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Aromatase Activity in Brain and Pituitary of Immature and Mature Atlantic Salmon (*Salmo salar* L.) Parr

E. ANDERSSON,* B. BORG,* AND J. G. D. LAMBERT†

*Department of Zoology, University of Stockholm, S-106 91, Stockholm, Sweden; and †Department of Experimental Zoology, University of Utrecht, Padualaan 8, Utrecht 3584 CH, The Netherlands

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Brain homogenates from Atlantic salmon parr converted tritiated androstenedione to testosterone, estrone, and 17β-estradiol. The formation of estrogens was markedly higher in homogenates of whole brains from mature parr males than from immature parr males. The highest estrogen synthesis was found in the telencephalon and diencephalon. In both of these parts the estrogen production was higher in mature males than in immature males. There was no difference in estrogen formation by pituitaries from immature female and immature male parr, whereas the formation of estrogens and testosterone was higher in the mature male parr. © 1988 Academic Press, Inc.

The Atlantic salmon, *Salmo salar,* is an anadromous fish. The young females and some of the young males migrate to the sea in a sexually immature state and return to the river for spawning after a few years. Some of the young males, however, mature already as parr and may breed at a small size, without having left the river. It is not known if and in what respects the neuroendocrine feedback control of the pituitary gonadotropic (GTH) cells differs between the two categories of parr males. Androgens can be aromatized to estrogens in the brains of most vertebrates. This conversion is particularily high in teleosts (Callard, 1982). Aromatization has been indicated as being of importance for androgen effects on the GTH cells in the young rainbow trout, *Salmo gairdneri* (Crim *et al.,* 1981) and in the adult African catfish, *Clarias gariepinus* (de Leeuw *et al.,* 1986). The object of the present study was to investigate this component in the neuroendocrine system in mature and immature Atlantic salmon parr. To that end brain homogenates and pituitaries from mature and immature Atlantic salmon parr were incubated with tritiated androstenedione and the steroids formed were analyzed.

MATERIAL AND METHODS

Animals. Two-summer-old Atlantic salmon parr of the Umeälven river stock were used in this investigation. The fish were kept at the Norrfors hatchery situated at the river Umeälven (64° N) Sweden in 2 × 2-m tanks supplied with through-flowing river water and at natural photoperiod. The fish were fed on EWOS salmon food. Mature and immature parr were dissected on the 14–15 of September, 1987. At this time the mature males had started to produce running milt and had a gonadosomatic index (GSI) ranging from 8.9 to 14% (mean GSI was 11.2%). The gonads of the immature males were thread-like and were too small for meaningful weighing (GSI $\ll$ 1%). RIA measurements of androgens from fish taken at this occasion gave mean levels of 5 ng/ml of 11-ketotestosterone and 2 ng/ml testosterone in mature parr and 0.4 ng/ml 11-ketotestosterone and 1 ng/ml testosterone in immature parr (I. Mayer and R. Schulz, unpublished).

Preparation of samples for incubation. After decapitation, the brains were removed and immediately frozen in plastic tubes on dry ice and stored at −70°. The brains were homogenized in whole or in parts with Teflon–glass homogenizers at 0° in 0.1 *M* phosphate buffer (pH 7.4) containing 0.25 *M* sucrose.

Chemicals. 7-[^{3}H]Androstenedione (7.6 Ci/mmol) was purchased from Radiochemical Centre, Amer-

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sham. The purity was controlled by thin-layer chromatography (TLC). Unlabeled steroids were obtained from Merck A.G.; NAD and NADPH were from Boehringer. All chemicals and solvents were of analytical grade.

Incubations. All incubations were carried out for 3 hr at ca. 20° in open tubes under continuous shaking. The 7-[^{3}H]androstenedione was first dissolved in propylene glycol (final concentration 5% brain parts, 17% whole brains), which was mixed with K-phosphate buffer (0.1 *M*, pH 7.4), with nicotinamide adenine dinucleotide (NAD, 2 m*M*) and reduced nicotinamide adenine dinucleotide phosphate (NADPH, 2 m*M*) added. The final steroid concentration was 0.13 μ*M* or 1.0 μCi/ml (except for the whole brains, 0.12 μ*M*, 0.9 μCi/ml).

Homogenates of whole brains and brains divided into major parts from mature and immature males were incubated in final volumes of 3 ml (whole brains) or 1.5 ml (brain parts).

Pituitaries were incubated individually in a volume of 0.07 ml. Pooled pituitaries, 15 from mature and 15 from immature, were both incubated in a final volume of 0.2 ml.

Analysis. The enzyme reactions were terminated by adding dichloromethane. Before extraction with dichloromethane the carriers androstenedione, testosterone, estrone, and estradiol were added. The tritium activity in the water fraction was determined with scintillation counting as described by Schoonen and Lambert (1986).

The combined organic extracts were evaporated and the residue was subjected to TLC on glass plates precoated with silica gel F254 (Merck) in saturated tanks, first three times in toluene–cyclohexane, 1:1 (system 1) to separate apolar compounds from steroids. The steroids remained localized on the base line. Consecutive TLC using diisopropylether–chloroform–hexane, 7:2:1 (3×) (system 2), separated the steroids. After development the carriers were located by uv absorption at 240 nm. The percentages of activity in different peaks on the TLC plates were determined using a Berthold LB2842 automatic TLC linear analyzer.

The areas corresponding to testosterone, estrone, and estradiol from some of the material were eluted and subjected to TLC using chloroform–ethanol, 95:5 (1X) (system 3). Testosterone, estrone, and estradiol formed by the brain from a mature male and by the pooled pituitaries from mature males were acetylated, rechromatographed in diisopropylether–chloroform–hexane, 7:2:1 (1X), and recrystallized to a constant specific activity as described by Schoonen and Lambert (1986).

RESULTS

By far most of the activity was found in the organic fraction. Only ca. 2% (highest value 4%) of the total activity was found in the water fraction.

The radiochromatograms after TLC in system 2 of the organic fraction from both the brain homogenate and the pituitary incubations showed peaks at the positions of the carriers androstenedione (Δ^4), testosterone (T), estrone (E1), and 17β-estradiol (E2). The identity of the formed T, E1, and E2 was confirmed successively by TLC in system 3, acetylation, and a rerun in system 2, and by recrystallization to a constant specific activity (Table 1). It was found that the T, E1, and E2 areas after a first separation in TLC system 2 contained no significant amounts of other radiolabeled substances. For this reason the amounts of T, E1, and E2 formed were calculated from these scanograms. There were also peaks

TABLE 1
IDENTIFICATION OF STEROIDS FORMED FROM ANDROSTENEDIONE BY BRAIN AND PITUITARIES

Tissue	Steroid	Specific activity (dpm/mg)			
		Original	1. Cryst.	2. Cryst.	3. Cryst.
Brain	T	126,467	116,481	124,023	124,208
	E1	36,642	35,312	36,057	36,063
	E2	87,347	87,171	87,509	85,548
Pituitary	T	7,558	7,446	6,993	7,291
	E1	10,109	9,139	10,144	9,748
	E2	4,405	4,123	4,324	4,169

Note. dpm = disintegrations per minute; T = testosterone; E1 = estrone; E2 = 17β-estradiol.

of unknown substances in the scanograms from both pituitary and brain incubations. The largest and most distinct of the unknown peaks from the homogenates of whole brains was found at a distance from the baseline two-thirds of that of T. It was larger in the scanograms from mature (12.8 ± 1.3% of organic fraction activity) than from immature brains (6.6 ± 0.4%). This peak was not present in the pituitary scanograms.

The results of the incubations of brains from immature and mature males are shown in Table 2. The yields of E1 and E2 were much higher in the mature than in the immature fish, whereas the opposite was the case with T. The percentages of both E1 and E2 were more than twice as high in all mature fish than in any of the immature ones. The estrogens (E1 + E2) constituted on an average 45% of the organic fraction from the mature brains and 13% from the immature brains. This corresponds to an aromatization of 159 and 45 pmol radiolabeled androgen/incubation, or 1.7 and 0.4 pmol/mg brain weight, respectively. The lower yield of T in the incubations of brains from the mature (27%) than from the immature fish (58%) is perhaps partly due to the higher formation of estrogens. The combined yield of T and E2, both formed by a 17β-hydroxysteroid dehydrogenase conversion, is 55% in the mature and 66% in the immature ones.

TABLE 2
STEROID CONVERSION IN HOMOGENATES OF WHOLE BRAINS FROM MATURE AND IMMATURE SALMON PARR

	Mature	Immature
n	8	7
Body weight (g)	25.6 ± 1.8	30.6 ± 1.7
Brain weight (mg)	96 ± 3	105 ± 3
Androstenedione (%)	10.1 ± 2.0	16.1 ± 2.5
Testosterone (%)	26.6 ± 3.0	58.4 ± 2.6
Estrone (%)	16.2 ± 1.1	4.7 ± 0.3
17β-Estradiol (%)	28.7 ± 1.7	8.0 ± 0.3
E1 + E2 (pmol/mg brain wt)	1.67 ± 0.10	0.43 ± 0.02

Note. Means and standard errors shown. Amount of steroids in percentage of total activity in organic fraction. The percentages of estrone, estradiol, and testosterone and the formation of E1 + E2/mg brain wt are different between the two groups. $P < 0.001$ in all four comparisons, Mann–Whitney U test.

When parts of a mature male brain were incubated a high synthesis of estrogens was found in the telencephalon and in the ventral diencephalon. A lower aromatization was found in the tectum opticum/dorsal diencephalon area, whereas the cerebellum and brainstem were practically negative. All parts showed considerable T formation. The results of individual incubations of telencephalon, ventral diencephalon ("diencephalon"), tectum opticum/dorsal diencephalon ("tectum") from mature and immature males are shown in Table 3. The tectum yields of T, E1, and E2 were about the same in mature and immature brains. In both telencephalon and diencephalon the yields of E1 and E2 were much higher (3–7 times) in the mature than in the immature ones. The yield of T was higher in the immature than in the mature ones, but these differences largely disappeared when the weights of the brain parts were considered. The average synthesis of radiolabeled estrogens via aromatization in the tectum was 0.3 pmol/mg in mature and 0.2 pmol/mg in immature fish, in the diencephalon it was 1.6 pmol/mg in mature and 0.2 pmol/mg in immature, and in the telencephalon it was 11.8 pmol/mg in mature and 2.0 pmol/mg in immature fish brains.

The yields of T, E1, and E2 in incubations with individual pituitaries from mature male parr and immature male and female parr are shown in Table 4. There was no difference between immature males and females. All three substances were formed in larger amounts by the pituitaries from the mature than from the immature fish.

DISCUSSION

Homogenates of salmon brains con-

TABLE 3
STEROID CONVERSION IN HOMOGENATES OF BRAIN PARTS FROM MATURE AND IMMATURE SALMON PARR

	Weight (mg)	Δ^4 (%)	T (%)	E1 (%)	E2 (%)	E1 + E2 (pmol/mg)
			Telencephalon			
Mature 1	5.1	7.6	49.6	16.1	15.6	12.3
Mature 2	6.5	5.6	44.0	22.8	14.3	11.3
Immature 1	7.3	14.9	68.0	5.0	4.0	2.4
Immature 2	6.4	10.9	73.9	3.3	2.6	1.8
			Diencephalon			
Mature 1	18.5	9.0	63.0	12.7	3.8	1.8
Mature 2	24.6	12.8	60.2	12.4	4.2	1.3
Immature 1	34.6	17.4	72.1	1.2	1.4	0.1
Immature 2	30.1	17.5	67.9	2.6	0.9	0.2
			Tectum			
Mature 1	19.6	17.4	70.5	1.7	1.4	0.3
Mature 2	19.0	20.7	68.2	1.5	0.9	0.2
Immature 1	29.6	27.1	61.8	1.4	1.8	0.2
Immature 2	25.5	32.6	54.8	0.9	1.4	0.2

Note. Individual values. Δ^4 = androstenedione; T = testosterone; E1 = estrone; E2 = 17β-estradiol. Amount of steroids as percentage of total activity in organic fraction.

verted Δ^4 to T, E1, and E2. This is in agreement with findings in several other teleosts (Callard *et al.*, 1981b; Pasmanik and Callard, 1985; Lambert *et al.*, 1982; Timmers and Lambert, 1987; Timmers *et al.*, 1987; Borg *et al.*, 1987a).

Although most of the Δ^4 precursor was converted during the incubations, there was always a considerable amount of aromatizable androgens (Δ^4 and T) left. The time course of conversion was not studied. However, in the African catfish, *C. gariepinus*, this conversion is linear for 4 hr with a similar method (Timmers and Lambert, 1987). It was not possible for us to examine the effect, if any, of freezing of the brains. Although the methods used may give some deviation from ideal values (i.e., underestimation), this is hardly of major importance for the comparison of the conversion between different brain areas and between mature and immature fish.

With the reservations above, the aromatase activity in whole brains was ca. 0.6 pmol mg wet wt^{-1} hr^{-1} in whole brains of mature parr, and up to ca. 3.9 pmol mg^{-1}

TABLE 4
STEROID CONVERSION BY PITUITARIES FROM MATURE MALES AND IMMATURE MALES AND FEMALES

	Fish weight (g)	T (%)	E1 (%)	E2 (%)
Mature males	23.5 ± 1.8	21.9 ± 1.2	12.2 ± 0.8	3.6 ± 0.4
Immature males	30.7 ± 1.6	12.8 ± 0.9	6.6 ± 0.3	1.2 ± 0.1
Immature females	31.8 ± 1.6	11.0 ± 1.2	6.1 ± 0.5	1.1 ± 0.1

Note. Mean and standard errors shown. T = testosterone; E1 = estrone; E2 = 17β-estradiol. Amounts of steroids expressed as percentage of activity in the organic fraction. Thirteen fish in each group studied. The amount of testosterone, estrone, and estradiol formed is larger in the mature males than in both immature males and females. $P < 0.001$ in all six comparisons, Mann–Whitney U test.

hr^{-1} in the brain area with the highest conversion. In the stickleback, *Gasterosteus aculeatus*, a conversion of ca. 0.1 pmol mg^{-1} hr^{-1} was found in the whole brain using similar methods (Borg *et al.*, 1987a). In homogenate incubations of major brain areas from other teleosts the maximum conversions were 1.6 pmol mg^{-1} hr^{-1} in the toadfish, *Opsanus tau*, 3.6 pmol mg^{-1} hr^{-1} in the goldfish, *Carassius auratus* (Pasmanik and Callard, 1985), and 0.1 pmol mg^{-1} hr^{-1} in the longhorn sculpin, *Myoxocephalus octodecimspinosus* (Callard *et al.*, 1981b). Incubations of punches from limited brain areas gave conversions of up to ca. 1 pmol mg^{-1} hr^{-1} in the stickleback (Borg *et al.*, 1987a) and up to 3.7 pmol mg^{-1} hr^{-1} (1–3 pmol mg^{-1} hr^{-1} in most areas in the telencephalon, diencephalon, and mesencephalon) in the African catfish (Timmers *et al.*, 1987). Thus it seems that the aromatase activity in the salmon parr is at least as high, if not higher, than that in other studied teleosts.

The conversion of androgens to estrogens was highest in the telencephalon and also high in the diencephalon. The tectum opticum displayed a lower aromatase activity and the cerebellum and brain stem were practically negative. A high aromatase activity in the diencephalon has also been found in all previously studied teleosts; longhorn sculpin, (Callard *et al.*, 1981b); rainbow trout, *S. gairdneri* (Lambert *et al.*, 1982); goldfish, toadfish (Pasmanik and Callard, 1985); African catfish (Timmers *et al.*, 1987), and three-spined stickleback (Borg *et al.*, 1987a). The aromatase activity in the telencephalon has also been found to be high in sculpin (Callard *et al.*, 1981b), goldfish (Pasmanik and Callard, 1985), and catfish (Timmers *et al.*, 1987), but not in stickleback (Borg *et al.*, 1987a). The tectum opticum/dorsal diencephalon displayed a moderate conversion in the Atlantic salmon. This was also the case in the stickleback (Borg *et al.*, 1987a), whereas the rainbow trout (Lambert *et al.*, 1982) and the catfish (Timmers *et al.*, 1987) had high estrogen formation in these regions. Brain stem–anterior spinal cord and cerebellum had a low conversion in the salmon as in most other studied fishes, except in the toadfish were a high conversion was found in the anterior spinal cord (Pasmanik and Callard, 1985).

The salmon pituitary was also capable of converting androstenedione into estrone and estradiol. A conversion of androgens to estrogens has also been found in other studied teleosts: sculpin (Callard *et al.*, 1981a; Olivereau and Callard, 1985), goldfish and toadfish (Pasmanik and Callard, 1985), catfish (de Leeuw *et al.*, 1985), and stickleback (Borg *et al.*, 1987a).

Comparing the aromatase activity in the brains of mature and immature parr it was found that much more estrogens were formed by homogenates of whole brains from mature parr males than from immature ones. This difference was also found in both telencephalon and diencephalon homogenates. Pituitaries from mature parr males displayed higher estrogen production than pituitaries from immature male and female parr. The brain homogenates from immature parr, however, produced more T than those from the mature parr.

Changes in brain aromatase activity according the reproductive state have been reported in a number of teleosts. For example, in the sculpin, aromatization changed seasonally in both the hypothalamus and the telencephalon (Callard *et al.*, 1981a). In the stickleback the aromatase activity in the nucleus preopticus–nucleus anterior periventricularis (but not in other regions) is strongly influenced by season (Borg *et al.*, 1987a). It must, however, be pointed out that the steroid conversion in the brain has, to our knowledge, not been studied in completely immature fish before. Thus, we do not know whether the dramatically higher estrogen formation in the mature parr than in the immature ones might be paralleled at puberty in other teleosts. A

higher aromatase activity in mature than in immature parr was found in both the telencephalon and the diencephalon. This suggests that the high conversion in both these regions is indeed connected with reproduction and makes it less likely that the high estrogen formation in teleost brains serves other needs. In mammals, several investigations (Callard, 1983; Michnovicz *et al.*, 1987) have shown a particularly high conversion early in ontogeny. However, this is difficult to compare with the present study, where immature and mature fish of the same age have been examined.

A number of studies performed in mammals have shown that only aromatizable androgens and estrogens are effective in stimulating male reproductive behavior, whereas androgens that cannot be converted to estrogens are ineffective (e.g., Beyer *et al.*, 1973). This is often also assumed to be the case in fishes, but experimental evidence is lacking. On the contrary, stimulatory effects of the nonaromatizable androgen 11-ketoandrostenedione on reproductive behavior have been found in the stickleback (Borg, 1987), and preliminary results indicate that this androgen also stimulates male sexual behavior in castrated Atlantic salmon parr (I. Mayer and I. Berglund, unpublished).

As in other vertebrates, aromatization appears to be of importance for negative feedback effects from the gonads on GTH secretion in the African catfish (de Leeuw *et al.*, 1985). A role for aromatase in the feedback systems has also been suggested in the African catfish and stickleback, where its anatomical distribution has been studied in more detail than in other fishes. In these two species the activity is particularly high in hypothalamic regions like the nucleus preopticus and the nucleus lateralis tuberis which probably control the GTH secretion (Peter, 1983). In young salmonids positive effects of steroids on accumulation of GTH in the pituitary have been reported (Crim and Evans, 1979; Crim *et al.*, 1981; Gielen *et al.*, 1982). Crim *et al.* (1981) found that several aromatizable androgens and estrogens had this effect, whereas nonaromatizable androgens and corticosteroids did not. An aromatase inhibitor partly suppressed the effect of testosterone. Van den Hurk *et al.* (1984), on the other hand, found that some corticosteroids and nonaromatizable 11-androgens also had stimulatory effects on GTH accumulation, though very much less than the aromatizable methyltestosterone, which was also tested. Ikuta *et al.* (1987) found the same stimulatory effect of high doses of testosterone and the nonaromatizable 11-ketotestosterone on pituitary GTH content in immature masu salmon, *Oncorhynchus masou*. The positive effect of androgen on GTH accumulation in young rainbow trout is due to a stimulation on the pituitary level (Fåhraeus-van Ree *et al.*, 1983; Gielen and Goos, 1983). Prolonged treatments also lead to elevated GTH levels in the plasma (Crim and Evans, 1983). Only aromatizable androgens have been used in the study of these aspects. The aromatase activity was higher in the pituitaries of the mature parr males than in the pituitaries of immature parr. The difference was, however, proportionally less than in the brain in general and is perhaps not larger than could be accounted for by the probable growth of the GTH cells. In the African catfish, aromatase activity was found to be present in this cell type (de Leeuw *et al.*, 1985).

The higher aromatase activity in the brain of the mature parr males might be related to changes in feedback mechanisms associated with maturation. It is not possible to say if differences in steroid metabolism determine which males will mature and which will not. Preliminary results from incubations with brains sampled in July, when the differentiation of the gonads first becomes visible, show a much smaller and less clear-cut difference between mature and immature males, indicating that this is not the case. Far more detailed studies are, however, needed. There is also the possibility that the gonads are influencing the ar-

omatase activity. Stimulatory effects of testes/androgens on brain estrogen formation have been found both in mammals (e.g., Roselli and Resko, 1984) and in the stickleback (Borg *et al.*, 1987b).

In the stickleback the testes stimulate an increase in aromatase activity in the nucleus preopticus–nucleus anterior periventricularis region (other brain areas were, however, not influenced) when winter fish are brought to breeding by long photoperiod and high temperature (Borg *et al.*, 1987b). The increased aromatase activity in the brain of the mature parr male might also be due to a stimulation from the gonads. In the stickleback morphological studies indicate that methyltestosterone stimulates the GTH cells in winter (Borg *et al.*, 1986) and have an inhibitory effect when the breeding season has already started (Borg *et al.*, 1985). The feedback from the gonads to the pituitary is largely negative in adult salmonids (e.g., van Putten *et al.*, 1981; Billard *et al.*, 1977). One might speculate that an increased aromatase activity in the brain in the maturing parr might change the general androgen effect on the GTH cells from a positive accelerating to a negative stabilizing feedback.

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