{H-T}$  was added to each tube and after mixing well the tubes were incubated for 1 h at 4°C. After this period, the tubes were mixed thoroughly and 400  $\mu$ l of each sample was pipetted onto a glass microfibre filter (Whatman GF/B, retention 1.0  $\mu\text{m}$ , 2.5 cm) mounted in a vacuum filtration manifold (Millipore Model 1225). A vacuum was applied to the filter and a 10.0 ml buffer wash was applied to each filter. Each filter was then placed in a 5.0 ml scintillation vial, 4.0 ml of scintillation fluid (Ecoscint A, National Diagnostics) were added, and the samples were counted under standard  $^3\text{H}$  conditions in a liquid scintillation counter (Canberra-Packard 1900TR).

### **2.5 The effect of protease treatment on the specific binding of $^3\text{H-T}$ to olfactory tissue membrane preparations**

The aim of this experiment was to demonstrate the proteinaceous nature of the specific binding sites for T in olfactory tissue membrane preparations. Aliquots of mature female rainbow trout olfactory tissue membrane fraction (400  $\mu$ l) were incubated together with 100,000 dpm (0.5 pmol) of  $^3\text{H-T}$  either with ( $B_{\text{NS}}$ ) or without ( $B_{\text{T}}$ ) 0.5 nmol of inert T. Half the total and non-specific binding assay tubes (12) also received 100  $\mu$ l of buffer containing sufficient trypsin (Sigma) to give a final concentration in the tube of 1.0 mg ml<sup>-1</sup>. The remaining tubes received 100  $\mu$ l of buffer alone. The tubes were vortex-mixed and incubated at 4°C for 24 h. At the end of this period, binding of  $^3\text{H-T}$  within each sample was determined by filtration as described above.

### **2.6 The tissue specificity of the specific binding of $^3\text{H-T}$ to membrane fractions from rainbow trout**

Membrane fractions were prepared as described in section 2.2 from brain, liver, spleen and muscle tissue of immature rainbow trout. Specific binding of  $^3\text{H-T}$  was quantified in each of these fractions as described in section 2.5. Each preparation was assayed in quadruplicate.

### **2.7 The ligand specificity of the $^3\text{H-T}$ binding sites in olfactory tissue membrane fraction and olfactory tissue cytosol**

The aim of this experiment was to determine whether the binding sites for  $^3\text{H-T}$  identified in trout

olfactory tissue membrane preparations and in trout olfactory tissue cytosol exhibited a narrow or broad range of ligand specificity. To 32 assay tubes containing 400  $\mu\text{l}$  of mature female olfactory tissue membrane fraction were added 100  $\mu\text{l}$  of buffer containing 1.0 pmol (200,000 dpm) of  $^3\text{H-T}$  and 1.0 nmol of either testosterone,  $11\beta$ -hydroxytestosterone, 11-ketotestosterone,  $17\alpha$ -hydroxyprogesterone,  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one, cortisol, estradiol- $17\beta$  (unlabelled). Each steroid was dispensed into 4 assay tubes. Four tubes received buffer and  $^3\text{H-T}$  only to estimate  $B_T$ . The tubes were vortex mixed and incubated at  $4^\circ\text{C}$  for 1 h. After this period, the samples were filtered and binding of the radiolabelled ligand was determined as described in section 2.3.

The specificity of binding in cytosol was assessed using the same protocol except that 300  $\mu\text{l}$  cytosol were employed in each tube. The tubes were mixed and incubated at  $4^\circ\text{C}$  for 2 h. Binding was determined as described in section 2.3.

A total of seven separate pools of membrane and cytosol preparation were employed, derived from both brown trout and rainbow trout.

### **2.8 The time-course of binding of $^3\text{H-T}$ to olfactory tissue membrane fraction and olfactory tissue cytosol**

The aim of this experiment was to determine the time required for binding of  $^3\text{H-T}$  to olfactory tissue to reach equilibrium. This information is necessary to carry out saturation analysis of the tissue fractions. A 400  $\mu\text{l}$  aliquot of mature female rainbow trout olfactory tissue membrane preparation was pipetted into each of 42 assay tubes, together with 100  $\mu\text{l}$  of buffer containing 0.5 pmol (100,000 dpm)  $^3\text{H-T}$ . Half of these tubes also received 0.5 nmol (144  $\mu\text{g}$ ) of inert T. The tubes were vortex mixed and incubated at  $4^\circ\text{C}$ . At 10, 20, 30, 45, 60, 120, 240 mins and 24 h after the start of the experiment, binding of  $^3\text{H-T}$  was determined in three  $B_T$  and three  $B_{NS}$  tubes, as described in section 2.3.

A similar procedure was carried out with olfactory tissue cytosol. In this case, 300  $\mu\text{l}$  aliquots of cytosol were added to each tube. The assay was carried out as for the membrane fraction and equilibration was terminated as described in section 2.3.



## **2.9 The time-course of dissociation of $^3\text{H-T}$ from olfactory tissue membrane fraction and from olfactory tissue cytosol**

The aim of this experiment was to determine that binding of  $^3\text{H-T}$  to each olfactory tissue fraction was reversible, a characteristic of receptor-ligand interaction. Aliquots of olfactory tissue membrane preparation (400  $\mu\text{l}$ ) were added to thirty-six assay tubes together with 100  $\mu\text{l}$  of buffer containing 0.5 pmol (100,000 dpm)  $^3\text{H-T}$ . Half of these tubes also received 0.5 nmol (144 pg) of inert T. The tubes were vortex mixed and incubated at  $4^\circ\text{C}$  for 2 h. After 2 h, binding of  $^3\text{H-T}$  was determined in three  $B_T$  and three  $B_{NS}$  tubes, as described above. At this point, 20  $\mu\text{l}$  of ethanol containing 0.5 nmol of inert T were added to 18 tubes, and 12 tubes received ethanol only. Binding of  $^3\text{H-T}$  was determined in the tubes which received additional inert T at 10, 20, 30, 45, 60 and 120 mins after the addition. Binding was determined in the control, ethanol only, tubes at 60 and 120 mins. Binding was quantified as described in section 2.3.

A similar procedure was followed to determine the time-course of dissociation in cytosol. Aliquots of 300  $\mu\text{l}$  were employed, and samples were initially incubated for 1 h before the addition of the additional excess inert T. Binding was quantified as described in section 2.3.

## **2.10 Saturation analysis of olfactory tissue membrane fractions, with $^3\text{H-T}$ as ligand**

The aim of these experiments was to estimate the equilibrium dissociation constant ( $K_d$ ) and abundance of specific binding sites for T ( $N_{\text{max}}$ ) in trout olfactory tissue membrane fraction. In order to conserve material, because of the limited quantities of olfactory tissue recovered from each fish ( $\sim 200$  mg wet weight fish $^{-1}$ ), saturation analyses were carried out over only 5 - 6 different concentrations of ligand. The range of concentrations of  $^3\text{H-T}$  over which the saturation analysis was carried out was 0.5 - 8.0 nM, equivalent to 50,000 - 800,000 dpm of  $^3\text{H-T}$  in a 500 $\mu\text{l}$  assay volume. Four assay tubes were assigned to each point on the saturation curve. An aliquot of 20 $\mu\text{l}$  of ethyl acetate containing 0.5 nmol inert T was added to two tubes within each group and evaporated under a vacuum. Then 100 $\mu\text{l}$  of buffer containing either 50, 100, 200, 400 or 800 K dpm of  $^3\text{-T}$  (see section 2.3 for details of specific activity etc.) was added to to each group of four tubes, two  $B_T$  and two  $B_{NS}$  tubes per concentration. A 500 $\mu\text{l}$  aliquot of membrane suspension (protein concentration  $\sim 2.0$  mg ml $^{-1}$ ) was pipetted into each tube and the tubes were vortex-mixed and incubated at  $4^\circ\text{C}$  for 1 h. After this period, the tubes were vortex-mixed and filtered to

separate membrane fragments from unbound steroid and radioactivity on the filters was quantified, as described in section 2.3.

### **2.11 Saturation analysis of olfactory tissue cytosol with $^3\text{H-T}$ as ligand**

The aim of these experiments was to estimate the equilibrium dissociation constant ( $K_d$ ) and abundance of specific binding sites for T ( $N_{\text{max}}$ ) in trout olfactory tissue cytosol. Initial attempts to obtain saturation curves for  $^3\text{H-T}$  with olfactory tissue cytosol were unsuccessful and it appeared to be the case that large amounts of T would be required to achieve saturating concentrations. To conserve  $^3\text{H-T}$ , a "spiked cold" approach was employed, in which increasing amounts of inert T were incubated together with a constant amount of  $^3\text{H-T}$ , both in the presence and absence of excess inert T. Four assay tubes were assigned to each point on the saturation curve. A 100 $\mu\text{l}$  aliquot of ethyl acetate either 0.5, 1, 2, 4, 8, 16, 32, or 64 pmols of inert T was added to each batch of tubes. two tubes from each batch of four also received 10 $\mu\text{l}$  of ethyl acetate containing 2 nmol inert T (576 ng). The solvent was evaporated under vacuum and 100 $\mu\text{l}$  of buffer containing 50,000 dpm  $^3\text{H-T}$  was added to each tube. A 200 $\mu\text{l}$  aliquot of cytosol (2.0 - 5.0 mg ml $^{-1}$ ) was pipetted into each tube and the tubes were vortex-mixed before being incubated at 4°C for 2 h. After incubation, a 200 $\mu\text{l}$  aliquot of DCC (see section 2.3) was added to each tube, tubes were mixed, incubated on ice for 10 mins, and then spun down in a refrigerated centrifuge. A 300 $\mu\text{l}$  aliquot of supernatant was removed, transferred to a 5.0 ml scintillation vial containing 4.0 ml scintillation fluid, and counted under standard  $^3\text{H}$  conditions.

### **2.12 Saturation analysis of olfactory tissue nuclear extract with $^3\text{H-T}$ as ligand**

The aim of these experiments was to estimate the equilibrium dissociation constant ( $K_d$ ) and abundance of specific binding sites for T ( $N_{\text{max}}$ ) in trout olfactory tissue nuclear extract. Saturation analysis of olfactory tissue nuclear extract was set up and carried out over the range of steroid concentrations described for the membrane fraction (section 2.10), but binding was quantified using the DCC method as described for cytosol (section 2.11).

### **2.13 Saturation analysis of olfactory tissue membrane fractions, with [ $^3\text{H}$ ]17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one 20-sulphate as ligand**

The aim of this study was to ascertain whether a steroidal conjugate, produced in abundance by

salmonid fish and known to elicit an EOG response in urine-primed male Atlantic salmon, was bound specifically by olfactory tissue of trout. [ $^3\text{H}$ ]17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one 20-sulphate was supplied by Dr A. Moore (MAFF Fisheries Laboratories, Lowestoft). The conjugate was synthesised from radiolabelled 17 $\alpha$ ,20 $\beta$ -P as described (for the inert compound) by Scott and Canario (1992). 17 $\alpha$ ,20 $\beta$ -P was prepared, in turn, from 17 $\alpha$ -hydroxy [1,2,6,7- $^3\text{H}$ ]progesterone by the method of Scott *et al.* (1982). It was assumed that the specific activity of the end-product was the same as that of the starting compound ( $\sim 2.44\text{TBq mmol}^{-1}$ ,  $\sim 66\text{ Ci mmol}^{-1}$ ,  $\sim 150,000\text{ dpm pmol}^{-1}$ ). The assay was carried out as for T (see 2.10), over a range of concentrations of 0.26, 0.53, 1.07, 2.13, and 4.3 nM (40 - 640 k dpm), and mature female brown trout membrane preparation was employed.

#### **2.14 The effect of pH on the specific binding of $^3\text{H}$ -testosterone to olfactory tissue membrane fraction**

Profound effects of pH have been reported on the EOG responses to T of precocious male Atlantic salmon parr (Moore, 1994) and the aim of this experiment was to determine whether these effects were matched by effects of pH on specific binding of  $^3\text{H}$ -T to trout olfactory tissue membrane preparation. A series of tris-HCl buffer solutions were prepared such that, on the addition of 250  $\mu\text{l}$  of pH-adjusted buffer to 350  $\mu\text{l}$  of assay buffer, the final pH was either 3.2, 4.7, 5.3, 6.4, 7.4, 8.3, or 9.3. Each sample of 250  $\mu\text{l}$  of membrane preparation was incubated with 250  $\mu\text{l}$  of pH-adjusted buffer and 100  $\mu\text{l}$  of assay buffer containing 500,000 dpm of  $^3\text{H}$ -T. Four assay tubes were designated to each pH, two of which contained 0.5 nmol of inert T. The samples were incubated at 4 $^{\circ}\text{C}$  for 1 h at which point binding in each tube was quantified by filtration as described in section 2.3.

#### **2.15 Electron microscopic examination of olfactory tissue membrane fraction**

After concentration by centrifugation, membrane fraction samples for electron microscope examination were fixed using a standard two-stage protocol. Initially samples were fixed for 60 min at room temperature in a 3% glutaraldehyde solution made up in 0.1M sodium cacodylate buffered at pH 6.8. This was followed by a 30 min fixation at room temperature in a 2% solution of osmium tetroxide made up in the same buffer. The fixed samples were then washed in fresh buffer and dehydrated through increasing concentrations of ethanol in distilled water, to 100%

ethanol. The samples were then embedded in Spurr resin over two days at room temperature before curing at 60°C. Sections were cut and triple stained with lead citrate/uranyl acetate/lead citrate before examination in a JEOL JEM 100CX electron microscope.

## **2.16 Electrophysiological response of rainbow trout to testosterone**

This study was carried out by Dr A. Moore, MAFF Lowestoft.

**2.16.1 Experimental Animals.** Rainbow trout (29 - 32 cm in length) were collected from Westacre Trout Farm (Narborough, Norfolk, UK) and transported to the Lowestoft Fisheries Laboratory. The fish were maintained under natural photoperiod in 1000 l tanks supplied with a constant flow (85 l min<sup>-1</sup>) of aerated, dechlorinated water (5.5 - 16.5° C; pH 7.5; alkalinity 156 mg HCO<sub>3</sub> l<sup>-1</sup>; total calcium 166 mg l<sup>-1</sup>; total hardness 405 mg l<sup>-1</sup> as CaCO<sub>3</sub>; aluminium <10-32 µg l<sup>-1</sup>; sodium 37.3 mg l<sup>-1</sup>; magnesium 12 mg l<sup>-1</sup>; NO<sub>3</sub> 0.2 - 49.1 mg l<sup>-1</sup>; SO<sub>4</sub> 10 µg l<sup>-1</sup>). Fish were fed to satiation daily with commercial salmon pellets.

The measurements were made between September and October 1994 (water temperature 8.9 - 13.8 °C). After each experiment (4 - 5h in duration) the fish were killed, sexed, and gonads were removed and weighed. Most of the fish tested were sexually immature with gonadosomatic indices of 0.026 ± 0.001% (mean ± SEM, n = 6).

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

The fish were anaesthetized with 2-phenoxyethanol (0.4 ml l<sup>-1</sup>) and skin and cartilage were removed to expose the olfactory rosettes. The fish were then immobilised with an intramuscular injection of gallamine triethiodide (0.3 mg kg<sup>-1</sup> of body weight) and placed in a V-shaped clamp within a Perspex flow-through chamber. The gills were constantly perfused with water containing 2-phenoxyethanol. Paired silver electrodes were attached subcutaneously to the fish to monitor heart rate and level of anaesthesia during each experiment. The output was continuously

displayed on an oscilloscope (Textronic 465B). 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 3M KCl. The tip of the pipette (diameter 80 - 100  $\mu\text{m}$ ) 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 and minimum response to dechlorinated water controls were normally 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 MX212) or digitised and stored for later analysis on an Apricot XEN-i 386/100 computer using Asystant+ software (Asyst Inc.).

A constant volume of the test substance (100  $\mu\text{l}$ ) 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 water over the olfactory epithelium ( $12 \text{ ml min}^{-1}$ ) and the EOG response recorded. The stimulus lasted 5 secs and the flow rate was unaltered by the addition of the test substance.

**2.16.3 Testing procedure.** Serial dilutions of testosterone (Sigma Chemicals), ranging from  $10^{-5}$  -  $10^{-12}$  M were prepared from a stock solution containing 500  $\mu\text{g ml}$  testosterone in absolute ethanol. The dilutions were freshly prepared before each experiment with water taken from the inlet pipe of the trout holding tank and allowed to stand at room temperature until required (room temperature 7.5 - 10.7  $^{\circ}\text{C}$ ). Increasing concentrations of testosterone were presented to the olfactory epithelium with a 2 min recovery interval between stimuli. The responses to  $10^{-5}$  M L-serine, ethanol, and water control were tested at the beginning and end of each test series. The amplitude of each EOG response was expressed as a percentage response of the initial L-serine standard.

**2.16.4 Data analysis.** The amplitude of each EOG response was measured from the baseline to the peak of each phasic displacement and expressed in millivolts (mV). Any replicates were then averaged and the values were expressed as a percentage of the response to the initial L-serine standard. The dechlorinated water control response level was subtracted from the EOG response

to each concentration of testosterone.

### **2.17 Protein determination**

Protein levels were determined by the method of Ohnishi and Barr (1978).

### 3. RESULTS

The main problem encountered during the course of these studies was the relatively limited amounts of olfactory tissue which could be retrieved from individual fish. This necessitated the use of tissue "pools" which inevitably results in an inability to discern fish-to-fish variation. From a 300 - 500g trout, approximately 0.12g (wet weight) of tissue could be removed. A batch of 36 fish provided enough material for approximately 20 ml of membrane fraction. Each saturation analysis requires 5.0 ml of membrane preparation.

#### 3.1 Preliminary experiments

Evidence was detected for the specific binding of  $^3\text{H-T}$  to both cytosolic and membrane fractions from the olfactory tissue of immature and mature female rainbow trout.

Table 1. The binding of  $^3\text{H-T}$  to olfactory tissue from mature female and immature rainbow trout.

Batch	Fraction	$B_T$ (dpm)	$B_{NS}$ (dpm)	$B_S$ (dpm)
Stannan imm.	membrane	10793	7432	3361
	cytosol	15738	2183	13555
Stannan mat. ♀	membrane	9199	5002	4197
	cytosol	16828	2604	14224
Tasman. imm.	membrane	10486	6345	4141
	cytosol	17542	2539	15003
Tasman. mat. ♀	membrane	7585	4570	3015
	cytosol	18535	3136	15399

#### 3.2 Effect of protein concentration on specific binding of $^3\text{H-T}$ to olfactory tissue membrane preparations

The results of this experiment are presented in Fig. 1. There was a clear linear relationship between the amount of specifically bound T and the dilution factor of the membrane preparation.

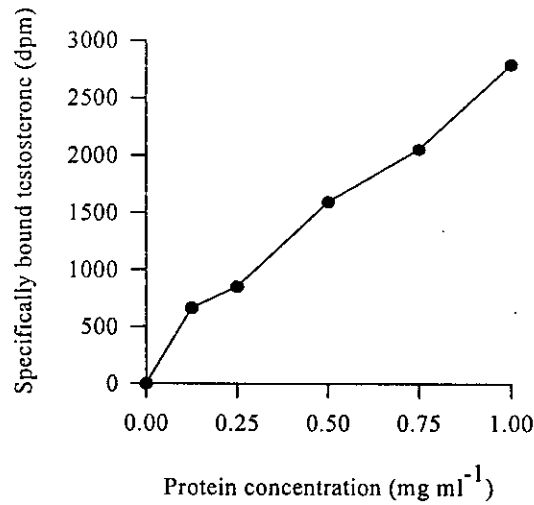


Fig. 1 The relationship between protein concentration and number of specific binding sites for <sup>3</sup>H-testosterone in rainbow trout olfactory tissue membrane preparation.

### 3.3 The effect of protease treatment on the specific binding of <sup>3</sup>H-T to olfactory tissue membrane preparations

Incubation with trypsin (1.0 mg ml<sup>-1</sup>) for 24 h completely abolished specific binding of <sup>3</sup>H-T to rainbow trout olfactory tissue membrane preparation.

Table 2. The effect of trypsin digestion on the binding of <sup>3</sup>H-T to olfactory tissue membrane preparation.

Treatment	B <sub>T</sub> (dpm)	B <sub>NS</sub> (dpm)	B <sub>S</sub> (dpm)
Buffer	4287	904	3383
Buffer + trypsin	628	505	123

### 3.4 The tissue specificity of the specific binding of <sup>3</sup>H-T to membrane fractions from rainbow trout

Specific binding of <sup>3</sup>H-T was detected in all tissue fractions except spleen, in which the difference between B<sub>T</sub> and B<sub>NS</sub> was minimal. The greatest amount of binding, normalised for



protein concentration, was observed in the whole brain preparation. The results are presented in full in Table 3. Significant differences in binding between preparations were determined by Students t-test.

Table 3. Specific binding of  $^3\text{H-T}$  to various tissue fractions from rainbow trout. The values are expressed as the mean  $\pm$  SEM, n = 4. a, binding sig. greater than all other preps  $p < 0.001$ ; b, binding sig. greater than in liver, muscle, and spleen,  $p < 0.001$ ; c, binding significantly greater than in muscle and spleen,  $p < 0.01$ .

Tissue	$B_s$ (dpm $\text{mg}^{-1}$ protein)
Brain	$17171 \pm 776^a$
Olfactory tissue	$1208 \pm 31^b$
Liver	$470 \pm 54^c$
Muscle	$154 \pm 30$
Spleen	$72 \pm 32$

### 3.5 The ligand specificity of the $^3\text{H-T}$ binding sites in olfactory tissue membrane fraction

The results of this experiment are presented in Fig. 2. In both membrane and cytosol,  $^3\text{H-T}$  was displaced most effectively by a 20-fold excess of unlabelled T. However, the extent to which other unlabelled steroids displaced  $^3\text{H-T}$  varied markedly between cytosol and membrane fractions. In cytosol, T displaced all but  $12.9 \pm 0.9\%$  (n=7) of specifically bound  $^3\text{H-T}$ , but 11-ketotestosterone,  $17\alpha$ -hydroxyprogesterone, and estradiol- $17\beta$  also displaced a substantial proportion of bound  $^3\text{H-T}$  (60 - 70 %; Fig. 2a). In contrast, in the membrane fraction, a twenty-fold excess of unlabelled T displaced all but  $2.3 \pm 1.1\%$  (n=7) of specifically bound  $^3\text{H-T}$  whereas 11-ketotestosterone,  $17\alpha$ -hydroxyprogesterone, and estradiol- $17\beta$  displaced only 25 - 30 % of bound  $^3\text{H-T}$ .

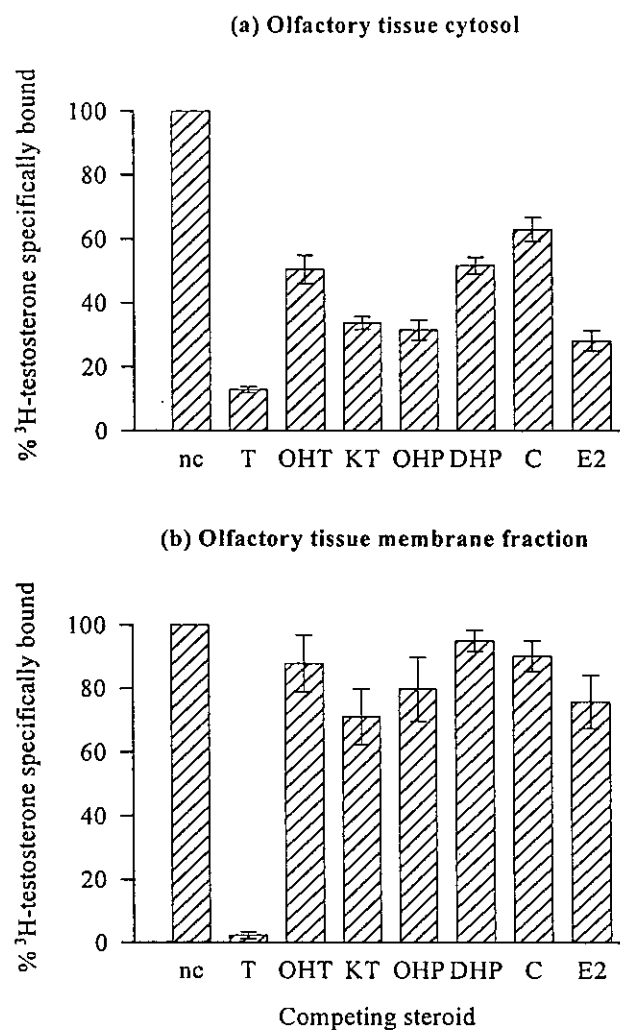


Fig. 2. The displacement of specifically bound <sup>3</sup>H-testosterone from (a) cytosolic and (b) membrane fraction binding sites by a range of steroids in 20-fold excess. T, testosterone; OHT, 11 $\beta$ -hydroxytestosterone; KT, 11-ketotestosterone; OHP, 17 $\alpha$ -hydroxyprogesterone; DHP, 17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one; C, cortisol; E2, estradiol-17 $\beta$ . Seven separate tissue pools were assayed. each estimate is the mean  $\pm$  SEM (n=7).

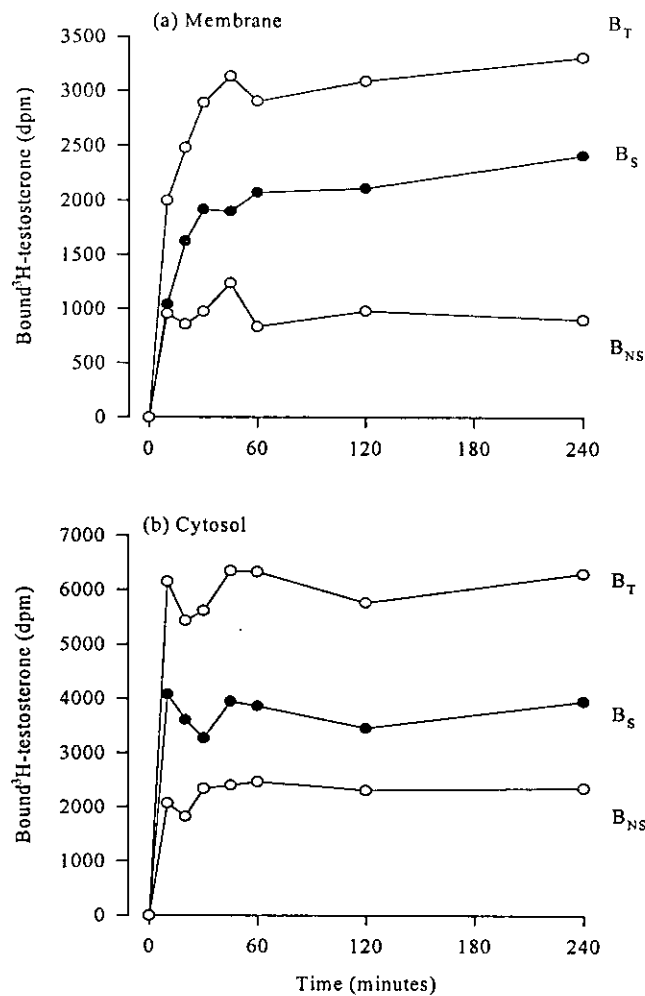


Fig. 3 The time-course of binding of  $^3\text{H}$ -testosterone to (a) membrane and (b) cytosol preparation of mature female rainbow trout olfactory tissue.  $B_T$ , total binding;  $B_{NS}$ , non-specific binding;  $B_S$ , specific binding. Each point is the mean of three determinations.

### 3.6 The time-course of binding of $^3\text{H}$ -T to olfactory tissue membrane fraction and olfactory tissue cytosol

The results of this study are presented in Fig. 3. Specific binding of  $^3\text{H}$ -T to olfactory tissue membrane fraction increased from time 0 to reach a maximum after 45 mins (Fig. 3a). This level of binding was maintained until at least 24 h after the start of the incubation (data not shown). Maximum specific binding of  $^3\text{H}$ -T was achieved more rapidly in cytosol (Fig. 3b). A stable maximum in specific binding was observed within 10 mins of the start of the incubation, and was maintained for 24 h (data not shown).

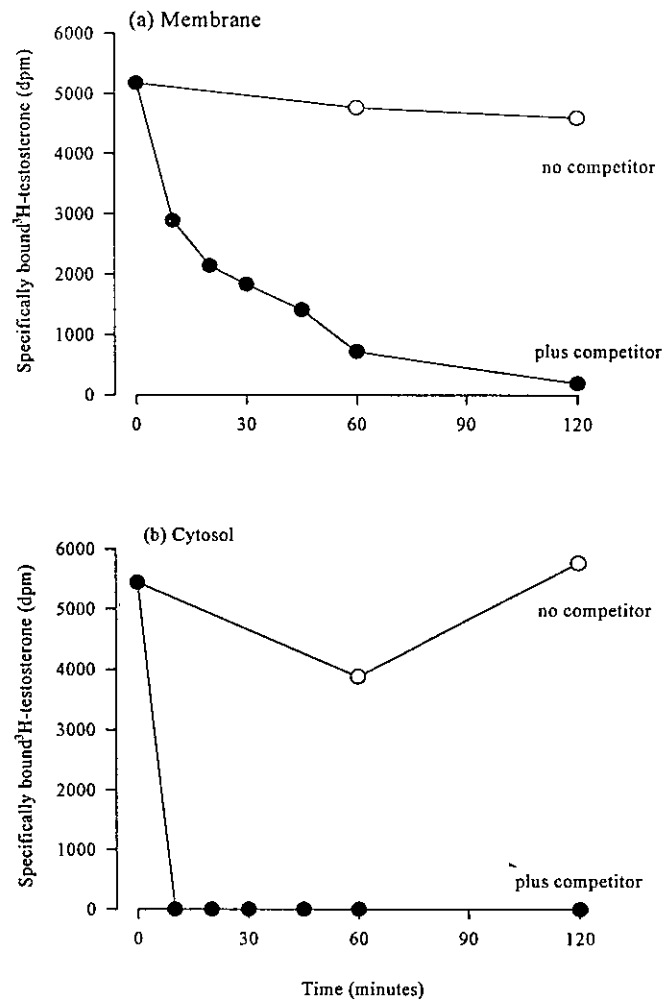


Fig. 4 The rate of dissociation of specifically bound  $^3\text{H}$ -testosterone from sites in (a) olfactory tissue membrane and (b) cytosol preparations.

### 3.7 The time-course of dissociation of $^3\text{H}$ -T from olfactory tissue membrane fraction and olfactory tissue cytosol

Following the addition of competing excess inert T, at least 2 h was required for all specifically bound  $^3\text{H}$ -T to dissociate from membrane fraction binding sites (Fig. 4a). In contrast, all specifically bound T was displaced within 10 mins of the addition of the competitor to the cytosol preparation (Fig. 4b).

### **3.8 Saturation analysis of olfactory tissue membrane fraction, cytosols, and nuclear extracts with $^3\text{H-T}$ as ligand**

For each species (brown and rainbow trout), sex (male or female), and tissue fraction (cytosol, membrane fraction, nuclear extract) saturation analyses were carried out employing  $^3\text{H}$ -testosterone as ligand. The analyses were of necessity carried out on preparations derived from tissue pools. Each pool comprised of olfactory tissue from approximately 35 fish and for each species/sex/fraction 3 - 5 separate pools were analysed. Representative saturation curves and Scatchard plots for each species/sex/fraction are presented in Figs 5 - 10. Mean binding parameters ( $K_d$ ,  $N_{\text{max}}$ ) derived from Scatchard analyses of each tissue pool are presented in Table 4.

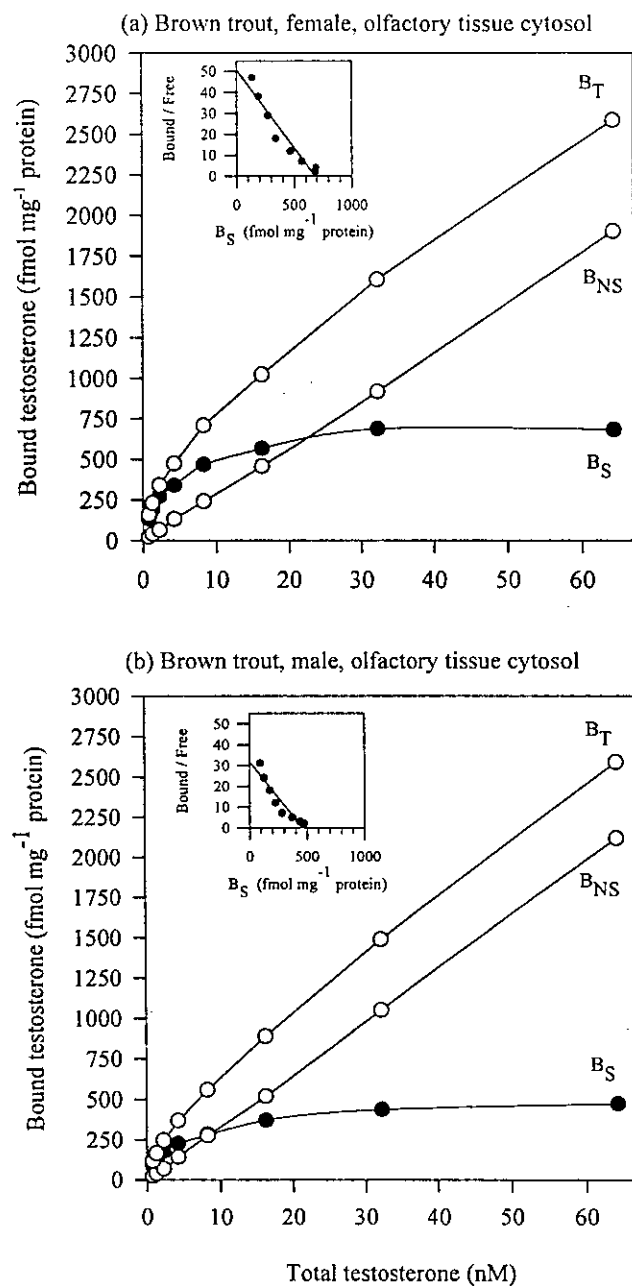


Fig. 5. Saturation analysis of the binding of  $^3\text{H}$ -testosterone to olfactory tissue cytosol from (a) female brown trout and (b) male brown trout. Scatchard plots for each saturation analysis are presented (insets). The analyses are of single pools, each comprising tissue from approximately 35 fish. The binding parameters derived from each plot are (a)  $K_d = 13.7$  nM,  $N_{\text{max}} = 690$  fmol  $\text{mg}^{-1}$  protein, and (b)  $K_d = 14.1$  nM,  $N_{\text{max}} = 441$  fmol  $\text{mg}^{-1}$  protein.

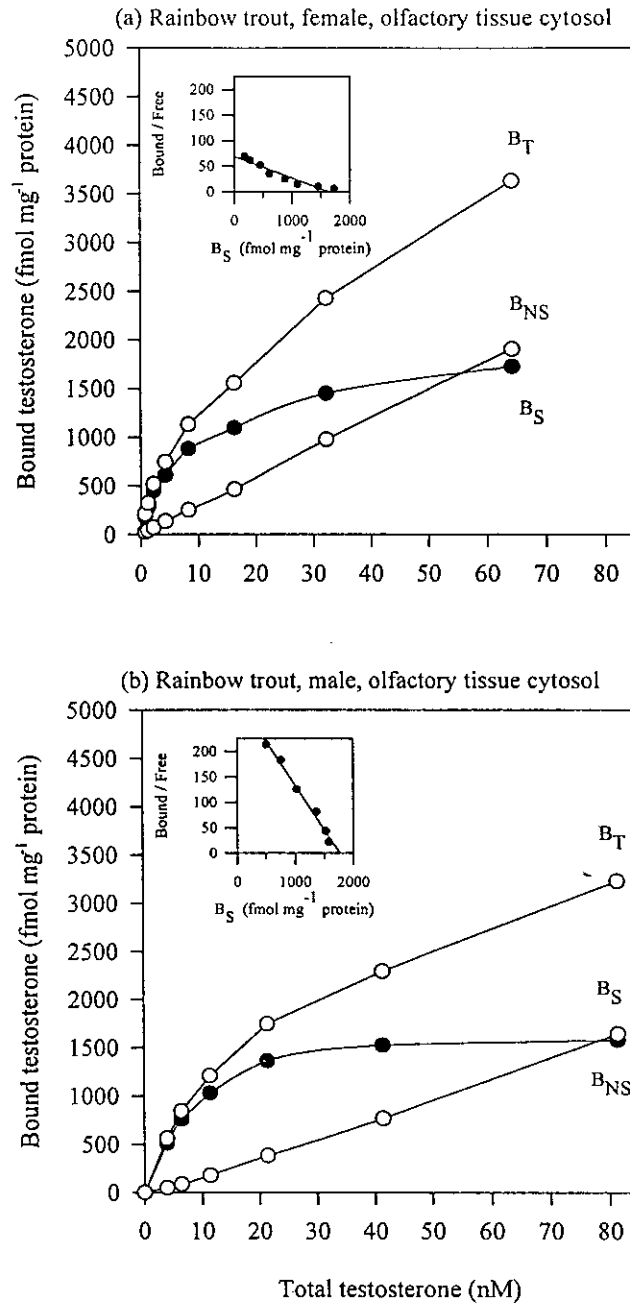
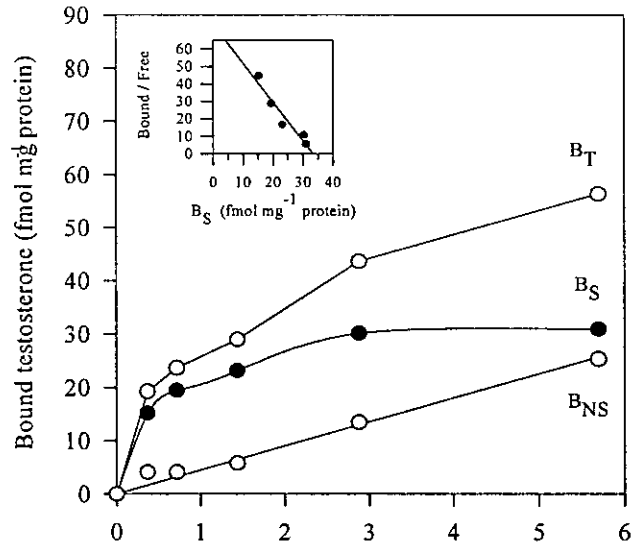


Fig. 6. Saturation analysis of the binding of  $^3\text{H}$ -testosterone to olfactory tissue cytosol from (a) female rainbow trout and (b) male rainbow trout. Scatchard plots for each saturation analysis are presented (insets). The analyses are of single pools, each comprising tissue from approximately 35 fish. The binding parameters derived from each plot are (a)  $K_d = 23.8 \text{ nM}$ ,  $N_{\text{max}} = 1636 \text{ fmol mg}^{-1} \text{ protein}$ , and (b)  $K_d = 5.75 \text{ nM}$ ,  $N_{\text{max}} = 1772 \text{ fmol mg}^{-1} \text{ protein}$ .

(a) Brown trout, female, olfactory tissue membrane fraction



(b) Brown trout, male, olfactory tissue membrane fraction

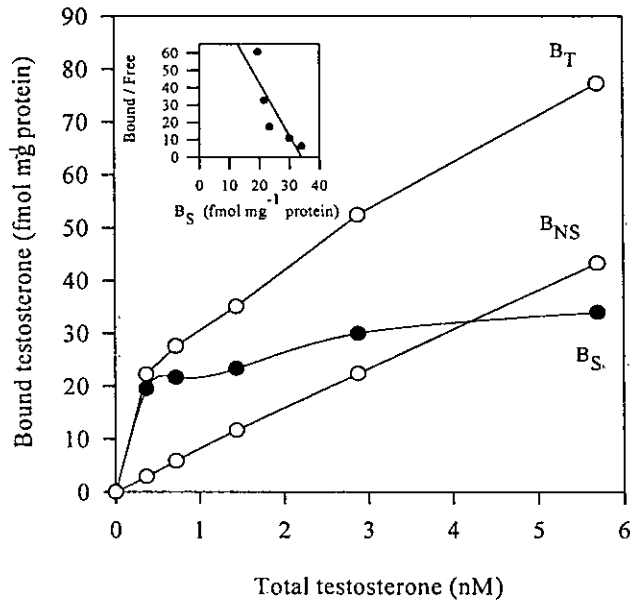
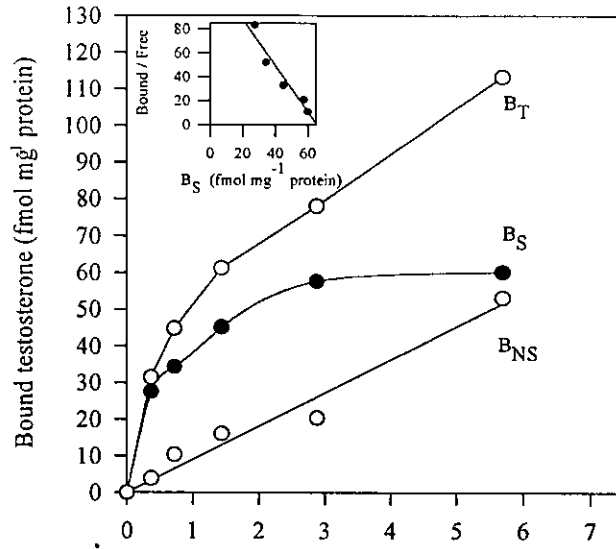


Fig. 7. Saturation analysis of the binding of  $^3\text{H}$ -testosterone to olfactory tissue membrane fraction from (a) female brown trout and (b) male brown trout. Scatchard plots for each saturation analysis are presented (insets). The analyses are of single pools, each comprising tissue from approximately 35 fish. The binding parameters derived from each plot are (a)  $K_d = 0.45$  nM,  $N_{\max} = 33.4$  fmol  $\text{mg}^{-1}$  protein, and (b)  $K_d = 0.33$  nM,  $N_{\max} = 34.1$  fmol  $\text{mg}^{-1}$  protein.



(a) Rainbow trout, female, olfactory tissue membrane fraction



(b) Rainbow trout, male, olfactory tissue membrane fraction

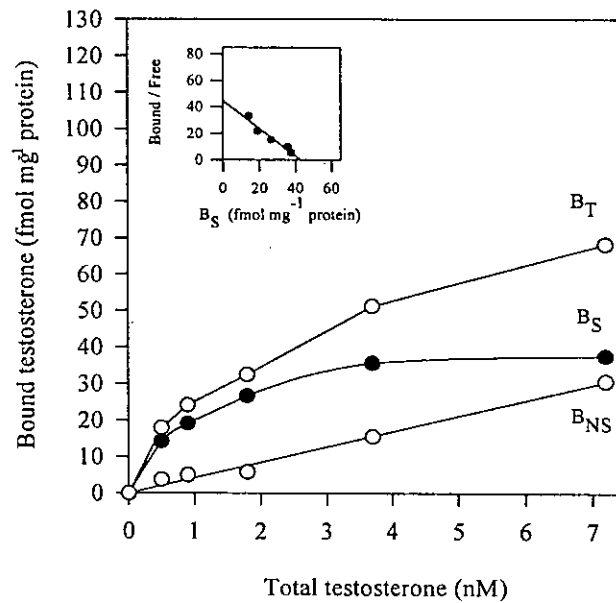


Fig. 8. Saturation analysis of the binding of <sup>3</sup>H-testosterone to olfactory tissue membrane fraction from (a) female rainbow trout and (b) male rainbow trout. Scatchard plots for each saturation analysis are presented (insets). The analyses are of single pools, each comprising tissue from approximately 35 fish. The binding parameters derived from each plot are (a)  $K_d = 0.75$  nM,  $N_{max} = 94.7$  fmol mg<sup>-1</sup> protein, and (b)  $K_d = 0.96$  nM,  $N_{max} = 42.9$  fmol mg<sup>-1</sup> protein.

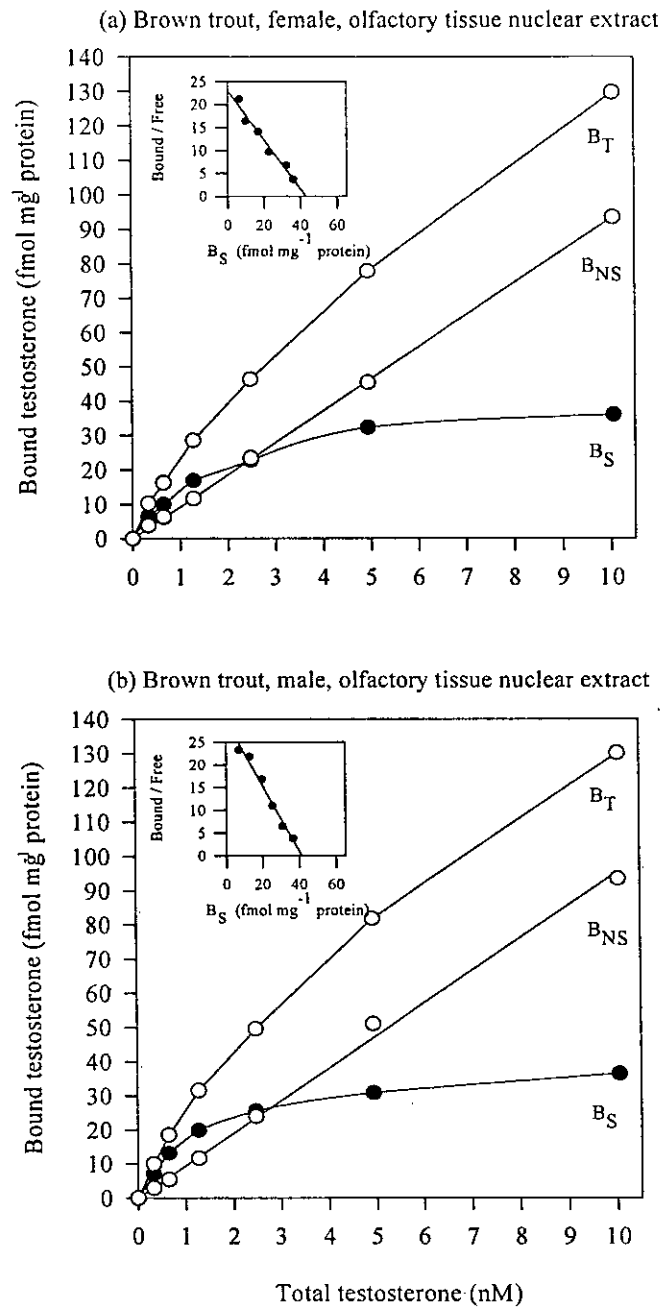


Fig. 9. Saturation analysis of the binding of  $^3\text{H}$ -testosterone to olfactory tissue nuclear extract from (a) female brown trout and (b) male brown trout. Scatchard plots for each saturation analysis are presented (insets). The analyses are of single pools, each comprising tissue from approximately 35 fish. The binding parameters derived from each plot are (a)  $K_d = 1.89 \text{ nM}$ ,  $N_{\text{max}} = 43.6 \text{ fmol mg}^{-1} \text{ protein}$ , and (b)  $K_d = 1.39 \text{ nM}$ ,  $N_{\text{max}} = 41.3 \text{ fmol mg}^{-1} \text{ protein}$ .

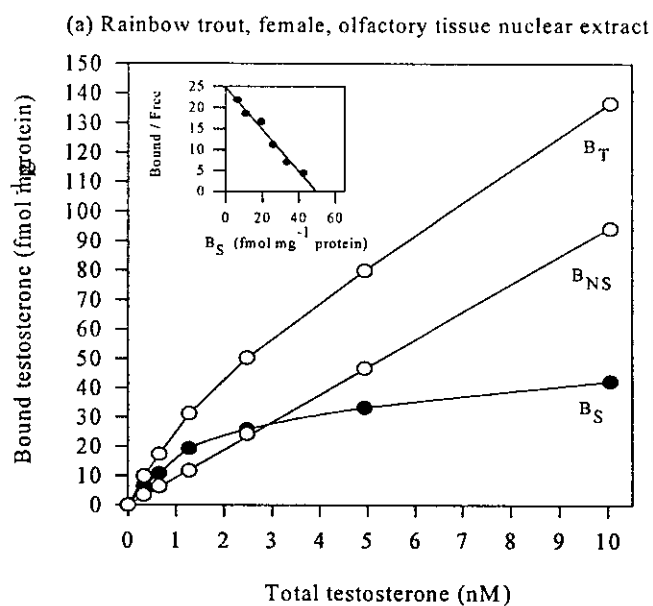


Fig. 10. Saturation analysis of the binding of  $^3\text{H}$ -testosterone to olfactory tissue nuclear extract from female rainbow trout. A Scatchard plot for the saturation analysis is also presented (inset). The analysis was of a single pool, comprising tissue from approximately 35 fish. The binding parameters derived from the plot are (a)  $K_d = 2 \text{ nM}$ ,  $N_{\text{max}} = 49.6 \text{ fmol mg}^{-1} \text{ protein}$ . No male rainbow trout olfactory tissue nuclear extracts were available.

Table 4. The equilibrium dissociation constant ( $K_d$ ) and maximum number of binding sites ( $N_{max}$ ) for  $^3\text{H}$ -testosterone in olfactory tissue cytosol, membrane fraction, and nuclear extract from male and female brown and rainbow trout. The data are expressed as mean  $\pm$  SEM.

Species	Sex	Fraction	n	$K_d$ (nM)	$N_{max}$ (fmol $\text{mg}^{-1}$ protein)
RT	♀	cytosol	5	$22.7 \pm 1.4$	$2943 \pm 721$
RT	♂	cytosol	3	$8.9 \pm 2.3$	$1543 \pm 374$
BT	♀	cytosol	5	$13.0 \pm 1.8$	$735 \pm 70$
BT	♂	cytosol	4	$15.1 \pm 1.5$	$473 \pm 41$
RT	♀	membrane	4	$1.6 \pm 0.6$	$60 \pm 19$
RT	♂	membrane	3	$1.9 \pm 0.7$	$30 \pm 7$
BT	♀	membrane	5	$1.6 \pm 0.6$	$36 \pm 3$
BT	♂	membrane	4	$0.5 \pm 0.1$	$30 \pm 2$
RT	♀	nuc. extract	3	$3.4 \pm 1$	$55 \pm 8$
RT	♂	nuc. extract	-	-	-
BT	♀	nuc. extract	5	$2.3 \pm 0.3$	$43 \pm 5$
BT	♂	nuc. extract	3	$2.8 \pm 1.2$	$49 \pm 14$

### 3.9 Saturation analysis of olfactory tissue membrane fractions, with [ $^3\text{H}$ ]17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one 20-sulphate as ligand

No evidence for specific binding of 17 $\alpha$ ,20 $\beta$ -P-sulphate to brown trout olfactory tissue was obtained. There was no difference between the level of binding of  $^3\text{H}$ -17 $\alpha$ ,20 $\beta$ -P-sulphate to membrane fraction in the presence and absence of excess inert 17 $\alpha$ ,20 $\beta$ -P-sulphate.

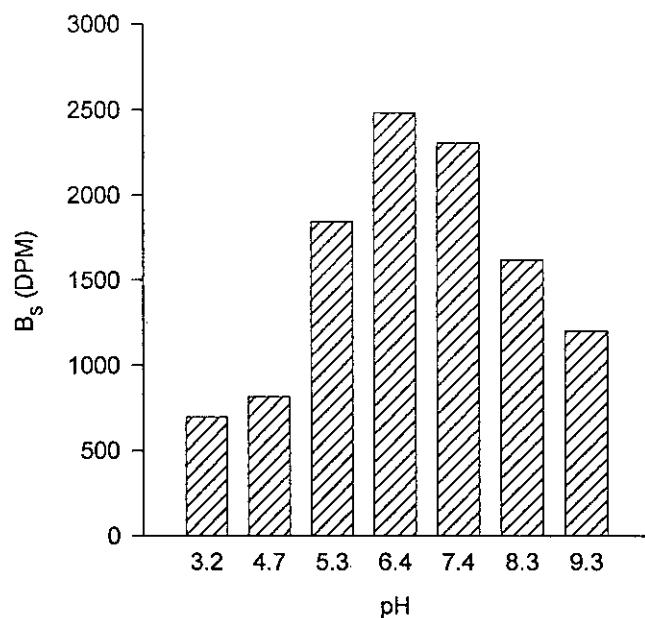


Fig. 11 Specific binding of  $^3\text{H}$ -testosterone to rainbow trout olfactory tissue membrane fraction at a range of pH values. Each value is the mean of two estimates.

### 3.10 The effect of pH on the specific binding of $^3\text{H}$ -testosterone to olfactory tissue membrane fraction

Highest levels of specific binding were observed at pH 6.4 and 7.4. Binding was inhibited by both low and high pH, lowest levels of specific binding being detected at pH 3.2 and pH 9.3.

### 3.11 Electron microscopic examination of olfactory tissue membrane fraction

Inspection of the membrane fraction revealed there to be no intact cells or nuclei present. The preparation consisted entirely of small ( $< 0.5 \mu\text{m}$ ) membrane micelles and included some apparent ciliary fragments, identifiable in cross-section by their characteristic arrangement of microtubules. In contrast, the pellet obtained from the first spin (1000 g) of the homogenate contained clearly identifiable cells, nuclei and organelles.

### 3.12 Electrophysiological response of rainbow trout to testosterone

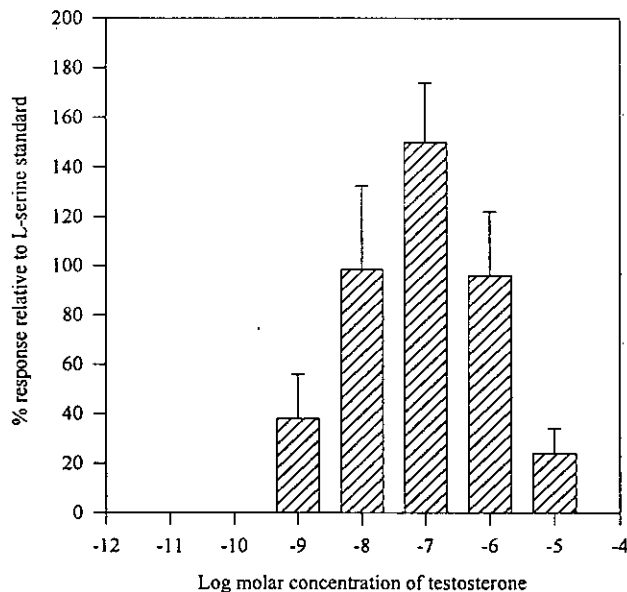


Fig. 12 The electrophysiological response of the olfactory epithelium of immature rainbow trout to various concentrations of testosterone. The amplitude of each response is expressed as a percentage of the response to a  $10^{-5}$  M L-serine standard. The data presented are the mean responses of six fish, the vertical error bar indicates the SEM.

Testosterone, at concentrations of  $10^{-9}$  M to  $10^{-5}$  M evoked an electrophysiological response from the olfactory epithelia of immature rainbow trout. The level of response varied according to the concentration of testosterone employed, minimal responses being observed at  $10^{-9}$  M and, surprisingly, at  $10^{-5}$  M. The EOG response did not appear to reach a plateau. Instead, exposure of the olfactory tissue to concentrations above  $10^{-7}$  M testosterone resulted in reduced EOG responses.

## 4. DISCUSSION

The rationale for undertaking this study was to identify and characterise a possible testosterone receptor in olfactory tissue of salmonid fish, capable of responding to external signals. While the "classical" genomic action of steroids requires a cytosolic/nuclear receptor via which the transduction of hormone signal to effect is a relatively slow process, pheromone reception arguably requires that the receptor be capable of initiating a rapid response. Because the transmission of pheromone-induced signals from the olfactory tissue is a rapid electrophysiological event, a receptor-mediated process which involves the initiation of transcriptional events within the nucleus is likely to be inappropriately slow. The initiation of events leading to rapid membrane depolarisation in the sensory neurone is likely to be best mediated by a receptor complex situated in the cell membrane. A membrane-associated receptor has been demonstrated to underly olfaction in those systems best studied to date, the best characterised of which comprise a receptor protein, GTP-binding protein, and adenylate cyclase which, via the production of cAMP, open a cationic channel in the plasma membrane (Shepherd, 1994). A second transduction mechanism, in which a G-protein-mediated increase in phospholipase C occurs, causing an increase in inositol triphosphate, which may in turn open  $\text{Ca}^{2+}$  channels in the cell membrane, has also been suggested to be associated with olfactory transduction (Farbman, 1994).

### 4.1 Steroid receptors located in the cell membrane.

In recent years it has become apparent that steroid hormones can act via non-classical mechanisms (Brann *et al.*, 1995). Many reports now exist of steroid hormone receptors in mammals which are localised within the cell membrane, rather than freely distributed within the cytosolic/nuclear compartments. Many of these studies have addressed the interaction of steroids, particularly corticosteroids but also estrogens and progestogens, with receptors located in the membrane fraction of neural tissues (McEwen, 1991; Horvat *et al.*, 1995; Ke and Ramirez, 1990; Orchinik *et al.*, 1991; Tischkau and Ramirez, 1993; Moore *et al.*, 1995). However, the range of vertebrate tissues which have been demonstrated to respond to steroid signals via membrane-located receptors also includes spermatozoa (Revelli *et al.*, 1994), liver (Grote *et al.*, 1993; Konoplya and Popoff, 1992), leucocytes (Wehling *et al.*, 1991, 1992) and the uterus (Pietras and

Szego, 1979). Direct evidence linking a membrane-bound steroid receptor with rapid changes in cellular ion flux has been presented for the progesterone-stimulated acrosome reaction in sperm (Blackmore, 1993; Wistrom and Meizel, 1993). Progesterone stimulates the influx of  $\text{Ca}^{2+}$  and increased  $\text{Cl}^-$  flux within seconds of the exposure of sperm to the steroid.

#### 4.2 Membrane-associated steroid receptors in fish.

As far as the author is aware, membrane-associated steroid binding sites have been reported from only two tissues in fish. The best characterised is the membrane receptor situated in the oocyte which binds a specific maturation-inducing steroid (Patino and Thomas, 1990; Maneckjee *et al.*, 1991; Yoshikuni *et al.*, 1993). The  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one ( $17\alpha,20\beta$ -DHP) binding site in rainbow trout oocytes has been shown to be associated with a G-protein sub-unit in the cell membrane. Whether this G-protein is coupled to the  $17\alpha,20\beta$ -DHP-induced decrease in adenylate cyclase and stimulation of oocyte maturation awaits confirmation (Yoshikuni and Nagahama, 1994). In addition to studies on the binding of steroids to oocyte membranes, there is also a single report of the binding of  $17\alpha,20\beta$ -DHP to olfactory tissue membrane preparations from goldfish, *Carassius auratus* (Rosenblum *et al.*, 1991).

In goldfish olfactory tissue membrane fraction Rosenblum *et al.* reported a binding site for  $17\alpha,20\beta$ -DHP which was of high affinity ( $K_d \sim 1.0$  nM) and moderate to low capacity ( $N_{\text{MAX}} \sim 1.4$  pmol  $\text{mg}^{-1}$  protein). The specificity of the binding site for  $17\alpha,20\beta$ -DHP was not absolute, both androgens and progestins showed a high level of displacement of  $17\alpha,20\beta$ -DHP in competition studies. However,  $17\alpha,20\beta$ -DHP binding sites were markedly more abundant in olfactory tissue membrane preparations, than in gut, liver or brain. Specific  $17\alpha,20\beta$ -DHP binding associated with particulate material was also detected in all subcellular fractions of the olfactory tissue preparations. The authors interpreted this as representing binding to membrane fragments and whole cells but did not exclude the possibility of binding associated with nuclei. The concentration of steroid at which saturation of the binding site was observed was within the range in which the EOG response to  $17\alpha,20\beta$ -DHP in goldfish is observed to saturate. The authors concluded that the binding site identified was involved in the transduction of pheromonal signals.



#### 4.3 Androgen binding in the olfactory tissue of brown and rainbow trout

The data obtained during the course of the present study indicate that both rainbow trout and brown trout olfactory tissue contains at least three specific binding sites for testosterone. These are located in the nuclear fraction, cytosolic fraction, and membrane fraction of olfactory tissue preparations. Binding of testosterone in the membrane fraction and nuclear fraction shows characteristics typical of binding to specific steroid receptors.

Binding to both the membrane and cytosol sites was saturable, reversible, and was eliminated by treatment of the preparations with a protease. The characteristics of testosterone binding to the membrane and nuclear extract fractions were similar for both rainbow and brown trout, and for both male and female fish. There was a greater discrepancy in the binding of testosterone to the cytosol fraction of olfactory tissue in rainbow trout. Female rainbow trout appeared to possess a greater number of cytosolic binding sites, with a lower affinity for testosterone, than male fish. The membrane binding-site showed a markedly greater specificity for testosterone relative to other potential ligands, than the cytosol site.

The equilibrium dissociation constant ( $K_d$ ) and maximum number of binding sites ( $N_{max}$ ) calculated for testosterone binding within the nuclear extract and the membrane fraction (Table 4) fall within the range considered indicative of a functional steroid receptor. Specific binding of testosterone to the cytosol fraction was of lower affinity and higher capacity, suggesting a non-receptor function for this site. Overall, these data represent the first report of specific androgen binding sites in the olfactory tissue of teleost fish, and the first report of a membrane-associated androgen binding site in fish.

#### 4.4 Has this study identified a pheromone receptor?

The results of the present study differ from that of Rosenblum *et al.* in several respects. The binding capacity of the trout olfactory tissue membrane fraction for testosterone (30 - 60 fmol  $mg^{-1}$  protein) is considerably lower than that of goldfish olfactory tissue membrane for  $17\alpha,20\beta$ -DHP (~ 1.4 pmol  $mg^{-1}$  protein) although the  $K_d$  is similar in both species (~ 1.0 nM). The ligand specificity appears to be greater for trout olfactory tissue membrane than goldfish. Of the range

of androgens, progestins, estrogen, and corticosteroid tested, only testosterone displaced bound  $^3\text{H}$ -testosterone in trout. In goldfish olfactory tissue, ligand specificity was not so clearly delineated. However, *tissue* specificity appeared to be greater in the goldfish. No other tissue tested, including brain, displayed as many membrane-located binding sites for  $17\alpha,20\beta$ -DHP as olfactory tissue. In the trout, however, membrane-associated testosterone binding was more abundant in brain membrane preparations than in olfactory tissue membrane fraction.

Goldfish display an EOG response to  $17\alpha,20\beta$ -DHP the characteristics of which closely parallel the characteristics of the membrane binding site, in terms of the concentration of ligand necessary to obtain threshold and saturated EOG responses. In rainbow trout, a measurable EOG response to testosterone is obtained, and the threshold for detection ( $10^{-9}$  M) corresponds to the  $K_d$  for testosterone binding to the membrane. However, increasing concentrations of testosterone do not produce a saturable EOG response. Instead, the response markedly declines at concentrations of testosterone of  $10^{-6}$  M and greater.

In a recent study, Moore (1994) demonstrated that exposure of the olfactory epithelia of mature Atlantic salmon parr to water at a range of pH values markedly affected the EOG response to testosterone. Exposure to water at pH of 5.5 and 4.5 severely reduced the EOG response and exposure to water at pH 3.5 abolished the response. Exposure to water at pH 8.5 and 9.5 also markedly reduced responsiveness to testosterone. The pH of the medium was also found to be a significant factor in determining binding of  $^3\text{H}$ -testosterone to binding sites within the olfactory tissue membrane fraction. Maximum binding was observed at pH 6.4 - 7.4 but declined at pH values above or below this range. The similarity of the effect of pH on EOG responsiveness of olfactory tissue, and on ligand binding within the olfactory tissue provides intriguing circumstantial evidence that the two processes are functionally linked.

On balance, the presence of specific testosterone-binding sites in the olfactory tissue membrane of rainbow and brown trout is consistent with their having a role in detecting testosterone in the environment. This interpretation is supported primarily by the ability of testosterone to evoke an EOG response in rainbow trout. However, three factors must be addressed. Testosterone has yet to be demonstrated to have a pheromonal or communicative role in trout. In the present study,

high levels of membrane-associated testosterone binding were detected in brain tissue. The EOG response of rainbow trout to testosterone is less sensitive than that of mature Atlantic salmon parr.

Much less is known of the role of pheromonal communication in salmonid fish than in cyprinid fish, and the identity of pheromonally active compounds is still under investigation. The high degree of olfactory sensitivity of precocious male Atlantic salmon parr to testosterone, albeit for a limited period, suggests that it is quite feasible for this steroid to play a role in chemical communication in salmonid fish. In this study, immature rainbow trout did not display an EOG response to concentrations of testosterone below  $10^{-9}$  M whereas mature Atlantic salmon parr display a threshold for detection of testosterone at  $10^{-14}$  M (Moore and Scott, 1991). Although the apparent sensitivity of the response is lower in rainbow trout, the response seems unlikely to have arisen by any means other than interaction of water-borne testosterone with a specific olfactory receptor. Scott and Moore (1992) tested a range of steroids and steroid conjugates for their ability to elicit an EOG response from the olfactory epithelium of mature male Atlantic salmon parr. The response of the tissue to each compound was clear and unambiguous; a potential was evoked or no response was observed at all. It seems unlikely that trout possess a mechanism allowing the detection of very low concentrations of testosterone ( $10^{-9}$  M = 288 ng l<sup>-1</sup> = 0.000000288 g) without there being some functional purpose. The apparently lower sensitivity of rainbow trout olfactory tissue to testosterone compared to Atlantic salmon may arise because immature, as opposed to sexually mature fish were employed in the EOG studies. Heightened olfactory acuity may be a feature associated with maturity. Alternatively, it is equally possible that the role of testosterone in trout requires a sensitivity within the range observed.

The presence of membrane-associated testosterone binding in the brain of rainbow trout does not preclude the possibility that binding within the olfactory tissue membrane represents a receptor system directed at stimuli external to the fish. Mammalian neural tissue is known to contain membrane-bound receptors for estradiol-17 $\beta$ , testosterone, progesterone and corticosterone (Haukkamaa, 1987). Steroid receptors in neuronal membranes are believed to mediate a number of rapid effects including alterations in firing rates of neurons, changes in neuronal sensitivity/excitability, and induction/suppression of specific behaviours (Brann *et al.*, 1995).

The membrane fraction of brain tissue collected from rainbow trout in the present study showed high levels of testosterone binding. It might be argued that the testosterone binding sites identified in trout olfactory tissue membrane fraction represent a phenomenon associated with neuronal tissue in general, and not olfactory tissue in particular. Why, then, should a specialised tissue such as the olfactory rosette, whose neurons terminate at the tissue surface as specialised sensory cells, possess a receptor which may be capable of mediating rapid effects when occupied by its ligand? It is conceivable that the binding of testosterone to olfactory tissue membrane represents a mechanism by which some functions of the olfactory tissue may be modified under androgenic influence. However, it seems equally plausible to suggest that a receptor system which is exploited for one purpose within the brain is also employed to detect signals within the aquatic environment. It is clear from the EOG studies that this system detects water-borne testosterone - if concerned only with an internal function, it would be highly susceptible to interference by testosterone released by conspecifics and present in the surrounding water.

#### **4.6 What is the significance of the cytosolic and nuclear testosterone binding sites in trout olfactory tissue?**

Cytosolic and nuclear binding sites which display characteristics consistent with specific androgen receptors have been identified in a number of tissues in several species of fish, including brown trout skin (Pottinger, 1987, 1988), goldfish brain (Pasmanik and Callard, 1988), and the electric organ of mormyrid fish (Bass *et al.*, 1986). In skin and the electric organ the presence of androgen receptors has been linked to sexually dimorphic patterns of development, while androgen binding in the brain is suggestive of a behavioural role. The binding of testosterone to nuclear extract and cytosol derived from trout olfactory tissue is very similar to that observed in the skin of brown trout. In the present study, binding of testosterone in the nuclear extract of olfactory tissue was of high affinity (~ 3.0 nM) while binding of testosterone in the cytosolic fraction was of lower affinity (~ 10-20 nM). In the skin of brown trout, nuclear binding is of high affinity (~ 1.0 - 3.0 nM) while cytosolic binding possesses a lower affinity for testosterone (~ 20.0 nM) (Pottinger, 1987, 1988). The similarity of these data suggest that the olfactory organ in fish may be a target tissue for androgens of internal origin and that olfactory tissue function or development may to some extent be androgen dependent. This interpretation is supported by the results of a recent study (Cardwell *et al.*, 1995) in which it was demonstrated

that the magnitude and sensitivity of EOG responses to prostaglandin were increased in cyprinid fish (*Puntis schwanenfeldi*) with artificially elevated blood androgen levels. It is likely that such an effect will be mediated by specific androgen receptors. The presence of androgen receptors in olfactory tissue from both sexes does not necessarily preclude a sex-specific role, the liver of male trout contains estrogen receptors (Pottinger, 1986) although the synthesis of vitellogenin, under estrogen stimulation, is a component of the female reproductive cycle. However, common to other intracellular androgen-binding sites identified in fish (Pottinger, 1987; Pasmanik and Callard, 1988), the olfactory tissue binding site has little affinity for 11-ketotestosterone, the (quantitatively) dominant androgen in male salmonids. Levels of 11-ketotestosterone in female salmonids are negligible compared to those in male fish (Scott *et al.*, 1980) but levels of testosterone in female plasma during the reproductive period may exceed those in male fish by up to fourfold (Scott and Sumpter, 1983; Baynes and Scott, 1985). Thus both male and female salmonids display elevated levels of plasma testosterone during the reproductive period. It is perhaps more likely, therefore, that the presence of intracellular testosterone binding in the olfactory tissue is involved with a response of the tissue to an overall change in reproductive status of the fish, rather than sex-specific effects.

## 5. CONCLUSIONS

1. Binding sites for testosterone have been detected in three compartments of olfactory tissue from brown and rainbow trout. This is the first report of androgen binding to olfactory tissue in fish, and the first report of a membrane-associated androgen binding site in fish.
2. Binding of  $^3\text{H}$ -testosterone to the membrane fraction of olfactory tissue is of high affinity ( $K_d = 0.5 - 1.9 \text{ nM}$ ) and limited capacity ( $N_{\text{max}} = 30 - 60 \text{ fmol mg}^{-1} \text{ protein}$ ). Binding is reversible, and eliminated by protease treatment. The binding site exhibits a high degree of ligand specificity,  $11\beta$ -hydroxytestosterone, 11-ketotestosterone,  $17\alpha$ -hydroxyprogesterone,  $17\alpha$ ,  $20\beta$ -dihydroxy-4-pregnen-3-one, cortisol, and estradiol- $17\beta$  all fail to displace testosterone at 20-fold excess. These features are characteristic of a specific steroid receptor.

3. Binding of testosterone within the cytosol fraction is of moderate affinity ( $K_d = 9.0 - 23.0$  nM) and high capacity ( $N_{max} = 0.5 - 2.9$  pmol mg<sup>-1</sup> protein) and shows less ligand specificity than the membrane site. The rate of association and dissociation of testosterone from the cytosolic binding site is markedly more rapid than the equivalent processes in the membrane fraction.
4. Binding of testosterone to the nuclear extract is of high affinity ( $K_D \sim 3.0$  nM) and limited capacity ( $N_{max} \sim 50$  fmol mg<sup>-1</sup> protein).
5. There are no marked differences between species or between sexes in the affinity or capacity of testosterone-binding sites in nuclear extract or membrane fraction. However, cytosolic testosterone-binding sites are three- to four-fold more abundant in rainbow trout than in brown trout, and female rainbow trout have more cytosolic binding sites than male rainbow trout, but a lower affinity for testosterone than male sites.
6. Rainbow trout display an EOG response to testosterone at a concentration which is consistent with the characteristics of the membrane-associated testosterone-binding site. An EOG response is obtained at concentrations of testosterone of 1.0 nM and higher. The equilibrium dissociation constant ( $K_D$ , the concentration of ligand at which half the binding sites are saturated) for membrane-associated testosterone binding is in the range 0.5 - 2.0 nM.
7. Binding of <sup>3</sup>H-testosterone to the membrane-associated site shows a pH dependency which is comparable to the effects of pH on the EOG response to testosterone in intact fish.
8. The data concerning the attributes of the membrane associated testosterone-binding site are consistent with the olfactory tissue of salmonids possessing the capability to respond to testosterone present in the aquatic environment. The intracellular testosterone-binding sites show characteristics common to testosterone receptors in other fish tissues which are known androgen target tissues. This suggests that the development and/or function of salmonid olfactory tissue may be open to influence by endogenous testosterone.

## **6. SUGGESTIONS FOR FUTURE WORK**

1. Application of the techniques described within this report to the olfactory tissue of sexually immature and mature Atlantic salmon parr should demonstrate whether it the presence of the binding site, or an upstream component of the putative receptor transduction system, which are responsible for the "window" during which mature males are sensitive to testosterone.
2. The techniques described within this report can equally well be applied to the study of binding sites for other candidate pheromones of both steroidal and non-steroidal nature.
3. The apparent scope for androgenic influence on developmental/functional aspects of the olfactory tissue in trout, suggested by the presence of intracellular testosterone-binding sites, could be further investigated. The effect on olfactory acuity of artificially elevating levels of androgen in immature fish, or blocking androgen effects in mature fish (e.g. by the use of cyproterone acetate) could be readily examined.
4. A major drawback of the techniques described within this report is the requirement for large amounts of olfactory tissue to carry out assay procedures. Effort could be directed at "scaling down" the procedures to allow measurement of binding within the olfactory organs of individual fish. This would permit evaluation of fish-to-fish differences in binding activity, and detailed study of seasonal and developmental changes in binding specificity and capacity. In addition, the presence of specific binding sites could be determined in individual fish following an EOG evaluation of sensitivity to odorants. Assessment of the degree of correlation between electrophysiological evidence of binding activity and biochemical evidence of binding activity would then be possible.

## **ACKNOWLEDGEMENTS**

The author wishes to thanks Mr T. R. Carrick (IFE) for assistance with sampling and tissue preparation, and Mr K. Clarke (IFE) for carrying out the electron microscopy. As already noted, the electro-olfactographic studies were carried out by Dr A. Moore (MAFF Fisheries Laboratory, Lowestoft) who also provided helpful input during the course of these studies.

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