

Dynamics of $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one and $17\alpha,20\beta,21$ -trihydroxy-4-pregnen-3-one in plasma and oocyte incubation media of catfish (*Clarias batrachus*) in response to salmon gonadotropin

R. Moses Inbaraj[†], S. Haider^{‡,*} and S. S. R. Baqri[‡]

[‡]Department of Zoology, Banaras Hindu University, Varanasi 221 005, India

[†]Department of Zoology, Madras Christian College, Chennai 600 059, India

Free and sulphated $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one ($17\alpha,20\beta$ -DP) and $17\alpha,20\beta,21$ -trihydroxy-4-pregnen-3-one ($17\alpha,20\beta,21$ -P) were separated by HPLC in blood plasma and oocyte incubation medium and measured by radioimmunoassay (RIA) in the female catfish, *Clarias batrachus* in response to salmon gonadotropin (SG-G100). A significant rise of both free (4.24 ± 0.44 ng/ml) and sulphated (46.08 ± 1.15 ng/ml) $17\alpha,20\beta$ -DP was noticed in the plasma of SG-G100 injected fish in comparison to the respective saline control values, 1.75 ± 0.14 ng/ml and 19.59 ± 0.58 ng/ml. In the oocyte incubation medium also, SG-G100 elevated the levels of both free and sulphated $17\alpha,20\beta$ -DP, but the level of the free steroid was much higher (131.07 ± 3.46 ng/ml) than the sulphated one (36.51 ± 2.02 ng/ml). On the other hand, levels of $17\alpha,20\beta,21$ -P in plasma (free, 5.50 ± 0.87 vs 2.49 ± 0.69 ng/ml; sulphated, 14.31 ± 1.32 vs 1.99 ± 0.92 ng/ml) as well as in the oocyte incubation medium (free, 2.59 ± 0.4 vs 2.08 ± 0.32 ng/ml; sulphated, 2.04 ± 0.23 vs 1.44 ± 0.22 ng/ml) were much lower than that of $17\alpha,20\beta$ -DP in response to SG-G100. The peak level of free $17\alpha,20\beta$ -DP in the oocyte incubation medium coincides with the oocyte maturational activity (earlier observations) in *C. batrachus*.

In several fish, $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one ($17\alpha,20\beta$ -DP) has been shown to be the most potent steroid for inducing final oocyte maturation¹⁻⁴. This steroid has been established as the naturally occurring physiologically active maturation-inducing steroid (MIS) in salmonid fish⁵, catfish⁶ and killifish⁷. Blood level of $17\alpha,20\beta$ -DP has also been found to rise in many species of fishes during final maturation^{2,8-15}. However, in Atlantic croaker¹⁶, spotted seatrout¹⁷ and striped bass¹⁸ $17\alpha,20\beta,21$ -trihydroxy-4-pregnen-3-one ($17\alpha,20\beta,21$ -P) has been identified as the natural MIS and is reported as the most effective inducer of oocyte maturation under *in vitro*. Thus, in most teleosts studied, either $17\alpha,20\beta$ -DP or $17\alpha,20\beta,21$ -P functions as the physiological MIS.

In the catfish, *Clarias batrachus*, $17\alpha,20\beta$ -DP was shown to be the most potent steroid in inducing oocyte

maturation *in vitro*¹⁹ and was identified as the major naturally occurring MIS from the media in which immature, full-grown folliculated oocytes have been incubated with salmon gonadotropin (SG-G100)⁶. However, Zairin *et al.*²⁰ observed that plasma levels of both $17\alpha,20\beta$ -DP and $17\alpha,20\beta,21$ -P increased prior to ovulation in human chorionic gonadotropin (HCG) injected *C. batrachus* and suggested that $17\alpha,20\beta$ -DP and/or $17\alpha,20\beta,21$ -P are the MIS(s) in this species. It is inferred that $17\alpha,20\beta$ -DP-sulphate is a 'metabolite' of $17\alpha,20\beta$ -DP, but its functional significance to ovarian physiology is not known. However, there are evidences that once released in the water it served as pheromone²¹. Keeping all these in view, we measured plasma levels of free and sulphated $17\alpha,20\beta$ -DP as well as $17\alpha,20\beta,21$ -P in SG-G100 injected *C. batrachus*. In addition, the free and sulphated levels of both these steroids were measured in the incubation medium of SG-G100-induced oocytes.

SG-G100 was a gift from E. M. Donaldson, Canada, and [³H]- $17\alpha,20\beta$ -DP and [³H]- $17\alpha,20\beta,21$ -P and their specific antisera were provided by A. P. Scott, UK. The steroid standards were purchased from Sigma, USA. Acetonitrile was of HPLC grade from E. Merck (India), while other chemicals were of analytical grade from Glaxo (India).

Gravid female *C. batrachus* were obtained from local suppliers in June/July and were maintained in the laboratory for seven days. Five fish were killed by decapitation, their ovaries were transferred to a polypropylene trough containing cool incubation medium²², and the oocytes were separated from each other. Only post-vitellogenic folliculated oocytes having centrally located germinal vesicle (GV) were selected for incubation. Approximately 2000-2500 oocytes were either incubated in medium alone or medium containing 1 µg/ml SG-G100 at $23 \pm 2^\circ\text{C}$ for 36 h. After incubation, the media from troughs were collected and stored at -20°C until use.

In the second experiment, five fish were injected with 15 µg/g BW SG-G100 (ref. 23). Equal number of fish injected with comparable quantity of solvent (0.6% saline) served as control. After 12 h of injection, blood samples (2 ml) were taken by caudal puncture with a heparinized syringe. Plasma was separated by centrifuging at 2000 g at 4°C and stored at -20°C until the assay.

Plasma and oocyte incubation media were passed through C₁₈ Sep-Pak cartridges (Waters chromatography) which were previously activated with 5 ml methanol followed by 5 ml distilled water. Cartridges were further washed with 5 ml distilled water and the free and conjugated steroids were eluted with 5 ml methanol. The methanol extracts were collected and evaporated at 45°C under a stream of nitrogen, and residues were either applied directly to the HPLC column or subjected to acid solvolysis.

The residue obtained after Sep-Pak extraction was redissolved in 1 ml assay buffer and treated with TFA

*For correspondence.

(trifluoroacetic acid/ethyl acetate; 1.4/100; v/v) and placed in a capped test tube overnight at 45°C. The solvent was evaporated on the following day and the residue was redissolved in sodium acetate buffer (0.5 M, pH 5.0). In this process, the sulphated steroids became free and were extracted with 4 ml diethyl ether. The extracts were evaporated and subjected to HPLC analysis.

The HPLC system consisted of an analytical μ Bondapak C₁₈ stainless-steel reverse-phase column (3.9 mm i.d., 30 cm long, 10 μ m particle size; Waters Assoc. Inc.). Waters 501 HPLC binary pump was used to deliver solvents through the column at a flow rate of 0.5 ml/min. Solvent A was 0.01% TFA in distilled water and solvent B was 70% acetonitrile + 0.01% TFA in distilled water. A gradient method was adopted to deliver solvents for different durations (0–10 min, 28.6% solvent B; 10–60 min, the gradation of 28.6 to 100% solvent B; and 60–80 min, 100% solvent B). The absorbance was measured at 254 nm.

At first, the retention time of individual steroids (17 α ,20 β ,21-P, 24 min; 17 α ,20 α -P, 30 min; 17 α ,20 β -DP, 31 min) was obtained and then a standard graph was plotted using a mixture of steroid standards (Figure 1). Sample extracts were reconstituted in 100 μ l acetonitrile/water/TFA (28/72/0.01; v/v/v) and then loaded onto the column and one-minute fractions were collected over 10 to 40 min. Solvent was evaporated by vacuum centrifugation and residues were redissolved in the assay buffer. Radioimmunoassay (RIA) was carried out in the respective tubes against 17 α ,20 β -DP and 17 α ,20 β ,21-P standards only.

Levels of 17 α ,20 β -DP and 17 α ,20 β ,21-P in the plasma and incubation media were measured using the RIA^{24,25} developed specifically for measuring the levels of these two steroids. At first, 100 μ l of sample and known concentrations (1–500 pg) of standard 17 α ,20 β -DP and 17 α ,20 β ,21-P were maintained in duplicate in separate tubes. The labelled steroids were prepared in the assay buffer and were mixed with their specific antisera in

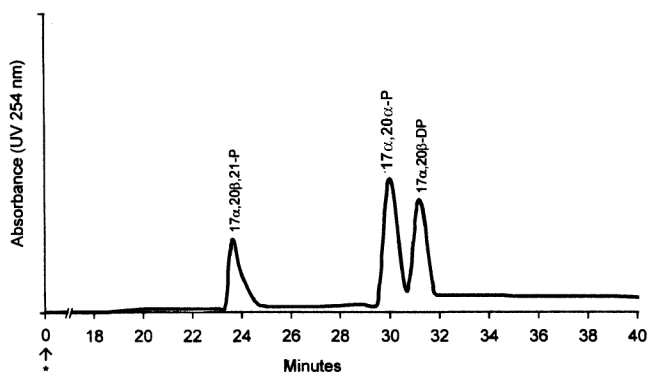


Figure 1. HPLC scan of three steroid standards showing their elution times. *Binary gradient method was adopted by using 0.01% TFA in distilled water as solvent A and 70% acetonitrile and 0.01% TFA in distilled water as solvent B. Flow rate was maintained as 0.5 ml/min.

appropriate concentrations (the amount of label added was such that each tube contained ca. 1200 cpm of tritium). 100 μ l of this mixture was added to all tubes. After overnight incubation at 4°C, 1 ml of ice-cold Dextran-coated charcoal solution (0.5 g charcoal, 0.05 g Dextran T-70 in 100 ml assay buffer) was added to each tube and after standing for 10 min, the tubes were centrifuged (2000 rpm for 10 min at 4°C) and the supernatant poured into scintillation vials containing 7.5–8.5 ml of scintillation fluid (10 ml of a solution of PPO, 4 g/l and POPOP, 100 mg/l in toluene). The vials were briefly vortexed and counted in a Beckman LS 1801 Liquid Scintillation Counter after a 24 h equilibration period. All data are expressed as mean \pm SEM and were analysed by Student's *t* test.

Plasma levels of both free and sulphated 17 α ,20 β -DP increased significantly in the SG-G100 injected female catfish (Figure 2 *a* and *b*). After 12 h of the injection, the level of free 17 α ,20 β -DP reached 4.24 \pm 0.44 ng/ml from 1.75 \pm 0.14 ng/ml (control). Similarly, the sulphated 17 α ,20 β -DP showed more than 2-fold increase in the plasma of SG-G100 injected fish (19.59 \pm 0.58 vs 46.08 \pm 1.15 ng/ml). Around a 10-fold increase was observed in sulphated plasma levels of 17 α ,20 β -DP when the data were compared with free 17 α ,20 β -DP levels either among the controls or among the SG-G100 injected fish. On the other hand, plasma concentrations of free and sulphated 17 α ,20 β ,21-P were much lower (free, 5.50 \pm 0.87 vs 2.49 \pm 0.69 ng/ml; sulphated, 14.31 \pm 1.32 vs 1.99 \pm 0.92 ng/ml) in response to the SG-G100 treatment (Figure 3 *a* and *b*).

When the incubation medium was run on HPLC and fractions were assayed, the levels of free 17 α ,20 β -DP obtained from the SG-G100 group showed a dramatic (12-fold) increase in comparison to the control group (10.94 \pm 0.54 vs 131.07 \pm 3.46 ng/ml) (Figure 4 *a*).

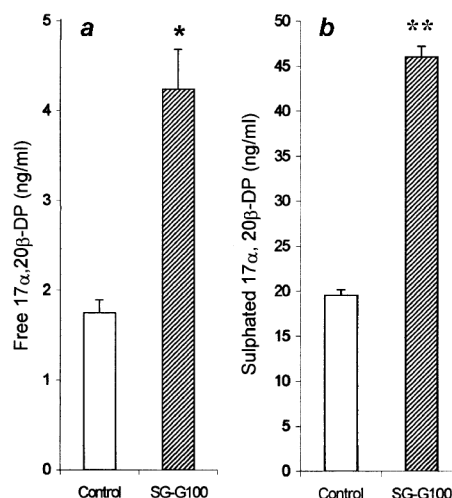


Figure 2. Levels of free (*a*) and sulphated (*b*) 17 α ,20 β -DP in blood plasma from SG-G100- and saline-injected female catfish. Values represent mean \pm SEM; **P* < 0.01; ***P* < 0.001.

Although a high level of sulphated $17\alpha,20\beta$ -DP was also observed in the SG-G100 incubate, the increase was only 2.7 times in comparison with the control incubate (13.56 ± 0.80 vs 36.51 ± 2.02 ng/ml) (Figure 4 b). Only low amounts of free (2.08 ± 0.32 ng/ml) and sulphated (1.49 ± 0.22 ng/ml) $17\alpha,20\beta,21$ -P were detected from the SG-G100 incubate and the levels were not significant in comparison with the control (Figure 5 a and b).

In *C. batrachus*, $17\alpha,20\beta$ -DP as well as deoxycorticosterone (DOC) are the most potent steroids in inducing oocyte final maturation *in vitro*¹⁹. Later, when the MIS in SG-G100-induced oocytes was purified, it coincided only with $17\alpha,20\beta$ -DP but not with DOC⁶. From these studies, it was concluded that $17\alpha,20\beta$ -DP is the major naturally occurring MIS in *C. batrachus*. However, Zairin *et al.*²⁰ observed that the plasma levels of $17\alpha,20\beta,21$ -P also

increased during several hours prior to ovulation in the HCG-treated *C. batrachus*. Since $17\alpha,20\beta,21$ -P is as effective as $17\alpha,20\beta$ -DP in inducing oocyte maturation in some teleost species², it was suggested that $17\alpha,20\beta$ -DP and/or $17\alpha,20\beta,21$ -P act as MIS(s) in this species²⁰. Because $17\alpha,20\beta,21$ -P is a form of corticoid, they also suggested the existence of a correlation between the ovary and interrenal, as was proposed earlier by Sundararaj and Goswami²⁶ in the Indian catfish, *Heteropneustes fossilis*. But in a later communication²⁷, they also reported that $17\alpha,20\beta$ -DP is more effective in inducing oocyte maturation in *H. fossilis*. In our present study, by using specific RIA, we found that SG-G100 effectively increased the $17\alpha,20\beta$ -DP levels in blood plasma and this increase was more prominent (nearly 12-fold) in the oocyte incubation medium. On the contrary, levels of $17\alpha,20\beta,21$ -P in plasma as well as in oocyte incubates in response to SG-G100 were much lower than those of $17\alpha,20\beta$ -DP. Thus, a clear cut discrepancy exists between the results obtained by us and those of Zairin *et al.*²⁰. A possible explanation for this discrepancy may be that we used fish directly from the wild, whereas Zairin's group used fish collected from Indonesia and maintained in Japan for experiments in a stock pond supplied with warm water (23–25°C).

We have also recorded a 10-fold increase in the level of sulphated $17\alpha,20\beta$ -DP (46.08 ± 1.15 ng/ml) in comparison to free $17\alpha,20\beta$ -DP (4.24 ± 0.44 ng/ml) in plasma after SG-G100 injection. However, in oocyte incubation medium the level of free $17\alpha,20\beta$ -DP (131.07 ± 3.46 ng/ml) was significantly higher than sulphated $17\alpha,20\beta$ -DP (36.51 ± 2.02 ng/ml) in response to SG-G100. The most likely explanation for this pattern is that the free $17\alpha,20\beta$ -DP is rapidly converted into conjugated $17\alpha,20\beta$ -DP in blood stream under the influence of sulphating enzymes, and due to the absence of predominant conjugation in the follicles, the level of sulphated

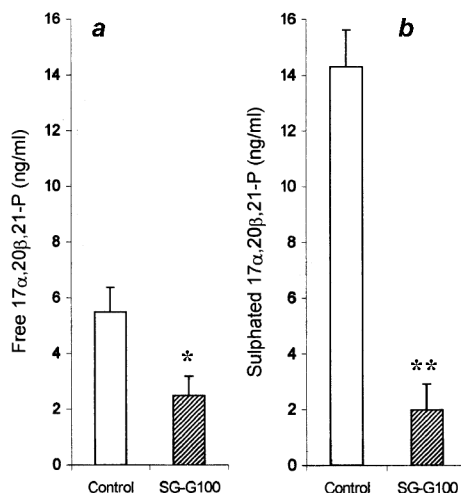


Figure 3. Levels of free (a) and sulphated (b) $17\alpha,20\beta,21$ -P in blood plasma from SG-G100- and saline-injected female catfish. Values represent mean \pm SEM; * $P < 0.05$; ** $P < 0.001$.

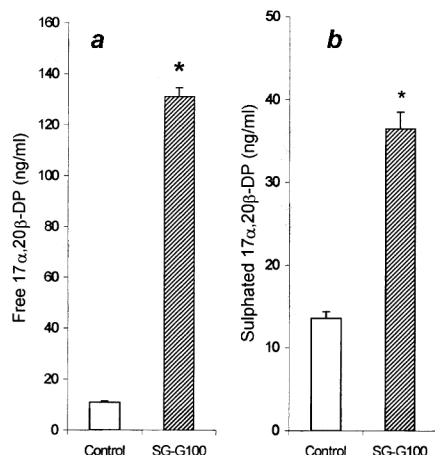


Figure 4. Levels of free (a) and sulphated (b) $17\alpha,20\beta$ -DP secreted by catfish oocytes incubated in the presence or absence of SG-G100 for 36 h. Values represent mean \pm SEM; * $P < 0.001$.

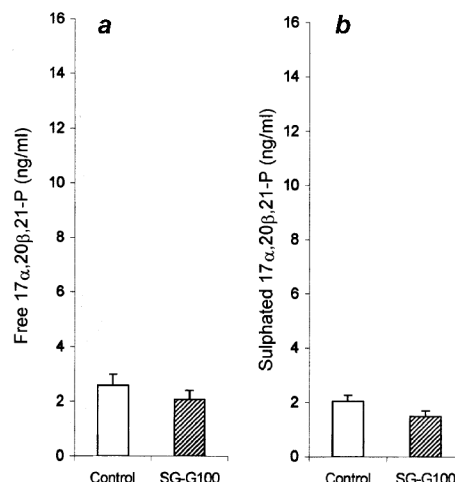


Figure 5. Levels of free (a) and sulphated (b) $17\alpha,20\beta,21$ -P secreted by catfish oocytes incubated in the presence or absence of SG-G100 for 36 h. Values represent mean \pm SEM.

$17\alpha,20\beta$ -DP is relatively insignificant in the incubation medium (for more details, see refs 28 and 29). Conjugated (sulphated and/or glucuronidated) steroids have also been demonstrated in sailfin molly¹¹, African catfish³⁰, carp³¹, zebrafish³², dab³³, goldfish³⁴ and plaice³⁵. Scott and Canario² have opined that the presence of conjugated steroids is a common feature of teleost gonads. Despite the high plasma concentrations of free and sulphated $17\alpha,20\beta,21$ -P that we have found in the saline-injected control catfish, these levels always remain low in response to SG-G100. In incubates of oocytes stimulated with SG-G100, the levels of free and sulphated $17\alpha,20\beta,21$ -P were extremely low and did not significantly differ from the control. This implied that $17\alpha,20\beta,21$ -P is a minor steroidal product of the catfish ovary and is probably not the steroid responsible for mediating the oocyte maturation process. However, its presence in low quantity would probably have some other role in the reproductive process.

In summary, the results of the present study together with earlier works^{6,19} reaffirm that $17\alpha,20\beta$ -DP acts as the natural MIS in the catfish, *C. batrachus*.

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ACKNOWLEDGMENTS. We are grateful to Dr A. P. Scott, Suffolk, England for providing [³H]- $17\alpha,20\beta$ -DP, [³H]- $17\alpha,20\beta,21$ -P and their specific antisera. We also thank the Head and Coordinator, Centre of Advanced Study in Botany, Banaras Hindu University for the use of HPLC. This research was supported by a research grant awarded to S.H. from CSIR, New Delhi. S.S.R.B. thanks CSIR for the award of a Junior Research Fellowship.

Received 24 July 2000; revised accepted 13 November 2000