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Seasonal variations in oestrogen and testosterone levels in the plasma of brown trout (Salmo trutta lacustris) and in the metabolism of testosterone in its skin

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Seasonal changes in the levels of oestrogens and testosterone in the plasma of mature brown trout (Salmo trutta lacustris) were determined using a radioimmunoassay technique. Steroid metabolism in the skin of brown trout was investigated in *in vitro* incubations using testosterone and 17α-hydroxyprogesterone as precursor and analysis in tlc. The metabolites were separated into ether and watersoluble fractions.

Levels of oestrogens and testosterone in females were low from February to May and were at their maximum in August. In males the levels of oestrogens were at their maximum and the level of testosterone at its minimum in February. In August the level of testosterone was at its maximum and the levels of oestrogens at their minimum in the plasma.

The metabolism of testosterone in the skin was at its minimum when the daylight period was shortest and at its maximum in May. In late August, when the day was becoming shorter the metabolic efficiency was reduced. The efficiency of 17α-hydroxyprogesterone metabolism seemed to decrease while that of testosterone increased. No watersoluble metabolites were detected.

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1. Introduction

The biosynthesis of fish sex steroids has been demonstrated using incubation and radioimmunoassay techniques (see Scott et al. 1980a, b). Both Δ-5 and Δ-4 pathways have often been shown to exist in the gonads of fish using *in vitro* techniques (Colombo et al. 1970, 1972; Kristoffersson et al. 1976). As an interesting feature, Eckstein & Eylath (1970) and Colombo et al. (1972) have shown the 11-hydroxylation of testosterone and androstenedione. Previous work indicates that at constant temperatures the photoperiod is one of the main factors influencing the reproductive process in salmonids (cf. Whitehead et al. 1978). In the extreme lighting and temperature conditions in Finnish hatcheries (60°N — 70°N) even the light intensity (continuous light during summer and continuous darkness in winter) and the low water temperatures in the ponds during winter (0.2°C) may affect the endocrine system, which forms a link between the external and internal environments of the organism. In addition to gonadal steroid metabolism the extreme external environments may also affect the steroid metabolism in skin by influencing the synthesis of keratohyaline and the elimination of steroid catabolites through the skin.

A radio-immunoassay method was used to investigate the changes in the levels of peripheral plasma testosterone, oestradiol and oestrone in male and female brown trout during their annual reproductive cycle. To investigate androgen metabolism in the skin of brown trout, skin samples from males and females were incubated with radioactive precursors (testosterone and 17α-

Table 1. The sampling time, weight, length and GSI for fish groups. The mean ± *SE* and number of animals (*n*) are given for both sexes.

Group	Time		*n*	weight (g)	length (cm)	GSI
1	21. II.	♀	6	1870±9	56.3±0.7	0.62±0.04
		♂	4	1410±10	52.0±1.1	0.38±0.08
2	4. V.	♀	3	1880±11	55.0±1.0	0.63±0.12
		♂	4	2290±9	57.5±0.9	0.33±0.01
3	27. VIII.	♀	4	2145±5	58.0±0.6	12.03±0.71
		♂	4	1610±9	53.0±1.0	4.43±0.35
4	26. X.	♀	8	2335±6	59.0±0.5	28.27±1.46

OH-progesterone) and analysed for metabolites with thin layer chromatography.

It is hoped that the results obtained from this study will provide an insight into the endocrine control of reproduction in fish and be of benefit in fishfarming.

2. Material and methods

The fish used were seven year old brown trout (*Salmo trutta lacustris*) from the experimental stock (JTR —67/8) of Laukaa Fish Culture Research Station in Central Finland. The average length, body and gonadal weights and gonado-somatic index of the animals in the various experimental groups are given in Table 1. The fish were reared under natural climatic conditions in outdoor ponds. Seven days before sampling they were transferred to indoor tanks supplied with unpolluted, oligotrophic lake water at a flow rate of about 2 l/kg/min. The average water quality was as follows in summer (figures in parentheses = in winter): specific conductivity c. 40μ S · cm^{-1} (36μ S · cm^{-1}) total hardness 0.9° dH (0.8° dH), pH 6.5—6.8 (6.8). The water temperature and day length varied as shown in Fig. 5a. The lakes were frozen from the beginning of December to the beginning of May and the outdoor ponds in the hatchery were partly covered with ice and snow from the beginning of January to the end of March. The light intensity in the ponds was not recorded. Because the sun at noon in midwinter rises only 4—6° above the horizon the light intensity below the ice is clearly minimal. One day before sampling, fish were allowed to swim into black, mesh-ended restrainers to reduce the stress from external stimuli and to prevent excessice movement (cf. Soivio et al. 1975). For sampling, the fish were stunned with a blow on the head. The blood was collected by heart puncture and the plasma obtained by centrifuging for ten minutes at 3000 g was analysed radio-immunologically (RIA) for oesterone (E_1), oestradiol (E_2) and testosterone (T) according to Kuoppasalmi et al. (1976).

The skin for steroid metabolism studies was collected from a site ventral to the lateral line and slightly superior to the pectoral fins. It was prepared free from all muscle tissue, and minced with scissors on ice.

Steroid

The trivial names, abbreviations and systematic names of the steroids used are as follows: testosterone (T) (17β-hydroxy-4-androsten-3-one), androstenedione (Δ^4A) (4-androstene-3,17-dione) and 17α-hydroxyprogesterone (17αOH-Δ^4P) (17α-hydroxypregn-4-ene-3,20-dione) were obtained from Schering A.G. (Berlin, Germany); androstandione (A) (5α-androstane-3,17-dione) and 5α-androstanediol (5αA) (3α/β, 17β-dihydroxy-5α-androstane) were obtained from Steroid Reference Collection, London. 4-^{14}C testosterone, specific activity 57 mCi/mmol and 4^{14}C 17α-hydroxyprogesterone, specific activity 61 mCi/mmol, were obtained from the Radiochemical Centre (Amersham, England). The purity of the steroids was tested using thin layer chromatography (tlc) before use.

NADH was supplied by Boehringer (Mannheim, Germany).

Solvents were analytical grade and redistilled before use.

Incubation

— 500 mg of tissue in 5 ml of Cortland saline (Wolf, 1963)
— 0.157 μCi (= 0.8 μg) 4-^{14}C testosterone of 4-^{14}C 17α-hydroxyprogesterone as substrate
— NADH at a concentration of 1.3 mmol
— at 10μC for 2 hours
— under a continuous flow of carbogen

Incubation was stopped by the addition of 1.5 ml of boiling acetone. Control incubations were carried out using acetone-denaturated homogenates and [4-^{14}C] testosterone or [4-^{14}C] 17α-hydroxyprogesterone as substrate (0.157 μCi/5 ml).

Isolation and characterization of the radioactive metabolites

For isolation, measuring and separation of the neutral metabolites of T and 17α-OH-Δ^4P the incubated samples were handled further as described by Teräväinen and Saure (1976).

The recoveries of radioactivity in ether and water phases are shown in Table 2.

The water-soluble radioactivities in testosterone incubations were measured from ether after $(NH_4)_2SO_4$ saturation of the water phases and extraction in ether but were not investigated further. The neutral radioactive metabolites were further characterized and identified by

Table 2. The recoveries of radioactivity extracted in ether and soluble in water (mean percentage of dose ± *SE*) are given after incubation with T or 17αOH-Δ^4P as substrates. For further details, see text.

Group		Testosterone as substrate			17αOH-Δ^4P as substrate	
		in ether	in water	tot.	in ether	in water
1	♀	62.8±4.2	24.6±5.2	87.4±0.5	87.2±1.0	—
	♂	64.5±4.6	25.6±4.1	90.1±0.5	87.4±0.4	—
2	♀	43.4±8.6	32.1±0.1	75.5±2.7	82.7±0.5	—
	♂	39.4±4.0	34.2±3.8	73.6±1.7	81.6±1.2	—
3	♀	56.4±5.3	33.7±2.7	90.1±1.5	93.3±1.4	—
	♂	56.9±5.3	34.9±3.1	91.8±1.1	91.3±0.7	—
4	♀	47.8±4.8	28.4±2.5	76.2±1.1	84.7±1.7	—

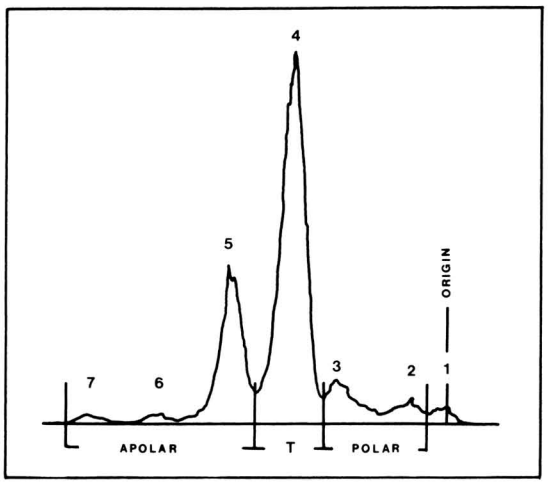

Fig. 1. Example of a scanning record of separation of testosterone and its metabolites in tlc (system 1). For details see text.

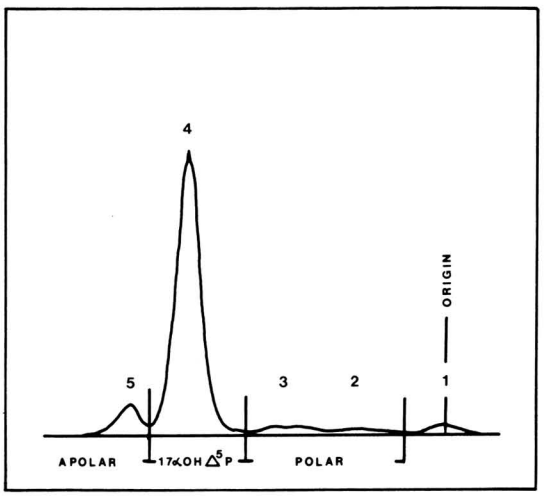

Fig. 2. Example of a scanning record of separation of 17α-hydroxy-progesterone and its metabolites in tlc (system 8). For details see text.

rechromatography on tlc, and by determination of constant specific activity using the repetitive tlc method described by Kaartinen et al. (1971). The solvent systems used in tlc were as follows:

1. Ether-chloroform 1:3
2. Ethanol-chloroform 1:19
3. Acetone-dichloromethane 1:5
4. Cyclohexane-ethyl acetate 1:1
5. Cyclohexane-ethyl acetate-ethanol 9:9:2
6. Ethanol-benzene 2:8
7. Cyclohexane-ethyl acetate 4:6
8. Acetone-chloroform 15:85
9. Ethanol-chloroform 1:9
10. Ethanol-benzene 1:9

Testosterone as precursor

In tlc I (system 1) the radioactivity was distributed in seven peaks (Fig. 1) which were pooled in each experimental group. In bidimensional tlc the radioactivity of metabolites in peaks 3, 4, 6 and 7 were isopolar with authentic unlabeled steroids in the systems used, as follows:

Peak 3; 5αA, systems 2 and 4, 83 % of pooled activity
Peak 4; T , —»— 6 » 7, 95 % of —»—
Peak 6; Δ^4A, —»— 1 » 2, 92 % of —»—
Peak 7; A , —»— 1 » 4, 81 % of —»—

The identity of the radioactivity in peaks 4 and 6 was also confirmed by the determination of constant specific activity (Table 3). Peak 1 represents the radioactivity retained at the origin of the chromatogram and was not investigated further. Peaks 2 and 5 could not be identified.

17α-hydroxyprogesterone as precursor

In tlc (system 8) the radioactivity was distributed in five peaks (Fig. 2). 96 % of the radioactivity of pooled peaks 4 co-migrated with authentic carrier 17αOHΔ^4P in bidimensional tlc (systems 5 and 2). The identity was confirmed by the determination of constant specific activity (Table 4). The other peaks were not identified. The distribution of radioactivity in the first tlc in different experimental groups are shown in Table 5 (T as precursor) and Table 6 (17αOH-Δ^4P as precursor).

Table 3. Determination of constant specific activity of testosterone (Peak 4) and androstenedione (Peak 6). (Repeated tlc method).

Chromatography no.	Solvent system	Testosterone (cpm/μg) ♀	Testosterone (cpm/μg) ♂	Androstenedione (cpm/μg) ♀ + ♂
Before chromatogr.		451	370	388
1	3	406	363	348
2	5	388	363	320
3	2	397	371	328
Mean ± *SD*		397±9	366±5	332±14

Table 4. Determination of constant specific activity of 17α-hydroxy-progesterone (Peak 4). (Repeated tlc method).

Chromatography no.	Solvent system	(cpm/μg) ♀	(cpm/μg) ♂
Before chromatogr.		430	428
1	5	411	400
2	9	403	385
3	10	381	374
Mean ± *SD*		398±15	386±13

Table 5. The percentage distribution (mean ± *SE*) of [4-^{14}C] testosterone and its neutral metabolites in various experimental groups in tlc I (system 1).

Group		P1	P2	P3	P4	P5	P6	P7
1	♀	2.5±0.8	4.4±1.4	9.4±0.4	55.8±2.0	24.4±1.3	3.5±0.7	—
	♂	2.1±1.0	2.9±0.8	7.6±1.8	62.9±5.4	21.4±3.8	3.2±0.6	—
2	♀	4.2±1.1	6.7±1.1	10.1±1.1	49.6±4.8	24.5±3.6	2.3±0.3	2.5±0.3
	♂	4.3±0.9	6.6±0.6	9.1±1.6	58.9±4.1	16.5±3.7	2.6±0.8	2.0±0.2
3	♀	2.8±0.5	5.1±0.2	10.2±0.8	51.2±4.9	27.4±4.2	1.5±0.4	1.8±0.4
	♂	2.4±0.5	4.7±0.4	11.2±4.1	62.6±4.3	15.9±1.8	1.8±0.5	1.5±0.4
4	♀	3.7±0.5	5.8±1.0	9.7±0.9	55.0±3.5	22.0±2.3	2.0±0.4	1.9±0.3

Table 6. The percentage distribution (mean ± *SE*) of [4-^{14}C] 17α-hydroxyprogesterone and its neutral metabolites in various experimental groups in tlc I (system 8).

Group		P1	P2	P3	P4	P5
1	♀	3.0±0.7	2.4±0.4	7.7±1.0	71.4±3.4	15.5±3.9
	♂	2.7±0.8	2.6±0.3	5.9±0.5	76.8±5.7	12.1±4.9
2	♀	3.3±0.5	3.2±0.5	8.8±0.4	73.8±1.0	10.9±1.1
	♂	3.3±1.0	3.4±0.2	7.0±1.9	77.5±2.6	8.9±1.8
3	♀	2.7±0.6	1.7±0.2	7.6±1.3	75.3±2.2	13.7±2.5
	♂	2.5±0.6	2.0±0.5	6.3±0.5	81.0±1.4	10.1±3.6
4	♀	3.0±0.5	2.6±0.4	8.2±1.1	77.2±2.5	8.9±1.7

3. Results

3.1. Steroid levels in plasma

The gonado-somatic index (GSI) for brown trout remained low and unchanged until May (Table 1). After the following three months the GSI increased by about 20 times for females and 13 times for males. The maximum GSI (28.3) for females was recorded in October-November, during the spawning season.

In females the measured steroid concentrations of the peripheral plasma (Fig. 3) remained low (E_1 60—90 pg/ml, E_2 40—320 pg/ml and T 70—80 pg/ml) from February until May. In August the steroid concentrations reached their maximum (E_1 500 pg/ml, E_2 2100 pg/ml and T 380 pg/ml) when the growth of the gonads was well under way. At the time of spawning in October when the GSI for females was at its maximum the steroid concentrations in the peripheral plasma reached their minimum (E_1 60 pg/ml, E_2 180 pg/ml, T 100 pg/ml). All plasma testosterone values refer to Mwenesi (1978).

In males (Fig. 4) E_1 (40—60 pg/ml) and E_2 (50—230 pg/ml) concentrations were at their maximum in late February, while T concentration (40—50 pg/ml) was in its minimum. In August the concentrations of oestrogens were minimal while the T concentration had reached its maximum (about 1000 pg/ml).

3.2. Skin incubations

Testosterone as substrate

The metabolism of testosterone in the skin was at its minimum when the day was shortest (Fig.

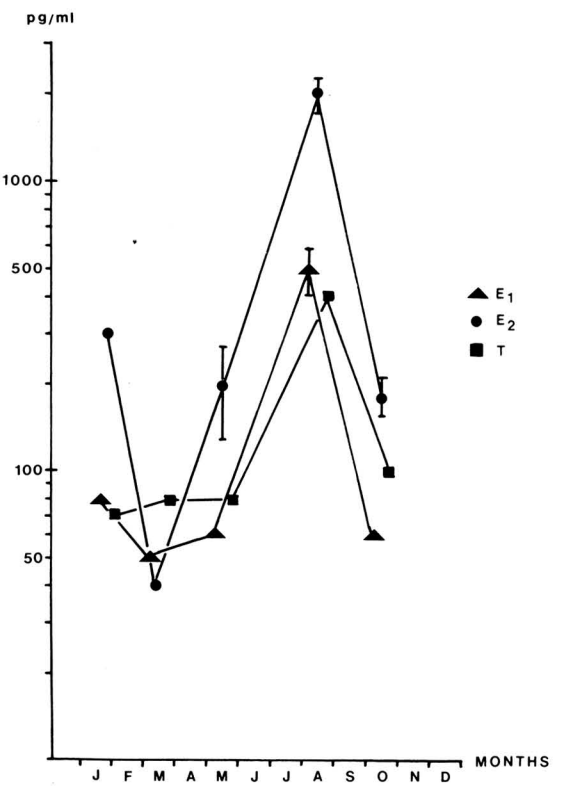

Fig. 3. Concentrations of oestrone, oestradiol and testosterone in the peripheral plasma of female brown trout from February to November.

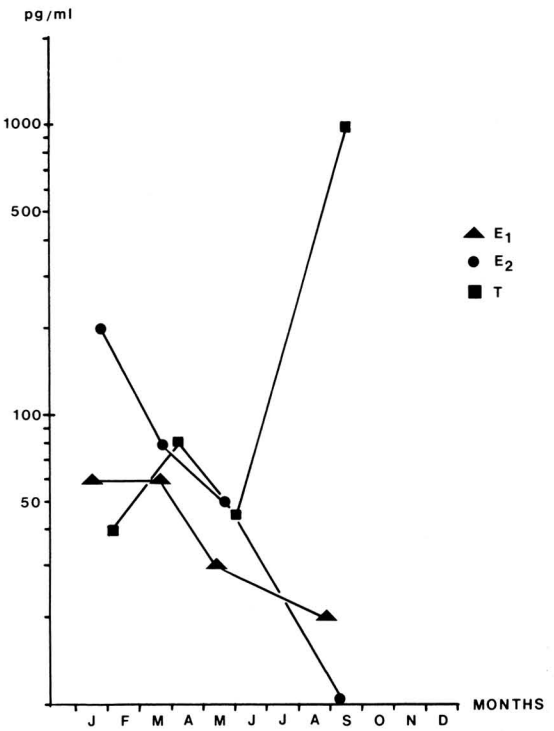

Fig. 4. Concentrations of oestrone, oestradiol and testosterone in the peripheral plasma of male brown trout from February to August.

5B). In late February only 60 % of the substrate was metabolized in female and 55 % in male skin. The metabolism was activated to its maximum (70 %) in May, when the water was beginning to become warm and the daylight period already exceeded 17 h. About two months before the spawning season in late August, when the water still remained warm but the daylight period was becoming shorter (14 h) the metabolic efficiency was particularly reduced in male skin (61 %). The decreasing trend in metabolic efficiency seemed to continue in females until the spawning season.

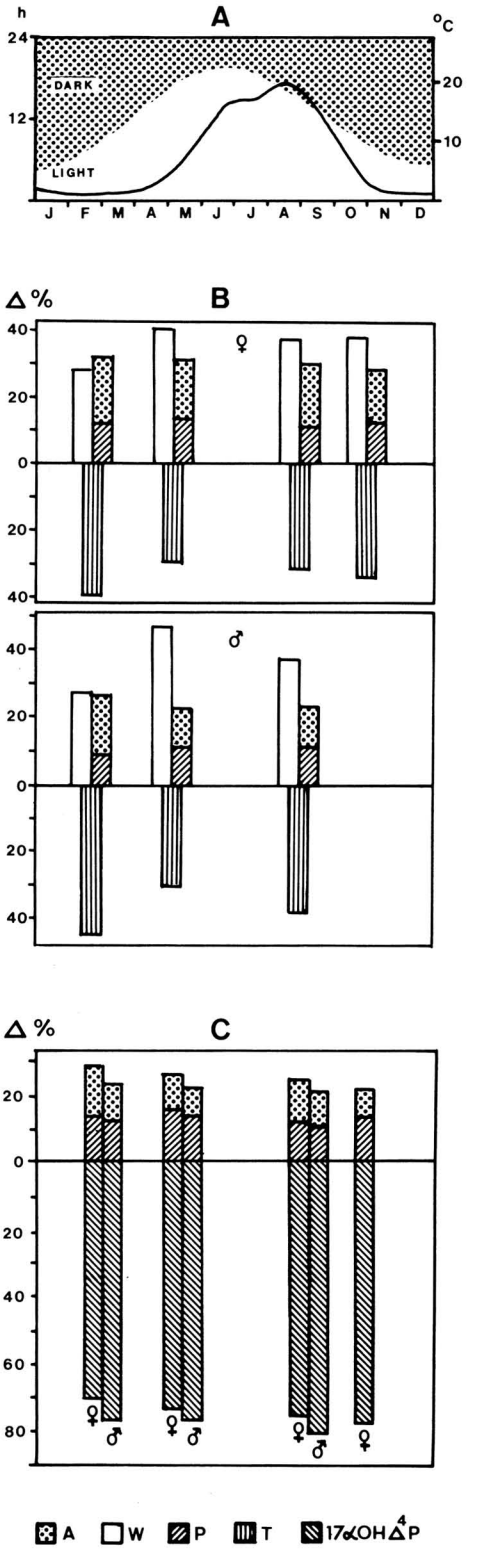

Fig. 5. — A. The annual variations in day length and water temperature during the experiment. — B. Metabolism of testosterone (T) in the skin of brown trout. Bars below the zero line represent the amount of unchanged precursor and above the zero line the water (W) and ether-soluble metabolites. The bar denoting ether-soluble metabolites is divided into apolar (A) and polar (P) fractions. — C. Metabolism of 17α-hydroxyprogesterone (17αOH-Δ^4-P) in the skin of brown trout. Explanations as for B. No water-soluble metabolites were detected.

About 48 % of the metabolites produced in the skin were water-soluble when the metabolic efficiency of testosterone was low. Of the ether soluble fractions, 34 % (♀) and 37 % (♂) were polar. When the efficiency increased in May a higher proportion of the metabolites was water-soluble (about 59 % for females and about 68 % for males) and at the same time the relative proportion of apolar metabolites seemed to decrease. During the spawning season the proportions of water-soluble and polar metabolites still remained relatively high.

17αOH-progesterone as substrate

A maximum of 29 % of the 17αOH-Δ^4P was metabolized by the skin in late February in females (Fig. 5C). The efficiency of 17αOH-Δ^4P metabolism seemed to decrease while that of T increased. In all incubations the quantity of metabolites produced by male skin was smaller than that produced by female skin. No water-soluble metabolites were detected.

4. Discussion

The variations in testosterone concentration in brown trout seem to agree with the observations of Scott et al. (1980a) on rainbow trout, indicating that the maximum concentrations precede the spawning season. On the other hand, the minimum concentrations of oestrone and oestradiol in male plasma in August fits well with their nature as metabolites of testosterone.

Testosterone has been identified in the plasma of several female teleosts (cf. Scott et al. 1980b), and our results agree well with the annual cycle reported by Scott et al. (1980b). However results from this study indicate that testosterone is not the main steroid in the brown trout female, as it seems to be in rainbow trout (Scott et al. 1980b). The highest concentration of oestradiol occurred just prior to ovulation and the levels of all steroids analysed decreased at about the time of ovulation. The oestrone and oestradiol increases do not coincide with the decrease in peripheral testosterone concentration.

The identification of fractions after tlc was successful for the precursors used and for androstenedione, 5α-androstanediol and androstadione from incubations with testosterone as precursor. Because the activities of the identified fractions were low we prefer the grouping of the metabolites as polar (fractions 2—3) and apolar (fractions 5—7) when compared to the migration of precursors. The testosterone metabolism of the skin seems to be most active at the time of increasing daylight, before the warming of water, when also the superficial mucous concentration is increasing and the epidemal thickness reach its maximum (Pickering 1977). At this time the induction of the reproductive cycle has evidently taken place (Whitehead et al. 1978), but the concentrations of plasma testosterone in males and oestrogen in females still remain low. When the hormone concentration in peripheral plasma reaches its maximum in August and the epidermis is at its thinnest (Pickering 1977), the efficiency of testosterone metabolism in the skin is decreasing, possibly due to the overloading of steroid receptors by the endogenous substrates.

Bonnin (1979) reports an annual cycle for conjugated testosterone in the plasma of *Gobius niger* (L.). It is interesting to note that in *Gobius niger* the maximum concentration of testosterone in plasma and maximum GSI precede the maximum amount of conjugated testosterone in plasma (Bonnin 1979). In our material the activity of conjugation (production of water-soluble metabolites) in both sexes increased c. 3—4 months prior to the maximum GSI and testosterone concentration in plasma.

The production of conjugated metabolites by the skin of brown trout agrees well the speculation of Bonnin (1979) that the origin of these water-soluble hormones might be extra-testicular.

The results of 17αOH-progesterone incubations reveal that there is no desmolase activity in the skin. Changes in the A-ring explain the existence of apolar and polar compounds. The lack of conjugated water-soluble metabolites indicate that this route of metabolism is not used in detoxication of the 17αOH-progesterone in skin.

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