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The cytochrome P450 system of Atlantic salmon (*Salmo salar*): II. Variations in hepatic catalytic activities and isozyme patterns during an annual reproductive cycle[1]

Håvard E. Larsen, Malin Celander* and Anders Goksøyr
*Laboratory of Marine Molecular Biology, University of Bergen, HIB, N-5020 Bergen, Norway; *Department of Zoophysiology, University of Göteborg, Medicinaregatan 18, S-413 90 Göteborg, Sweden*

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Abstract

A group of Atlantic salmon (*Salmo salar*) was followed through their first year of maturation and spawning. At monthly intervals, starting with juvenile fish in December, 5-7 fish of each sex were killed, and liver and plasma were sampled. The last sampling point was of spawning fish in November a year later. Variables in the cytochrome P450 (P450) system were studied in hepatic microsomes, and estradiol 17β was measured in the plasma of females to assess the maturational status. The P450 1A1-mediated 7-ethoxyresorufin O-deethylase (EROD) started at high levels in winter, but decreased to non-detectable activities in pre-spawning females. Decreases, but not to the same extent, were also observed during this period in total cytochrome P450, cytochrome b_5, NADPH-cytochrome P450 reductase, and in the content of two immunochemically determined P450 isozymes. At the same time, LSI levels increased in maturing females (starting in July), and GSI levels increased in both sexes (starting in May). Sex specific differences were observed in pre-spawning fish in September and October, with levels of total P450, b_5, NADPH-cytochrome P450 reductase, EROD and P450 isozymes significantly lower in females. At the same time, plasma estradiol-17β levels reached peak values in females. The results point to the important role of sex steroids such as estradiol-17β as major factors in the regulation of final sexual maturation. However, this study also indicates that there may be estradiol-17β independent events of equal importance in the early stages of gonadal maturation that may involve the P450 system. The changes observed in the P450 system (as a major drug and steroid metabolizing system) of Atlantic salmon during sexual maturation may be of importance both in the endogenous transduction of hormonal signals, and as a pharmacological basis for designing therapeutic treatment of diseases in the aquaculture industry.

Introduction

The cytochrome P450 (CYP or P450) system plays an important part in the oxidative metabolism of a number of endogenous compounds such as steroids, bile acids, fatty acids and prostaglandins. In addition, a wide range of foreign compounds (xenobiotics) including environmental pollutants, drugs and antibiotics are metabolized by this system (Nebert *et al.* 1987). In many cases, this biotransformation can lead to reactive intermediary metabolites with toxic and carcinogenic effects.

In fish, the P450 system has been described in molecular detail in only a few species (reviewed by Goksøyr and Förlin 1992; Stegeman 1989), but certain species-differences have become apparent. Several physiological and environmental factors have also been show to affect the enzyme system.

[1]Parts of this work were presented at the 5th International Symposium on Responses of Marine Organisms to Pollutants, April 1989 in Plymouth, United Kingdom (Larsen and Goksøyr 1989).

Correspondence to: Anders Goksøyr, Laboratory of Marine Molecular Biology, University of Bergen, HIB, N-5020 Bergen, Norway

Such factors include sex, reproductive status and steroid levels (Förlin and Hansson 1982; Stegeman *et al.* 1982; Andersson 1989; Förlin and Haux 1990), changes in season and temperature (Lindström-Seppä 1985; Snegaroff and Bach 1990), and exposure to certain types of environmental pollutants (Payne *et al.* 1987; Goksøyr *et al.* 1991a).

After decades of intensive rearing, the Atlantic salmon (*Salmo salar*) is of enormous economic importance in the world's aquaculture industry today. However, very little is known about the steroid and drug biotransformation enzymes of this species. In our first report on the P450 system of Atlantic salmon (Goksøyr and Larsen 1991), we described the basic characteristics of this enzyme system and its response to inducing agents, with special emphasis on the P450 1A-(CYP 1A)-subfamily, the subfamily most responsive to organic pollutants in fish (Goksøyr *et al.* 1991b). In this report, we describe the seasonal variation in enzyme activities and isozyme levels in a group of salmon entering the sexual maturation and spawning cycle (first-time spawners). Accordingly, plasma levels of estradiol-17β were measured as a parameter of maturation status, and these levels were correlated with the other variables investigated, including the microsomal electron transport components NADPH-cytochrome P450 reductase, cytochrome b_5 and total cytochrome P450 protein, and catalytic and immunochemical studies of two P450 isozymes from different subfamilies. The nomenclature applied to the P450 system in this article is in accordance with the recently updated recommendations of Nebert *et al.* (1991).

A better knowledge of the drug and steroid biotransformation systems in Atlantic salmon may give an important pharmacological basis for designing therapeutic treatment of disease with drugs and antibiotics in this species. Also, knowledge of the changes occurring during maturation may point to important factors in the biochemistry of salmon reproduction.

Materials and methods

Chemicals

7-Ethoxyresorufin and resorufin were purchased from Pierce Eurochemie. NADPH, β-naphthoflavone (BNF), 4-chloronaphthol, o-phenylenediamine-hydrochloride, and sodium dodecyl sulfate were from Sigma Chemical Co. Equipment, other chemicals, and secondary antibody (goat anti-rabbit horseradish peroxidase, GAR-HRP) for Western blotting and ELISA, were purchased from Bio-Rad. Anti-17β estradiol-6-BSA was from ICN (ImmunoBiologicals). Microtiter plates were from Nunc. All other chemicals were of the highest commercial grade available.

Fish and sampling

Atlantic salmon (*Salmo salar*) for the experiments were taken from a genetically homogeneous group hatched and bred at the Matre Aquaculture Station in 1985, and transferred to seawater net pens at the Austevoll Aquaculture Station in the summer of 1986. Both of these stations are in the vicinity of Bergen on the Norwegian west coast. The photoperiod was natural for this area (latitude 60° 30' N). The salmon were expected to spawn for the first time in October-November 1988. Due to an outbreak of vibriosis, the fish were treated with oxytetracycline (100 mg/kg/day in soft pellets for 12 days) from March 12, 1988. Every 35-40th day from December 1987 to November 1988 12 individuals (5-7 of each sex) were killed by a blow to the head, livers were taken out, put in ice-cold 0.1 M Na-phosphate buffer (pH 7.4) with 0.15 M KCl, and transported on ice to the laboratory where homogenization and microsome preparation started within 3 hours. Blood was drawn from the heart using heparinized syringes, transferred to eppendorf vials, centrifuged (5000 rpm for 5 min to remove blood cells) and kept on ice for a few hours before storage in a -20°C freezer.

Preparation of microsomes

Liver samples were homogenized in 4 vol of 0.1 M Na-phosphate buffer (pH 7.4) with 0.15 M KCl, and microsomes were prepared by ultracentrifugation essentially as described by Förlin (1980). Microsomes were resuspended in 0.1 M Na-phosphate buffer (pH 7.4), containing 1 mM EDTA, 1 mM DTT, and 20% glycerol, and stored at -80°C until used.

Measurement of protein and microsomal electron transport components

Total cytochrome P450 was analyzed by the dithionite-difference procedure of Matsubara *et al.* (1974). Cytochrome b_5 was analyzed by NADH-difference (Omura and Sato 1964). Cytochrome content was determined using extinction coefficients of 91 $mM^{-1}cm^{-1}$ for P450 and 185 $mM^{-1}cm^{-1}$ for cytochrome b_5. NADPH-cytochrome P450 reductase was assayed at room temperature with cytochrome c as electron acceptor, at optimal conditions as described previously (Goksøyr and Larsen 1991). All of these measurements were performed on a Perkin-Elmer Model 554 dual-beam spectrophotometer. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Measurements were simplified using a Titertek Multiskan Plus MkII (Flow Laboratories) for absorbance readings.

Assays of cytochrome P450 monooxygenase activities

7-Ethoxyresorufin O-deethylase (EROD) activity was determined fluorometrically at room temperature (20°C) as described previously (Goksøyr and Larsen 1991). A known amount of resorufin was added as internal standard to each reaction series, using the extinction coefficient of 73 $mM^{-1}cm^{-1}$ at 572 nm (Klotz *et al.* 1984) to quantitate the standard. Fluorometric measurements were performed on a Perkin-Elmer LS-5 Luminescence Spectrometer at room temperature.

Immunochemical studies

Rabbit anti-cod P450 1A1 (=cod P-450c, Goksøyr 1985) IgG purified on a Protein A-Superose column on the Fast Protein Liquid Chromatography system (Pharmacia) was used as primary antibody in the study of P450 1A1-cross-reacting proteins. We have previously shown that this antibody cross-reacts with a BNF-inducible 58,000 Da protein in salmon liver microsomes (Goksøyr and Larsen 1991). Rabbit anti-rainbow trout P450con (Celander *et al.* 1989) serum was used to study cross-reactions of this P450 form. This antibody cross-reacts with a 56,000 Da protein in salmon liver (Goksøyr and Larsen 1991). Indirect ELISA was performed by the method of Goksøyr (1991), employing either anti-P450 1A1 IgG or anti-P450con serum as primary antibodies. In all cases, horseradish-peroxidase conjugated goat-anti-rabbit IgG (GAR-HRP, Bio-Rad) was used as secondary antibody.

Estradiol-17β assay

Estradiol-17β was extracted by a 5:1 mixture of diethylether and n-heptane and subjected to radioimmunoassay (RIA) measurements. Estradiol-17β was assayed using specific antibodies. Procedures, isotopes and antiserum were obtained from Chemical Credential, ICN (ImmunoBiologicals) and Amersham International.

Statistical analysis

Statistical analyses were performed using the JMP Software for Statistical Visualization (SAS Institute Inc.) on a Macintosh computer. Differences between groups (sexes) were analyzed by a standard t-test. Effects of sampling time were analyzed by an ANOVA using a second-degree polynomial fit. The level of significance was set at $p<0.05$ in all cases.

Results

Temperature observations from the Aquaculture stations were the salmon was kept are presented in Fig. 1, showing an increase from May to a peak in

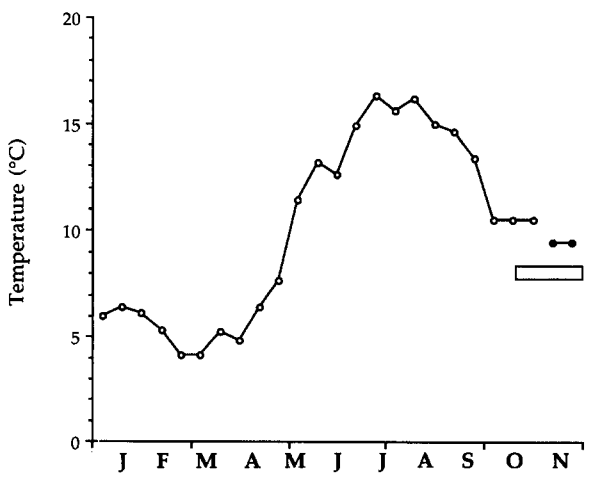

Fig. 1. Temperature observations in 1988 from the Aquaculture Stations in Austevoll (open circles) and Matre (filled circles), where Atlantic salmon were held in net pens for the annual cycle studies. The bar indicates the period of spawning.

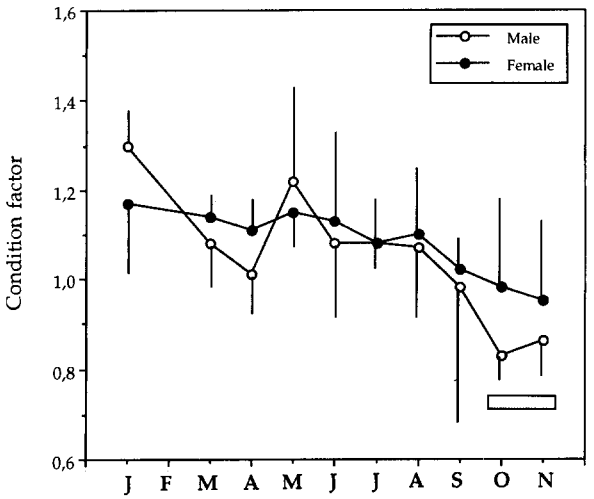

Fig. 2. Condition factor (body weight × 100/length^3, Bagenal and Tesch 1978) of the Atlantic salmon used in this study. Each data point represents the mean ± SD of 5-7 individual fish of each sex.

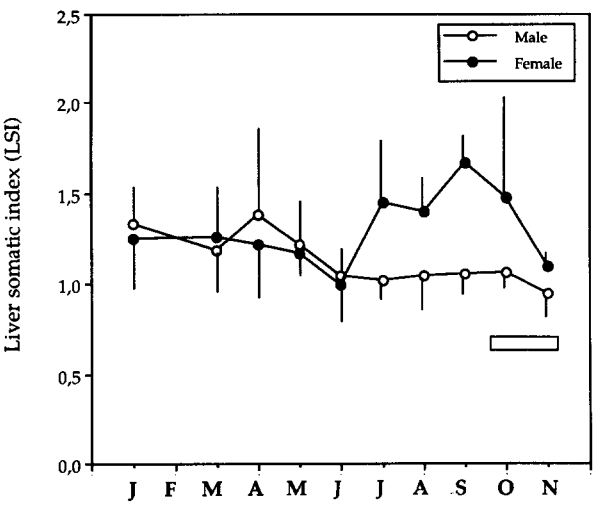

Fig. 3. Liver somatic index (LSI) of first-time spawning Atlantic salmon during an annual reproductive cycle.

July-August. The condition factor (Bagenal and Tesch 1978) of the salmon studied decreased through the year-cycle, most pronouncedly in males (Fig. 2). A slight decrease was also observed in the liver somatic index (LSI) in males, whereas in females values peaked from August to November (Fig. 3). The gonadosomatic index (GSI) values were low in both males and females from January to August (Fig. 4A). However, an increasing trend was observed starting in May (Fig. 4B). The highest values were found for both males and females in October and November.

Yield of microsomal protein/g liver increased slightly and peaked in September, whereafter it decreased and significant differences between the sexes became apparent (Fig. 5). The total amount of cytochrome P450 was stable through the winter season for both sexes, and no significant sex differences were observed. In August, the cytochrome P450 level started to increase, and in September there was a significant difference between males and females (Fig. 6). At this stage the cytochrome P450 level in males was approximately twice that in females. After spawning (October-November) a slight increase was observed in the cytochrome P450 levels in females. Nearly the same pattern appeared for the level of cytochrome b_5, where we also observed no significant differences between the sexes except in September. For both sexes a

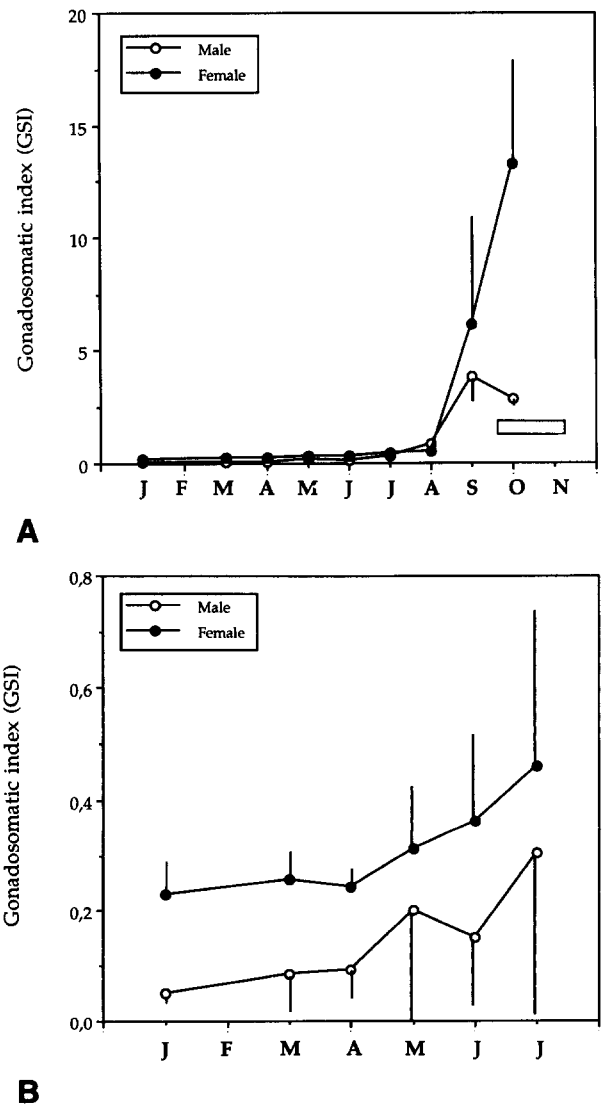

Fig. 4. Changes in gonadosomatic index (GSI) in Atlantic salmon during an annual reproductive cycle (A), and changes occurring from January to July (B).

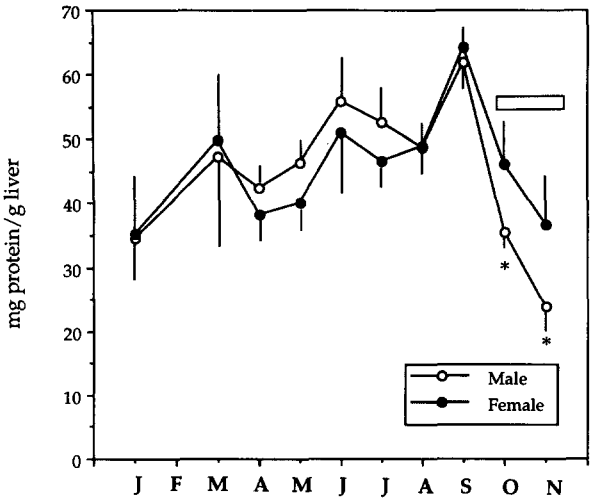

Fig. 5. Yield of microsomal protein per g liver in Atlantic salmon during an annual cycle. *females significantly different from males ($p<0.05$).

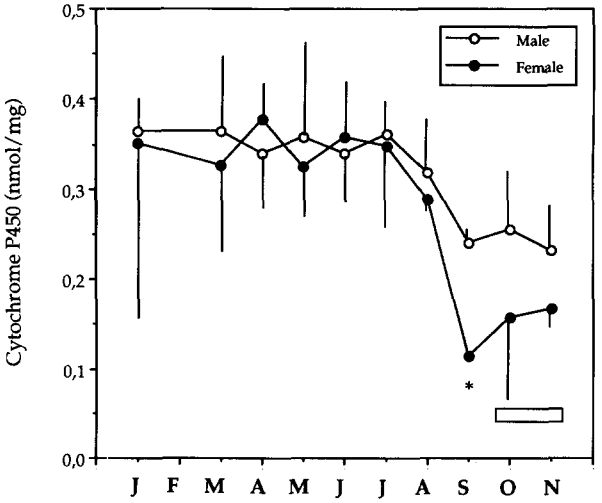

Fig. 6. Changes in the levels of total hepatic cytochrome P450 (nmol/mg microsomal protein) in Atlantic salmon during an annual reproductive cycle. *females significantly different from males ($p<0.05$).

slight decrease appeared in the total amount of cytochrome b_5 from July and towards spawning (Fig. 7). The NADPH-cytochrome P450 reductase activity fluctuated somewhat during the winter season, but started to decrease in July and significant sex differences were observed in October (Fig. 8). Microsomal protein, total P450, cytochrome b_5, and NADPH-cytochrome P450 reductase showed a significant effect of sampling time in an ANOVA ($p<0.05$).

The 7-ethoxyresorufin O-deethylase (EROD) activity stayed at high levels during the winter season, and no sex differences were detectable. After a conspicuous peak in May, the enzyme activities were decreasing towards spawning in October (Fig. 9A). Overall a significant effect of sampling time was observed by ANOVA. To better illustrate the differences in the EROD activities between each salmon during spawning, we have presented the individual values from the last part of the season (Fig. 9B). Just before spawning in September the EROD activities in females were dropping below the detection limit for the assay setup (0.5 pmol/min/mg). The EROD activity in ovulated females was significantly higher than in pre-spawning females ($p<0.05$ with zero activities set at detec-

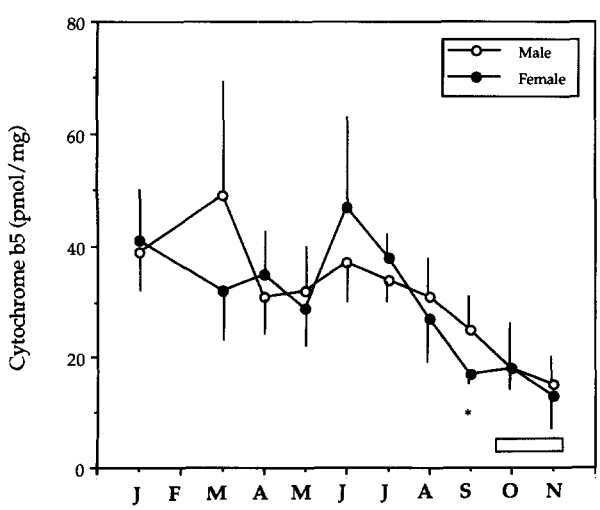

Fig. 7. Changes in the levels of hepatic cytochrome b_5 (nmol/mg microsomal protein) in Atlantic salmon during an annual cycle. *females significantly different from males ($p<0.05$).

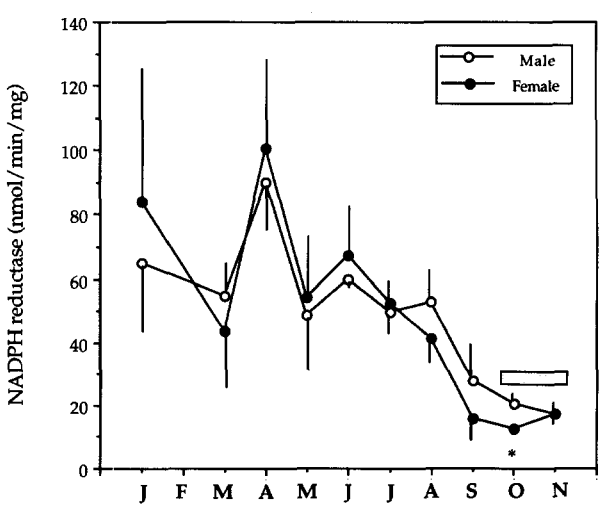

Fig. 8. Changes in NADPH-cytochrome P450 reductase activity in Atlantic salmon liver microsomes during an annual cycle. *females significantly different from males ($p<0.05$).

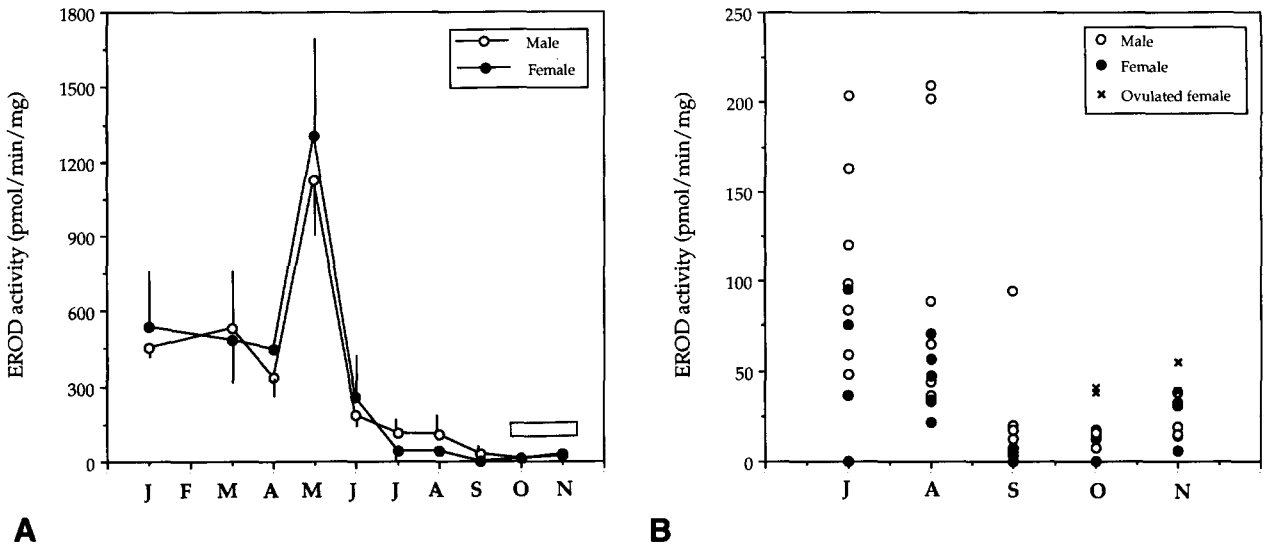

Fig. 9. Changes in 7-ethoxyresorufin O-deethylase (EROD) activity in Atlantic salmon liver microsomes during an annual reproductive cycle (A). Changes occurring during the latter part of the season, presented as individual values, with males, females, and ovulated females symbolized as indicated (B).

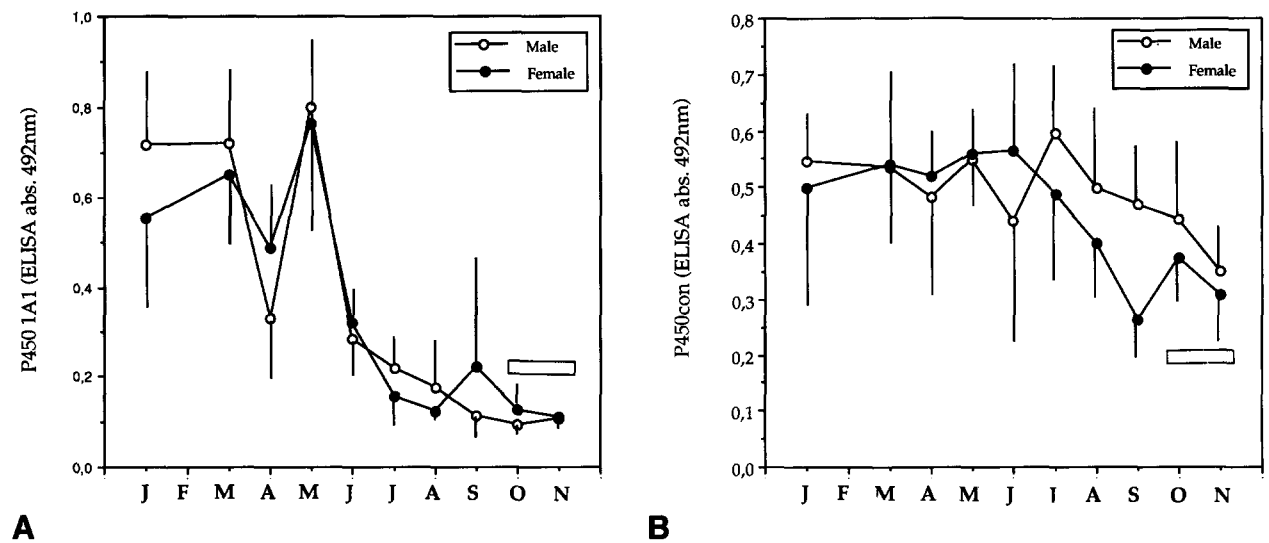

Fig. 10. Changes in the levels of two P450 isozymes, determined immunochemically as indirect ELISA absorbance values, in Atlantic salmon liver microsomes during an annual reproductive cycle: (A) P450 1A1, and (B) P450con. *females significantly different from males ($p<0.05$).

tion limit) (Fig. 9B).

The level of P450 1A1 determined by ELISA was high during the winter season, dropped somewhat in April before peaking in May and decreasing towards spawning, but no significant sex differences were detectable (Fig. 10A). The level of a constitutive P450 form, the P450con cross-reacting protein (CYP2), followed a different pattern with a smoother decrease towards spawning. Significant sex differences were observed in September due to a drop in female levels (Fig. 10B). Both immunochemically determined isozymes varied significantly with sampling time according to ANOVA ($p<0.05$).

In females, plasma levels of the sex steroid estradiol-17β were low and relatively stable from January to July, but started to increase in August and peaked in September-October (Fig. 11). Corre-

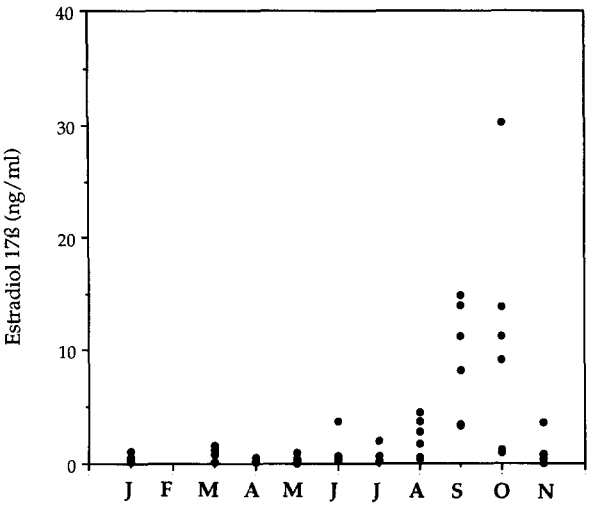

Fig. 11. Changes in estradiol-17β levels in plasma of female Atlantic salmon during an annual reproductive cycle, presented as individual values.

lation analysis revealed that there was a significant correlation between EROD and the inverse of estradiol-17β levels (log-transformed), with a correlation coefficient of 0.58 ($p<0.0001$).

Discussion

This study attempts to delineate the changes occurring in the drug and steroid metabolizing cytochrome P450 system in the liver of Atlantic salmon during sexual maturation and first-time spawning. The parameters studied include both components of the microsomal electron transport system (NADPH-cytochrome P450 reductase, cytochrome b_5, and total cytochrome P450), and P450 isozyme patterns using model substrates for catalytic activities and specific antibodies against fish P450 forms from two different families. This is the first report of seasonal changes in P450 isozymes using immunochemical probes in any fish species. In addition, plasma estradiol-17β levels were measured in females to evaluate the reproductive status of individual fish. The results are generally in accordance with the concept that changes in the cytochrome P450 system occur during sexual maturation in fish, and that these in part are sex-dependent (Förlin and Haux 1990; Pajor *et al.* 1990). In addition, the data presented give new information about the molecular changes occurring under natural temperature and photoperiod conditions in an important salmonid species that has not been studied in any detail previously.

In the group of Atlantic salmon followed here, sexual maturation is initiated in May, when GSI levels start to rise. The surge of vitellogenesis appears in July, with elevated LSI levels in females due to increased synthesis in the liver of vitellogenin (reviewed by Mommsen and Walsh 1988) and eggshell proteins, as recently shown by Oppen-Berntsen *et al.* (1991a). From July onwards, gonadal growth accelerates, plasma estradiol-17β levels start to rise, and other gonadal hormones, such as gonadotropin (GtH) I, as well as eggshell proteins, become abundant in the plasma of these fish (Oppen-Berntsen *et al.*, unpublished data). On the basis of these observations, it seems reasonable to divide this discussion into two parts: the first part considering the events taking place prior to LSI level rise in July (December to June), the second part focusing on the period July to November when final maturation and spawning occur.

During this first period, both total P450 and cytochrome b_5 remain at stable levels with no differences between the sexes. NADPH-cytochrome P450 reductase activities fluctuate somewhat, but also with no sex differences. EROD activity, catalyzed by P450 1A1 (Goksøyr *et al.* 1991b), is stable through April, but at the May sampling point a peak in activity appears, followed by decreasing levels through the summer. The May samples were reassayed several times, both alone and in parallel with other sampling points, always confirming the pattern shown here. To further substantiate this finding, we followed a new group the year after, again observing a peak in EROD activity in May (results not shown). This pattern of P450 1A1 activity is partly reflected in the P450 1A1 protein levels, as assessed by ELISA measurements. However, when studied at this level, it can be seen that a drop in P450 1A1 protein takes place in April (most pronounced in males), before the peak in May, and in parallel with a peak in NADPH-reductase activities.

The levels of another P450 isozyme, the P450con cross-reacting protein, seem stable, although with large individual variations, through June. This isozyme initially characterized by Celander *et al.* (1989) is the major constitutive isoform in juvenile rainbow trout liver. P450con was slightly induced

by cortisol and pregnenolone-16α-carbonitrile in this species, which implies that it might be a member of the CYP3A subfamily. On the other hand, antibodies raised against rainbow trout P450con recognized a protein band in rat liver that is constitutively expressed and that seems to be slightly induced by starvation or acetone, and more by diabetes. In addition to important sex differences in mature rainbow trout these findings indicate that P450con belongs to the CYP2 family. Besides, the molecular weight of P450con and localization in rainbow trout kidney (data not shown) indicate that our P450con is similar or identical to P450LMC2 characterized by Miranda *et al.* (1990) which is immunochemically related to rat CYP 2B1. However, further work is needed to specify which gene subfamily P450con belongs to.

In this first period then, the major event occurring at the physiological level is the initiation of gonadal development in May. At the biochemical level, a peak in P450 1A1 protein and catalytic activity (EROD) is observed at the same sampling point. Whether these two events (and/or the drop in P450 1A1 protein and peak in NADPH-reductase observed in April) are related in one way or another, we do not know at the moment, but it is known that P450 isozymes participate in steroid and fatty acid metabolism, opening for possible signal transduction mechanisms at the biochemical level (the physiological substrate of P450 1A1 has yet to be identified). Another possible explanation for the surge in P450 1A1 activity could be to relate it to a concurring rise in environmental temperature, which has been shown to influence P450 activities (Snegaroff and Bach 1990). This alternative seems difficult to accept, since the increase was only 2°C (5-7°C from April to May), and the temperature continued to rise to 13-15°C in June-July (Fig. 1), without any similar effect. However, the influence of temperature and photoperiod on this enzyme system under natural conditions is still not sufficiently characterized. A third alternative is the possibility of environmental pollutants in the area at this time, causing induction of P450 1A1 (Goksøyr *et al.* 1991a). The treatment with antibiotics in March should have had its effects, if any, already in the April sampling (April 12). We have not been able to locate any other events that could have led to this sort of effect in the area, and so the cause of the P450 1A1 peak in May remains to be elucidated. The finding of a similar peak the subsequent year does, however, imply that the contribution of environmental pollution can be eliminated.

In the second period of this study, from July to November, several changes occur, and a certain sexual differentiation becomes apparent. Female LSI levels are significantly elevated compared to males, and GSI levels rise exponentially from September, coinciding with a peak in liver microsomal protein. Through this period there is a decrease in total P450 levels, especially in females, with significant differences between sexes occurring in September. Similar decreases are observed in cytochrome b_5 levels and in NADPH cytochrome P450 reductase activities. Here, significant differences between sexes are observed in September and October, respectively. EROD activity in July is already much lower than in winter, but activities continue to drop to undetectable levels in female pre-spawning fish. In October and November a couple of females had ovulated eggs in the ovary, and these fish had regained measurable EROD activity. P450 1A1 protein levels determined by ELISA indicate that the protein is present, also in the samples with no EROD activity (no difference in absorbance values). In contrast, the P450con counterparts display significantly lower levels in (pre-spawning) females, especially at the September sampling point.

In the second part of the season, although there is a gradual decrease in activities, several events seem to take place concurrently in pre-spawning females. In September, both cytochromes P450 (total) and b_5, and the P450con form, are significantly reduced in females. In addition, NADPH-cytochrome P450 reductase is lower (significant only in October), and EROD activity is virtually absent in these females. At the same time, plasma estradiol-17β reach peak values.

Apparently, the transient disappearance of EROD activity may be due to the presence of an endogenous inhibitor of enzyme activity, and not a suppression of protein synthesis. The level of the single steroid hormone studied in the plasma of these females, estradiol-17β, peaks in pre-spawning (low-EROD) fish, whereas in females with ovulated eggs values have returned to basal levels. There is a significant inverse correlation between plasma

estradiol-17β levels and EROD activities in all the female fish (log-transformed data, r=0.58, p<0.0001). This raises the possibility that estradiol-17β itself is the inhibitor of EROD activity. *In vitro* studies have indeed shown that estradiol-17β and other steroids, such as testosterone and androstenedione, are able to inhibit EROD activity in salmon liver microsomes in the μM range (Goksøyr, unpublished results), and preliminary examinations have shown estradiol (as well as testosterone) to be present in the microsomal preparations from livers of mature fish (not shown). Also, to fulfill its function as vitellogenin and eggshell protein inducing factor (Mommsen and Walsh 1988; Oppen-Berntsen *et al.* 1991a), estradiol-17β must be able to enter the hepatocyte and bind to its intracellular receptor. These results all indicate that estradiol-17β, or other steroids, can affect the catalytic activity of P450 isozymes directly. Such *in situ* regulation of enzyme activity may be of critical importance in the signal transduction processes regulating maturation and spawning.

Alternatively, the presence of immunoreactive P450 1A1 protein in the absence of EROD activity in pre-spawning females may be explained by degradation products or other cross-reacting proteins in these samples.

In addition to a possible direct effect, several studies have shown estradiol-17β and other steroids to be involved in the regulation of gene expression of components in the P450 system. In a recent study with brook trout (*Salvelinus fontinalis*), Pajor *et al.* (1990) demonstrated that estradiol-17β and testosterone treatment resulted in feminization of immature fish, with reduced levels of cytochromes P450 (total) and b_5, NADPH-cytochrome P450 and NADH-cytochrome b_5 reductase activities, and several forms of P450 isozymes. Similar results were observed with estradiol-17β, but not with testosterone, in a previous study with brook trout (Stegeman *et al.* 1982), and with estradiol-17β in rainbow trout (Hansson and Gustafsson 1981). In studies of seasonal variations in the cytochrome P450 system of fish, the general pattern is indeed down-regulation of activities during sexual maturation with females consistently attaining the lowest levels (Stegeman and Chevion 1980; Koivusaari *et al.* 1981; Walton *et al.* 1983; Tarlebø *et al.* 1985; Edwards *et al.* 1988; Förlin and Haux 1990), as in the present study. Williams *et al.* (1986) discovered that one form of P450 in rainbow trout called P450 LM2 was specifically expressed in the male kidney, along with several monooxygenase activities. Based on immunochemical cross-reactivity, this form has recently been assigned to the CYP2 family (Miranda *et al.* 1990), possibly being identical to the P450con studied here (discussed above). However, the differences observed in the sexual regulation of the two forms in rainbow trout and Atlantic salmon, respectively, may suggest that this is not the case.

The different patterns observed between the two P450 forms can also be presented by comparing their proportions of total P450 during the annual cycle. When each of the forms are correlated with total P450 in individual samples, it becomes apparent that P450con is much better correlated with total P450 than with P450 1A1 (r=0.58 vs. 0.46). In both cases the correlation is significant at p<0.0001. This points at independent regulation of the forms, indicating different roles in the reproductive cycle.

The present study confirms the pattern of sexual regulation of cytochrome P450 activities in maturing fish, and lends further support to the evidence in favour of estradiol-17β as a major regulating factor in the events occurring late in the spawning cycle. However, this study also indicates that there may be events of equal importance in the early stages of gonadal maturation (from May onwards) that are independent of estradiol-17β, and that may involve the P450 system. In addition, several aspects of the P450 system of Atlantic salmon are shown to differ from those of other fishes. Among these are the much higher activities observed during winter, as discussed in our first report of these studies (Goksøyr and Larsen 1991). Furthermore, the simultaneous decrease in activities in both male and female fish during maturation is different from that observed in other seasonal studies with fish.

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