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Relationship Between Seasonal Plasma Estradiol-17β and Testosterone Levels and In Vitro Production by Ovarian Follicles of Amago Salmon (Oncorhynchus rhodurus)

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ABSTRACT

Plasma estradiol-17β and testosterone levels were assessed by radioimmunoassay during the sexual maturation of female amago salmon (*Oncorhynchus rhodurus*). Estradiol-17β levels gradually increased during vitellogenesis (June to September), reached a peak in September (about 16 ng/ml) and rapidly decreased in mature and ovulated fish (about 3–4 ng/ml) in October. The seasonal pattern of plasma testosterone levels lagged behind and followed that of estradiol-17β during vitellogenesis, but levels remained high in mature and ovulated fish (90–110 ng/ml). Estradiol-17β levels and the gonadosomatic index (GSI) values correlated well during vitellogenesis: GSI values showed a linear increase, and reached a peak (29.9 ± 1.4) in October. Values were extremely low in ovulated fish (1.2 ± 0.2).

In vitro production of estradiol-17β and testosterone by ovarian follicles in response to partially purified chinook salmon gonadotropin (SG-G100) was examined monthly using 18-h incubations. Throughout the vitellogenic period SG-G100 stimulated both estradiol-17β and testosterone production: the steroidogenic response of follicles increased from June (about 2 ng/ml estradiol-17β; 0.1 ng/ml testosterone) to September (about 10 and 14 ng/ml, respectively). In October full-grown immature follicles which could be induced to mature in vitro by hormone treatment produced large amounts of testosterone (about 130 ng/ml) but not estradiol-17β. Postovulatory follicles also produced testosterone but the values were low (10 ng/ml) compared with full-grown immature follicles. Very low levels of estradiol-17β were produced by postovulatory follicles.

These results are discussed in relation to other studies on steroidogenesis in the amago salmon, with particular emphasis on the physiological role of testosterone in reproduction in female teleosts.

INTRODUCTION

Estradiol-17β has been identified in the plasma of many female teleost fishes (see Lance and Callard, 1978) and its role in the synthesis and secretion of hepatic vitellogenic protein is well established (see van Bohemen and Lambert, 1981). We reported that teleost gonadotropin directly stimulated estradiol-17β production by early vitellogenic ovarian follicles of amago salmon, *Oncorhynchus rhodurus*, in vitro (Kagawa et al., 1982a; Young et al., 1982b). Furthermore, in the same species, a "two-cell-type model" for the production of follicular estradiol-17β has been proposed, the thecal layer contributing to estradiol-17β production by synthesizing aromatizable androgens which are metabolized in the granulosa layer to estradiol-17β (Nagahama et al., 1982; Kagawa et al., 1982b; Young et al., 1982c).

High levels of testosterone are produced by the isolated thecal layer of amago salmon. This finding, combined with other observations, suggested that testosterone may be the major aromatizable androgen produced by follicles of amago salmon in vitro (Kagawa et al., 1982b). Although testosterone has also been identified in the ovarian tissue and the plasma of a number of other species (see Lance and Callard, 1978; Schmidt and Idler, 1962; Katz and Eckstein, 1974; Schreck and Hopwood, 1974; Campbell et al., 1976, 1980; Wingfield and Grimm, 1977), the functional significance of this steroid in female fish is unknown, apart from its postulated role as one of the major aromatizable androgens of amago salmon.

The present study was conducted to obtain fundamental data on the relationship between seasonal plasma estradiol-17β and testosterone levels and in vitro production by the ovarian follicles of amago salmon, as a basis for further studies on the mechanisms controlling steroidogenesis in this species.

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MATERIALS AND METHODS

Amago salmon were taken from the breeding stock of the Gifu Prefectural Fisheries Experimental Station, Japan. Plasma samples were taken every month (twice in October) from the caudal veins of at least 5 females from June to October 1980. The ovaries were dissected out, and the gonadosomatic index (GSI) calculated as follows:

$$GSI = \frac{\text{total gonad weight}}{\text{body weight}} \times 100.$$

Oocytes surrounded by intact follicle layers (intact follicles) from the ovary of a randomly selected female were then isolated by dissection. In September and October "squeezed follicles" (yolk expelled from intact follicles) were used, because by this time oocytes were too large to incubate in groups of 10 in 1 ml Ringer's. Postovulatory follicles were also obtained in October. Groups of 10 follicles were incubated in 1 ml trout Ringer's in the presence or absence of partially purified chinook salmon gonadotropin (SG-G100; 0.01, 0.1 and 1 μg/ml; see Donaldson et al., 1972) for 18 h at 15°C. Three replicate incubations were made for each concentration of SG-G100 and the hormone-free Ringer's control. Full details of the incubation procedure are given in Kagawa et al. (1982a).

Radioimmunoassay

Estradiol-17β and testosterone concentrations in plasma were measured by radioimmunoassay according to the method of Kagawa et al. (1981, 1982b). Data on the validation of the assay of plasma have been published previously (Kagawa et al., 1982b). Rabbit anti-estradiol-17β-6-(O-carboxymethyl)-oxime-bovine serum albumin (BSA) and anti-testosterone-11-hemisuccinate-BSA serum were obtained from Teikokuzoki Pharmaceutical Co., Tokyo. The anti-estradiol-17β serum cross-reacts with estradiol-17β, estradiol-17α, estrone, estriol and testosterone at 100, 0.80, 3.20, 1.77 and 0.29% levels, respectively (determined by Dr. Kambegawa, Teikyo University, Tokyo). The anti-testosterone serum cross-reacts with testosterone, androstenedione, dehydroepiandrosterone, progesterone and estradiol-17β at 100, 2.24, 0.017, 0.065 and <0.009% levels, respectively (determined by Dr. Kambegawa). [2,4,6,7-^{3}H] Estradiol-17β and [1,2,6,7-^{3}H] testosterone (New England Nuclear) were used as antigens. In the present study, the assays were validated for incubation media. Media of 10 different incubates with different levels of estradiol-17β and testosterone were used for comparison of concentrations of each steroid before and after chromatography on Sephadex LH-20 columns. The correlation coefficient of estradiol-17β and testosterone between two methods of analysis was r=0.99 and 0.95, respectively. Thus, the data in this study were obtained by radioimmunoassay without chromatography. Both estradiol-17β and testosterone standards of 20 pg/ml could be reliably distinguished from the buffer blank, but for practical purposes medium aliquots reading less than 30 pg/ml were considered nondetectable. To test the accuracy of the assays, known weights of estradiol-17β or testosterone (200, 400, 800, 1600 pg) were added to 1 ml media after incubation of follicular tissues with SG-G100. The correlation coefficient between the observed and expected values was r=0.99 (P<0.001) for estradiol-17β and r=0.97 (P<0.001) for testosterone. The amounts of estradiol-17β and testosterone were thus very closely related with the added steroid.

Statistical Analyses

All data are expressed as mean ± SEM. Data were statistically analyzed by analysis of variance followed by Student-Newman-Keul's multiple range test.

RESULTS

Ovarian Maturity

To evaluate the state of maturity of the ovary, the gonadosomatic index (GSI) and the average diameter of oocytes was calculated every month during the experimental period; oocyte diameter data were obtained from 10–20 oocytes of each female (Fig. 1). Vitellogenesis had already started in June fish. Oocytes were about 1.5 mm in diameter and contained small amounts of yolk globules (GSI: 1.0 ± 0.1). Thereafter GSI values and oocyte diameter showed a linear increase and reached a peak (GSI: 29.9 ± 1.4, oocyte diameter: 5.3 ± 0.5 mm) on October 24. The ovaries from fish collected on October 9 contained numerous large oocytes, ranging in diameter from 4.5 to 5.0 mm with abundant yolk globules (GSI: 23.3 ± 1.3). The ovaries of some of the fish collected on October 24 contained mature oocytes, 5.0–5.5 mm in diameter, in which the germinal vesicle was absent and the ooplasm was translucent. After ovulation, the GSI showed a rapid decrease to 1.2 ± 0.2. The ovaries after ovulation were occupied by flattened and opaquely white oval postovulatory follicles.

Changes in Plasma Estradiol-17β and Testosterone Levels

Estradiol-17β levels in plasma (Fig. 2) gradually increased from June 20 (3.0 ± 0.6 ng/ml) to August 27 (7.8 ± 1.2 ng/ml), reached a peak of 16.4 ± 1.3 ng/ml on September 26 (P<0.01) and rapidly fell to 4.1 ± 0.8 ng/ml in mature fish and 3.0 ± 0.3 ng/ml in ovulated fish (P<0.01).

Testosterone levels (Fig. 3) reached a peak of 56.0 ± 8.6 ng/ml on September 26 (P<0.01) and, in contrast to estradiol-17β, remained at high levels in mature and ovulated fish. Testosterone levels were much higher than those of estradiol-17β throughout the study period.

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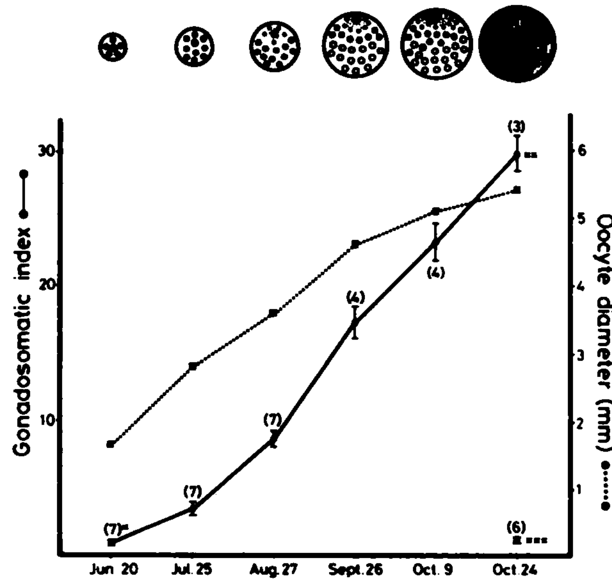

FIG. 1. Changes in the gonadosomatic index (*solid line*) and oocyte diameter (*dotted line*) during sexual maturation of female amago salmon (*Oncorbyncbus rbodurus*). Each value represents the mean ± SEM of the indicated number of samples. *=Number of fish examined. **=Samples from fish with mature oocytes. ***= Samples from fish containing ovulated oocytes. The relative increase in oocyte size is shown drawn to scale at the top of the figure. Small *open circles* represent yolk globules; the *closed circle* in each oocyte represents the position of the germinal vesicle. Shading represents the massed yolk present after germinal vesicle breakdown.

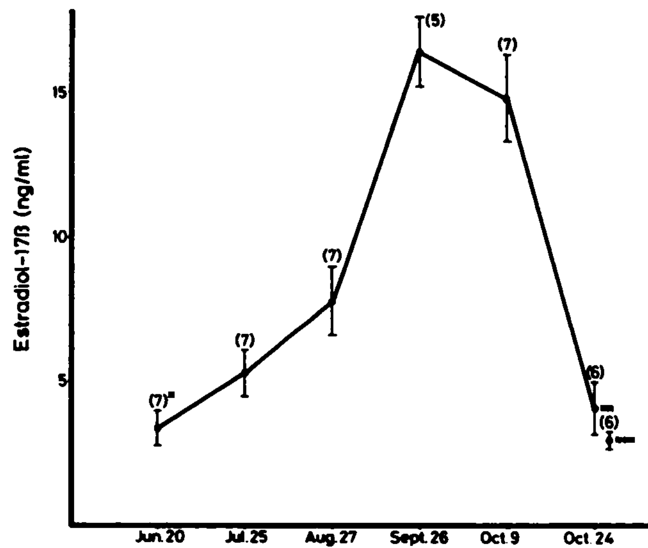

FIG. 2. Changes in plasma estradiol-17β levels during sexual maturation of female amago salmon (*Oncorbyncbus rbodurus*). Each value represents the mean ± SEM of the indicated number of samples. *=Number of fish examined. **=Plasma samples from fish with mature oocytes. ***=Plasma samples from fish containing ovulated oocytes.

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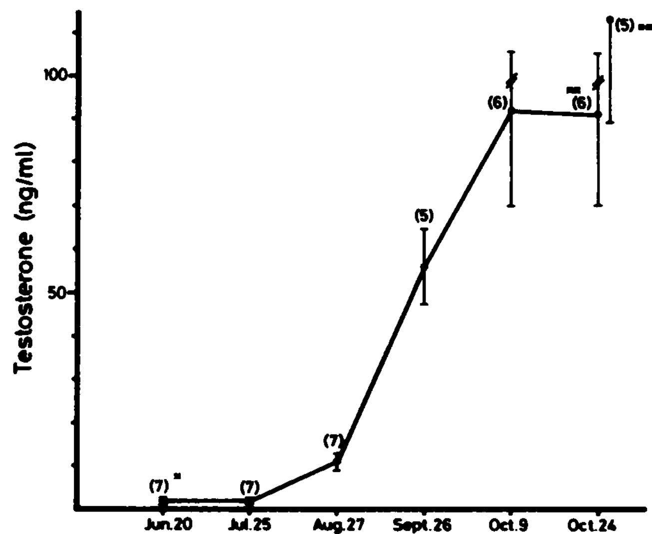

FIG. 3. Changes in plasma testosterone levels during sexual maturation of female amago salmon (*Oncorhynchus rhodurus*). Each value represents the mean ± SEM of the indicated number of samples. *=Number of fish examined. **=Plasma samples from fish with mature oocytes. ***=Plasma samples from fish containing ovulated oocytes.

Changes in In Vitro Estradiol-17β and Testosterone Production

Changes in in vitro estradiol-17β and testosterone production are shown in Figs. 4 and 5, respectively. Experiments were performed in 1980 and 1981, and since the results obtained were essentially the same, the present study described the data from 1980. In the June, July, and August experiments, SG-G100 (0.1 and 1 μg/ml) stimulated estradiol-17β production by intact follicles ($P<0.01$). In the September experiment, only 1 μg/ml of SG-G100 stimulated estradiol-17β production by squeezed follicles ($P<0.01$). Values of estradiol-17β in media (1 μg/ml SG-G100 groups) increased from June (1.8 ± 0.1 ng/ml) to August (3.0 ± 0.03 ng/ml) and reached a peak in September (9.9 ± 1.0 ng/ml). Basal (Ringer's) control values also increased from June (about 0.1 ng/ml) to September (4.0 ± 0.2 ng/ml). However, in the October experiment, using full-grown immature follicles which could be induced to mature with 1 μg/ml SG-G100, estradiol-17β production was not stimulated by any concentration of SG-G100 ($P>0.05$). Basal control values were still high (3.8 ± 0.1 ng/ml), and comparable to September levels. A small amount of estradiol-17β (below 0.1 ng/ml) was produced by postovulatory follicles with every concentration of SG-G100.

In the June and July experiments, SG-G100 (1 μg/ml) slightly but significantly stimulated testosterone production ($P<0.05$). In the August experiment, testosterone production by intact follicles was significantly ($P<0.01$) stimulated by SG-G100 at a concentration of 0.1 and 1 μg/ml. In September, only 1 μg/ml SG-G100 enhanced testosterone production by squeezed follicles ($P<0.01$), and in October, SG-G100 stimulated testosterone production by squeezed immature follicles at concentrations of 0.1 and 1 μg/ml ($P<0.01$). Values of testosterone in media (1 μg/ml SG-G100 groups) gradually increased from June (about 0.1 ng/ml) to September (14.0 ± 1.8 ng/ml) and then markedly increased in October (129.9 ± 12.6 ng/ml). Basal (Ringer's) control values also increased from June (below 0.1 ng/ml) to October (2.0 ± 0.1 ng/ml). In the postovulatory follicle, only 0.1 μg/ml SG-G100 enhanced testosterone production ($P<0.01$), but the value was low (19.3 ± 4.4 ng/ml) compared to levels produced by immature follicles in October.

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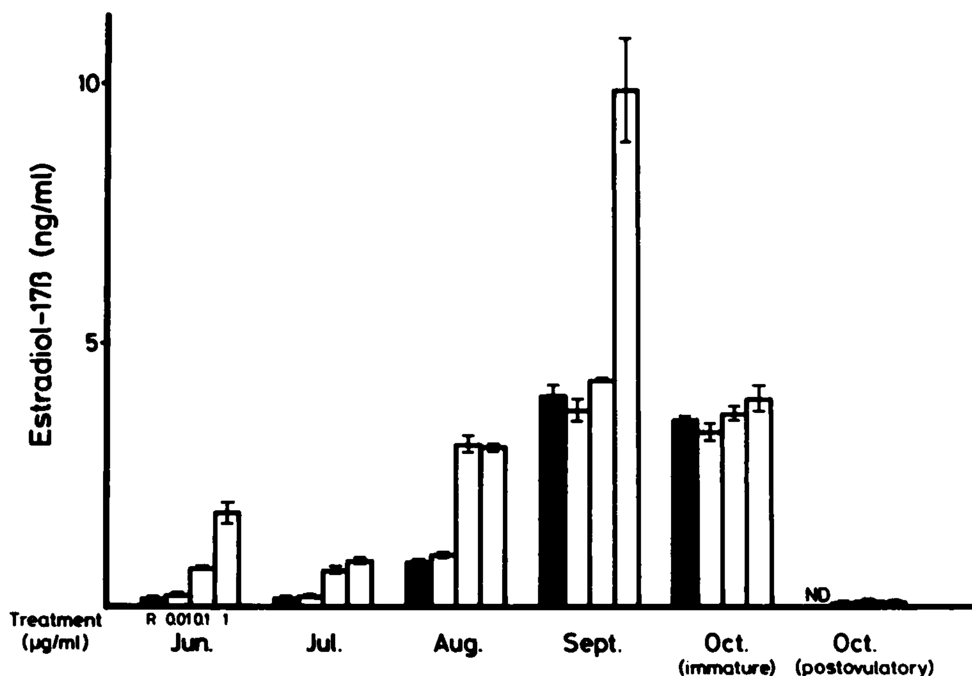

FIG. 4. In vitro production of estradiol-17β by ovarian follicles of amago salmon (*Oncorhynchus rhodurus*) at different stages of sexual maturation. Follicles were incubated in Ringer's alone (*R*) or with chinook salmon gonadotropin (SG-G100) at concentrations of 0.01, 0.1 or 1 µg/ml for 18 h at 15°C. Each *vertical bar* represents the mean ± SEM of three replicate incubations. In October, production was examined in two different groups of follicles; full-grown immature follicles (*immature*) which could be induced to mature in vitro by hormone treatment and follicles 1–2 days after ovulation of oocytes (*postovulatory*).

DISCUSSION

The amago salmon, like other anadromous salmon (see de Vlaming, 1982), has an ovary of the total synchronism type; their oocytes develop synchronously and they spawn once and then die. GSI values climbed during vitellogenesis and were very low after ovulation; oocytes increased in size during vitellogenesis to reach about 4.5–5 mm in diameter in October, just prior to final maturation. GSI values are probably a reliable indicator of the degree of ovarian development in species of this type.

Values for plasma estradiol-17β found in the amago salmon are of the same order as those reported for other salmonids (Billard et al., 1978; Crim and Idler, 1978; Fostier et al., 1978; Kagawa et al., 1981; van Bohemen and Lambert, 1981). In the present study, there was a good correlation between plasma estradiol-17β levels and GSI values during the vitellogenic period, with indirect evidence implicating estradiol-17β in the control of the synthesis of vitellogenic proteins (see van Bohemen and Lambert, 1981). Similarly, a peak in plasma estradiol-17β levels associated with the synthesis of vitellogenic proteins has been described in several teleosts (Schreck et al., 1973; Wingfield and Grimm, 1977; Crim and Idler, 1978; Lambert et al., 1978; Kagawa et al., 1981; van Bohemen and Lambert, 1981).

Plasma testosterone levels gradually increased during vitellogenesis and reached a peak before maturation in the present study. Maximal levels were maintained in mature and ovulated fish. Seasonal changes in plasma testosterone levels similar to those in the amago salmon have been reported in plaice (Wingfield and Grimm, 1977) and goldfish (Schreck and Hopwood, 1974). In amago salmon, the seasonal pattern of plasma testosterone lags behind but follows that of estradiol-17β, suggesting a close relationship between these two steroids.

In vitro production of estradiol-17β by teleost ovarian follicles in response to gonadotropin (amago salmon, Kagawa et al., 1982a) or

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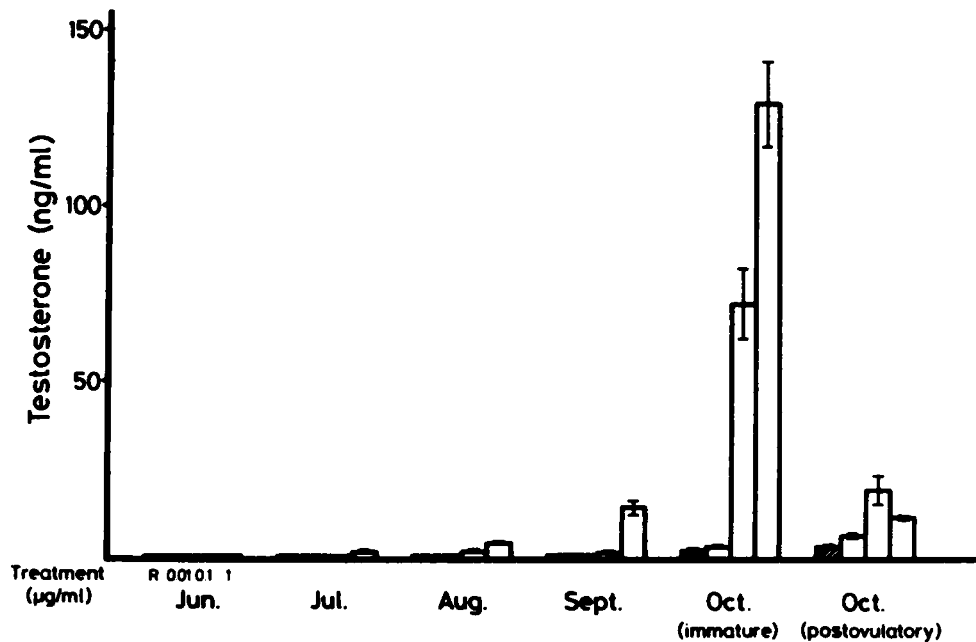

FIG. 5. In vitro production of testosterone by ovarian follicles of amago salmon (*Oncorhynchus rhodurus*) at different stages of sexual maturation. Follicles were incubated in Ringer's alone (*R*) or with chinook salmon gonadotropin (SG-G100) at concentrations of 0.01, 0.1 or 1 µg/ml for 18 h at 15°C. Each *vertical bar* represents the mean ± SEM of three replicate incubations. In October, production was examined in two different groups of follicles, full-grown immature follicles (*immature*) which could be induced to mature in vitro by hormone treatment and follicles 1–2 days after ovulation of oocytes (*postovulatory*).

homologous pituitary extracts (plaice, Yaron and Barton, 1980) has been reported previously, and Young et al. (1982b) have demonstrated that stimulation of estradiol-17β production by gonadotropin is dependent on the presence of Δ^5-3β-hydroxysteroid dehydrogenase in the follicle of the amago salmon. In the same species, testosterone is secreted by ovarian follicles in response to gonadotropin (Kagawa et al., 1982b). Follicles obtained each month during the vitellogenic period (June to September) produced both estradiol-17β and testosterone when incubated with gonadotropin. The follicular capacity to produce these steroids in terms of responsiveness to SG-G100 gradually increased during vitellogenesis and showed a strong correlation with plasma levels of these steroids. The significance of increasing testosterone levels in the plasma and increased production in vitro by follicles in the course of vitellogenic growth probably results from the hypothesized function of the thecal layer to produce aromatizable androgens which are metabolized to estradiol-17β in the granulosa cells (Kagawa et al., 1982b; Young et al., 1982c).

During the period of final oocyte maturation (October), levels of plasma estradiol-17β were very low in mature and ovulated amago salmon, a finding similar to those reported in other salmonids (Fostier et al., 1978; Kagawa et al., 1981; van Bohemen and Lambert, 1981). This decrease in plasma estradiol-17β in amago salmon is in good agreement with the data on in vitro estradiol-17β production; at this stage estradiol-17β production was not stimulated by SG-G100. van Bohemen and Lambert (1981) have similarly reported a decreased estrogen synthesis in the ovaries of rainbow trout after vitellogenesis. The loss of the ability of the follicle to produce estradiol-17β seems largely due to the loss of aromatase activity in the granulosa cell (Young et al., 1983b), since maximal testosterone production was observed at this time. The drop in plasma estradiol-17β may be related to the increase of gonadotropin

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during final oocyte maturation (Fostier et al., 1978).

In mature and ovulated amago salmon, plasma testosterone was still maintained at high levels, while the concentration of estradiol-17β was low. High concentrations of plasma testosterone during the spawning period have been reported in female salmonids (Schmidt and Idler, 1962; Campbell et al., 1980) and plaice (Campbell et al., 1976; Wingfield and Grimm, 1977). Moreover, plasma testosterone levels in mature or ovulated female amago salmon (about 100 ng/ml) were much higher than those of precocious males during the spawning season (less than 15 ng/ml) (Ueda et al., 1983). Similarly, high plasma concentrations of testosterone in females have been reported in other species (Schmidt and Idler, 1962; Campbell et al., 1976, 1980). Testosterone may not be the major testicular steroid secreted by male teleosts, since the concentration of plasma 11-ketotestosterone is much higher than testosterone in several salmonids (Schmidt and Idler, 1962; Idler et al., 1971; Campbell et al., 1980; Hunt et al., 1982). Thin-layer chromatography experiments have failed to identify 11-ketotestosterone as a metabolite produced by amago salmon ovarian tissue (Ueda and Nagahama, unpublished data).

The maximally high plasma testosterone levels in mature and ovulated females are in good agreement with the enhanced capacity of preovulatory follicles to produce this steroid in October. The apparent observed increase in the capacity of the follicle to produce testosterone could conceivably be related to decreased aromatase activity (Young et al., 1983b). However, a true increase in testosterone production is indicated by the observation that isolated thecal layers (from the same ovary used in the present study) incubated in vitro with SG-G100 produce maximal amounts of testosterone in October (Kagawa, 1982). Thecal layers cannot produce estradiol-17β; their major products are testosterone and 17α-hydroxyprogesterone (Kagawa, 1982; Kagawa et al., 1982b).

A major question raised by this study is the functional significance of enhanced follicular testosterone production and elevated plasma testosterone levels during the period of final oocyte maturation. Although in teleosts testosterone is effective in inducing in vitro final oocyte maturation only at very high concentrations (Hirose, 1972; Jalabert, 1976; Goetz and Bergman, 1978; Iwamatsu, 1978; Iwamatsu and Katoh, 1978; Young et al., 1982a), it has been reported to enhance the effectiveness of gonadotropin on the induction of final oocyte maturation in rainbow trout (Jalabert, 1976) and amago salmon (Young et al., 1982a). This suggests that elevated plasma testosterone levels during the period of oocyte maturation in part may be directly associated with the induction of maturation.

Estradiol-17β and testosterone may have important regulatory influences on steroidogenesis in the teleost ovarian follicle, as in mammals (Leung and Armstrong, 1980), although data is lacking. Recent studies on steroid metabolism in the brain have revealed that aromatizable androgens, particularly testosterone, may elevate pituitary gonadotropin levels in sexually immature salmon (Crim and Peter, 1978; Crim and Evans, 1979; Crim et al., 1981). Whether these findings are applicable to sexually mature fish remains to be clarified. In the scalpin, aromatase activity in the anterior hypothalamus, an area implicated in the regulation of gonadotropin secretion (Peter and Crim, 1979), is highest during the period of oocyte maturation and spawning (Callard et al., 1981a). Aromatase activity has also been detected in other areas of the brain which have been implicated in the control of sexual behavior or spawning (Callard et al., 1981a). High plasma testosterone levels during the maturation and spawning period of amago salmon may be related to an accumulation of gonadotropin in the pituitary prior to the rapid elevation of plasma gonadotropin levels which occurs at the time of oocyte maturation and ovulation (Young et al., 1983a), and to spawning behavior. It should also be noted that the sculpin pituitary also shows a high aromatase activity (Callard et al., 1981b).

Postovulatory follicles of amago salmon, like full-grown preovulatory follicles, do not produce estradiol-17β in response to SG-G100 (see Nagahama and Kagawa, 1982). These results are in agreement with the relatively low levels of plasma estradiol-17β after oocyte maturation and ovulation in this species, and also in the white-spotted char (Kagawa et al., 1981). Although SG-G100 stimulated testosterone production by the postovulatory follicle, levels were very low (18 ng/ml) compared with those of the full-grown preovulatory follicle (130 ng/ml). This result suggests a decreased activity in one or both of the thecal layer enzymes involved in the conversion of 17α-hydroxy-

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progesterone to testosterone ($C_{17}-C_{20}$ lyase and 17β-hydroxysteroid dehydrogenase), since SG-G100 stimulated the production of high levels of 17α-hydroxyprogesterone by the same postovulatory follicles (Kagawa, 1982).

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