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Production of fertilizable oocytes from follicles of rainbow trout (*Salmo gairdnerii*) following *in vitro* maturation and ovulation

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Summary. Trout ovarian follicles were taken *in vivo* with oocytes showing a peripheral germinal vesicle (fig. 1 : stage 1°, before natural induction of maturation) and incubated at 10 °C with hormones to complete maturation. $PGF_{2\alpha}$ was added after 6, 7 or 8 days of incubation and ovulations were recorded 24 hours later. By this means, trout pituitary extract, pure trout gonadotropin t-GtH, or 17 α-hydroxy-20 β-dihydro-progesterone (17 α-20 β P) was equally effective for production of fertilizable oocytes after 7 + 1 days of incubation. After only 6-day maturation incubation, $PGF_{2\alpha}$ was already able to induce successful ovulation, but fertilizability was near zero ; after 8-day maturation incubation, responsiveness to $PGF_{2\alpha}$ was lost (tables 1, 2, 3). The dose of 17 α-20 β P required for further successful ovulation response to $PGF_{2\alpha}$ was more than 10 times higher than required for GVBD completion only (fig. 2), and a 24-hour exposure of follicles to 17 α-20 β P action at the beginning of incubation was more efficient than a 4-hour exposure or continuous exposure (table 4).

When follicles with oocytes already engaged in the process of maturation *in vivo* (incubation started at any time between stage 1^+ and GVBD) were transferred *in vitro*, addition of t-GtH or 17 α-20 β P enhanced further ovulation response to $PGF_{2\alpha}$.

These facts show that 17 α-20 β P, the most likely mediator of oocyte maturation in trout, is also able to induce follicle preparation to ovulation. But this preparation to ovulation needs higher doses and longer exposure to 17 α-20 β P action than oocyte maturation induction does ; this also implies longer exposure to high doses of t-GtH *in vivo*.

Fertilization of eggs produced in the above conditions shows that *in vitro* maturation and ovulation must be very similar to the natural processes *in vivo*.

In rainbow trout, intrafollicular maturation (resumption of meiosis) of oocyte with a peripheral germinal vesicle (PGV) can be triggered *in vitro* by fish pituitary extracts and some steroids (Jalabert, Breton and Bry, 1972 ; Jalabert *et al.*, 1973). This response was used as a bioassay for fish gonadotropin (Jalabert, Breton and Billard, 1974) and allowed purification of a specific gonadotropin, t-GtH (Breton, Jalabert and Reinaud, 1976). Among steroids, 17 α hydroxy-20 β dihydroxyprogesterone (4 pregnen-17 α, 20 β diol-3 one ; Abr. 17 α-20 βP) was shown to be the most potent steroid-inducer of trout oocyte maturation (Fostier, Jalabert and Terqui, 1973) and

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appeared as the most likely mediator produced by the follicular envelopes in response to t-GtH action (Jalabert, 1976).

On the other hand, ovulation (active expulsion of oocyte from the mature follicle) of trout follicles matured *in vivo* can be induced *in vitro* by $PGF_{2\alpha}$ (Jalabert and Szöllösi, 1975). But in the non-sterile incubation conditions employed so far, sufficient at 10 °C or 15 °C for studying maturation of the oocyte protected by follicular envelopes and the chorion, ovulation could not be triggered at the end of *in vitro* maturation, probably because of alteration of the functional integrity of follicular envelopes. On the contrary, Sakun and Gureeva-Preobrazhenskaya (1975) claim to have observed spontaneous ovulation *in vitro* in rainbow trout following simple treatment by pink salmon pituitary extract or progesterone.

The present work using long-term sterile incubation techniques was aimed at production of fertile eggs from follicles successively induced to mature and to ovulate by pure hormones in order to :

1) show that the process termed until now as maturation, is normal and non-abortive ;
2) understand the modification of hormone action so that ovulation can follow *in vitro* maturation ;
3) try to clarify the apparent contradictions between our data and observations by Sakun and Gureeva-Preobrazhenskaya (1975).

Materials and methods.

Ovaries were removed from 2-3 year old rainbow trout (*Salmo gairdnerii*) during the normal spawning season from November to February. Females were chosen according to the state of maturity of a few oocytes squeezed out by abdominal stripping. The different maturation stages selected for the experiments are schematically shown

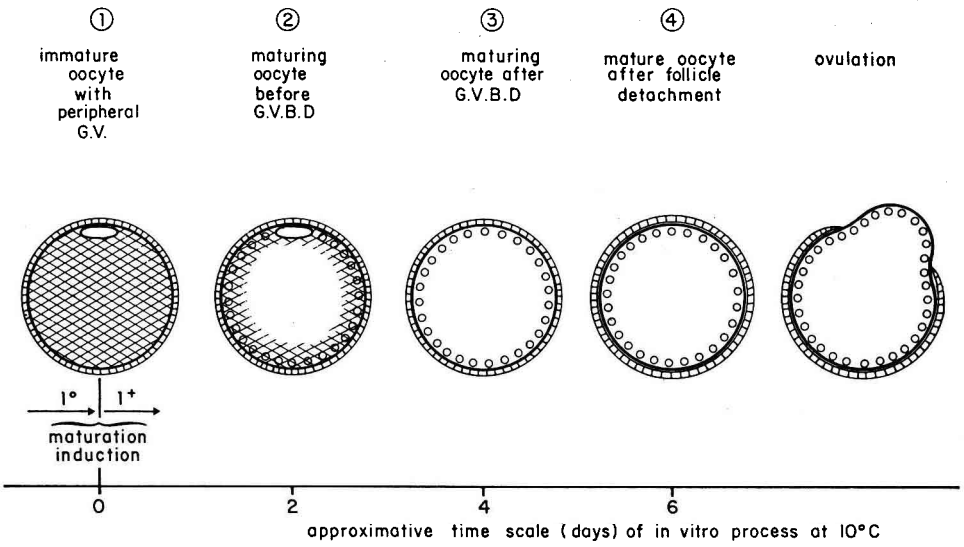

FIG. 1. — *Schematic representation o f the different stages of oocyte maturation as function of time at* 10 °C in vitro. G. V. : germinal vesicle ; GVBD : Germinal Vesicle Breakdown.

on figure 1. Stage 1 (peripheral germinal vesicle, opaque yolk) has been subdivided into two parts which can be distinguished only *a posteriori* from the evolution of control follicles during incubation : follicles at stage 1° never mature *in vitro* without hormone addition ; follicles at stage 1+ are able to complete germinal vesicle breakdown (GVBD) *in vitro* without hormone and are supposed to be taken just after *in vivo* natural gonadotropic induction of maturation but before any morphological change becomes obvious in yolk structure.

Incubations were carried out at 10 °C under an atmosphere of 1 p. 100 CO_2 — 49 p. 100 O_2 — 50 p. 100 N_2 in Trout balanced solution (TBSS) buffered around pH 8 with $NaHCO_3$ (NaCl 7.51 g/l ; KCl 0.23 g/l ; $MgSO_4$ (7 H_2O) 0.07 g/l ; $MgCl_2$(6 H_2O) 0.20 g/l ; $CaCl_2$ 0.50 g/l ; $NaHCO_3$ 2.055 g/l ; glucose 1 g).

Trout pituitary extract (TPE) and purified trout gonadotropin (t-GtH) were prepared according to Breton, Jalabert and Reinaud (1976). 4 Pregnen-17 α, 20 β diol-3 one (17 α-20 βP) was prepared from 17 α hydroxyprogesterone and purified as described in Jalabert *et al.* (1977). Those hormones were added in TBSS at the beginning of incubation at doses known to induce 100 p. 100 maturation (TPE : 5.10^3 ng/ml ; t-GtH : 3.10^2 ng/ml ; 17 α-20 βP : 3.10^{-6} M) unless otherwise mentioned in fig. and tables.

The ability of follicles to ovulate was checked at the end of incubation 24 hrs after addition of prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$: 10^{-5} M) from the Upjohn Co. (Kalamazoo, Michigan).

After artificial insemination with diluted sperm (1/100) according to the technique described by Billard *et al.* (1974), fertilizability of ovulated oocytes was quantified from the proportion of eggs with normal embryo after 10 days of development.

TABLE 1

Incubation for 7 + 1 days from stage 1° (germinal vesicle peripheral)

Female Nr.	Number of follicles	Incubation for maturation (7 days)			p. 100 ovulation 1 day after addition of $PGF_{2\alpha}$
		Treatment	p. 100 degenerating oocytes	p. 100 maturation	
50	196	Control	25,0 **	0	0
	194	TPE	7,7	92,3	31,3
	197	t-GtH	6,6	93,4	37,0
	175	17 α-20 βP	6,9	93,1	10,4
51	200	Control	10,5	0	0
	198	TPE	1,5 *	98,5	39,0
	190	t-GtH	7,9	92,1	33,7
	170	17 α-20 βP	7,1	92,9	43,7
46	168	Control	10,7	0	0
	166	t-GtH	16,3	83,9	6,5
	168	17 α-20 βP	3,0 **	97,0	92,6
47	203	Control	24,6	0	0
	204	17 α-20 βP	9,8 **	90,2	61,4

Statistical analysis of results calculated as per cent of response (p) in the same number of follicles (usually 200) was carried out by analysis of variance after transformation arc sin $\sqrt{\frac{p}{100}}$.

Results.

Maturation and ovulation responses of follicles from 4 different females incubated *in vitro* from stage 1° are shown on table 1. Control follicles without hormones never matured or ovulated, but exhibited a proportion of degenerating oocytes significantly higher than hormone-treated groups in which all other oocytes matured. Either TPE t-GtH or 17 α-20 βP seems to be able to prepare for a positive ovulatory response to $PGF_{2\alpha}$; while some differences appear in the relative efficiency of the different treatments in individual females, no overall tendency is found.

TABLE 2

Mean p. 100 of ovulation observed 1 day after addition of $PGF_{2\alpha}$ *following incubation with hormones for 6, 7 or 8 days (control follicles never mature or ovulate).*
The number of females and the number of follicles per female from which mean ovulation percentage is calculated are shown in brackets

Hormonal treatment	Duration of incubation prior to addition of $PGF_{2\alpha}$		
	6	7	8
Trout pituitary extract..	No assay	33,6 (2 × 200)	No assay
t-GtH	10,0 (2 × 200)	24,6 (3 × 200)	4,8 (2 × 200)
17 α-20 βP	45,8 (3 × 200)	48,8 (4 × 200)	3,8 (2 × 200)

Table 2 shows that the ability of the follicle to respond to $PGF_{2\alpha}$ is already acquired after 6 days of incubation and decreases by 8 days. On the other hand, fertilizability is close to zero after 6 + 1 days of incubation and appears quite normal compared to *in vivo*-ovulated oocytes after incubation for 7 + 1 days (table 3).

The relationship between optimal dose of 17 α-20 βP for successful maturation (GVBD) and optimal dose for subsequent successful ovulation using $PGF_{2\alpha}$ has been investigated in 2 females (fig. 2). In female 1, the Median efficient dose (MED) of 17 α-20 βP to prepare for ovulation is around 10 times higher than the MED for maturation alone ($\simeq 380.10^{-9}$ M against $\simeq 30.10^{-9}$ M), and around 30 times higher in female 2.

TABLE 3

Mean p. 100 of embryonic development in eggs fertilized after in vitro *maturation from stage* 1°, *and* PGF_2*-induced ovulation.*
The number of inseminated eggs from 3 different females is shown in brackets

Maturation treatment	Duration of incubation prior to addition of $PGF_{2\alpha}$ (days)	
	6	7
TPE	No assay	86,4(132)
t-GtH	0(28)	89,8(127)
17 α-20 βP	0,85(1 049)	79,1(96)

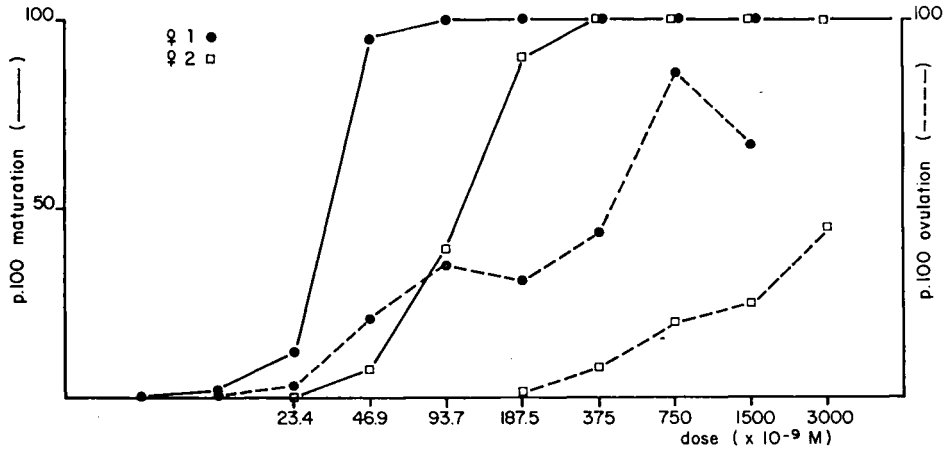

FIG. 2. — *Relationship between dose of* 17α-20 βP (*continuous exposure* in vitro *from stage* 1°), p. 100 *of oocyte maturation after 7-day incubation and* p. 100 *of ovulation* 24 hrs *after* $PGF_{2\alpha}$ *addition in two different females.* Each value was obtained on 75 follicles.

TABLE 4

p. 100 ovulation induced by $PGF_{2\alpha}$ *after 7-day incubation from stage* 1° *as a function of exposure time to* 17 α-20 βP *at the beginning of incubation*
(around 200 follicles per treatment and per female. Follicles are from 3 different females)

Duration of exposure to 17 α-20 βP	Female Nr.		
	38	43	47
4 hrs	57,6	54,8	51,5
24 hrs	65,7	91,8	80,1
Permanent	55,2	68,9	61,4

At the beginning of incubation, follicles were exposed to 17 α-20 βP for different durations ; ovulation response is shown in table 4. The ovulation rate after 7-day incubation is significantly higher when follicles were exposed to 17 α-20 βP for 24 hrs rather than 4 hours only or continuously.

When follicles were incubated from stage 1+ after *in vivo* induction of maturation (fig. 1, table 5), some of the controls without hormone were able to ovulate after $PGF_{2\alpha}$, but t-GtH or 17 α 20 βP treatment during incubation greatly enhances ovulation response to $PGF_{2\alpha}$.

TABLE 5

p. 100 ovulation following addition of $PGF_{2\alpha}$ *after 5 days incubation of follicles taken in vivo with oocytes at stage 1+ and incubated with or without hormones*
(all control and experimental oocytes from this stage reach GVBD without any hormone addition).
Each value is obtained on 200 follicles

Treatment during maturation (5 days)	Ovulation treatment (1 day)	Female		
		Nr 33	Nr 34	Nr 48
Control	Control ..	0	0	0
	$PGF_{2\alpha}$	25	28	4
t-GtH	Control ..	0	0	0
	$PGF_{2\alpha}$	72 **	38 *	82 **
17 α-20 βP	Control ..	0	0	0
	$PGF_{2\alpha}$	78 **	42 **	95 **

TABLE 6

p. 100 ovulation following addition of $PGF_{2\alpha}$ *after 4 days incubation of follicles taken in vivo with oocytes at stage 2 and incubated with or without hormones.*
Each value is obtained on 200 follicles

Treatment during maturation (4 days)	Ovulation treatment (1 day)	Female		
		Nr 48	Nr 44	Nr 39
Control	Control ..	0	0	0
	$PGF_{2\alpha}$	45	2,2	25
t-GtH	Control ..	10	0	No assay
	$PGF_{2\alpha}$	55	56 **	No assay
17 α-20 βP	Control ..	6	3,5	3
	$PGF_{2\alpha}$	70 **	43 **	45 **

When incubation started from stage 2 (fig. 1, table 6), the same favorable effect of t-GtH or 17 α-20 βP upon ovulation preparation is seen. Moreover, following these treatments, some spontaneous ovulations occurred even without $PGF_{2\alpha}$ addition.

Finally, when incubation was started with stage 3 follicles after *in vivo* GVBD (fig. 1, table 7), ovulation rate after $PGF_{2\alpha}$ was equally high in control or in hormone-treated follicles so that no additional hormonal action was required any more for ovulation preparation.

TABLE 7

p. 100 ovulation following addition of $PGF_{2\alpha}$ *after 4 days incubation of follicles taken in vivo with oocytes at stage 3 and incubated with or without hormones.*
Each value is obtained on 200 follicles

Treatment during incubation (3 days)	Ovulation treatment	Female	
		Nr 39	Nr 45
Control	Control ..	21	6
	$PGF_{2\alpha}$	83	88
t-GtH	Control ..	No assay	5
	$PGF_{2\alpha}$	No assay	86
17 α-20 βP	Control ..	19	4
	$PGF_{2\alpha}$	79	82

Discussion.

The main point demonstrated here is that fertile trout eggs can be produced from follicles induced to mature and to ovulate entirely *in vitro* by successive hormonal treatments. Thus, the maturation processes induced *in vitro* are necessarily very similar to normal processes *in vivo*. They can be triggered by a crude trout pituitary extract, by t-GtH (a trout gonadotropin purified in trout oocyte maturation assay) or by 17 α-20 βP, known to be the most likely steroid mediator of oocyte maturation in trout (Jalabert, 1976) produced by folliclar envelopes in response to t-GtH stimulation.

The observation that success of embryonic development is much better when ovulation is induced after 7-day incubation rather than after only 6-day incubation shows that acquisition of developmental capacity occurred later than GVBD (3-4 days), as already shown by Iwamatsu (1965) in Medaka. However, the delay is much longer in trout due to the lower temperature. In trout, $PGF_{2\alpha}$ appears able to artificially induce ovulation before developmental capacity is acquired. On the other hand, the follicular envelopes seem to lose their ability to respond to $PGF_{2\alpha}$ stimulation after 8-day incubation, and fertilizability of those oocytes was not assayed.

Incubation started at different stages of *in vivo* maturation demonstrated that t-GtH or 17 α-20 βP action is required until stage 3 (after GVBD) to prepare for ovulation. This fact has two main implications regarding the mode of hormonal

action during follicle maturation (both oocyte maturation and preparation of follicular envelopes for ovulation) :

1) 17 α-20 βP action must be continuous or renewed until stage 3. This assumption is in good agreement with the fact that a 24-hr exposure to 17 α-20 βP is more efficient than a 4-hr exposure for stage 1° follicles, but it is in apparent contradiction with the fact that 24-hr exposure is more efficient than continuous exposure. A main criticism of our « continuous exposure experiment » is that 17 α-20 βP was added to the incubation medium only once at the beginning of incubation, and was not renewed : 17 α-20 βP might be metabolized by the follicle and eventually transformed into more or less inhibitory derivatives. This was shown to be the case for progesterone in *Xenopus* oocyte maturation (Ozon *et al.*, 1975). However, even a 24-hour optimum exposure is very different from the few minutes of exposure to the same dose of 17 α-20 βP required for subsequent successful GVBD in oocytes (Jalabert, 1976). Thus, the mode of 17 α-20 βP action in preparing the follicle for ovulation, supposedly by stimulation of enzymatic processes promoting detachment between oocyte and granulosa cells (Jalabert and Szöllösi, 1975 ; Jalabert, 1976), is probably completely different from oocyte maturation induction. On the other hand, the doses of 17 α-20 βP required for ovulation preparation are much higher than those for oocyte maturation, which might imply a non-specific action in preparation for ovulation. However, recently Campbell *et al.* (in preparation) have identified 17 α-20 βP in trout plasma during maturation at concentrations of the same order of magnitude.

2) t-GtH action must also be continuous or renewed as it exhibits the same positive action as 17 α-20 βP on ovulation success of follicles taken after *in vivo*-induced maturation. This induction is supposed to be triggered first by a rise in t-GtH secretion. However, from our experiments, it seems obvious that the follicular tissue does not then acquire an inertia for the secretion of the necessary amount of 17 α-20 βP required for ovulation, but needs persistant stimulation by high levels of t-GtH. This is in good agreement with *in vivo* observations by Jalabert *et al.* (1976) showing a progressive rise in mean level of plasma t-GtH during *in vivo* natural maturation from stage 1 to stages 2 and 3.

The main contradiction between our data and those of Sakun and Gureeva-Preobrazhenskaya (1975) remains. Despite the lack of exact information concerning the yield of spontaneous ovulations observed *in vitro* by those authors, such spontaneous ovulations after incubation from stage 1 rarely occurred in our incubation conditions, and $PGF_{2\alpha}$ action was always necessary to induce a significant number of ovulations after maturation. Moreover, follicles taken *in vivo* at stage 3 a short time before normal ovulation do not ovulate *in vitro* in the absence of $PGF_{2\alpha}$ or Epinephrine stimulation (Jalabert and Szöllösi, 1975 ; Jalabert, 1976), even when treated with high levels of pituitary extracts. The difference in the nature of the incubation medium is probably not a good explanation, as Goetz (1976) using the same medium as Sakun *et al.* (Cortland's solution) did not observe spontaneous ovulation after complete *in vitro* maturation of brook trout follicles. Some other differences in methodology could be responsible for the different ovulation results. Sakun *et al.* used heavy doses of progesterone, 50 times higher than our routine dose of 17 α-20 βP, and pink salmon pituitary extract ; they renewed the incubation medium after 48 hrs. This could have eliminated any inhibitory metabolism products.

Two hypotheses thus remain ; one is that trout pituitary extract, t-GtH and 17 α-20 βP, although specific for induction of normal oocyte maturation, are non-specific sources for induction of subsequent ovulation and thus need to be assisted by extra-physiological $PGF_{2\alpha}$. The other hypothesis is that our methodology dissociates and reveals some physiological features like $PGF_{2\alpha}$ involvement in trout ovulation, involvement which has also been hypothesized in goldfish ovulation by different methods (Stacey and Pandey, 1975).

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Résumé. Des follicules ovariens de Truite arc-en-ciel présentant des ovocytes au stade de la vésicule germinative périphérique (fig. 1, stade 1° avant induction naturelle de la maturation) ont été incubés à 10 °C en présence d'hormones destinées à induire la maturation. L'ovulation a été induite par PGF 2 α ajoutée au milieu pendant 24 h, après 6, 7 et 8 jours d'incubation. Dans ces conditions, l'extrait hypophysaire de Truite, la gonadotropine pure t-GtH ou la 17 α hydroxy-20 β dihydroprogestérone (17 α-20 βP) se sont révélées aptes à produire des ovocytes fécondables après 7 + 1 jours d'incubation. Après seulement 6 jours d'incubation, l'ovulation en réponse à l'action de PGF 2 α est déjà possible, mais la fécondabilité est quasi nulle. Après 8 jours, la sensibilité à PGF 2 α diminue (tabl. 1, 2, 3). La dose de 17 α-20 βP nécessaire à l'ovulation ultérieure sous l'action de PGF 2 α est plus de 10 fois supérieure à celle qui serait nécessaire à la seule maturation ovocytaire (fig. 2). Une exposition de 24 h en début d'incubation à l'action de la 17 α-20 βP est plus efficace qu'une exposition de 4 h ou continue (tabl. 4).

Lorsque des follicules sont placés en incubation alors que la maturation ovocytaire est déjà engagée *in vivo* (à n'importe quel moment depuis le stade 1+ jusqu'à l'éclatement de la vésicule germinative ; voir fig. 1), l'action de t-GtH ou de la 17 α-20 βP augmente le pourcentage d'ovulations induites par PGF 2 α.

Ces faits démontrent que la 17 α-20 βP qui est le médiateur le plus vraisemblable de la maturation ovocytaire chez la Truite peut aussi induire la préparation du follicule à l'ovulation ; celle-ci requiert cependant des doses et des temps d'action supérieurs à ceux strictement nécessaires à l'induction de la maturation ovocytaire, ce qui implique aussi que l'action de t-GtH *in vivo* doit s'effectuer à un niveau et avec une durée supérieurs.

La fécondation des œufs produits dans ces conditions démontre que les processus de maturation et d'ovulation contrôlés *in vitro* doivent être très voisins des processus naturels.

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