J. steroid Biochem. Vol. 23, No. 5A, pp. 583–591, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

PROGESTOGENS, ANDROGENS AND THEIR GLUCURONIDES IN THE TERMINAL STAGES OF OOCYTE MATURATION IN LANDLOCKED ATLANTIC SALMON

Y. P. So, D. R. Idler*, B. Truscott and J. M. Walsh
Marine Sciences Research Laboratory, Memorial University of Newfoundland, St John's, Newfoundland, Canada A1C 5S7

(*Received* 13 *March* 1985)

Summary—Peripheral serum levels of free and conjugated steroids were correlated with seven terminal stages of oocyte maturation in female landlocked Atlantic salmon. Pregnenolone levels were maximal at stage 1, and there was a surge in testosterone after initiation of germinal vesicle migration. Testosterone remained high, above 25 ng/ml until ovulation, and its glucuronide was always lower than the corresponding free form. 17α,20β-Dihydroxy-4-pregnen-3-one increased progressively from 0.71 to 68 ng/ml from oocyte stage 1 to 6, with the glucuronide predominating in stages 1 to 4 and the free steroid in stages 5 to 7. Thus, very low levels of free 17α,20β-dihydroxy-4-pregnen-3-one were present when migration of the germinal vesicle was initiated but considerable free steroid was present at and after germinal vesicle breakdown. Negligible amounts of progesterone, 17α-hydroxypregnenolone, 5-pregnene-3β,17α,20β-triol were found in the serum during final maturation. In male salmon with expressible milt, 17α,20β-dihydroxy-4-pregnen-3-one was present with a gradual increase of both free and conjugated form at the advent of spawning, suggesting that this steroid may also play a role in reproduction in male fish. The action of steroids *in vitro* on oocytes at various stages was consistent with conclusions based on blood levels.

INTRODUCTION

Various steroids can accelerate oocyte maturation in teleosts [1], the most potent being 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P). This steroid was first identified in the plasma of mature adult sockeye salmon [2] and Atlantic salmon [3]; it accelerates oocyte maturation *in vivo* in trout, carp and coho salmon [4–6] and *in vitro* in goldfish, rainbow trout, northern pike [1] and yellow perch [7]. Further evidence that this steroid participates in oocyte maturation is the increase in its blood levels immediately before and/or around ovulation in rainbow trout [8–12], Atlantic salmon [12] and goldfish [13, 14]. Other steroids have also been implicated to a lesser degree in oocyte maturation and these include corticoids [3, 15, 16], testosterone [17, 18] and progesterone derivatives [19]; for a recent review, see Goetz[20].

A principal purpose of the present study was to correlate serum levels of 17α,20β-P and other steroids with the precise state of oocyte development prior to ovulation. Does the rise in serum level of these steroids, particularly 17α,20β-P coincide with germinal vesicle migration, or if not, with what stage do they coincide? A second objective was to see if the serum levels of 17α,20β-P-glucuronide (17α,20β-P-G) suggested a role for this steroid in regulating biological activity. A preliminary report of 17α,20β-P and 17α,20β-P-G in landlocked salmon has been given in abstract form [21]. An *in vitro* study was included to compare the effect of some serum steroids on salmon oocytes at precise terminal stages of development.

*To whom correspondence should be addressed.
M.S.R.L. Contribution Number 579.

EXPERIMENTAL

Experiment 1 was conducted on two and a half year old mature female landlocked Atlantic salmon *Salmo salar* Ouananiche from a laboratory stock, with an average weight of 105 g. Serial blood samples, collected from caudal vessels, were centrifuged and the sera stored at −80°C until analysed. Samples were taken every 2 days from November 5–13, 1981 and every 4 days from November 13–27, 1981.

Experiment 2

One and a half year old mature landlocked Atlantic salmon, with an average weight of 40 g were randomly chosen in the period from December 23, 1981 to January 8, 1982. This was the period when these late spawners were close to spawning. Serum samples were prepared following caudal puncture. Immediately after sampling, the ovary from each fish was removed and weighed. Following treatment with oocyte clearing fixative, a mixture of acetic acid–glycerol–teleost balanced salt solution (TBSS) [17] in volume ratios of 4:6:90, the oocytes were observed within 10–15 min for stages of maturation as described by Ng and Idler[22]. The oocytes were rated as follows: stage 1, germinal vesicle (GV) at centre; stage 2, GV slightly off centre; stage 3, GV

midway to periphery; stage 4, GV peripheral; stage 5, GV breakdown (GVBD); stage 6, oocyte clearing and increase in size, oil droplets peripherally attached and coalesced; stage 7, oocyte translucent and free in body cavity or easily extruded when gentle pressure applied to abdomen.

The average stage rating for a group of oocytes was computed from the equation:

$$S = \frac{\Sigma ni\ Si}{\Sigma ni}$$

where ni is the number of oocytes with the stage Si. Blood samples were taken from males of the same stock on the same days as the females were sampled. These fish were mature with expressible milt.

Experiment 3

Two-year old mature landlocked Atlantic salmon were used in October, 1983. After removing the ovary, oocytes were mechanically isolated in ice-cold TBSS. The stage development of the oocytes before incubation was detemined on approx $\frac{1}{5}$ of the total as described above. The remaining oocytes were placed in 30 ml beakers containing 5 ml of TBSS for every 20 oocytes. Controls had TBSS only, while the experimental had steroids at a concentration of 50 ng/ml TBSS. The steroids studied were pregnenolone (preg), testosterone, 17β-hydroxy-5α-androstan-3-one (5αDHT) and 17α,20β-P. They were incubated at 9°C with constant agitation and moist oxygen. After incubation for 42 h, the oocytes were fixed in oocyte fixing solution to check the location of the germinal vesicles. The resulting average stage for each incubate was then calculated.

Radioimmunoassays

The preparation and characterization of an antiserum to 17α,20β-P and of [^{3}H]17α,20β-P were described in an earlier publication [23]; this antiserum did not cross react with corticosteroids, androgens, or other progestogens expected in fish blood serum and there was less than 10% reaction with possible metabolites of 17α,20β-P. However, subsequent tests have shown 30% cross reactivity with 5-pregnene-3β,17α,20β-triol (17α,20β-preg).

Antisera for the measurement of testosterone, progesterone, 17α-hydroxy-4-pregnen-3-one (17α-OH-P), pregnenolone, and 17α,3β-dihydroxy-5-pregnen-20-one (17α-OH preg) were purchased from Radioassay Systems Laboratories, Inc., Carson, CA. Tritiated steroids for use as label and recovery tracer were purchased from New England Nuclear, Boston, MA except [^{3}H]17α-OH preg which was supplied by Radioassay Systems. Reduction of [^{3}H]17α-OH preg with 20β-hydroxysteroid dehydrogenase (Sigma, St Louis, MO) and purification of the product by paper chromatography (PC) gave the tritiated 20β-dihydro derivative which could not be separated from authentic 17α,20β-preg. Since there was 30% cross-reactivity between 17α,20β-preg and the antibody against 17α,20β-P, the latter with displacement of [^{3}H]17α,20β-preg was used to measure 17α,20β-preg isolated from serum samples. Except for the measurement of 17α,20β-P in some samples (see below) all radioimmunoassays were performed on steroid fractions isolated by chromatography. Recovery of steroids through extraction and purification was determined by the addition of tracer amounts of the appropriate tritiated steroids to the serum samples.

Assay for 17α-OH-P, 17α,20β-P and 17α,20β-P-G in salmon sera

In Experiment 1, 17α-OH-P and 17α,20β-P were isolated by PC and measured by radioimmunoassay [23]. In Experiment 2, solvent extracts of sera were assayed by direct RIA, i.e. no chromatography, for 17α,20β-P before and after β-glucuronidase hydrolysis. To determine levels of 17α,20β-P-G, the total 17α,20β-P in the serum was measured by the above assay after the samples were incubated with bovine liver β-glucuronidase (EC 3.2.1.31, Sigma) at 1000 Fishman U/ml 0.1 M sodium acetate buffer pH 4.5 at 37°C for 24 h to convert the glucuronide to free steroid. The level of glucuronide was calculated as the difference between amounts of 17α,20β-P with and without treatment with the enzyme. In order to compare results obtained by direct RIA and after PC of 17α,20-P, aliquots of 100 μl from a pool of serum taken at random from salmon in stages 1–7 were extracted with ethylacetate:ether, 1:1 and assayed for 17α,20β-P, in triplicate, with and without PC. Similarly, 17α,20β-P-G was quantified with and without PC.

The antibody to 17α,20β-P was known to cross-react with 17α,20β-preg but any contribution the latter compound might make to the 17α,20β-P values should be eliminated by PC since the pregnenolone derivative is more polar than and separable from 17α,20β-P.

Assay for testosterone, pregnenolone, 17α-OH preg, 17α,20β-preg and progesterone

Sera from salmon at each terminal stage of oocyte maturation were analyzed for these five steroids after their isolation by PC; the glucuronic acid conjugates of all except progesterone were also measured. Extracts of sera, with and without hydrolysis, were applied to multiple-strip paper chromatograms. The position of the separated steroid fractions on the strips were determined by radioscan of radioactive reference strips as proposed by Frölich *et al.*[24]. Duplicate reference strips were used since separation of all five compounds required serial chromatography with two solvent systems. Development of the chromatogram with heptane–80% methanol (v/v) for 4 h separated progesterone, pregnenolone and testosterone. The appropriate areas of the sample strips, as indicated by radioscan of the outer reference strips, were cut from the sample strips and eluted with

ethanol. The pregnenolones remained at or near the origin. New arms were stapled to the original paper head, which included the second pair of reference strips. The reconstructed paper chromatogram was then developed with cyclohexane–toluene–methanol–water (100:70:100:25). Again according to the radioscan, the separated 17α-OH-Preg and 17α,20β-Preg areas of the sample strips were cut and eluted with ethanol. The ethanolic steroid extracts were evaporated to dryness and redissolved in assay buffer. An aliquot was removed for determination of recovery through the procedure and the remainder set up for radioimmunoassay with the appropriate antibody [23].

RESULTS

Radioimmunoassay for 17α,20β-P

Results on 17α,20β-P obtained by direct RIA and after paper chromatography were compared. The mean by direct assay was 15.8 ng/ml and was 15.3 ng/ml after paper chromatography. A second random pool of plasma gave mean values of 13.7 and 13.3 ng/ml for triplicate analyses of 17α,20β-P after β-glucuronidase hydrolysis, with and without paper chromatography.

No 17α,20β-preg was detected in sera from fish in stages 1–5 and values of only 4.5 ng ± 1.2 ($n = 7$) and 5.8 ng ± 1.2 ($n = 17$) were found in stages 6 and 7 respectively using the 17α,20β-P antibody after paper chromatography. The level of detection of 17α,20β-P in the RIA was 15 pg/tube. Thus, there was no significant contribution of 17α,20β-preg to the direct RIA for 17α,20β-P in these experiments.

Experiment 1

17α-OH-P and 17α,20β-P were measured in female landlocked Atlantic salmon during the spawning season. Of eight individual fish examined only four showed an increase in 17α,20β-P (up to 117 ng/ml), one of which ovulated (Fig. 1) on the day 16 of sampling. Very low levels of 17α-P (up to 4.8 ng/ml) were detected even in the ovulating fish.

Experiment 2

The female landlocked Atlantic salmon used in this study also were found to be not necessarily in the same state of maturation although they were reared under identical conditions. When levels of free and conjugated 17α,20β-P were examined according to the dates that fish were sampled, no correlation could be shown (Fig. 2). However, when the serum levels of steroids were plotted according to the stages of oocyte maturation (Fig. 3), we observed a rise of free 17α,20β-P from 0.7 ng/ml to 6.8 ng/ml when the germinal vesicles started to migrate towards the oocyte periphery. It attained a level of 46 ng/ml during GVBD and continued to increase to 68 ng/ml

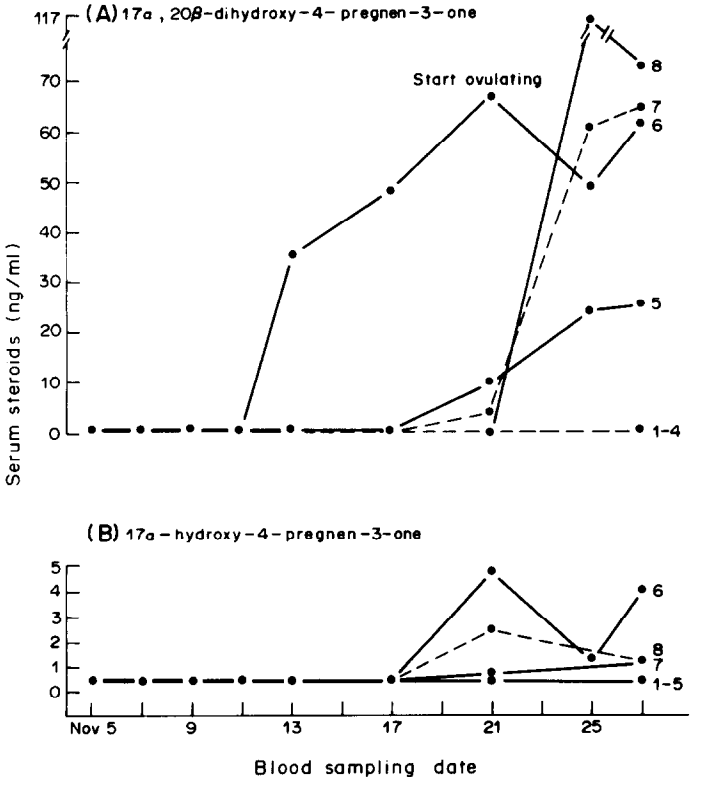

Fig. 1. Levels of (A) 17α,20β-P and (B) 17α-P in serial serum samples from 8 individual $2\frac{1}{2}$ year old landlocked Atlantic salmon.

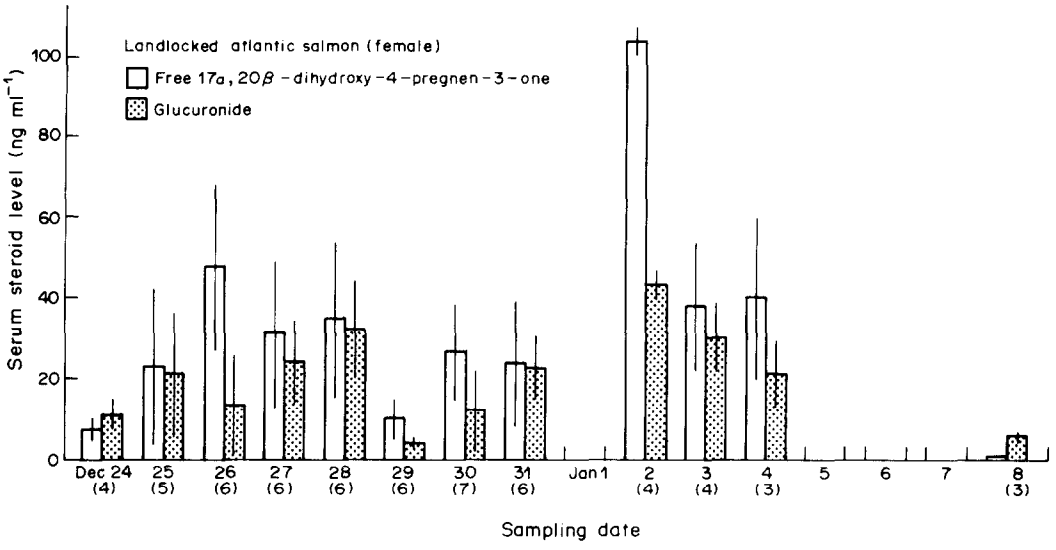

Fig. 2. Serum levels of free 17α,20β-P and 17α,20β-P-G in daily samples from $1\frac{1}{2}$ year old female landlocked Atlantic salmon near spawning. Vertical lines represent the standard error of the means; number of animals in brackets above bars.

when the oocytes cleared and increased in size. There was a slight drop of free steroid during ovulation.

The absolute amount of the glucuronide increased along with the increase of free steroid before the onset of GVBD was higher than the free steroid from stage 1 to 3. After GVBD the glucuronide increased greatly in quantity but the concentration was only half that of the free steroid in the last three terminal stages.

Table 1 shows the serum levels of testosterone, pregnenolone, progesterone, 17α-OH preg and 17α,20β-preg at different stages of oocyte maturation. Less than 1 ng/ml of progesterone and 17α-OH-Preg were detected in all stages. The highest level of pregnenolone occurred in the initial stage (11 ng/ml) while testosterone started to peak at stage 2 and remained high for the rest of the development. 17α,20β-Preg was undetectable in the initial 5 stages and present only in the last two stages when the oocyte started to clear and be ready for ovulation.

When the gonadosomatic index (GSI) of each fish was evaluated according to the terminal stage of oocyte maturation, a gradual increase of the index was observed in fish with oocytes developing from stages 1 to 7 (Fig. 4). In stage 7, the fish attained a maximum GSI of 19.7% although it was not statistically different from the preceding two stages.

During the sampling period, male salmon ($n = 46$) had expressible sperm and had similar serum levels of free 17α,20β-P (14.9 ± 0.9 ng/ml) and its glucuronide [15.8 ± 0.5 ng/ml (Fig. 3)]. Interestingly, when the

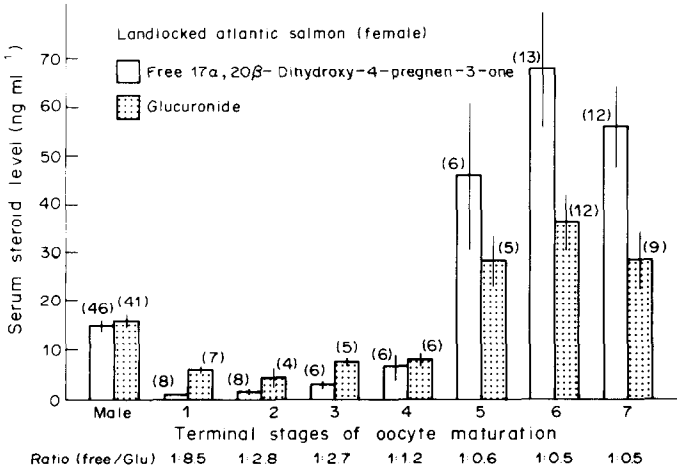

Fig. 3. Change in serum levels of free 17α,20β-P and 17α,20β-P-G in $1\frac{1}{2}$ year old female landlocked Atlantic salmon in relation to the terminal stages of oocytes maturation. Vertical lines represent the standard error of the means; number of animals in brackets above bars.

Table 1. Steroids concentrations in serum samples of *Salmo salar* Ouananiche with oocytes at different terminal stages of maturation

Terminal stages	1	2	3	4	5	6	7
(a) *Free steroids*							
Testosterone	8.4 ± 2.2 (6)*	30 ± 8 (6)	27 ± 4 (15)	25 ± 4 (15)	30 ± 2 (25)	35 ± 8 (20)	36 ± 8 (22)
Progesterone	< 1.0 (6)	< 1.0 (6)	< 1.0 (11)	< 1.0 (15)	< 1.0 (25)	< 1.0 (20)	< 1.0 (22)
Pregnenolone	11 ± 3 (6)	1.4 ± 0.2 (5)	< 1.0 (11)	3.1 ± 0.8 (5)	3.1 ± 0.5 (5)	1.4 ± 0.2 (9)	1.1 ± 0.3 (10)
17α-OH Preg	< 1.0 (6)	< 1.0 (6)	< 1.0 (11)	< 1.0 (15)	< 1.0 (25)	< 1.0 (20)	< 1.0 (20)
17α,20β-Preg	< 1.0 (6)	< 1.0 (6)	< 1.0 (11)	< 1.0 (15)	< 1.0 (25)	4.5 ± 1.2 (7)	5.8 ± 1.2 (17)
(b) *Steroid glucuronide (G)*							
Test-G	6.7 ± 1.1 (6)	11 ± 3 (6)	8.5 ± 2.2 (11)	7.3 ± 1.2 (15)	9.1 ± 1.1 (25)	17 ± 3 (20)	14 ± 3 (22)
Preg-G	< 1.0 (6)	< 1.0 (5)	< 1.0 (11)	4.2 ± 1.4 (5)	2.9 ± 0.9 (6)	< 1.0 (20)	< 1.0 (22)
17α-Preg-G	< 1.0 (6)	< 1.0 (6)	< 1.0 (11)	< 1.0 (15)	< 1.0 (25)	< 1.0 (20)	< 1.0 (22)
17α,20β-Preg-G	< 1.0 (6)	< 1.0 (6)	< 1.0 (11)	< 1.0 (15)	< 1.0 (25)	< 1.0 (20)	< 1.0 (22)

*Steroid concentrations in ng/ml; numbers of fish sampled in brackets.

steroids levels in fish from every 2 or 3 consecutive days in the sampling period were grouped and calculated, a gradual increase was seen of both the free steroid and glucuronide from 8.2 and 7.2 ng/ml respectively on December 24–25 (Fig. 5). Then they plateaued in the range of 17 ng/ml from December 30 to January 8, 1982.

Experiment 3

Figure 6 shows the rate of germinal vesicle migration in terms of net change of terminal stages in oocytes after *in vitro* incubation. It appears that both pregnenolone and 5αDHT were able to induce GVM when the oocytes were at stage 2, with 5αDHT being more potent than pregnenolone. Their effectiveness decreased with the increase in stage of the oocytes. 17α,20β-P was able to induce GVM in oocytes at stage 2, but the rate of development was highest when the oocytes initially had peripheral germinal vesicles. While the effect of testosterone at stage 2 and 3 was not determined, the steroid could induce, to a lesser degree than 17α,20β-P, GVBD in oocytes with initially peripheral germinal vesicles. No effect of testosterone was observed in oocytes at stage 5 and 6.

DISCUSSION

In salmonids, maximal levels of 17α,20β-P are found in the plasma during the late pre-ovulatory stage of maturation. In the present study, when following the serum levels of 17α-OH-P and 17α,20β-P in the 2-year old salmon (Fig. 1), 17α,20β-P appeared to be related to the later stages of final maturation. A definitive identification of 17α,20β-P by double isotope derivative assay on a pool of plasma from mature rainbow trout revealed this level was among the highest yet reported in female salmonids [8]. The surge of 17α,20β-P around the ovulatory period has also been investigated in

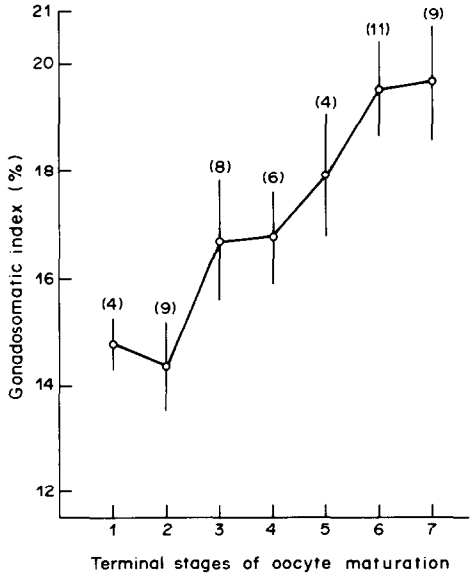

Fig. 4. The gonadosomatic indices in 1½ year old female landlocked Atlantic salmon in relation to terminal stages of oocyte maturation. Vertical lines represent the standard error of the means; number of animals in brackets above the means. The GSI was calculated by: gonad weight × 100 ÷ (total body weight − gonad weight).

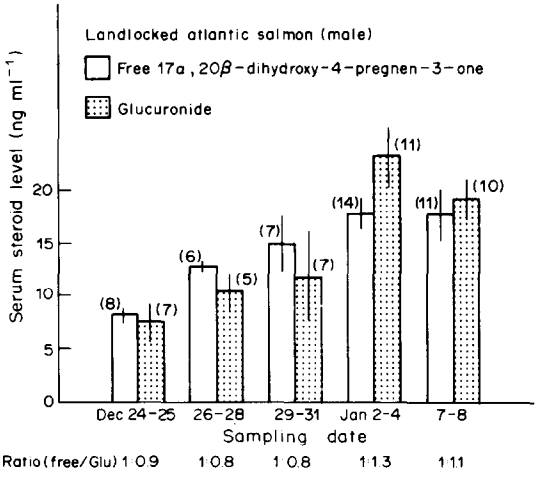

Fig. 5. Serum free 17α,20β-P and 17α,20β-P-G in 1½ year old male landlocked Atlantic salmon. Average taken from fish sampled on every two or three consecutive days. Vertical lines represent the standard error of the mean; number of animals in bracket above bars.

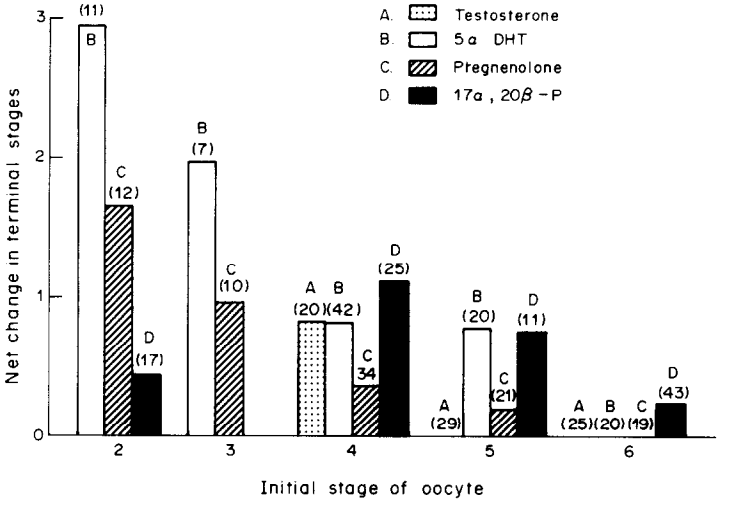

Fig. 6. *In vitro* effects of four steroids on the net change of terminal stages in oocytes from 2-year old landlocked Atlantic salmon. Numbers above bars represent total number of oocytes in incubation. Net change was calculated as the difference between the resulting change in stages in oocytes 42 h after incubation with or without respective steroids.

rainbow trout sampled monthly [25], weekly [11], 4 day intervals [18] and every two days [9]. Its level was low until a few days prior to spawning and the peak extended over 1–2 weeks after spawning. Because of asynchronous development of final maturation among the same batch of fish and in order to pinpoint the exact change of steroid levels in relation to the development towards maturation and ovulation, some of the above authors followed the change in serum levels in individual fish and assigned the levels from the day of ovulation. A similar asynchronous situation occurred in the landlocked Atlantic salmon used in the present study (Figs 1 and 2). In the population of fish that were hatched in the same period and brought up in the same environment in our laboratory, we observed a wide variation of serum levels of 17α,20β-P on a given date. Consequently a plot of the mean value on each date did not reveal a clear progression of serum 17α,20β-P levels through the final maturation (Fig. 2). Serial sampling on individual fish was not feasible because of the small size of the salmon. In spite of variation among individuals, the serum levels of 17α-OH-P were consistently lower than that of 17α,20β-P in the same fish (Fig. 1).

In the present study, serum concentrations of 17α,20β-P and its glucuronide and the GSI have been assessed at precise reproductive stages near ovulation (Figs 3 and 4). By associating the sequence of events occurring in the maturation and ovulation of oocytes with serum steroid levels, we were able to detect a steady rise of free 17α,20β-P in the serum before the surge at stage 5 where GVBD occurred.

Thus the surge occurs at the oocyte stage which appears to be most sensitive to steroid. Oocytes used for the *in vitro* bioassay of steroids and gonadotropins are usually selected when the germinal vesicle is in a peripheral position, i.e. immediately before GVBD [1, 26]. However, oocytes at stage 4 do not appear to have been selected for all assays in which positive results are reported [27]. The elevation of 17α,20β-P coincident with GVBD does not eliminate the possibility that the steady increase in this steroid during the earlier phases may play a role in migration of the GV (Fig. 3). However, it is interesting that the glucuronide predominated during stages 1–4. If the conjugate is biologically less active or inactive then this may be a mechanism for controlling the concentration of "free" 17α,20β-P until the steroid is required for stage 5. We have no direct evidence for the relative potency of a steroid and its glucuronide in salmon but 50 ng/ml of either testosterone or its glucuronide stimulated GV migration and GVBD up to stage 4 in winter flounder (unpublished). The glucuronides of some biologically active steroids should be synthesized and tested.

The conjugated steroids measured in this study are desginated as glucuronides since hydrolysis was effected by a highly purified bovine liver β-glucuronidase; no attempt was made to isolate and confirm the structure of these compounds. The efficacy of the β-glucuronidase was checked in each experiment by including a sample of authentic testosterone glucuronide and the subsequent analysis of the solvent extractables for testosterone. Also there were no structural studies done on the conjugated form of 17α,20β-P present in the landlocked salmon. However, plasma from a Pacific salmon, *Oncorhynchus* sp., was found to contain a conjugated form of 17α,20β-P, again hydrolyzable by liver β-glucuronidase but not so in the presence of the inhibitor, glucosaccharo-1,4-lactone. Further experiments on

the Pacific salmon plasma indicated that the glucuronic acid moiety was attached to the 20β-hydroxyl group: oxidation of the plasma with 20β-hydroxy steroid dehydrogenase did not produce a compound immunoreactive with the antibody to 17α-OH-P whereas [^{3}H]17α,20β-P added to the plasma was oxidized to [^{3}H]17α-OH-P (unpublished data).

In considering the possible biological significance of 17α,20β-P levels at stages 1 to 4, it would have been useful to measure 17α,20β-P in ovarian effluent and peripheral blood of the same fish. Since there is considerable evidence that 17α,20β-P is produced in the ovary [28] and is stimulated by gonadotropin [9, 29, 30], the peripheral levels may represent only a fraction of the amount present in the ovary. However the landlocked Atlantic salmon was too small to sample ovarian blood. In anadromous Atlantic salmon, the gonad effluent contained substantially more androgenic steroid than did peripheral plasma [31]. This suggests that a larger amount of 17α,20β-P may be available to the oocytes in the early stages in spite of the relatively low peripheral levels.

Some other steroids are elevated when the germinal vesicle is migrating and these must also be considered as potential agents involved in the stages before GVBD. Pregnenolone and testosterone are the obvious candidates since their serum levels are elevated earlier than 17α,20β-P (Table 1a). As serum pregnenolone was elevated at stage 1 and testosterone rose between stages 1 and 2 and remained high throughout the development towards maturation and ovulation, these steroids might be involved in the migration of the germinal vesicle before the surge of 17α,20β-P between stages 4 and 5. It has been reported in rainbow trout [10, 18] that serum testosterone increases before the rise of 17α,20β-P prior to ovulation. Some direct evidence for the involvement of other steroids was obtained by the study of the effect *in vitro* of pregnenolone, testosterone, 5α-DHT and 17α,20β-P on the oocytes (Fig. 6). At stages 2–5, both pregnenolone and 5α-DHT migrated the GV and were more effective than 17α,20β-P at the same dose. In addition, testosterone and 5α-DHT had some activity as did pregnenolone to a much less extent in inducing GVBD. 17α,20β-P was still effective when oocytes were mature (stage 6) whereas pregnenolone, testosterone and 5α-DHT exerted no action by that time. We know of no other study where migration of the germinal vesicle has been quantified. However, the possible participation of pregnenolone and testosterone in GVBD has been reported elsewhere. Pregnenolone was able to induce the breakdown *in vitro* at a dose greater than 500 ng/ml [1, 32] and testosterone induced GVBD *in vitro* in oocytes of amago salmon, but only at a level of 1 μg/ml [17, 30]. In the present study, we found that induction by testosterone can be observed (though only 30% efficient) with a concentration of 50 ng/ml in the incubation medium. This is more in line with van Ree *et al.*[27] who showed a similar effect in zebra fish oocytes *in vitro* with approx 90 ng/ml testosterone. At stage 4, 5α-DHT and testosterone were equally effective and neither was active on stage 6 oocytes (Fig. 6). In summary, given the concentrations of serum steroid determined in this study, it seems not unreasonable to suggest that testosterone and its metabolite and pregnenolone stimulate GV migration and 17α,20β-P brings about GVBD.

It is interesting to note that the naturally occurring serum levels of 17α,20β-P and testosterone remained high when the oocytes were further advanced than stage 4 (Table 1a), while in the *in vitro* study 17α,20β-P, testosterone and 5α-DHT were effective in stages 4 or 5 (Fig. 6). The presence of both steroids may indicate synergism in the stimulation of final oocyte maturation, i.e. GVBD. Goetz has pointed out that synergism or facilitation by corticosteroids may play an important role in stimulation of final oocyte maturation by progestogens and he cites examples [20]. Whether the progestogen and androgen are playing a similar role in later stages of final maturation remains to be explored.

Only a few results on blood levels of 17α,20β-P have been reported for Atlantic salmon. In wild Atlantic salmon levels of 17α,20β-P ranged from ca 45 to 340 ng/ml in fish treated with pituitary extract and in naturally ovulating fish [12]. In the present work on landlocked Atlantic salmon, we observed serum levels of 112 ng/ml in some individuals at stage 6. In a study on three Pacific coho salmon, *Oncorhynchus* sp., 17α,20β-P (225–370 ng/ml) appeared after induction with pituitary extract [12]. It is obvious from Fig. 3 that serum levels of 17α,20β-P changed rapidly depending on the precise state of oocyte development.

To our knowledge one other study has attempted to relate plasma levels of unconjugated 17α,20β-P to a stage of oocyte development on a limited number of samples [33]. Amago salmon showed modest levels (ca. 4 ng/ml) of 17α,20β-P in plasma taken from fish when the germinal vesicle was fully migrated to the periphery and higher levels were found in fish of the same date with mature or ovulated oocytes (*ca* 50 and 70 ng/ml). The plasma levels are similar to those found in stages 4–7 in landlocked Atlantic salmon.

The present investigation shows that in the male salmon, there was a significant increase of free 17α,20β-P in the serum from the beginning until the end of the 2 week sampling period; this strongly indicates a role in the spermiating fish. It has been shown that the change in free 17α,20β-P may not be associated with any change in the production of expressible milt [12]. However, from the correlation between the amount of plasma 17α,20β-P and potassium concentration in seminal fluid in rainbow trout, Scott and Baynes suggested that the major function of this steroid in males is the control of the concentration of potassium ion in the seminal fluid [10], and hence, the inhibition of spermatozoan motility [34].

Also again based on the timing of the increase of 17α,20β-P, it has been suggested that this steroid is involved in the hydration and thus fluidity of sperm [35].

The peripheral plasma steroid concentration represents a net balance between the rate of production and the metabolic clearance rate. Although testosterone glucuronide is formed in the liver of the landlocked salmon [36] it is also known to be product of the male gonad since its concentration was higher in the testicular than in the peripheral plasma, suggesting glucuronides may play a role in regulating reproductive activity [31].

In the brown trout the ratio of testosterone to testosterone glucuronide increased in the males from August to November but in the female the ratio remained constant [37]. In the present study on the female landlocked salmon, testosterone levels were measured only during the period of final maturation and, except for stage 1, the ratio of free to glucuronide bound was constant at 1:0.3 through stages 2–7. However, the ratio of 17α,20β-P to its glucuronide in the female landlocked salmon increased slowly from 1:8.5 at stage 1 to 1:1.2 at stage 4, then dramatically to 1:0.5 for stages 5–7 (Fig. 3). In the mature male fish the ratio of free to conjugated 17α,20β-P was more or less constant at 1:1 through the 2 week sampling period (Fig. 5). The role of the glucuronides in reproduction of teleosts is not known. The rapid surge of free steroid before final maturation and spawning could suggest a mechanism which can promote biotransformation rather than proliferation of appropriate steroid secreting cells in the oocytes. The interconversion of free steroid to glucuronide through the control of glucuronyl transferase may be one of the mechanisms involved.

Acknowledgement—This work was supported by NSERC grant No. A6732 to D.R.I.

REFERENCES

1. Jalabert B.: *In vitro* oocyte maturation and ovulation in rainbow trout (*Salmo gairdnerii*), northern pike (*Esox lucis*) and goldfish (*Carassius auratus*). *J. Fish. Res. Board Can.* **33** (1976) 974–988.
2. Idler D. R., Fagerlund U. H. M. and Ronald A. P.: Isolation of pregn-4-ene-17α,20β-diol-3-one from the plasma of Pacific salmon (*Oncorhynchus nerka*). *Biochem. biophys. Res. Commun.* **2** (1960) 133–137.
3. Schmidt P. J. and Idler D. R.: Steroid hormones in plasma of salmon at various stages of maturation. *Gen. comp. Endocr.* **2** (1962) 204–214.
4. Jalabert B., Bry C., Breton B. and Campbell C.: Action de la 17α hydroxy-20β dihydroprogestérone et de la progestérone sur la maturation et l'ovulation *in vivo* et sur le niveau d'hormone gonadotrope plasmatique t-GtH chez la Truite Arc-en-ciel *Salmo gairdneri. C.r. hebd. Séanc. Acad. Sci., Paris* **283** Ser. D (1976) 1205–1208.
5. Jalabert B., Breton B., Bruzuska E., Fostier A. and Wieniawski J.: A new tool for induced spawning: the use of 17α-hydroxy-20β-dihydroprogesterone to spawn carp at low temperature. *Aquaculture* **10** (1977) 353–364.
6. Jalabert B., Goetz F. W., Breton B., Fostier A. and Donaldson E. M.: Precocious induction of oocyte maturation and ovulation in coho salmon, *Oncorhynchus kisutch. J. Fish. Res. Board Can.* **35** (1978) 1423–1429.
7. Goetz F. W. and Theofan G.: *In vitro* stimulation of germinal vesicle breakdown and ovulation of yellow perch (*Perca flavescens*) oocytes. Effects of 17α-hydroxy-20β-dihydroprogesterone and prostaglandins. *Gen. comp. Endocr.* **37** (1979) 273–285.
8. Campbell C. M., Fostier A., Jalabert B. and Truscott B.: Identification and quantification of steroids in the serum of rainbow trout during spermiation and oocyte maturation. *J. Endocr.* **85** (1980) 371–378.
9. Fostier A., Breton B., Jalabert B. and Marcuzzi O.: Evolution des niveau plasmatiques de la gonadotropine glycoproteique et de la 17α-hydroxy-20β-dihydroprogestérone au cours de la maturation et de l'ovulation chez la truite arc-en-ciel, *Salmo gairdnerii. C.r. hebd. Séanc. Acad. Sci. Paris* **293,** Sér. III (1981) 817–820.
10. Scott A. P. and Baynes S. M.: Plasma levels of sex steroids in relation to ovulation and spermiation in rainbow trout (*Salmo gairdneri*). In *Proceedings of the International Symposium on Reproductive Physiology of Fish* (Edited by C. J. J. Richter and H. J. T. Goos). Pudoc, Wageningen, Netherlands (1982) pp. 103–106.
11. Scott A. P., Sheldrick E. L. and Flint A. P.: Measurement of 17α,20β-dihydroxy-4-pregnen-3-one in plasma of trout (*Salmo gairdneri* Richardson): Seasonal changes and response to salmon pituitary extract. *Gen. comp. Endocr.* **46** (1982) 444–451.
12. Wright R. S. and Hunt S. M. V.: A radioimmunoassay for 17α,20β-dihydroxy-4-pregnen-3-one: its use in measuring changes in serum levels at ovulation in Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Salmo gairdneri*). *Gen. comp. Endocr.* **47** (1982) 475–482.
13. Kagawa H., Young G. and Nagahama Y.: Changes in plasma steroid hormone levels during gonadal maturation in female goldfish *Carassius auratus. Bull. Jap. Soc. sci. Fish.* **49** (1983) 1783–1787.
14. Stacey N. E., Peter R. E., Cook A. F., Truscott B., Walsh J. M. and Idler D. R.: Changes in plasma concentrations of gonadotropin, 17α-estradiol, testosterone, and 17α-hydroxy-20β-dihydroprogesterone during spontaneous and brain lesion induced ovulation in goldfish. *Can. J. Zool.* **61** (1983) 2646–2652.
15. Columbo L., Columbo B. P. and Arcarese G.: Emergence of ovarian 11-deoxycorticosteroid biosynthesis at ovulation time in the sea bass, *Dicentrarchus labrax* L. *Ann. Biol. anim. Biochem. Biophys.* **18** (1978) 937–941.
16. Cook A. F., Stacey N. E. and Peter R. E.: Periovulatory changes in serum cortisol levels in the goldfish, *Carassius auratus. Gen. comp. Endocr.* **40,** (1980) 507–510.
17. Nagahama Y., Kagawa H. and Tashiro F.: The *in vitro* effect of various gonadotropins and steroid hormones on oocyte maturation in amago salmon *Oncorhynchus rhodurus* and rainbow trout *Salmo gairdnerii. Bull. Jap. Soc. sci. Fish.* **46** (1980) 1097–1102.
18. Scott A. P., Sumpter J. P. and Hardiman P. A.: Hormone changes during ovulation in the rainbow trout (*Salmo gairdneri* Richardson). *Gen. comp. Endocr.* **49** (1983) 128–134.
19. Nagahama Y., Hirose K., Young G., Aduchi S., Suzuki K. and Tamaoki B.: Relative *in vitro* effectiveness of 17α,20β-dihydroxy-4-pregnen-3-one and other pregnene derivatives on germinal vesicle breakdown in oocytes of 4 species of teleost, ayu (*Plecoglossus altivelis*), amago salmon (*Oncorhynchus rhodurus*), rainbow trout (*Salmo gairdneri*) and goldfish (*Carassius auratus*). *Gen. comp. Endocr.* **51** (1983) 15–23.
20. Goetz F. W.: Hormonal control of oocyte final maturation and ovulation in fishes. In *Fish Physiology*

(Edited by W. S. Hoar, D. J. Randall and E. M. Donaldson). Academic Press, New York, Vol. IXB (1983) pp. 117–170.
21. So Y. P., Idler D. R. and Walsh J. M.: Plasma levels of 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P) in relation to stages of oocyte maturation in *Salmo salar* Ouananiche. *Can. Soc. Zool.* Annual Meeting, May 15–18, 1983. Ottawa, Canada, Abstr. p. 68.
22. Ng T. B. and Idler D. R.: "Big" and "little" forms of plaice vitellogenic and maturational hormones. *Gen. comp. Endocr.* **34** (1978) 408–420.
23. Peter R. E., Sokoloswka M., Truscott B., Walsh J. and Idler D. R.: Secretion of progestogens during induced ovulation in goldfish. *Can. J. Zool.* **62** (1984) 1946–1949.
24. Frölich M., Termorshuizen W., Kenter E. and Moolenaar A. J.: Steroid radioimmunoassay: Contribution of standards to blank values. *Steroids* **24** (1974) 1–13.
25. Scott A. P. and Sumpter J. P.: A comparison of the female reproductive cycles of autumn-spawning and winter-spawning strains of rainbow trout (*Salmo gairdneri* Richardson). *Gen. comp. Endocr.* **52** (1983) 79–85.
26. Hirose K.: A bioassay for teleost gonadotropin using the germinal vesicle breakdown (GBVD) of *Oryzias latipes* oocytes *in vitro*. *Gen. comp. Endocr.* **41** (1980) 108–114.
27. Van Ree G. E., Lok D. and Bosman G.: *In vitro* induction of nuclear breakdown of oocytes of the zebrafish *Branchydanio rerio* (Ham. Buch.). Effects of the composition of the medium and of the protein and steroid hormones. Proceedings of the Koninklijke Nederlandse Akademie van Wetenschappen, Amsterdam, series C, 80 (1977) pp. 353–371.
28. Suzuki K., Tamaoki B. and Nagahama Y.: *In vitro* synthesis of an inducer for germinal vesicle breakdown of fish oocytes, 17α,20β-dihydroxy-4-pregnen-3-one by ovarian tissue preparation of amago salmon (*Oncorhynchus rhodurus*). *Gen. comp. Endocr.* **45** (1981) 533–535.
29. Suzuki K., Tamaoki B. and Hirose K.: *In vitro* metabolism of 4-pregnenes in ovaries of a freshwater teleost, the Ayu (*Plecoglossus altivelis*): production of 17α,20β-dihydroxy-4-pregnen-3-one and its 5β-reduced metabolites, and activation of 3β and 20β-hydroxysteroid dehydrogenase by treatment with a fish gonadotropin. *Gen. comp. Endocr.* **45** (1981) 473–481.
30. Young G., Kagawa H. and Nagahama Y.: Oocyte maturation in the amago salmon (*Oncorhynchus rhodurus*): *In vitro* effects of salmon gonadotropin, steroids, and cyanoketone (an inhibitor of 3α-hydroxy-Δ^5-steroid dehydrogenase). *J. exp. Zool.* **224** (1982) 265–275.
31. Idler D. R., Horne D. A. and Sangalang G. B.: Identification and quantification of the major androgens in testicular and peripheral plasma of Atlantic salmon (*Salmo salar*) during sexual maturation. *Gen. comp. Endocr.* **16** (1971) 257–267.
32. Goetz F. W. and Bergman H. L.: The effects of steroids on final maturation and ovulation of oocytes from brook trout (*Salvelinus fontinalis*) and yellow perch (*Perca flavescens*). *Biol. Reprod.* **18** (1978) 293–298.
33. Young G., Crim L. W., Kagawa H., Kambegawa A. and Nagahama Y.: Plasma 17α,20β-dihydroxy-4-pregnen-3-one levels during sexual maturation of amago salmon (*Oncorhynchus rhodurus*): correlation with plasma gonadotropin and *in vitro* production by ovarian follicles. *Gen. comp. Endocr.* **51** (1983) 96–105.
34. Baynes S. M., Scott A. P. and Dawson A. P.: Rainbow trout, *Salmo gairdneri*, spermatozoa: effect of cations and pH on motility. *J. Fish Biol.* **19** (1981) 259–267.
35. Ueda H., Young G., Crim L. W., Kambegawa A. and Nagahama Y.: 17α,20β-dihydroxy-4-pregnen-3-one: plasma levels during sexual maturation and *in vitro* production by the testes of amago salmon (*Oncorhynchus rhodurus*) and rainbow trout (*Salmo gairdneri*). *Gen. comp. Endocr.* **51** (1983) 106–112.
36. Truscott B.: Steroid metabolism in fish. II. Testosterone metabolites in the bile of the marine winter flounder *Pseudopleuronectes americanus* and the freshwater Atlantic salmon *Salmo salar*. *Gen. comp. Endocr.* **51** (1983) 460–470.
37. Kime D. E. and Manning N. J.: Seasonal patterns of free and conjugated androgens in the brown trout *Salmo trutta*. *Gen. comp. Endocr.* **48** (1982) 222–231.