

SEASONAL RESPONSE OF CARP (*CYPRINUS CARPIO*) OVARIAN CELLS TO STIMULATION BY VARIOUS HORMONES AS MEASURED BY STEROID SECRETION: TISSUE CULTURE APPROACH.

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Objective. To study seasonal variation of steroid secretion by dispersed carp ovarian follicular cells as influenced by carp pituitary homogenate (CPH), human chorionic gonadotropin (hCG) or $17\alpha, 20\beta$ -dihydroxy-4-pregnen-3-one ($17\alpha, 20\beta$ OH-P).

Methods. Carp ovaries were collected each month throughout the year from January to December. The cells of ovarian follicles were dispersed by trypsin treatment and cultivated as monolayers. Control cultures were grown in medium M199 alone. Media for experimental cultures were supplemented either with CPH, hCG or ($17\alpha, 20\beta$ OH-P). The direct effect of these three factors on progesterone (P_4), androgens and estradiol (E_2) secretion by isolated follicular cells in cell culture was assayed by appropriate RIA.

Results. In control cultures the highest levels of P_4 , A and E_2 appeared before spawning time and at the time of vitellogenesis. Under the influence of CPH the secretion of P_4 increased in December. Significant increase of androgen secretion was seen in May, while that of E_2 appeared in February, May, September and December. In December, February and November the secretion of E_2 was suppressed by hCG. The most intense stimulation of steroidogenesis was observed after $17\alpha, 20\beta$ OH-P treatment. It increased the level of P_4 at the time of its maximum in the control cultures; androgen level was elevated throughout the whole year; E_2 secretion was stimulated only during the spawning time, while during vitellogenesis E_2 level declined.

Conclusion. It was shown that steroid secretory pattern of follicular cells changed during the year and that the three factors used (pituitary homogenate, hCG and $17\alpha, 20\beta$ OH-P) exerted direct influence upon steroids secretion.

Key words: Carp ovarian cells – Progesterone – Estradiol – Androgens – hCG – Seasonal variation – Tissue culture – Pituitary homogenate – $17\alpha, 20\beta$ OH-P

It is known that follicular cells isolated by enzyme treatment from carp ovarian follicles survive in cell culture and secrete steroid hormones (STOKLOSOWA and EPLER 1985; PETRINO et al. 1989 a,b). GALAS and BIENIARZ (1989) showed characteristic seasonal fluctuations of three ovarian steroids (progesterone, androgens and estradiol) as measured by their level in the carp plasma and ovarian tissue homogenates. Ovarian steroids, as reported by JALBERT and FOSTIER (1984 a,b), SUZUKI et al. (1991), BIENIARZ and EPLER (1992), FUKADA et al. (1994)

are involved in fish oocyte maturation and vitellogenesis.

Carp pituitary homogenate containing two fish gonadotropins (GtH-I, GtH-II) is known to be responsible for estradiol- 17β production, stimulating in turn $17\alpha, 20\beta$ OH-P secretion (TYLER et al. 1991).

Human chorionic gonadotropin (hCG) was also used in experiments on fish reproduction. It was shown that this hormone stimulated germinal vesicle breakdown (GVBD) in oocytes of several fish species (PATINO and THOMAS 1990; DEGANI and BOKER 1992; YORK

et al. 1993) and steroid production in vitellogenic and full-grown ovarian follicles (SRIVASTAVA and VAN-DEKRAAK 1994). As about $17\alpha,20\beta\text{OH-P}$, DEGANI and BOKER (1992) observed that it stimulated GVBD in *Trichogaster* ovaries during reproductive season.

$17\alpha,20\beta\text{OH-P}$ is formed by the conversion from P_4 in ovarian follicles (NAGAHAMA 1987; SANGALANG et al. 1988; KIME et al. 1991) and is known as the steroid stimulating last stages of oocyte maturation in fish. By several authors it is called MIS (maturation-inducing steroid) or MIH (maturation-inducing hormone). The most recent data by NAGAHAMA (1997) show that $17\alpha,20\beta\text{OH-P}$ initiates the translation of cyclin B mRNA in the oocyte.

Data concerning the seasonal secretory variation of follicular steroids as well as their regulation are scarce. Since the follicle is the most hormonally active ovarian compartment, a model of isolated follicular cell culture was chosen to study the steroid secretion, apart from the complex structure of the whole ovary. The sensitivity of follicular cells to CPH, hCG and $17\alpha,20\beta\text{OH-P}$ during the annual reproductive cycle expressed in terms of steroid secretion was of special interest.

Materials and Methods

Animals. The material for culture were ovaries of 6-year-old female carp spawners (average weight 3 kg) from one breed. The stage of ovarian maturity was established according to SAKUN and BUCKA (1968). Altogether 48 females were used in the experiments. The animals were reared at the Experimental Fish Farm, Agriculture University at Krakow-Mydlniki, Poland. The ovaries were collected each month throughout the year.

Isolation of follicular cells. Immediately after sacrifice the ovaries were aseptically removed. Follicles were dissected from the surrounding connective tissue stroma and rinsed several times in sterile phosphate buffered saline (PBS). To obtain a suspension of single follicles vigorous pipetting was applied. The largest follicles (0.8-1.2 mm in diameter) were collected. Further procedure followed the protocol used by STOKLOSOWA and EPLER (1985).

Briefly, obtained cell suspension was inoculated in Leighton tubes. The initial inoculum contained

2×10^6 cells per 1.5 ml of medium. Cells were cultured as monolayers in M199 medium supplemented with 10 % calf serum, penicillin (120 IU/ml) and streptomycin (0.1 $\mu\text{g/ml}$). Viability of cells assessed by trypan blue exclusion test was 95 %. The cells were cultured at 37 °C which appeared to be the optimal temperature for satisfactory cell growth.

Cell culture. Following types of cultures were used: 1. control culture in medium M199 alone, 2. culture treated with carp pituitary homogenate (CPH) dissolved in the medium to a concentration of 50 $\mu\text{g/ml}$. Lyophilized CPH was prepared by a routine in house procedure, 3. cultures with addition of 10 IU hCG/ml medium, 4. cultures supplemented with $17\alpha,20\beta\text{OH-P}$ (150 ng/ml of medium). The cultures were grown in quadruplicates and replicated 3 times. After 2 days the media were collected and frozen for further steroid analysis.

Steroid analysis. Progesterone (P_4), oestradiol (E_2) and androgens (A) were analysed by specific radioimmunoassays described elsewhere (STOKLOSOWA et al. 1982).

Progesterone was estimated using [$1,2,6,7\text{-}^3\text{H}$]progesterone (spec. act. 96 Ci/mmol: Amersham International plc) as a tracer and an antibody induced in sheep immunized against 11α -hydroxyprogesterone succinyl:BSA (a gift from Prof. B. Cook, University of Glasgow, Glasgow, Scotland). The lower limit of sensitivity was of the order of 50 pg. Cross reaction was 1.8 % with pregnenolone, 1.5 % with corticosterone, 0.8 % with 17α -hydroxyprogesterone, and 0.1 % with testosterone. Binding of four related steroids such as 20α -dihydroprogesterone, 20β -dihydroprogesterone, 17α -hydroxy- 20β -dihydroprogesterone, $17\alpha,20\alpha$ -hydroxyprogesterone, and other steroids was below 0.01 %. Coefficients of variations within and between assays were below 5.0 % and 9.8 %, respectively.

Oestradiol- 17β was determined using [$2,4,6,7,16\text{-}^3\text{H}$]oestradiol (spec. act. 140 Ci/mmol: Amersham International plc, Amersham, Bucks, UK) as a tracer and an antibody raised in a rabbit against oestradiol- 17β -6-carboxymethyloxime:BSA (a gift from Prof. R. Rembiesa, Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland). The lower limit of sensitivity of the assays was of the order of 5 pg. Cross-reaction was 1 % with keto-oestradiol- 17β , 0.8 % with oestrone, 0.8 %, with oestriol and

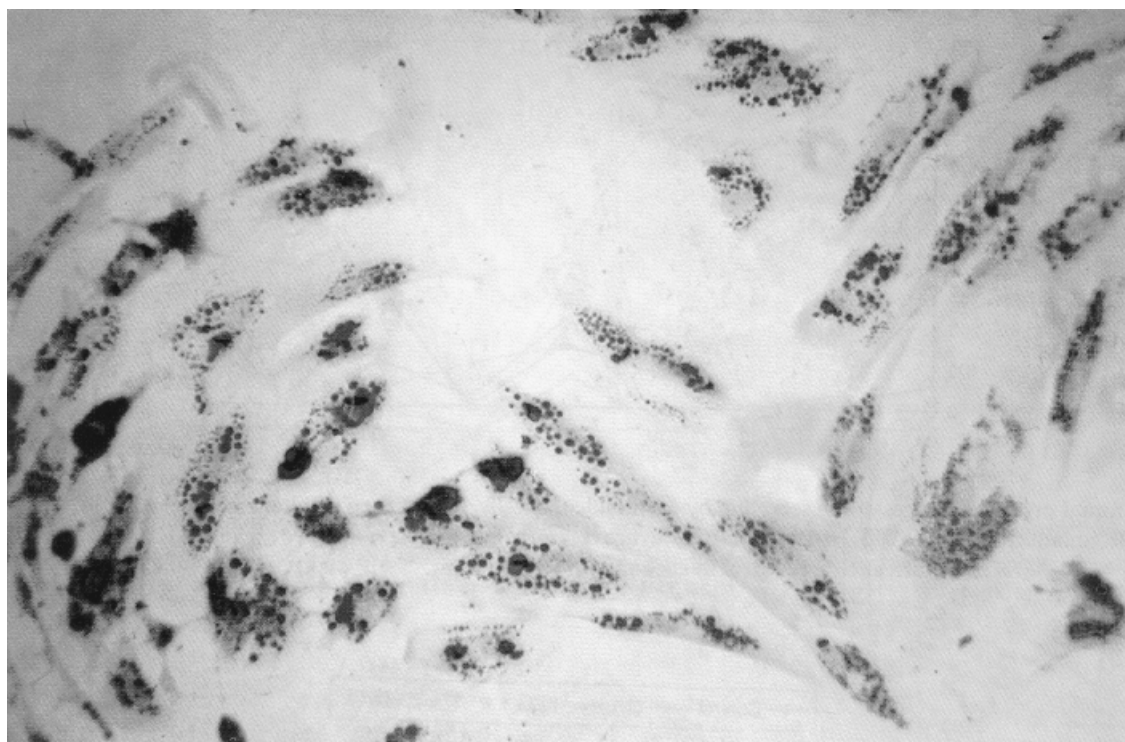


Fig. A Carp follicular cells in two day culture. Cells were stained with Oil Red O (ORO) to visualize lipids (red droplets) in cytoplasm, x 150.

0.01 % with testosterone, other major ovarian steroids showing less than 0.1 % cross-reaction. Coefficients of variations within and between assays were below 4 % and 7.5 %, respectively.

Androgens were measured using [1,2,6,7-³H]testosterone (spec. act. 89 Ci/mmol:Amersham International plc) as a tracer and an antibody raised in a rabbit against testosterone-3-⁰-CMO:BSA (a gift from Dr.B.Ricarova, Institute of Radiology, Czech Academy of Sciences, Prague, Czech Republic). The lower limit of sensitivity was of the order of 5 pg. Cross-reaction of this antibody was 18.3 % with dihydrotestosterone and 0.1 % with androstenedione, while with other major ovarian steroids it was less than 0.1 %. Since this antibody bound also 18.3 % dihydrotestosterone, the measured steroids were referred to as androgens rather than testosterone. Coefficients of variations within and between assays were below 5.0 % and 9.7 %, respectively.

Oestradiol and progesterone were detected directly in the media, while androgens were extracted with ethyl ether. All samples were assayed in duplicate.

Statistical evaluation. The concentrations of steroids were computed in pg/ml of culture medium and were expressed as means±S.E. Statistical differences were calculated by Kruskal-Wallis range test (ZAR 1974).

Results

The cells attached to the bottom of culture dishes after 6 hrs in vitro and after further 18 hrs formed colonies (Fig A) similar to that described and shown by STOKLOSOWA and EPLER (1985).

Progesterone secretion: A. control culture. Sixty pg of P₄ per ml of the medium was secreted in December and then gradually declined to an undetectable concentration in March (Fig. 1). From April until May the level of secreted hormone increased up to 125 and 218 pg/ml respectively, then it fluctuated from 25 pg/ml in June through 112 pg in July to 180 pg in November (Fig. 1).

B. Experimental cultures. The presence of CPH in the culture medium stimulated P₄ secretion 5 fold (300 pg/ml; P≤ 0.05) only in December, while its

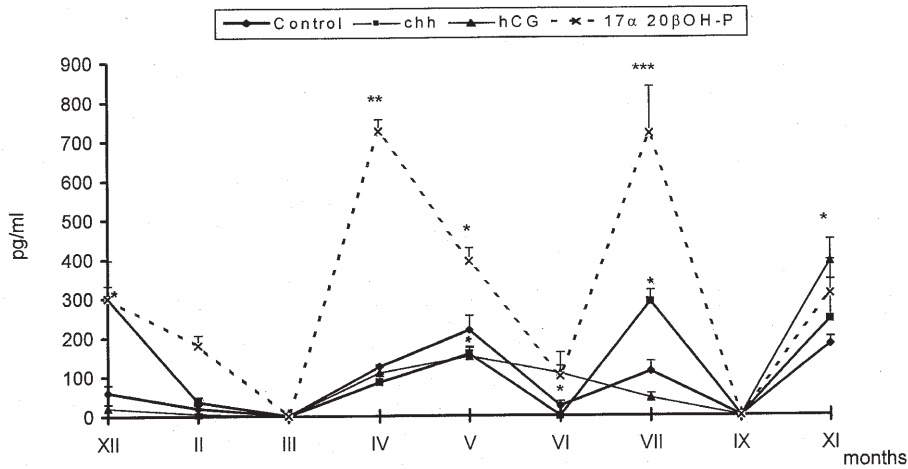


Fig. 1 Annual profile of progesterone secretion by carp follicular cells. Chh = carp pituitary homogenate, hCG = human chorionic gonadotropin, $17\alpha,20\beta\text{OH-P}$ = $17\alpha,20\beta$ dihydroxy-4-pregnen-3-one. Values are means \pm S.E.; asterisks show the level of significance vs. control in each month (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

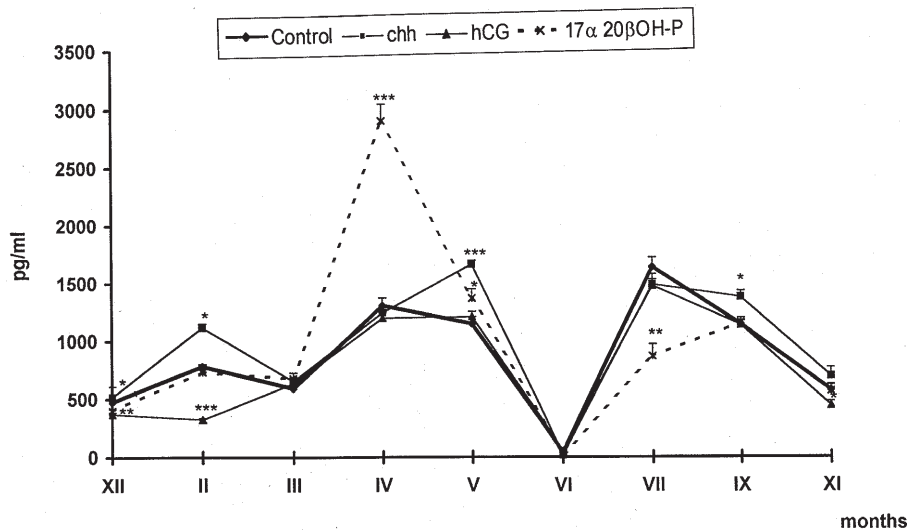


Fig. 2 Annual profile of estradiol secretion by carp follicular cells. Chh = carp pituitary homogenate, hCG = human chorionic gonadotropin, $17\alpha,20\beta\text{OH-P}$ = $17\alpha,20\beta$ dihydroxy-4-pregnen-3-one. Values are means \pm S.E.; asterisks show the level of significance vs. control in each month (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

secretion apparently declined from February until April and then June down to the undetectable concentration ($P \leq 0.05$) (Fig. 1). The cultures supplemented with hCG did not differ in P_4 secretion when compared to the control, although in June and November they showed a certain insignificant increase. The presence of $17\alpha,20\beta\text{OH-P}$ in the medium exerted a visible effect on P_4 secretion raising it up to 725 pg/ml in April, 394 pg in May, and 718 pg in

July ($P \leq 0.01$, $P \leq 0.05$, $P \leq 0.001$, respectively) (Fig. 1).

Oestradiol secretion: A. control culture. The secretion of E_2 in control follicular cells isolated from carp ovaries in December was 476 pg/ml of control medium. It increased in February up to 780 pg/ml, to 1307 pg in April and to 1146 pg in May. In June, however, the secretion of E_2 declined dramatically to 40 pg. Maximal secretion (1638 pg/ml) was ob-

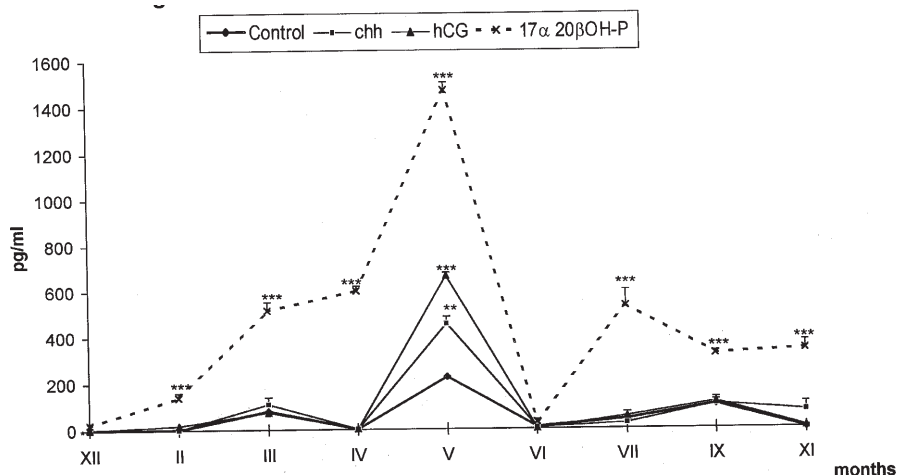


Fig. 3 Annual profile of androgen secretion by carp follicular cells. Chh = carp pituitary homogenate, hCG = human chorionic gonadotropin, $17\alpha,20\beta\text{OH-P}$ = $17\alpha,20\beta$ dihydroxy-4-pregnen-3-one. Values are means \pm S.E.; asterisks show the level of significance vs. control in each month (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

served in July, after which it gradually decreased to 572 pg/ml in November (Fig. 2).

B. Experimental cultures. Cultures treated with CPH showed an increase in E_2 secretion in December, February, May and September – 517, 1114, 1664, 1376 pg/ml ($P \leq 0.05$, 0.05, 0.001, 0.05, respectively) (Fig. 2). On the other hand, hCG suppressed E_2 secretion in cultures in December, February, and November – 370, 322, 442 pg/ml, ($P \leq 0.01$, 0.001, 0.05, respectively) (Fig. 2). The presence of $17\alpha,20\beta\text{OH-P}$ in the culture medium caused an increase of E_2 secretion by cultures of cells collected in April and May – 2905 pg/ml versus control 1307 pg/ml ($P \leq 0.001$) and 1369 pg versus control 1146 pg ($P \leq 0.05$), respectively. In July and December the secretion of E_2 declined to 864 pg/ml ($P \leq 0.001$) and 402 pg/ml ($P \leq 0.05$), respectively (Fig. 2).

Androgen secretion: A. control culture. Control cultures secreted the highest amount of androgen in March (80 pg/ml), May (226 pg/ml), and September (103 pg/ml) (Fig. 3).

B. Experimental cultures. CPH added to the control medium significantly stimulated secretion of androgens in May only (456 pg/ml; $P \leq 0.01$) (Fig. 3). hCG added to the culture medium did not exert any effect on androgen secretion. On the other hand, $17\alpha,20\beta\text{OH-P}$ added to the medium markedly stimulat-

ed androgen secretion in all (except June) investigated months ($P \leq 0.001$) (Fig. 3).

Discussion

Our research model of cell culture allows the investigation of secretory potency of isolated components of the ovary apart from the complexity of the entire organ. Based on the data obtained in mammals which showed that ovarian cells in culture secreted steroid hormones for 48 hrs *in vitro* in a manner reflecting *in vivo* pattern at the time of cell harvest (SZOLTYS et al. 1982; MADEJ 1986), steroid secretion by follicular cells during a 2-day culture was studied also in this experiment. Specific *in vitro* dynamics of steroid secretion by isolated carp ovarian follicular cells during the annual cycle was showed for the first time in this experiment. Similar annual fluctuations were described in blood plasma and ovarian tissue homogenates by GALAS and BIENIARZ (1989). In the present study E_2 was a dominant hormone secreted to the culture medium by carp follicular cells. Maximal amount was secreted in April and May, thus before and during spawning time. The results obtained previously by GALAS and BIENIARZ (1989) are in agreement with these reported in the present work. Another maximum of E_2 secretion was observed in cultures of follicular cells collected in

July and September. This high level of secretion was thus closely correlated with vitellogenesis – a very important stage of fish reproduction. The role of E_2 in this process was described elsewhere (JALABERT and FOSTIER 1984b; BURZAWA-GERARD 1991; NAGLER and IDLER 1992; KWON et al. 1993). The ovarian origin of E_2 in fish was well documented (STOKLOSOWA and EPLER 1985; PETRINO et al. 1988b; NAGLER et al. 1992; VENKATESH et al. 1992). KAGAWA et al. (1982b) proposed two cell type model of E_2 secretion based on studies on the wall of *Salmon* ovarian follicle where E_2 secretion results from the interactions between granulosa and theca cells. Both cell types contain active aromatase, although in different amounts. Thecal aromatase is less active. However, it has not been yet established in carp which cell type of ovarian follicle has the ability to aromatize testosterone, since so far such two cell types have not been discerned. In follicular cell suspension prior to culture as well as later in monolayers STOKLOSOWA and EPLER (1985) observed two cell types, dark cells showing stronger activity of steroid dehydrogenase ($\Delta^5, 3\beta$ HSD) and light cells showing weaker enzyme activity.

Simultaneously with preovulatory maximum of E_2 also maximal level of androgen secretion was observed. This was in accord with the findings by MANNING and KIME (1984). Another increase of androgens secreted to the culture medium was seen in September along with intense vitellogenesis. It is possible that this steroid could be used as the substrate for aromatization to E_2 .

The smaller amount of androgens, with simultaneous increase in E_2 , speaks for active conversion. As about P_4 , an important metabolite in steroidogenesis, its maximum occurred in April and May, which might be related to its intense conversion to $17\alpha, 20\beta$ OH-P essential for final oocyte maturation (CANARIO and SCOT 1988, 1990; SUZUKI et al. 1991; BIENIARZ and EPLER 1992; VENKATESH et al. 1992). YOSHIKUNI and NAGAHAMA (1994) showed that $17\alpha, 20\beta$ OH-P acts on an external surface receptor of rainbow trout oocytes to induce maturation.

Recently, PINTER and THOMAS (1995) showed the presence of both cytosolic and nuclear P_4 receptors in the ovary of spotted sea trout. However, they did not perform the experiment during the annual cycle but collected samples arbitrarily once in Spring and

once in Autumn. It is possible that steroid receptors in fish exhibit similar time dependent changes during the annual cycle as receptors in rodents during the sexual cycle (RICHARDS et al. 1974). This would explain the fluctuations in sensitivity of follicular cells to stimulating factors applied.

The effect of carp pituitary homogenate and hCG on annual steroid secretion by follicular cells was of interest. DRORI et al. (1994) and YARON (1995) observed that the action of pituitary extracts on steroid secretion was correlated with the stage of oocyte maturation. Mature carp females injected with CPH to induce spawning showed a 10-fold increase in E_2 concentration and a 20-fold increase of androgen level in carp plasma (GALAS and BIENIARZ, unpublished; EPLER et al. 1987; EPLER et al. 1989). The crude carp hypophyseal gonadotropin used contains both gonadotropins GtH-I and GtH-II and their binding to follicular cells is modulated by steroids and GH (QUESNEL and BRETON 1995). In the present experiment CPH stimulated mainly E_2 secretion by carp follicular cells in culture. That stimulation was especially significant in February, May, September and December. This could suggest that at that time (spawning and vitellogenesis) the cells were more responsive to that homogenate.

Human chorionic gonadotropin, proved to stimulate maturation of the gonads of several fish species (BARANNIKOVA et al. 1975; SCOTT and CANARIO 1990; KAGAWA et al. 1994). JAMAL-UDDIN and BHATTACHARYA (1986), showed hCG binding sites in fish ovary. MUGNIER et al. (1997) showed a decrease of testosterone and E_2 secretion by fish ovarian fragments incubated with hCG. Suppressive action of hCG upon E_2 secretion during 24 hrs culture of *Limanda limanda* ovaries was also observed by CANARIO and SCOTT (1990). Similar correlation was observed in this experiment (Fig. 2).

In the past, hCG was extensively used for inducing spawning in fish hatcheries. However, recent reports indicate that repeated hCG treatment induces hCG antibodies which impair fish fertility. Moreover, BARANNIKOVA et al. (1975) showed that hCG did not affect oocyte maturation in carp what was also supported by EPLER et al. (1986) who did not observe any induction of spawning by hCG in the carp. The results of this work could support their results that hCG is not effective in carp reproduction. This

could be a result of species specificity of the hormone or of a different mechanism of hCG action on fish follicle.

$17\alpha,20\beta\text{OH-P}$ production by fish ovaries was shown by PETRINO et al. (1989b), CANARIO and SCOTT (1990) and SUZUKI et al. (1991). BILLARD et al. (1992) showed fluctuations in the secretion of this steroid by carp testes from 17th until 41st month of life. GOETZ et al. (1997) showed that $17\alpha,20\beta\text{OH-P}$ stimulated ovulation *in vitro* and prostaglandin (PG) synthesis by yellow perch follicles incubated with fragments of interstitial tissue.

There is no information so far concerning the reactivity of ovarian cells to this steroid. It may well be induced in para- or rather autocrine fashion, similarly as E_2 affects granulosa cells in mammalian follicles (RICHARDS 1974). In the presence of $17\alpha,20\beta\text{OH-P}$ in the culture medium, secretion of P_4 showed two distinct maxima in April and July (Fig. 1). Since there was no significant cross-reactivity of $17\alpha,20\beta\text{OH-P}$ with P_4 in RIA assay, the increase of P_4 could result from stimulation by exogenous $17\alpha,20\beta\text{OH-P}$ (Fig. 1).

At the presence of $17\alpha,20\beta\text{OH-P}$ androgen secretion was higher than in the control cultures in all the studied months except for June. It is difficult to explain this finding. It cannot be excluded that $17\alpha,20\beta\text{OH-P}$ serves as a substrate for androgen synthesis. Interesting biphasic action was exerted by $17\alpha,20\beta\text{OH-P}$ on E_2 secretion, which was stimulated before ovulation and inhibited at the beginning of vitellogenesis (July) and then in December.

On the other hand, JALABERT and FOSTIER (1984a) showed that in *Oncorhynchus mykiss* a high level of E_2 significantly suppressed secretion of $17\alpha,20\beta\text{OH-P}$ by the follicles. They concluded that high levels of E_2 could also prevent oocyte maturation before vitellogenesis was completed. In the present experiment it was observed that $17\alpha,20\beta\text{OH-P}$ in December and in July suppressed aromatization, because the increase in A secretion was accompanied by the decrease in E_2 . Thus, this progestagen could suppress aromatization like P_4 does in mammalian ovarian follicles (FORTUNE and VINCENT 1983). Also in fish the progestagens inhibit the aromatization before ovulation. It seems likely that $17\alpha,20\beta\text{OH-P}$, apart from its action as MIS, could be an important modulator of follicular steroidogenesis in fish. It would

be interesting to detect in another experiment, how much $17\alpha,20\beta\text{OH-P}$ is secreted by ovarian cells during the annual cycle.

Acknowledgements

This investigation was supported by the State Committee for Research (KBN), DS and BW, as well as by SMA grant of the World Health Organization Special Programme of Research Development and Research Training in Human Reproduction. Dr. Maria Szoltys consultations and help in performing radioimmunoassays is acknowledged.

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Accepted: June 15, 1999