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STEROIDS INVOLVED WITH FINAL OOCYTE MATURATION IN THE WINTER FLOUNDER

BERYL TRUSCOTT,† YING P. SO, JAMES J. NAGLER and DAVID R. IDLER*
Marine Sciences Research Laboratory, Ocean Sciences Centre, Memorial University of Newfoundland, St John's, Newfoundland, Canada A1C 5S7

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Summary—A number of androgens and progestogens including 17α,20β-dihydroxy-4-pregnen-3-one (17,20-P) were examined in female winter flounder as possible maturation inducing steroids (MIS). During final oocyte maturation serum levels of testosterone (T) and 17β-hydroxy-5β-androsten-3-one (5β-T) peaking at over 200 ng/ml and pregnenolone (PE) at 40 ng/ml were the predominant steroids found from each major group. High levels of T and 5β-T were correlated with oocyte stages characterized by germinal vesicle migration. Of the PEs measured, maximum serum levels of PE, 3β,17α-hydroxy-5-pregnen-20-one (17-PE) and 3β,17α,20β-dihydroxy-5-pregnene (17,20-PE) were found during later oocyte stages associated with germinal vesicle breakdown. Levels of 17,20-P, an established MIS in most fish, were almost non-detectable (<0.1 ng/ml serum) in females throughout all stages of final oocyte maturation. Incubations of ovarian follicles *in vitro* with physiological concentrations of T and 5β-T indicated that these steroids could induce all stages of final oocyte maturation. Similar *in vitro* incubations showed that 17-PE and 17,20-PE were only effective on germinal vesicle breakdown. The principal conclusions are that T, 5β-T and the PEs can be considered as MISs in winter flounder and the PE pathway predominates during the final stages of oocyte maturation in winter flounder in contrast to progesterones which predominate in other fish species, mostly salmonids, studied to date.

INTRODUCTION

Final oocyte maturation in fish begins with the resumption of meiosis and is characterized by two distinct morphological events. The first is migration of the nucleus or germinal vesicle (GV) from a central position to the periphery of the oocyte and secondly the dispersion of the GV referred to as GV breakdown (GVBD) [1]. Steroid hormones particularly C_{21} steroids with a 20β-hydroxyl group are potent inducers of final oocyte maturation in fish. One steroid, 17,20-P[1], has been shown to be very effective as a maturation inducing steroid (MIS) in most fish studied [2]. It was established as the MIS on the basis of high serum levels (5–1000 ng/ml) during final maturation and studies conducted on ovarian follicles *in vitro* demonstrating it's effects on GV migration (GVM) and GVBD [2].

However 17,20-P may not be the MIS in all fish. The Atlantic croaker (*Micropogonius undulatus*) is a notable exception and 17,20,21-P is believed to be the MIS [3]. In the Order Pleuronectiformes (marine flatfishes) although 17,20-P is usually the most active steroid if introduced experimentally [4] it is found at low levels in the blood and is not synthesized by the ovary to any extent [5]. Therefore the search has begun for other C_{21} steroids that could act as MISs in these fish.

In most *in vitro* studies GVBD has been used as the morphological criterion to assess hormonal effects on final oocyte maturation presumably due to the relative ease with which it can be quantified. The measurement of GVM, tending to be a more subjective parameter and exhibiting some species differences, has been used less frequently [1]. There is evidence though that T and PE are more effective on GVM than 17,20-P in landlocked Atlantic salmon (*Salmo salar ouananiche*) [6]. It may be important to consider both GVM and GVBD when studying hormonal regulation of final

†Deceased
*Author to whom correspondence should be addressed.
Abbreviations: 17,20-P = 17α,20β-dihydroxy-4-pregnen-3-one; 17,20,21-P = 17α,20β,21-trihydroxy-4-pregnen-3-one; T = testosterone; PE = pregnenolone; 5α-T = 5α-dihydrotestosterone; 5β-T = 17β-hydroxy-5β-androsten-3-one; DHA = 3β-hydroxy-5-androsten-17-one; 17-PE = 3β,17α-hydroxy-5-pregnen-20-one; 17,20-PE = 3β,17α,20β-dihydroxy-5-pregnene; 20-P = 20β-hydroxy-4-pregnen-3-one; 17-P = 17α-hydroxy-4-pregnene-3,20-dione; P = progesterone; E_2 = estradiol-17β.

oocyte maturation in fish as these two events could be separately regulated.

The present study is intended as an examination of steroids that may act as MISs in the winter flounder (*Pleuronectes americanus*) a marine flatfish with group synchronous ovarian development. Serum androgen and progestogen levels were correlated with all stages of final oocyte maturation. Incubations of ovarian follicles (OF) were conducted *in vitro* to determine the effect of steroids on GVM and GVBD.

EXPERIMENTAL

Female winter flounder were obtained before the spawning season (May–June) in each of three separate years and held under laboratory conditions as previously described [7]. Throughout the spawning season blood was sampled by needle and syringe from caudal vessels, the sera separated by centrifugation, and aliquots stored at −60°C until analysis. Ovaries were biopsied or the fish were killed by decapitation and ovaries removed to determine the oocyte stage [8]. Usually about 20 OF were dissected free of connective tissues, cleared with oocyte clearing solution (OCS), and staged according to the scheme in Ref. [8]. Briefly, the oocytes were rated as follows: stage 1 (1.0–1.9), GV central; stage 2 (2.0–2.9), GV slightly off centre; stage 3 (3.0–3.9), GV midway to periphery; stage 4 (4.0–4.9), GV, peripheral; stage 5 (5.0–5.9), GV breaking down (GVBD); stage 6 (6.0–6.9), oocyte clearing and increasing in size, oil droplets peripherally attached and coalesced; stage 7, ovulated, eggs translucent. The average stage rating for a group of oocytes was computed from the equation:

$$\text{stage} = \Sigma n_i \, S_i / \Sigma n_i$$

where n_i is the number of oocytes with the stage S_i [6].

The following steroids were measured in blood sera: T, 5α-T, 5β-T, DHA, PE, 17-PE, 17,20-PE, P, 20-P, 17-P and 17,20-P. All steroids were extracted from serum with either diethyl ether or diethyl ether–ethyl acetate (1:1). Solvent extracts were dried down under N_2, reconstituted in 100% ethanol and the free steroids usually isolated by paper chromatography (PC) before quantification with RIA [7]. Recovery of steroids through extraction and purification were determined by addition of tracer amounts of the appropriate tritiated steroids (NEN DuPont, Boston, MA or Radioassay Systems Ltd., Carson, CA, U.S.A.) to the serum samples. The individual methodologies for preparation and quantification of each steroid are given below. The androgenic steroids T, 5α-T and 5β-T were separated from other steroids by PC in heptane–80% methanol and the individual steroids obtained by TLC in toluene–ethyl acetate–cyclohexane (80:50:20). Since the T antibody produced here cross-reacted equally with T, 5α-T and 5β-T, all androgenic steroids were quantified after separation by the T RIA procedure in Ref. [7]. No androgen levels were determined in postspawned fish. DHA in sera was isolated by chromatography on Sephadex LH-20 [7] and quantified by an RIA protocol identical to the T RIA using DHA antisera and tritiated DHA purchased from Radioassay Systems Ltd. The PC separation and RIA protocols followed for all the progestogens with the exception of 20-P are described in Ref. [6]. For 20-P it was first separated from other progestogens by PC in heptane–80% methanol and then treated with 20β-hydroxysteroid dehydrogenase to convert it to P which was then measured in the P RIA outlined in Ref. [6].

Incubations were performed *in vitro* using isolated OF from fish over five spawning seasons (i.e. 5 years) to which the following hormones were separately added at either 50 or 250 ng/ml: T, 5β-T, DHA, PE, 17-PE, 17-P, 17,20-PE and 17,20-P. The doses 50 and 250 ng/ml were chosen based on a dose–response curve for 17,20-P developed in Ref. [9]. Subsequently, incubations *in vitro* were conducted testing PE and 17-PE at doses of 2.5, 20 and 40 ng/ml and 17,20-PE at 2.5, 5 and 10 ng/ml which represent physiological concentrations based on blood levels. Each assay consisted of 60–100 OF manually dissected from the ovaries of each freshly killed fish which were placed in triplicate wells of a plastic culture plate (Linbro, Flow Laboratories, Rockville, MA, U.S.A.) in 1 ml of incubation medium (FO solution [10]) with (treated) or without (control) added hormone. Incubations were conducted at 6–7.5°C in a shaking water bath under humidified O_2 for either 24 or 48 h. At termination OF were fixed in OCS and staged. To quantify GVM all OF with stages between 1 and 4 were rated and the stage calculated to determine any differences between treated and control groups. The proportion (%) of OF

exhibiting GVBD (i.e. stages 5–7) relative to the total number of OF were calculated to allow comparisons between groups.

One-way ANOVA followed by Tukey's test for multiple comparisons were used to determine statistical significance ($P < 0.05$) between the means of treated and control groups [11]. Since no significant differences were observed between years data for all years were combined in the results.

RESULTS

The androgens were more prevalent than progestogens in the blood of female winter flounder during all stages of final oocyte maturation (Fig. 1). T was the most abundant androgen and fish with stage 3 oocytes had the highest levels of T (212 ng/ml) [Fig. 1(a)]. Serum levels of 5β-T were even higher than T in fish with stage 3 and 4 oocytes, at 225 and 96 ng/ml, respectively, although levels were significantly lower than T at stages 1, 2 and 6. The serum levels of DHA (96 ng/ml) and 5α-T (6.5 ng/ml; data not shown) also peaked at stage 3. Amongst the progestogens the PEs predominated over the Ps with levels significantly higher at all stages [Fig. 1(b, c and d)]. Within the PE group PE itself was dominant attaining the highest blood levels during stage 4 at 40 ng/ml and generally remained elevated through to stage 7 [Fig. 1(b)]. As well 17-PE and 17,20-PE tended to increase with advanced oocyte stages reaching 39 and 6.6 ng/ml, respectively by stage 7 [Fig. 1(c and d)]. The MIS 17,20-P was <0.1 ng/ml in all fish throughout the period of final oocyte maturation. Serum levels of 20-P were <2 ng/ml and unchanged during final oocyte maturation (data not shown). All serum progestogen levels measured dropped dramatically in postspawned fish. No fish was observed to have oocytes at stage 5 (undergoing GVBD).

Only data from OF incubations conducted *in vitro* and sampled after 48 h are shown since results after 24 h were comparable. All the steroids tested at doses of 50 and 250 ng/ml significantly advanced GVM after 48 h with the exception of 17-P and 17-PE at 50 ng/ml, and DHA at both doses (Fig. 2). At the lower doses that PE, 17-PE and 17,20-PE were tested no significant differences in GVM were found. GVBD was significantly advanced after 48 h with all steroids tested at 50 and 250 ng/ml except DHA at 50 ng/ml (Fig. 3). The lower doses of 17-PE at 40 ng/ml and 17,20-PE at 5 and 10 ng/ml also significantly increased the number of follicles undergoing GVBD (Fig. 4).

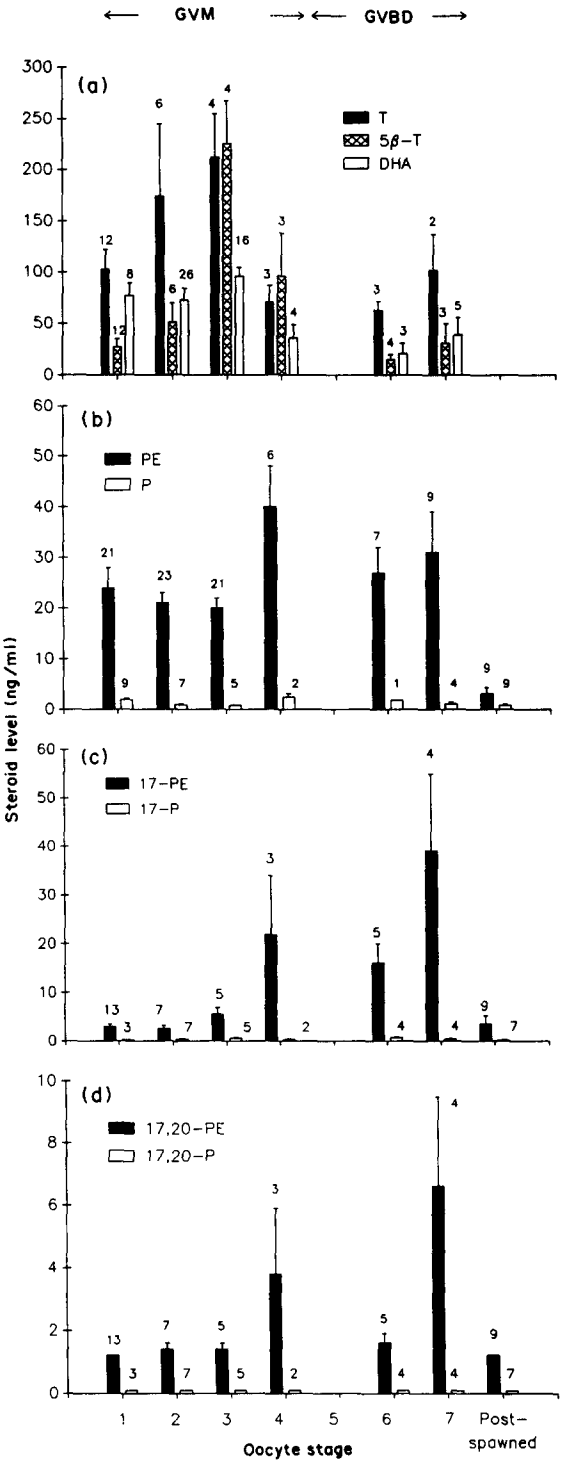

Fig. 1. Serum steroid levels in female winter flounder at different stages of final oocyte maturation. Bars represent the mean steroid level and standard error. The number of fish sampled is indicated above each bar. Bars without standard errors were too small for the scale. Statistically significant differences are discussed in the text.

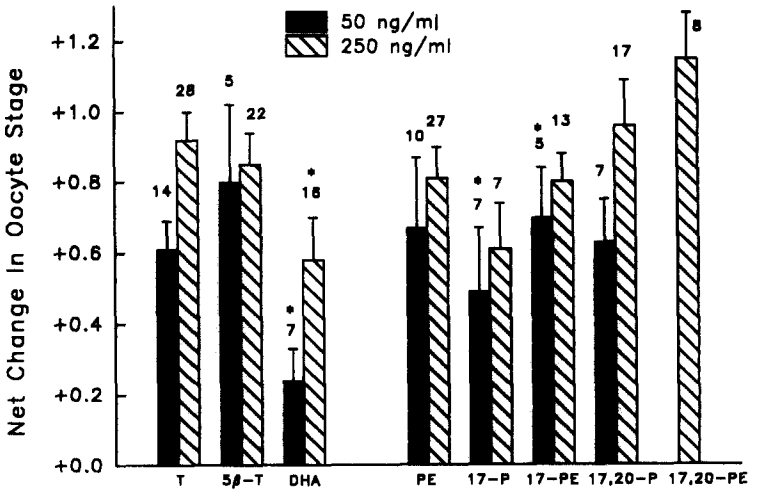

Fig. 2. The effect of steroids, at two different doses, on GVM in winter flounder OF incubated for 48 h *in vitro*. Bars represent the mean net change in oocyte stage relative to the control and standard error. The number of incubations (each representing a different fish) is indicated above each bar. *Not significantly different ($P > 0.05$) from the control group.

The mean of the coefficients of variation within the two *in vitro* bioassays, measuring advancement of GVM and GVBD, were 0.12 and 0.31, respectively.

DISCUSSION

These results indicate that 17,20-P, the MIS previously described in many teleosts, is likely not to be the active MIS in winter flounder. This statement is based on the virtual absence of 17,20-P in the peripheral blood of naturally spawning females during all stages of final oocyte maturation. Two other groups of steroids, the androgens and the PEs from the progestogen group, are present in the blood during final maturation at substantial levels. Of the androgens T was usually the most abundant while amongst the PEs PE was elevated particularly during the latter oocyte stages. While blood steroid levels may not be the only indicator of involvement of a steroid in final oocyte maturation they must be considered especially when high levels coincide with maturational events.

It could be argued that local intrafollicular steroid concentrations may attain transient levels sufficient to be active during final oocyte

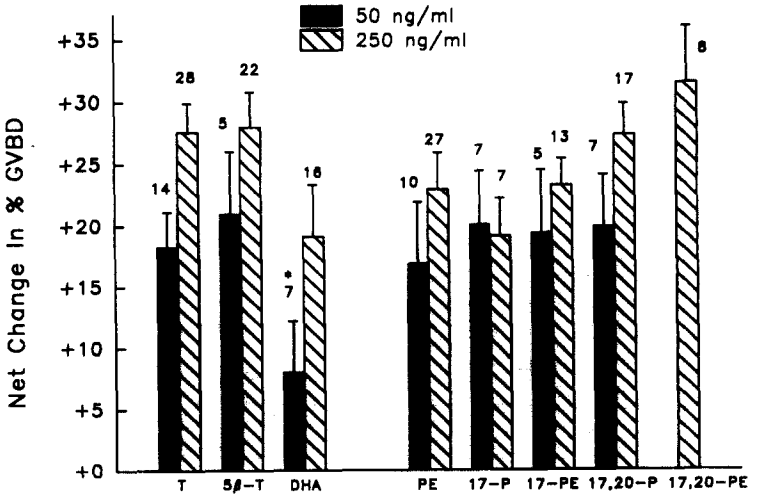

Fig. 3. The effect of steroids, at two different doses, on GVBD in winter flounder OF incubated for 48 h *in vitro*. Bars represent the mean net change in percent GVBD relative to the control and standard error. The number of incubations (each representing a different fish) is indicated above each bar. *Not significantly different ($P > 0.05$) from the control group.

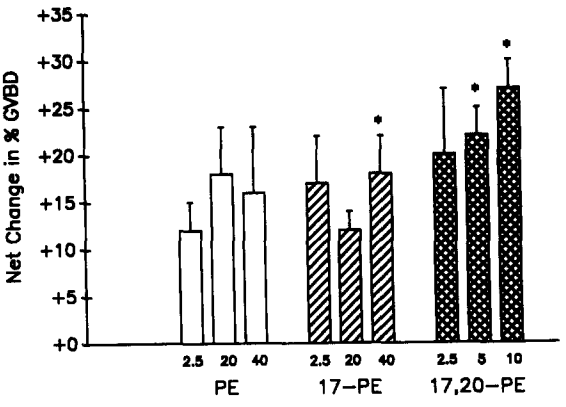

Fig. 4. The effect of steroids (ng/ml) on GVBD in winter flounder as in Fig. 3. The number of incubations (each representing a different fish) is 6 in each case. *Significantly different ($P < 0.05$) from the control group.

maturation but not be found to any extent in peripheral blood. In the instance of 17,20-P this has yet to be proven. Considering it takes about 3–4 weeks for final oocyte maturation to occur in the winter flounder it is difficult to accept that a principal MIS would not be measurable in the peripheral blood during this time. But more importantly if steroids such as T, 5β-T and the PEs are being produced by the ovary to the extent that high blood levels are achieved we can assume that intrafollicular levels of these steroids are as high or higher. Theoretically, since T is produced by OF and is biologically active *in vitro* (Figs 2 and 3) at the concentrations demonstrated in the serum (Fig. 1) and is capable of positively affecting both GVM and GVBD at these levels there is no reason to believe that T is not the MIS. In addition studies have shown that hypophysectomy of female winter flounder reduces the subsequent ovarian production of T *in vitro* (Nagler and Idler, unpublished) and that addition of winter flounder pituitary extract to ovarian fragments during final maturation *in vitro* will significantly enhance T synthesis above control levels [12].

In winter flounder ovaries the major progestogen biosynthetic pathway appears to be the PE pathway leading from PE to 17,20-PE. Two possible explanations can be given for the lack of a significant progesterone pathway in winter flounder. First that Δ^5-3β-hydroxysteroid dehydrogenase is compartmentalized with adequate levels in thecal cells to produce T but very low levels present in granulosa cells where 17,20-P would be produced within the ovarian follicle. This is in contrast to studies on two other pleuronectids (dab, *Limanda limanda* and plaice, *Pleuronectes platessa*) which indicate that substantial levels of Ps are formed in these fish [13,14]. However, it is interesting to note that 17,20-PE was one of the most effective steroids relative to 17,20-P in dab and plaice *in vitro* GVBD assays with potencies of 0.97 and 1.79, respectively [4]. A second explanation relates to Δ^5-3-ketosteroid isomerase and the competitive inhibition effects of estradiol-17β (E_2) with androst-5-ene-3,17-dione for this enzyme that have been documented [15]. Unlike salmonids during final oocyte maturation where serum levels of E_2 are low [16], in winter flounder serum E_2 levels attain annual maximums shortly before final oocyte maturation [12]. Since the granulosa cell layer is the site of E_2 synthesis high levels would be present locally and could inhibit the formation of P at this point of biosynthesis.

The fact that no stage 5 oocytes, representing GVBD in progress, were evidenced from winter flounder sampled immediately after death or by biopsy was unexpected. However, this observation may be explained by the very rapid dissolution of the GV in winter flounder oocytes *in vivo* in contrast to salmonids where GVBD occurs at a slower rate and from which this staging system for oocytes was adapted. During incubations of OF *in vitro* stage 5 oocytes were observed indicating that GVBD may be slower during *in vitro* culture.

The pattern of steroid levels during the progression of final oocyte maturation in winter flounder and their effectiveness at physiological concentrations during *in vitro* incubation suggest that androgens (T and 5β-T) have biological effects on early oocyte stages likely influencing GVM (i.e. peaking at stage 3). Patino and Thomas [17] have also concluded that T may have a role during early final oocyte maturation in Atlantic croaker being primarily involved in the facilitation of gonadotropic regulation of ovarian maturational competence. The PEs attain their highest levels during latter oocyte stages (i.e. stages 4–7) and 17-PE and 17,20-PE are effective on GVBD *in vitro* at concentrations found in the blood. At this point it is suggested that the androgens and PEs act together during final oocyte maturation in this fish, particularly during the later stages when they could combine in a synergistic fashion to induce GVBD.

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