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Steroidogenesis in rainbow trout (*Salmo gairdneri*) at various preovulatory stages: changes in plasma hormone levels and *in vivo* and *in vitro* responses of the ovary to salmon gonadotropin

Alexis Fostier and Bernard Jalabert

Laboratoire de Physiologie des Poissons, INRA, Campus de Beaulieu, 35042 Rennes, Cédex, France

Keywords: rainbow trout, meiotic maturation, ovulation, ovarian steroidogenesis, gonadotropin, oestradiol, 17 α -hydroxy-20 β -dihydroprogesterone

Abstract

In order to specify the timing of some changes in ovarian steroid production during the transition from vitellogenesis to ovulation, plasma hormones levels and *in vivo* and *in vitro* responses of the ovary to salmon gonadotropin (s-GtH) or dibutyl-cyclic adenosine mono-phosphate (db-cAMP) were recorded in relationship with the state of germinal vesicle migration in the oocyte. *In vivo*, a small, but significant, increase of plasma 17 α -hydroxy-20 β -dihydroprogesterone (17 α , 20 β -OH-P) level was detected earlier (at the "subperipheral germinal vesicle" stage) than the increase of GtH level (detectable at the "peripheral germinal vesicle" stage) and the decline of oestradiol-17 β (E2-17 β) (also detectable at the "peripheral germinal vesicle" stage). Negative correlations were established between E2-17 β levels and GtH ($\rho = -0,53$) or 17 α ,20 β -OH-P ($\rho = -0,43$) levels while a positive correlation occurred between 17 α ,20 β -OH-P and GtH levels ($\rho = +0,54$). *In vivo* no action of GtH on the decline of E2-17 β levels was detected GtH did not stimulate 17 α ,20 β -OH-P production, within 72h, in females at the "end of vitellogenesis" stage. It had significant effect in females at other stages closer to ovulation, but the pattern of responses changed according to the stage. *In vitro* db-cAMP like GtH was able to stimulate 17 α ,20 β -OH-P output from ovarian follicles. The greatest response was observed at the later stage. (GVBD). Testosterone output was also increased by GtH, but the lowest response was observed at the later stage (GVBD). Androstenedione output was lower than testosterone output. *In vitro*, a small but significant decline of E2-17 β output was induced by GtH. We conclude that substantial changes occur during the very last stages prior to ovulation, both in the steroidogenic potential of the ovary and in the ovarian sensitivity to GtH. 20 β -oxydoreductase is probably progressively induced during GV migration when GtH basal levels are increasing but still relatively low. Without minimizing the role of discrete pulses of GtH on this induction, we could expect synergic actions of other hormones. Thus a high testosterone/oestradiol ratio in the follicle environment favours 17 α ,20 β -OH-P secretion.

Introduction

In the past few years a good deal of interest has centered on the endocrine control of oocyte maturation and ovulation in fish (see review Goetz 1983). Such works help to improve new techniques to induce spawning (Breton *et al.* 1980; Fostier and

Jalabert 1982; Lam 1982; Donaldson and Hunter 1983). Furthermore, the study of the transition from vitellogenesis into meiotic maturation, two stages of ovogenesis under very different specific endocrine control, makes fish an original model for physiological research.

Changes in ovarian steroidogenesis occur during

this transition (see review Fostier *et al.* 1983). The present study was conducted to specify the timing of some of these changes. Plasma hormones levels were measured in relationship with the state of oocyte nucleus (germinal vesicle = GV) migration, which offers us a morphological criterion to follow the ovarian evolution from the end of vitellogenesis until the resumption of meiotic maturation (germinal vesicle breakdown = GVBD). In addition, changes in terms of ovarian steroid production were recorded, *in vivo* or *in vitro*, in responses to pure salmon gonadotropin (s-GtH). The *in vitro* response to dibutyryl-cyclic adenosine monophosphate (db-cAMP) was also tested.

Materials and methods

In vivo experiments

Two year old rainbow trout females, weighing 660 ± 220 g, were kept in a recirculating water system and submitted to natural temperature and photoperiod. Ovulation occurred during december or January. Fish were anesthetized in phenoxyethanol (Merck) and plasma were sampled at 09.00 h, as described by Fostier *et al.* (1978). Before plasma sampling a few oocytes were collected and their stages determined as described by Jalabert and Fostier (1984 a, b); i.e. 'end of vitellogenesis' (EV, stage 1), 'subperipheral germinal vesicle' (SP-GV, stage 2), 'peripheral germinal vesicle' (P-GV stage 3), 'germinal vesicle breakdown' (GVBD, stage 4), 'ovulated oocyte' (OV, stage 5). Ovulation was checked twice times weekly.

Some of the fish were used to study the *in vivo* response of ovarian steroid secretion to s-GtH. For each ovarian stage two groups of 4 to 7 fish were identified by marking them with individual tags (Floy-Tag). One group received $5 \mu\text{g/kg}$ body wt of s-GtH diluted in saline and administered via an intracardiac injection. The other group was used as a control and received only saline (0.5 ml/kg body wt). The fish were bled 3h, 6h, 24h, 48h and 72h after the GtH or saline injection. Plasma samples were stored at -30°C .

In vitro experiment

Four females, three year old, weighing 1900 ± 350 g, were used. They belonged to a 'spring' strain (spawning between 15 January and 15 April). The procedure to collect the ovary, to incubate follicles *in vitro* and to estimate the median efficient dose of GtH for maturation (theoretical dose expected to induce 50% of the maximal responses) are described by Jalabert and Fostier (1984a). However the present work was performed under sterile conditions and no antibiotics were used, since they have been shown to depress steroid production (Fostier and Jalabert 1984). Vials containing 25 follicles per 2 ml of trout balanced salt solution received, in duplicate, either various doses of s-GtH (7, 15, 60, 250 and 1000 ng/ml) or various doses of db-cAMP (0.5, 1, 2, 4 and 8 mM). A control was done with no hormones in four replicates. Two hundred μl of the incubation medium was pipetted at various time after the beginning of incubations (12h, 24h, 48h and 72h). The incubation volume was maintained by adding fresh trout balanced solution. Samples were frozen at -30°C until analysis.

Hormones

The pure salmon (*Oncorhynchus tschawytscha*) gonadotropin (s-GtH) was prepared in the laboratory by Breton (Breton *et al.* 1976). Steroids were purchased from Steraloids (USA) and N^6, O^2 -dibutyryl adenosine-3':5'-cyclic monophosphate sodium salt (db-cAMP) was obtained from Sigma (USA). 5β -Pregnane- $3\beta, 17\alpha, 20\beta$ -triol and 5β -pregnane- $17\alpha, 20\beta$ -diol-3-one, the cross reactivities of which have been tested in our $17\alpha, 20\beta$ -OH-P radioimmunoassay, were kindly given by Professor B.I. Tamaoki (NIRS, Japan).

Steroids measurements

Plasma or incubation media were extracted with cyclohexane: ethyl acetate (50:50) and the organic phases were evaporated under air. The dry residues were subjected to chromatography on Sephadex

LH-20 (Pharmacia) columns ($\emptyset = 0.5$ cm, $h = 8$ cm) with dichloromethane : methanol (95 : 5) as eluant.

17 α -Hydroxy-20 β -dihydroprogesterone (17 α , 20 β -OH-P): The radioimmunoassay was performed as described earlier (Fostier *et al.* 1981a, Jalabert and Fostier 1984b). The main crossreactivities, expressed as the ratio of the mass of 17 α ,20 β -OH-P to the mass of steroid required to decrease bound level of the tracer to half its value without competitor, were with: 20 β -dihydroprogesterone (2%), 5 β -pregnan-17 α , 20 β -diol-3-one (1%), 17 α -hydroxy-20 α -dihydroprogesterone (1%), 5 β -pregnan-3 β ,17 α ,20 β -triol (0.4%); the other steroids tested did not cross-react significantly ($< 0.1\%$) (see Jalabert and Fostier 1984b).

Oestradiol-17 β (E2-17 β): The radioimmunoassay was performed as described by Jalabert and Fostier (1984b). The anti-oestradiol-6-O-carboxymethoxyme-BSA was prepared by Dr Terqui (INRA). The main cross-reactivities were with oestrone (29%), 16-ketooestradiol-17 β (1%), 16 epiestriol (11%). The specificity of the assay is improved by the chromatographic step.

Testosterone (T): The antibody was prepared in rabbit against testosterone-3-carboxymethoxyme-BSA. The main cross-reactivities were with 5 α -dihydrotestosterone (41%), 11-ketotestosterone (31%), androstenedione (14%), 5 α -androstane-3 β ,17 β -diol (2,2%), 5 α -androstane-3 α ,17 β -diol (6,2%). Low cross reactivities occurred with 11 β -hydroxytestosterone, 11 β -hydroxyandrostenedione, androsterone, adrenoesterone, dehydroepiandrosterone ($< 1\%$). Androstenedione, but not 11-ketotestosterone was separated from testosterone on LH-20 columns, however only very low levels of 11-ketotestosterone have been detected in rainbow trout females during oocyte maturation (Campbell *et al.* 1980). The testosterone RIA was performed as for 11-ketotestosterone (Fostier *et al.* 1982).

Androstenedione ($\Delta 4$): the antibody was prepared by Dr. Terqui (INRA) against androstenedione-11 α -succinyl-BSA. The main cross reactivities were with: adrenoesterone (54%), 11 β -hydroxyandrostenedione (15%); low cross-reactivities occurred with testosterone, 11 β -hydroxytestosterone, 11-ketotestosterone, dehydroepiandroste-

Fig. 1. Plasma oestradiol-17 β (E2), gonadotropin (t-GtH) and 17 α -hydroxy-20 β -dihydroprogesterone (17 α ,20 β -OH-P) in rainbow trout at various ovarian stages. 17 α ,20 β -OH-P scale is logarithmic. Numbers of fish used for each measurement are given in the histogram blocks. Stage 1: End of vitellogenesis (EV). Stage 2 = subperipheral germinal vesicle (SP-GV) – Stage 3 = peripheral germinal vesicle (P-GV) – Stage 4 = germinal vesicle breakdown (GVBD) – Stage 5 = ovulated oocytes (OV).

rone ($< 1\%$). The androstenedione RIA was performed as for 11-ketotestosterone (Fostier *et al.*, 1982).

GtH radioimmunoassay was performed as described by Breton and coauthors (1983).

Statistics

Two factors variance analysis and Student's test were used to compare groups of values.

Results

A. Plasma hormones levels in relationship with preovulatory ovarian stages (Fig. 1)

Significant evolutions of plasma E2-17 β and GtH levels were synchronous: a significant decrease of E2-17 β could be detected only from the 'subperipheral germinal vesicle' stage (SP-GV, stage 2) to the 'peripheral germinal vesicle' stage (P-GV, stage 3) then from this last stage to the 'germinal vesicle breakdown' stage (GVBD, stage 4), while GtH increased significantly between the same stages. As far as the whole population was concerned, in-

Fig. 2–6. Plasma changes in GtH, E2-17 β and 17 α ,20 β -OH-P levels ($\bar{X} \pm \text{SE}$) after intracardiac injection of either s-GtH (5 $\mu\text{g}/\text{kg}$ b.w. —) or saline (0.5 ml/kg b.w. - -) in rainbow trouts at various ovarian stages: EV (Fig. 2), SP-GV (Fig. 3), P-GV (Fig. 4), GVBD (Fig. 5), OV (Fig. 6). * or ** = significant ($p < 5\%$ or $p < 1\%$) variations, within a group of fish, during the time of sampling. S* or S** (or ns) = significant difference (or not) between two groups of fish.

dividual values for GtH and E2 were highly significantly and negatively correlated: $\rho = -0.53$ ($n = 64$).

The first significant increase of plasma 17 α ,20 β -OH-P levels was detected from the 'end of vitellogenesis' stage (EV, stage 1) to the SP-GV stage, then an other significant increase, with a higher amplitude, occurred from the P-GV to the GVBD, stage. Fish found with ovulated oocytes (OV, stage 5) had lower 17 α , 20 β -OH-P level. For the whole population, a highly significant and negative correlation was calculated between E2-17 β and 17 α , 20 β -OH-P levels: $\rho = -0.43$ ($n = 64$); while a

Fig. 3. (see legend to Fig. 2)

positive one was found between GtH and 17 α ,20 β -OH-P levels: $p = 0.54$ ($n = 69$).

B. Changes in plasma concentrations of oestradiol-17 β and 17 α -hydroxy-20 β -dihydroprogesterone in response to in vivo intracardiac injection of pure s-GtH, at different periovulatory ovarian stages (Fig. 2–6)

GtH levels reached their maximum within 6h following injection (peak detected at 3h sampling), then came back to the control level within 48h. When all the stages were considered, no effects of GtH were detectable on E2-17 β levels, but a significant increase of 17 α ,20 β -OH-P levels was obtained (variance analysis, $p < 5\%$). No effect of GtH could be shown on the decline of E2-17 β levels at any stage.

Fig. 4. (see legend to Fig. 2)

As far as $17\alpha,20\beta$ -OH-P was concerned, no stimulation by GtH was detected at the EV stage (Fig. 2). For the next two stages (SP-GV and P-GV) a significant increase was induced by GtH, 48h or 72h after injection (Fig. 3–4). The patterns of the responses were different for the two last stages studied (GVBD and OV), since a significant increase occurred within the first 6h following injection, then the progestin levels came back to those of the control, within the next 24h for the GVBD stage, and only within the next 18h for the OV stage (Fig. 5–6).

C. In vitro steroid output from ovarian follicles, at various preovulatory stages, in response to db-cAMP or s-GtH stimulation

The estimated median efficient doses of GtH on *in*

Fig. 5 (see legend to Fig. 2)

vitro maturation were well related with the ovarian stages. Female 1: EV, MED = 114 ng/ml; female 2 SP-GV, MED = 30 ng/ml; female 3: SP-GV, MED = 22 ng/ml; female 4: P-GV, MED = 9 ng/ml.

s-GtH and db-cAMP stimulated $17\alpha,20\beta$ -OH-P output (Fig. 7) and these stimulations were dose-dependent (Fig. 8). Both phenomena were very highly significant ($p < 1\%$). When all the incubations with GtH are considered (5 doses and 4 incubation times) only the $17\alpha,20\beta$ -OH-P production of female 1 (EV) was highly significantly lower than the others. However, kinetics were different (Fig. 7) and at 24h the $17\alpha,20\beta$ -OH-P productions of the four females were very highly significantly different: female 1 < female 2 < female 3 < female 4. After 72h of incubation this hierarchy was maintained only for physiological doses of GtH (less than 250 ng/ml) (Fig. 8).

Fig. 6. (see legend to Fig. 2)

Low doses of db-cAMP gave the same ranking between females (Fig. 7, 8). Maximal secretions appeared higher under GtH than under db-cAMP action.

Testosterone, androstenedione and oestradiol outputs were measured after 72h incubation with various doses of s-GtH (Fig. 9). Testosterone output was increased with increasing doses of GtH. The lowest response occurred with the most advanced stage (female 4, P-GV, $p < 1\%$). A lower, but highly significant ($p < 1\%$), stimulation was observed on androstenedione output in females 1 (EV) and female 4 (P-GV); the ranking between females being: female 1 > females 3 > female 2 > female 4.

Oestradiol output was higher with follicles from female 4 ($p < 1\%$). Besides, a small, but significant ($p < 1\%$), decrease of this output was observed with increasing doses of GtH.

Fig. 7. Kinetics of $17\alpha,20\beta$ -OH-P output by rainbow trout ovarian follicles stimulated *in vitro* by either 2mM db-cAMP or 250 ng/ml s-GtH. Females at various ovarian stages were used: female 1 (EV-MED for s-GtH = 114 ng/ml, ▲▲), female 2 (SP-GV, MED = 30 ng/ml ●●), female 3 (SP-GV, MED = 22 ng/ml, ○○), female 4 (P-GV, MED = 9 ng/ml, △△).

Fig. 8. *In vitro* output of $17\alpha,20\beta$ -OH-P by rainbow trout ovarian follicles stimulated *in vitro* by various doses of either db-cAMP or s-GtH. Incubation duration = 72h. Females at various ovarian stages were used (see legend Fig. 7).

Discussion

Numerous studies have been performed on members of the suborder salmonoides (salmonidae and plecoglossidae: Goetz 1983; Nagahama 1983; Fostier *et al.* 1983), thus our discussion will be mainly restricted to these species. Plasma GtH levels reach their highest values during the peri-

Fig. 9. *In vitro* output of oestradiol-17 β (E2), androstenedione (Δ 4) or testosterone (T) by rainbow trout ovarian follicles stimulated by various doses of s-GtH. Incubation duration = 72h. Females at various ovarian stages were used (see legend Fig. 7).

ovulatory period in pink salmon, *Oncorhynchus tshawytscha* (Crim *et al.* 1973), sockeye salmon, *O. nerka*, brook trout, *Salvelinus fontinalis* (Crim *et al.* 1975), brown trout, *Salmo trutta* (Crim and Idler 1978; Breton *et al.* 1983), rainbow trout, *S. gairdneri* (Billard *et al.* 1978; Bromage *et al.* 1982; Whitehead *et al.* 1983, Scott and Sumpter 1983; Lou *et al.* 1984) and Atlantic salmon, *S. salar* (Crim *et al.* 1975; Stuart-Kregor *et al.* 1981). When frequent samplings have been done during the spawning season a slight increase of plasma GtH levels is first detected during the week or the two weeks, prior to ovulation (rainbow trout: Fostier *et al.* 1978, 1981; Fostier and Jalabert 1982; Scott *et al.* 1983; coho salmon, *O. kisutch*: Jalabert *et al.* 1978; Atlantic salmon: Crim and Glebe 1984). This first increase is coincident with the transition from the 'subperipheral germinal vesicle' stage to the 'peripheral germinal vesicle' stage (rainbow trout:

Weil 1981 and the present work; amago salmon, *O. rhodurus*: Young *et al.* 1983a). At this stage GtH levels are relatively low (around 5 to 10 ng/ml in the various studies), and the preovulatory increase remains limited in comparison with postovulatory levels (Jalabert and Breton 1980; Fostier *et al.* 1981b; Scott *et al.* 1983). This may be related to a negative feedback of 17 α ,20 β -OH-P at the pituitary level (Jalabert *et al.* 1976). All these results, however, must be considered as only indicative since daily continuous fluctuations of GtH levels have been recorded during the periovulatory period in cannulated rainbow trout. Two distinct GtH surges occur, one at early photophase and the other during the mid scotophase (Zohar, 1982; Zohar *et al.* 1982a, b). These fluctuations being relatively synchronous between individual fish and related to the photoperiod, we have performed our bleedings at the same time in the day. Furthermore, the time of the day which has been chosen (09:00h) coincided with the GtH peak; thus our comparison between stages is probably still valid. A few studies have been done *in vitro* on the ovarian metabolism of radiolabelled steroid precursors during the spawning season (rainbow trout: Lambert and Van Bohemen 1979; ayu: Suzuki *et al.* 1981b; amago salmon: Suzuki *et al.* 1981a; brook trout: Theofan and Goetz 1983). The following enzymatic activities have been demonstrated: 17 α - and 21-hydroxylase, C-17-C-20-lyase, 3 α -, 3 β -, 17 β - and 20 β -oxydo-reductase, Δ 5- Δ 4-3-cetoisomerase, 5 β -reductase and aromatase. In addition, various steroids have been identified at the time of spawning in rainbow trout plasma using double isotope derivative assay (Campbel *et al.* 1980) or mass spectrometry coupled with gas chromatography (Diederick and Lambert 1982); only few of them have been studied further.

In 1960, 17 α ,20 β -OH-P was isolated for the first time from the plasma of sockeye salmon (Idler *et al.* 1960). Two years later the first quantitative data were published showing that this progestin could be related to spawning (Schmidt and Idler 1962). The first direct evidences of the predominant effectiveness of 17 α ,20 β -OH-P on oocyte maturation were obtained in our laboratory (Fostier *et al.* 1973; Jalabert 1976) using an *in vitro* intrafollicular

incubation of rainbow trout oocytes (Jalabert *et al.* 1972). This activity was confirmed in other salmonoides species (brook trout: Duffey and Goetz 1980; coho salmon: Sower and Schreck 1982a; amago salmon and ayu: Nagahama *et al.* 1983). Recently, Nagahama and Adachi (1985) demonstrated that the major maturation-inducing activity, isolated from media in which folliculated oocytes of amago salmon had been incubated, was found in the $17\alpha,20\beta$ -OH-P fraction. In addition, the development of radioimmunoassays for the progestin enabled its follicular secretion during *in vitro* maturation to be observed (rainbow trout: Fostier *et al.* 1981b, amago salmon: Young *et al.* 1982; Atlantic salmon: Zhao and Wright 1985). *In vivo*, a tremendous surge of $17\alpha,20\beta$ -OH-P occurs at the end of the sexual cycle (rainbow trout: Scott *et al.* 1982, Scott and Sumpter 1983; amago salmon: Young *et al.* 1983a; chum salmon, *O. keta*: Ueda *et al.* 1984). More accurately, the first increase is detected within ten days prior to ovulation, and levels usually peak before the day when ovulation can be detected (rainbow trout: Fostier *et al.* 1981b, Fostier and Jalabert 1982, Scott *et al.* 1983, Springate *et al.* 1984; Atlantic salmon: Wright and Hunt 1982; masu salmon: Yamauchi *et al.* 1984; coho salmon: Van der Kraak *et al.* 1984). Our own results show a decrease of $17\alpha,20\beta$ -OH-P level in fish which have ovulated within the last 3 days (Fig. 1), and at least no significant increase in the 3 days following GVBD, the period in which ovulation occurs (control group Fig. 5). These data, together with our earlier report on individual hormone profiles (Fostier *et al.* 1981b), indicate to us that the highest $17\alpha,20\beta$ -OH-P level coincides with completion of meiotic maturation. Like plasma GtH, rhythmic circadian fluctuations have been observed in plasma $17\alpha,20\beta$ -OH-P levels (Zohar 1982; Zohar *et al.* 1982b). However, the amplitude of such fluctuations does not prevent the detection of the basal level increase. Furthermore, our bleedings were performed at the same time in the day (09:00 h).

The preovulatory plasma oestradiol decline we reported earlier in rainbow trout (Fostier *et al.* 1978) was confirmed in the same species (Whitehead *et al.* 1978, 1983; Scott *et al.* 1980, 1982;

Van Bohemen and Lambert 1981, Fostier and Jalabert 1982) as in others (brown trout: Soivio 1982, Breton *et al.* 1983; white spotted char, *Salvelinus leucomaenis*: Kagawa *et al.* 1981; Atlantic salmon: Stuart Kregor *et al.* 1981; coho salmon: Jalabert *et al.* 1978, Sower and Schreck 1982b, Van der Kraak *et al.* 1984; masu salmon: Yamauchi *et al.* 1984; amago salmon: Kagawa *et al.* 1983). Plasma E2- 17β levels begin to decrease from the vitellogenesis period, with a major decline in levels occurring within the two weeks prior to ovulation (the present work, Jalabert *et al.* 1978, Van der Kraak *et al.* 1984), thus low values are reached during GVBD (Fig. 1).

The regulation of this E2- 17β decline is not yet well understood. *In vivo* injection in coho salmon of partially purified salmon gonadotropin (sG-G100) or of gonadotropin-releasing hormone analog depress plasma E2- 17β levels when ovulation occurs (Sower *et al.* 1984; Van der Kraak *et al.* 1984). We did not find such a significant effect in rainbow trout, even at the 'EV' stage (Fig. 2), and the negative correlation calculated between E2- 17β and GtH levels may reflect a negative feedback of E2- 17β at the pituitary level (Bommelaer *et al.* 1981). However, we detected a significant decrease of the *in vitro* ovarian output of E2- 17β under the action of s-GtH (Fig. 9). Such a decrease was also observed in a perfusion system in which follicles were exposed continuously to physiological levels of GtH (Zohar 1982; Zohar *et al.* 1982b). In amago salmon the decline of E2- 17β production was associated with a decrease in aromatase activity but no inhibition by GtH was detectable *in vitro* (Kagawa *et al.* 1983, Young *et al.* 1983b). In Atlantic salmon the *in vitro* release was slightly reduced by s-GtH (Zhao and Wright 1985). However, these various data do not allow us to conclude definitively that GtH inhibits aromatase. Such inhibition has been found in rainbow trout by Sire and Depêche (1981), but they were working on previtellogenic or vitellogenic stages. *In vivo*, the E2- 17β decline may be related also to the regulation of the metabolic clearance. We recently found in our laboratory that metabolic clearance rate for E2- 17β tends to be higher at the end of the sexual cycle than during vitellogenesis (J.F. Baroiller, A. Fostier, Y. Zohar

and O. Marcuzzi, unpublished data). *In vitro*, measurement of steroid levels in the incubation medium gives the result of a net effect between secretion and uptake by follicle layers and, if present, by the oocyte. Actually, Kagawa *et al.* (1982) found a significant drop of E2-17 β levels in medium containing vitellogenic amago salmon ovarian follicles between 12h and 24h of incubation. Furthermore, this equilibrium could be under regulation since a frog pituitary homogenate has been shown to stimulate steroid uptake by *Rana pipiens* ovarian follicles (Snyder and Biggers 1975). Further studies are needed to know the different levels at which oestradiol concentrations could be regulated to control its preovulatory decline.

It is now well established from both *in vivo* (coho salmon: Van der Kraak *et al.* 1984; ayu: Hirose *et al.* 1983) and *in vitro* (rainbow trout: Fostier *et al.* 1981a; amago salmon: Young *et al.* 1983a, c; Atlantic salmon: Zhao and Wright, 1985) experiments, that glycoprotein gonadotropin stimulates 17 α ,20 β -OH-P production from ovarian follicles. *In vivo* injection of SG-G100 induced the key enzyme of 17 α ,20 β -OH-P synthesis, i.e. 20 β -oxydoreductase, in ovaries of the ayu (Suzuki *et al.* 1981). Previous studies on amago salmon have shown that the specific steroidogenic potential of the ovary for maturation, thus at least 20 β -oxydoreductase activity, was established within the few weeks before ovulation in amago salmon (Young *et al.* 1983a). This would imply a specific differentiation of granulosa cells (Young *et al.* 1983a; Nagahama 1983).

Our present *in vivo* and *in vitro* results demonstrate that the specific steroidogenic potential for maturation is established progressively during GV migration. Kinetics of the response to *in vivo* s-GtH stimulations indicate that enzymes are probably already active at 'SP-GV' and 'P-GV' stages but at a low level, since a small 17 α ,20 β -OH-P secretion occurred shortly (within 6h) after GtH injection (but not statistically significant). However for these stages most of the activities have to be induced to give a higher significant 17 α ,20 β -OH-P secretion within 48 to 72h after s-GtH injection (Fig. 3–4). Once maturation was completed, s-GtH amplified the specific secretion within a short period of time

(3h) without inducing more enzyme activities (Fig. 5–6). The relationship between stages and kinetics of the response to s-GtH was also observed *in vitro* (Fig. 7). Considering Suzuki *et al.*'s (1982) results and the fact that 17 α -hydroxy-progesterone, i.e. a substrate for 20 β -oxydoreductase, is stimulated *in vitro* before 17 α ,20 β -OH-P (Zhao and Wright 1985) we may assume that what was mainly observed in the present kinetics was the induction (or activation) of 20 β -oxydoreductase. The suppression of C-17-C-20-lyase which has been hypothesized (Scott and Baynes 1982; Zohar *et al.* 1982; Scott *et al.* 1983) could amplify the response but is probably not the key factor since high plasma testosterone levels are found *in vivo* when 17 α ,20 β -OH-P levels rise (Fostier and Jalabert 1982; Scott and Baynes 1982; Scott *et al.* 1982, 1983) and testosterone is still secreted when 17 α ,20 β -OH-P output occurs *in vitro* (Fig. 9; Zohar 1982; Zhao and Wright 1985). Besides, testosterone by itself has a synergic positive effect with s-GtH on maturation and 17 α ,20 β -OH-P output by ovarian follicles, while E2-17 β has an adverse effect (Jalabert 1975; Jalabert and Fostier 1984b). Comparable oestrogen inhibition was recently observed in *Rana pipiens* (Lin and Schuetz 1985). These findings suggest that a high testosterone/oestradiol ratio is favorable to the surge in 17 α ,20 β -OH-P secretion.

GtH has been shown to stimulate adenyl-cyclase activity in immature fish ovary (Fontaine *et al.* 1972; Idler *et al.* 1975) and we recently demonstrated an intrafollicular peak of cAMP after adding GtH in incubation of trout isolated follicles at 'P-GV' stage (A. Fostier, B. Jalabert, B. Finet, unpublished data). In addition, *in vitro* 17 α ,20 β -OH-P production by isolated follicles of amago salmon was enhanced by db-cAMP (Young *et al.* 1983c). Consequently, cAMP is considered to be an intracellular mediator of GtH action, as in mammals. Using follicles from the same females we have stimulated the output of 17 α ,20 β -OH-P *in vitro* by various doses of db-cAMP and s-GtH. Comparable responses were observed with db-cAMP and s-GtH in the relationship between the stages of the females kinetics of the responses (Fig. 7–8). For example the follicles of the 'EV' females gave the lowest 17 α ,20 β -OH-P output following stimulation by

either db-cAMP or s-GtH. Thus, the increasing secretion of $17\alpha,20\beta$ -OH-P under the GtH action prior to ovulation is connected, at least in part, to regulatory mechanisms following the stimulation of GtH receptors in the follicle. However, is GtH the physiological predominant factor in regulating these preovulatory steroidogenic changes? *In vivo*, relatively low plasma GtH levels were measured during GV migration (Fig. 1). One injection of s-GtH that induced levels higher than those naturally found at this time, did not enhance $17\alpha,20\beta$ -OH-P secretion within 72h in females with oocytes at 'EV' stage (Fig. 2). *In vitro*, unphysiological concentrations of GtH were necessary to induce such a rise, at the same stage (Fig. 8). As mentioned above, a daily rhythm in plasma GtH level has been shown in rainbow trout (Zohar 1982, Zohar *et al.* 1982a). Treatment of follicles with this pattern of GtH signal, during a few days, could be more effective than our present experimental stimulation to induce the appropriate enzyme system for $17\alpha,20\beta$ -OH-P secretion (Zohar *et al.* 1982b). However, as it was shown for example with testosterone/oestradiol balance, we are tempted to assume that ovulation is prepared by progressive subtle changes of a complex of intra and extra ovarian factors.

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