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Precocious induction of oocyte maturation and ovulation in rainbow trout (*Salmo gairdneri*) : problems when using 17 α-hydroxy-20 β-dihydroprogesterone

par B. JALABERT, (1) B. BRETON, A. FOSTIER (1)

with the technical assistance of Pierrette REINAUD and Micheline HEYDORFF

Laboratoire de Physiologie des Poissons, I. N. R. A.
78350 Jouy en Josas, France

Summary. The efficiency of 17 α-hydroxy-20 β-dihydroprogesterone (17 α-20 β P) administered alone or after pituitary priming, was investigated *in vivo* before peripheral migration of the germinal vesicle 4 to 8 weeks prior to natural ovulation. Treatmert with 17 α-20 β P alone (2 injections of 3 mg/kg at a 2-day interval) induced oocyte maturation in 94 p. 100 of the fish, but only 25 p. 100 ovulated. Treatment with 17 α-20 β P (3 mg/kg once) 2 days after pituitary priming (1 ml/kg of trout pituitary extract, TPE, containing 3.25×10^{-3} mg/ml of trout gonadotropin, t-GTH) induced maturation in all fish, 59 p. 100 of which ovulated. In both cases, fish in which ovulation did not follow oocyte maturation were killed 15 days after the first injection ; ovarian follicles were either dissected by hand to remove mature oocytes, or incubated *in vitro* with prostaglandin $F_{2\alpha}$ which induced successful ovulation. In all cases, oocytes obtained from *in vivo* ovulation, *in vitro* ovulation by $PGF_{2\alpha}$, or manual dissection were fertilized to some extent.

These observations demonstrate that :

1. Fertilizable mature oocytes can be produced 4 to 6 weeks in advance of natural spawning by injection of 17 α-20 β P *in vivo* ;
2. Although ovulation can occur *in vivo*, or *in vitro* with $PGF_{2\alpha}$, the specific stimulus for ovulation is lacking in most of the fish injected with 17 α-20 β P only, and appears to some extent when a pituitary priming is given prior to 17 α-20 β P.

The possible involvement of gonadotropin in the synthesis and storage of some mediator (or its precursor) specific for the induction of ovulation is discussed in relation to the plasma gonadotropin level in the different groups of females.

Introduction.

Previous work on rainbow trout (Jalabert *et al.*, 1976) has already shown that 17 α-hydroxy-20 β-dihydroprogesterone (4 pregnen-17 α, 20 β diol-3 one ; abbreviation : 17 α-20 β P) is able to induce normal oocyte maturation, i. e. resumption of meiosis characterized by clearing of the yolk and germinal vesicle breakdown (GVBD), and ovulation when injected to females presenting oocytes with the germinal vesicle

(1) Present address : Laboratoire de Physiologie des Poissons, I. N. R. A. Université de Rennes-Baulieu, BP 25 A, 35031 Rennes Cedex, France.

(GV) in subperipheral position. But recent work in Coho salmon (*Oncorhynchus kisutch*) (Jalabert *et al.*, 1978) and in northern pike (*Exos lucius*) (de Montalembert, Jalabert and Bry, 1978) has shown that injection of 17 α-20 β P alone before GV peripheral migration results in oocyte maturation without ovulation. On the other hand, 17 α-20 β P is completely ineffective in carp (*Cyprinus carpio*) when administered alone, but induces successful maturation and ovulation in marginal temperature conditions when given after pituitary priming (Jalabert *et al.*, 1977).

The present experiment was undertaken in trout before peripheral migration of GV, 3 to 8 weeks in advance of expected natural ovulation. We tried to answer the following questions : — Does 17 α-20 β P induce oocyte maturation without ovulation when injected at these precocious stages, as it does in Coho salmon and in pike ? — What is the nature of the blockade between maturation and ovulation, if any ? — Using a low dose of pituitary extract, what is the effect of preliminary pituitary priming on further 17 α-20 β P action ?

Material and methods.

The experiment was carried out in December 1976 using 2-year old rainbow trout (*Salmo gairdneri*) weighing 400 to 600 g and kept in water of about 12 °C. Sixty-six females were chosen according to the state of maturity of a few oocytes ; the criterium used was that the oocytes be 3.5 to 4.5 mm in diameter and without apparent GV at the periphery. They were squeezed out by abdominal stripping after anesthesia in a 0.5 p. 100 aqueous solution of 2-(phenoxy) ethanol (Merck). As seen in control fish without handling, the experimental fish would have ovulated naturally 3 to 8 weeks later. They were separated into 4 groups and submitted to different treatments (table 1) on day 0 (beginning of the experiment) and on day 2 (2 days later). The dose was injected intraperitoneally and calculated in order to always introduce the same volume of vehicle (physiological saline, 1 ml/kg).

TABLE 1

Experimental treatments

Lot	No. of fish	Treatments on day 0	Treatments on day 2
1	15	Physiological saline	Physiological saline
2	17	TPE (0.5 mg/kg)	Physiological saline
3	16	17 α-20 β P(3 mg/kg)	17 α-20 β P(3 mg/kg)
4	17	TPE (0.5 mg/kg)	17 α-20 β P(3 mg/kg)

17 α-20 β P : 17 α-hydroxy-20 β dihydroprogesterone (4 pregnen-17 α, 20 β diol-3 one).

TPE : Trout pituitary extract from dry acetonic powder. 0.5 mg/kg is equivalent to 3.25×10^{-3} mg/kg of pure t-GTH measured by *in vitro* trout maturation assay.

Trout pituitary extract (TPE) is a crude preparation made by homogenizing, in a glass-teflon homogenizer, acetone-dried trout pituitary powder suspended in physiological saline and taken from females at peripheral GV stage. The dose injected (1 ml/kg) contains 3.25×10^{-3} mg/ml of pure t-GTH (Breton, Jalabert and Reinaud, 1976), as measured by *in vitro* trout maturation assay (Jalabert, Breton, and Billard, 1974) ; it represents 1/10th of the dose known to induce maturation and ovulation in submature fish. Pure 17 α-20 β P was prepared according to Fostier *et al.* (1973).

Before each injection and at varied intervals until day 15 after the first injection, the fish were anesthetized and submitted to ovarian and blood sampling. Blood samples of 0.3 ml were taken from a caudal vessel in the tail by puncture using a 1 ml syringe previously rinsed with an heparine solution (700 IU/ml). The plasma obtained after centrifugation was kept frozen until subsequent determination of trout gonadotropin (t-GTH) by radioimmunoassay according to Breton and Billard (1977). When treatment resulted in oocyte maturation (characterized by GVBD) without ovulation, the fish were killed after 15 days and the ovaries removed. Mature oocytes were then either dissected out of the follicle using watchmaker's forceps to tear off the follicular envelope, or incubated *in vitro* within the follicle during 48 hrs. at 10 °C in trout balanced salt solution (TBSS) (Jalabert, 1978) with or without prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$ 10^{-5} M, Upjohn Co., Kalamazoo, Michigan) to induce ovulation.

Matured oocytes obtained after normal ovulation *in vivo*, manual dissection, or after ovulation *in vitro* by $PGF_{2\alpha}$ were inseminated with diluted sperm (1/100) according to Billard *et al.* (1974) ; fertilizability was estimated from the proportion of eggs with apparently normal embryos after 10 days of development.

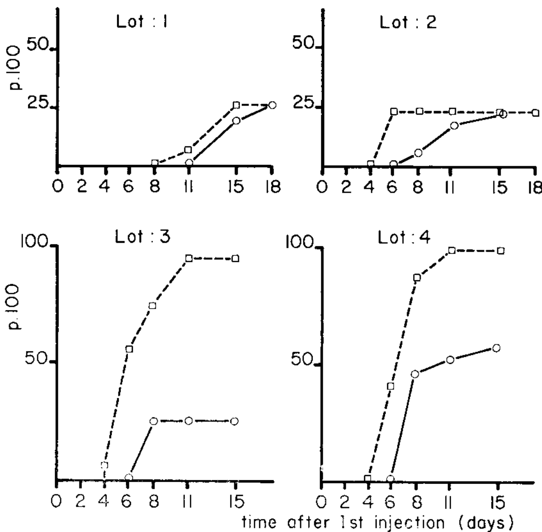

FIG. 1. — *p. 100 fish exhibiting complete oocyte maturation* (GVBD : ——□——) *or complete ovulation* (—— o ——) *in the 4 experimental groups.*

Lot 1, day 0, day 2 : physiological saline.
Lot 2, day 0 : TPE 0.5 mg/kg ; day 2 : saline.
Lot 3, day 0, day 2 : 17 α-20 β P 3 mg/kg.
Lot 4, day 0 : TPE 0.5 mg/kg ; day 2 : 17 α-20 β P 3 mg/kg.

Results.

Maturation and ovulation responses of fish in the different lots are shown on figure 1. 27 p. 100 of control fish (lot 1) mature and ovulate after 15 to 18 days ; in lot 2 (TPE priming only), roughly the same proportion of animals mature and ovulate, but are slightly precocious. In both cases, ovulation always follows maturation. In lot 3 (2 injections of 17 α-20 β P), apparently normal maturation occurs in all fishes within 6 to 11 days, but ovulation follows in only 25 p. 100. When priming treatment with TPE is given before 17 α-20 β P (lot 4), maturation still occurs in all fishes, but the proportion of those ovulating reaches 59 p. 100.

Plasma gonadotropin levels are presented in figure 2. In controls, basal levels are around 3.5 ng/ml at the beginning of the experiment and reach about 17 ng/ml in ovulating fish. In both lots 2 and 4, TPE priming injection induces a surge in plasma t-GTH up to 100 ng/ml, followed by a slow decrease. In lot 3 (2 injections of 17 α-20 β P), there is a small rise in gonadotropin beginning on day 6 and reaching 12 ng/ml on day 15.

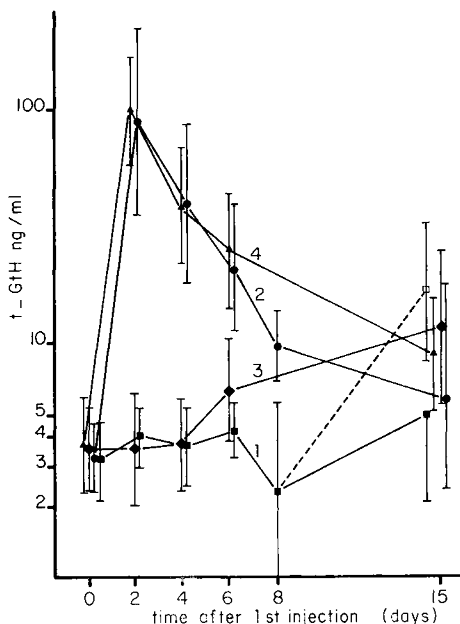

FIG. 2. — *Mean plasma gonadotropin level* (t-GTH) *in the 4 experimental groups.* Vertical bars show the standard deviation.

Symbols for the different groups : 1 : —■— (non-maturing controls) or □ (2 maturing and ovulating controls) ; 2 : —●— ; 3 : —◆— ; 4 : —▲—.

Table 2 shows the results of incubation *in vitro* of follicles from fish exhibiting oocyte maturation without ovulation and killed on day 15 (lots 3 and 4). Even though some spontaneous ovulation occurs, $PGF_{2\alpha}$ very significantly enhances the proportion of ovulation *in vitro*.

TABLE 2

In vitro *ovulation of follicles from fish exhibiting oocyte maturation without ovulation* (100 *follicles per fish incubated for* 48 *hrs at* 10 °C *in* TBSS *with or without prostaglandin* $PGF_{2\alpha}$, 10^{-5} M)

Lot	Treatment *in vivo*	No. of fish	Incubation of follicles *in vitro*		
			Treatment	Mean p. 100 ovulation	
3	17 α-20 β P/17 α-20 β P	7	control	8.5	**
			$PGF_{2\alpha}$	77.4	
4	TPE/17 α-20 β P	4	control	6.3	**
			$PGF_{2\alpha}$	74.1	

TABLE 3

Mean percent of embryonic development in eggs from in vivo *ovulation* (200 eggs per fish)

Lot	Treatment	Number of fish	Mean p. 100 of successful embryonic development
1	Phys. saline/Phys. saline	4	80.4
2	TPE/Phys. saline	4	93.2
3	17 α-20 β P/17 α-20 β P	4	90.9
4	Phys. saline/17 α-20 β P	10	87.5

TABLE 4

p. 100 *embryonic development in eggs inseminated either after manual dissection, or after* in vitro *ovulation by* $PGF_{2\alpha}$

Lot		Fish No.	Manual dissection		Ovulation *in vitro* by $PGF_{2\alpha}$	
No.	*in vivo* treatment		No. of eggs	p. 100 embryonic development	No. of eggs	p. 100 embryonic development
3	17 α-20 β P/17 α-20 β P	16	54	0	57	8.8
		21	53	75.5	20	33.3
		31	65	66.2	77	2.6
		36	23	0	48	4.4
		41	49	69.4	82	43.9
		66	55	43.6	79	5.1
		71	47	25.5	—	—
		74	50	0	78	1.3
4	TPE/17 α-20 β P	03	49	10.2	82	20.7
		23	45	4.4	49	2.0
		63	52	21.2	85	0
		81	53	9.4	16	0

Regarding fertilization data, eggs from *in vivo* ovulation (table 3) exhibit a normal amount of embryonic development without any significant difference between experimental and control groups. In eggs from matured but non-ovulated fish (table 4), inseminated after manual dissection or *in vitro* ovulation, the success of embryonic development is more irregular. Although a high percentage of development can be observed in the eggs of many fishes, particularly after manual dissection, eggs from some females give poor results and sometimes none. But comparison of data after manual dissection or after *in vitro* ovulation by $PGF_{2\alpha}$ shows that fertilization and development can occur in the eggs from any non-ovulated fish in groups 3 and 4. This fact is shown in the particular case of fish No. 71 (table 4) in which 50 p. 100 of the oocytes were found to remain in an immature state, while the others were fully matured. The latter, dissected out of the follicle, exhibited 25.5 p. 100 development after insemination.

Discussion.

Induction of oocyte maturation without ovulation after injection of steroid hormones has already been observed in the amphibian *Discoglossus pictus* by Alonso-Bedate *et al.* (1971) and in various fishes : *Misgurnus fossilis* (Kirshenblat, 1952) ; northern pike (de Montalembert, Jalabert and Bry, 1978).

The present experiment using 17 α-20 β P, which appears as the most likely mediator of oocyte maturation in trout (Jalabert, 1976), demonstrates that this steroid is also able to induce oocyte maturation well in advance of the natural process (3 to 8 weeks) and that these oocytes can be fertilized and develop normally until at least 10 days. However, this maturation is not necessarily followed by ovulation, in which case the mature oocytes to be inseminated must be removed from the follicular envelope by artificial means.

It must be underlined that the fishes chosen for the experiment were certainly heterogeneous as to expected time of natural spawning because of the absence of reliable criteria ; the follicle size only gives an approximation due to individual variations, and the non-peripheral position of the GV only indicates that natural maturation would normally occur more than 2 weeks later. This may explain why 25 p. 100 of the fish mature and also ovulate after treatment by 17 α-20 β P only, since this is the kind of response already found in females with oocytes at subperipheral GV stage (Jalabert *et al.*, 1976). Moreover, roughly the same proportion of fish ovulate at TPE priming only (lot 2), or spontaneously in controls after 15 to 18 days (lot 1). In fact, probably due to severe handling stress, these are in advance as compared with controls without such regular handling (anesthesia, blood and oocyte sampling). Thus, it can be assumed that a same proportion of fish in every group was advanced enough to mature and ovulate normally in response to 17 α-20 β P, TPE priming or handling stress.

The rate of embryonic development is normally high in eggs from normally ovulated fish, whatever the treatment ; this confirms that these females are probably closer to natural maturation, as discussed above. More surprising is the fact that embryonic development is always found in eggs of fish with mature but non-ovulated oocytes, some of them being probably very far from natural maturation. The discrepancy between data either after manual dissection or ovulation *in vitro* by $PGF_{2\alpha}$

emphasizes that the conditions were not optimum in either case for taking mature oocytes out of their follicle. In addition, mature oocytes were kept *in vivo* in the follicle much longer than they normally would be after natural maturation in order to make sure that ovulation was really blocked before killing the fish ; thus, some aging could have occurred within the follicle. Despite these unfavorable conditions, it remains that some embryonic development was always found in the eggs from all experimental females after 17 α-20 β P-induced maturation (lots 3 and 4). However, the success of embryonic development was evaluated by fixation in Stockard's solution as soon as 10 days after insemination, and it is not known if these embryos would have developed normally to hatching or further.

Another interesting point is that follicles containing mature oocytes, which do not ovulate spontaneously *in vivo* after *in vivo* 17 α-20 β P treatment, are able to contract and ovulate *in vitro* in response to $PGF_{2\alpha}$ as in naturally mature fish (Jalabert and Szöllösi, 1975). This implies that the lack of *in vivo* ovulation cannot be attributed to insufficient differentiation of the smooth muscle cells of the theca, but more probably to the absence of a specific mediator initiating follicle contraction, or of a precursor which should be synthesized and stored before 17 α-20 β P action. As preliminary priming with a low dose of TPE enhances the proportion of fish which ovulate in response to 17 α-20 β P treatment, it can be hypothesized that gonadotropin t-GTH is the pituitary factor which, at low doses, favors the synthesis and storage of such an ovulation mediator. This hypothesis coincides with the fact that 17 α-20 β P was able to induce maturation followed by successful ovulation when administered to fish at a later stage (subperipheral GV), characterized by plasma t-GTH levels of about 6 to 7 ng/ml (Jalabert *et al.*, 1976) as compared to 3 to 5 ng/ml at the beginning of the present experiment.

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Résumé. L'efficacité de la 17 α-hydoxy-20 β dihydroprogestérone (17 α-20 β P) seule ou après sensibilisation par un extrait hypophysaire a été testée chez des animaux dont le stade ovocytaire était antérieur à la migration périphérique de la vésicule germinative (V. G.) (4 à 8 semaines avant ovulation naturelle). Le traitement par la 17 α-20 β P (2 injections de 3 mg/kg à 2 jours d'intervalle) a induit la maturation ovocytaire (reprise de la méïose caractérisée par l'éclaircissement du vitellus et l'éclatement de la V. G.) chez 94 p. 100 des poissons, dont 25 p. 100 seulement ovulèrent normalement. Le traitement par la 17 α-20 β P (3 mg/kg) après sensibilisation hypophysaire (Extrait hypophysaire de Truite, 1 ml/kg, contenant l'équivalent de 3,25 $\times$ 10^{-3} mg/ml de gonadotropine de Truite, t-GtH) a induit la maturation ovocytaire chez tous les poissons, parmi lesquels 59 p. 100 ovulèrent. Dans les deux cas, les poissons chez lesquels l'ovulation ne se produisit pas normalement après maturation furent sacrifiés 15 jours après la 1re injection ; les follicules ovariens furent soit disséqués pour extraction des ovules mûrs, soit incubés *in vitro* en présence de prostaglandine $PGF_{2\alpha}$ (10^{-5} M) qui induisit l'ovulation avec succès. Les ovules mûrs récoltés après ovulation *in vivo* présentèrent une fécondabilité (estimée d'après le p. 100 de développements embryonnaires 10 jours après insémination) normalement élevée. Les ovules des animaux maturés mais non ovulés, récoltés après dissection manuelle ou ovulation *in vitro*, présentèrent une fécondabilité plus irrégulière, en fonction de la technique d'obtention, mais tous les animaux eurent des œufs fécondés.

Ces observations démontrent que :

1. Des ovules mûrs fécondables peuvent être produits 4 à 8 semaines en avance sur la fraie naturelle par l'injection de 17 α-20 β P.

2. Bien que l'ovulation puisse se produire (spontanément *in vivo* chez certains animaux, ou sous l'action de $PGF_{2\alpha}$ *in vitro* chez les autres), le stimulus spécifique de l'ovulation paraît faire défaut chez les poissons recevant la 17 α-20 β P seule, et réapparaît dans une certaine mesure lorsqu'une injection de sensibilisation par un extrait hypophysaire est administré avant la 17 α-20 β P.

L'implication possible de l'hormone gonadotrope t-GtH dans la synthèse et le stockage d'un médiateur (ou d'un précurseur) spécifique de l'induction de l'ovulation est discutée, en liaison avec les niveaux de gonadotropine plasmatique dans les différents groupes de femelles.

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