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Hydration of rainbow trout oocyte during meiotic maturation and *in vitro* regulation by 17,20β-dihydroxy-4-pregnen-3-one and cortisol

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Summary

Although oocytes of many teleost fish, especially marine species, are subjected to a hydration process during meiotic maturation, which leads to an important volume increase, no noticeable hydration of the preovulatory oocyte has ever been reported in rainbow trout (*Oncorhynchus mykiss*). In the present study, oocyte water content and dry mass were monitored using consecutive samples taken *in vivo* from the same female rainbow trout, from 4–5 days prior to ovulation to up to 7 days post-ovulation. In addition, yolk protein electrophoretic patterns were compared between oocytes sampled prior to germinal vesicle breakdown (GVBD) and unfertilized eggs. Furthermore, the effect of the maturation-inducing steroid (17,20β-dihydroxy-4-pregnen-3-one, 17,20β-P), cortisol and 11-deoxycorticosterone (DOC) on oocyte dry and wet masses, as well as GVBD occurrence was assessed *in vitro*. Finally, mRNA expression profiles of glucocorticoid and mineralocorticoid receptors as well as 11β-hydroxysteroid dehydrogenase (11β-HSD) were monitored in the periovulatory ovary by real-time PCR. Both *in vivo* and *in vitro* data showed, for the first time in rainbow trout, that a significant oocyte hydration occurs during oocyte maturation. In addition, an intra-oocyte dry matter increase was reported *in vivo* during the periovulatory period. However, yolk protein migration patterns were similar in preGVBD oocytes and unfertilized eggs, suggesting that no or little yolk proteolysis occurs during oocyte maturation. We also showed that oocyte hydration can be induced *in vitro* by 17,20β-P and cortisol but not by DOC. In contrast, GVBD was only observed after 17,20β-P stimulation. Finally, real-time PCR analysis showed an up-regulation of 11β-HSD and glucocorticoid receptor 2 transcripts in the ovary at the time of oocyte maturation. Together, these results suggest that cortisol could participate in the control of oocyte hydration and possibly in other periovulatory ovarian functions.

Key words: trout, *Oncorhynchus mykiss*, oocyte, meiotic maturation, hydration, cortisol.

Introduction

In marine teleosts, one of the main features of oocyte maturation (meiosis resumption) is a pronounced volume increase caused by hydration. Oocyte swelling due to water entry may reach about two- to fivefold the initial volume (Greeley et al., 1986; Selman and Wallace, 1983; Watanabe and Kuo, 1986). This results in the adjustment of the buoyancy of ovulated eggs either to pelagic or to benthic characteristics, depending on the species (Finn et al., 2002b; Finn et al., 2002a; Fyhn et al., 1999). Hydration appears to be induced by the combination of two different biological processes leading to a transient increase of yolk osmolarity. It was shown that lipoproteic yolk is deeply reorganized (Carnevali et al., 1992; Iwamatsu et al., 1992; Matsubara et al., 1999) under the action of proteolytic enzymes such as cathepsins (Carnevali et al., 1999). In fact, proteolytic liberation of free amino acids was reported in several species during oocyte maturation (Finn et al., 2002a; Fyhn et al., 1999). In addition, an uptake of inorganic ions also occurs during oocyte maturation (Craik and Harvey, 1987; Finn et al., 2002a; Greeley et al., 1991; LaFleur and Thomas, 1991; Wallace et al., 1992; Watanabe and Kuo, 1986). In freshwater species, changes in egg size or osmolality during maturation have been studied in several species. A limited oocyte volume increase was reported during the periovulatory period in *Barbus tetrazona* and *Rasbora trilineata*, but not in *Cichlasoma nigrofasciatum*, goldfish (*Carassius auratus*), *Gymnocorymbus ternetzi* and powan (*Coregonus lavaretus*) (Greeley et al., 1986). In rainbow trout, data obtained from a limited number of females sacrificed either before or after oocyte maturation suggested the absence of hydration (Craik and Harvey, 1984). It is therefore unclear if oocyte hydration actually occurs or not during the preovulatory period of some freshwater species, including rainbow trout.

In teleost species, oocyte meiotic maturation is triggered by a maturation-inducing steroid (MIS) produced by ovarian follicles in response to a gonadotropic stimulation. In rainbow trout, 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) is the natural MIS (Fostier et al., 1973). It is possible that 17,20β-P is also involved in the control of some oocyte hydration possibly occurring concomitantly with meiosis resumption in trout, since this progestin is known to promote both oocyte hydration and maturation *in vitro* in the marine species *Fundulus heteroclitus* (Wallace et al., 1992). However, it is also known that corticosteroids are present during oocyte maturation in salmonid fish (Campbell et al., 1980). Although their putative roles remain unclear, DOC (4-pregnen-21-ol-3, 20-dione, or 11-deoxycorticosterone) is strongly detected in the blood of rainbow trout during oocyte maturation (Campbell et al., 1980) and cortisol is detected during the periovulatory period in rainbow trout even though maximum levels are not reached until ovulation (Bry, 1985). High concentration of DOC can promote germinal vesicle breakdown (GVBD) of follicle-enclosed oocytes in brook trout (*Salvelinus fontinalis*) (Duffey and Goetz, 1980), but not in rainbow trout (Jalabert et al., 1972). Similarly, cortisol alone is unable to trigger intrafollicular oocyte maturation but it can enhance the action of gonadotropin and steroids on the induction of GVBD in rainbow trout (Jalabert, 1975). Besides, injections of high doses of cortisol have been reported to promote ovulation and ovarian tissue hydration associated with an increase of sodium content in the ayu (*Plecoglossus altivelis*) (Hirose et al., 1974) and in the cichlid *Tilapia nilotica* (Babiker and Ibrahim, 1979). Finally, a rise of 11β-hydroxylase mRNA levels, an enzyme controlling cortisol synthesis, was observed in the rainbow trout ovary during oocyte maturation (Bobe et al., 2004). Thus, it is also possible that not only cortisol but also DOC are involved in the control of some oocyte hydration in rainbow trout.

Finally, little is known about corticosteroid receptivity in the fish preovulatory ovary. Most physiological effects of corticosteroids are mediated through binding to nuclear receptors acting as ligand-dependent transcription factors. In trout, cortisol binds to two different glucocorticoid receptors: rtGR1 and rtGR2 (Bury et al., 2003; Ducouret et al., 1995). In addition, a mineralocorticoid receptor (rtMR) with a high affinity for cortisol was isolated in rainbow trout (Colombe et al., 2000). Moreover, DOC has been shown to be a potent agonist for this receptor, enhancing transcriptional activity of rtMR at lower concentrations than other corticosteroids (Sturm et al., 2005). Interestingly enough, an 11β-hydroxysteroid dehydrogenase (11β-HSD) cDNA was cloned and characterized in rainbow trout. Northern blot and in situ hybridization analysis showed that 11β-HSD gene expression is observed in many tissues including the ovarian follicle. This enzyme is homologous to mammalian 11β-HSD2 which metabolizes cortisol into cortisone, which is inactive on MR subsequently allowing aldosterone accessing to this receptor (Kusakabe et al., 2003). In fish, a similar mechanism may occur and allow a ligand less abundant than cortisol, such as DOC, to activate rtMR. Thus, the presence of 11β-HSD gene expression in ovarian follicle raises the question of whether potential oocyte hydration during maturation in trout could be regulated through DOC or cortisol activation of rtMR.

Therefore, the present investigation aimed to: (1) measure *in vivo* oocyte hydration during the natural oocyte maturation process in order to ascertain whether this phenomenon occurs in rainbow trout; (2) describe eletrophoretic patterns of yolk proteins before and after oocyte maturation; (3) determine whether hydration could be induced *in vitro* to the same extent by 17,20β-P, cortisol and DOC; (4) assess the possible implication of glucocorticoid and mineralocorticoid receptors in the process by measuring their mRNA levels in the ovary.

Materials and methods

Animals and tissue collection

Investigations and animal care were conducted according to the guiding principles for the use and care of laboratory animals and in compliance with French and European regulations on animal welfare. Late-vitellogenic rainbow trout (*Oncorhynchus mykiss*) were obtained during their first reproductive season from INRA/SEDI fish farm (Sizun, France) and were maintained under natural photoperiod in a recirculated water system at 12°C in INRA experimental facilities (Rennes, France).

For *in vivo* experiments, follicle and egg samples were collected by gentle manual stripping performed under anesthesia (2-phenoxyethanol, 0.05%). *In vitro* experiments on oocyte hydration were conducted using preGVBD oocytes (see below), incubated within surrounding follicular layers. Fish were over-anesthetized in 2-phenoxyethanol, killed by a blow on the head and bled by gill arch section. Ovaries were then dissected out of the body cavity under sterile conditions.

In vivo monitoring of oocyte mass

After manual stripping, the developmental stage of oocytes was assessed under a binocular microscope according to previously described criteria (Jalabert et al., 1978; Jalabert and Fostier, 1984).

Prior to germinal vesicle breakdown (preGVBD): subperipheral or peripheral germinal vesicle before any noticeable morphological changes in yolk structure due to the process of meiosis resumption; MAT: during oocyte maturation, i.e. after yolk clarification and around the time of GVBD; OV: ovulated eggs are present in the body cavity.

Four females were stripped daily from preGVBD until ovulation, or one week after ovulation (for three females), to obtain about 20–30 follicles or eggs from each one. Non-ovulated oocytes were mechanically expelled from their follicle by gentle manual pressure as previously described (Finet et al., 1988). To prevent air desiccation, defolliculated oocytes were then rapidly weighed individually for determination of wet mass (WM). Dry mass (DM), was measured after heating defolliculated oocytes for 24 h at 105°C

in a drying oven. Oocyte water content (WC) was calculated by subtracting DM from WM.

In vitro oocyte maturation and hormonal treatments

For each female assayed, clusters of one to four follicles were dissected out from the ovary, washed in incubation medium (IM8/300: 133 mmol l^{-1} NaCl, 3.09 mmol l^{-1} KCl, 0.28 mmol l^{-1} $MgSO_4$, 2.1 mmol l^{-1} $MgCl_2$, 4.5 mmol l^{-1} $CaCl_2$, 5.6 mmol l^{-1} glucose, 20 mmol l^{-1} Hepes, pH 8.0, 300 mOsm) and subsequently incubated at 12°C under gentle agitation (50 r.p.m.) in six-well culture plates (Corning, Schiphol-Rijk, The Netherlands) at a ratio of 25 follicles per 3 ml of IM8/300. After a 3 h pre-incubation step, each group of 25 follicles was exposed to a steroid treatment added in 5 μl ethanol vehicle. For each female, a group treated with ethanol alone (5 μl) was used as a 'negative control'. In a first experiment, performed using the follicles of seven different females, the following treatments were assayed: MIS (17,20β-P: 40 ng ml^{-1}), DOC (5 ng ml^{-1}), cortisol (12 ng ml^{-1}), cortisol (12 ng ml^{-1}) + MIS (40 ng ml^{-1}). In a second experiment, performed using the follicles of five different females, cortisol and DOC were assayed at three concentrations: 5, 12 and 50 ng ml^{-1}, in combination or not with MIS (40 ng ml^{-1}).

After a 60 h incubation and for each experimental treatment, 20 oocytes out of 25 were defolliculated. After individual wet mass (WM) measurement, oocytes were incubated in a drying oven for 24 h and subsequently weighed (dry mass; DM). For each female, a group of 20 oocytes, sampled before *in vitro* incubation to measure wet and dry masses (WM_0 and DM_0, respectively), was used as 'initial control'. In order to analyze data originating from different females, WM/WM_0 and DM/DM_0 ratios were calculated. In addition, the effect of each treatment on oocyte maturation was evaluated by monitoring GVBD, at 60 h, in each group.

Total RNA extraction and reverse transcription

Expression profiles were studied in the preovulatory ovary at the following ovarian stage: late vitellogenesis (LV, six females, approximately 3–4 weeks before expected ovulation); prior to germinal vesicle breakdown (preGVBD, eight females); during oocyte maturation (MAT, six females). Aliquots of ovaries were frozen in liquid nitrogen and stored at –80°C until RNA extraction. Total RNA was extracted from whole ovarian tissue using TRIzol reagent (Invitrogen, Cergy Pontoise, France) at a ratio of 1 ml per 100 mg of ovarian tissue. RNA concentration was estimated on the basis of absorbance at 260 nm and samples were diluted to a final concentration of 0.25 μg μl^{-1}.

Reverse transcription was performed using 2 μg of total RNA (in a volume of 8 μl) according to the following procedure: RNA was incubated in 25 μl of a RT reaction mixture: 5 μl of 5×RT buffer, 1.25 μl of dNTPs (Promega, Madison, WI, USA; 10 mmol l^{-1}), 2 μ1 of random primers (Promega; 500 μg ml^{-1}), 0.6 μl RNasin (Promega; RNase inhibitor, 40 U μl^{-1}), 1.25 μl of MMLV reverse transcriptase (Promega; 200 U μl^{-1}) and 6.9 μl of RNase-free water. Briefly, RNA and dNTPs were denatured for 5 min at 70°C and then chilled on ice for 5 min before the reverse transcription master mix was added. After a 10-min step at 30°C, reverse transcription was performed at 37°C for 60 min. Reverse transcriptase was inactivated by heating samples at 95°C for 10 min. cDNAs were then stored at –20°C until real-time PCR analysis.

Real-time PCR

Real-time PCR was performed using an iCycler iQ™ (Bio-Rad Laboratories, Hercules, CA, USA) thermocycler. Reverse transcription products were diluted to 1/50 and 5 μl of diluted RT products were used for each PCR reaction. Real-time PCR analysis was performed in triplicate using a SYBR Green PCR Master Mix (Eurogentec, Seraing, Belgium) in a total volume of 20 μl per PCR well and using 600 nmol l^{-1} of primers. After a 10 min incubation step at 95°C, 40 cycles of PCR were performed. Amplification parameters were as follows: each cycle consisted in denaturation at 95°C for 15 s and annealing/extension at 60°C for 40 s. CT (cycle threshold) values correspond to the number of cycles at which the fluorescent signal monitored in real-time is detected above threshold. A standard curve generated using serial dilutions of a pool of reverse transcribed ovarian RNA was used to calculate, using the I-cycler IQ software, the relative abundance of target cDNA within analyzed samples. This curve was also used to assess PCR efficiency. A melting curve analysis was performed to check that a single PCR product was generated. In order to do so, the following protocol was performed after the initial PCR program: 10 s holding followed by a 0.5°C increase from 55°C to 95°C. Elongation factor 1α (EF1α) and ribosomal 18S levels were monitored and used as internal standards. Primer sequences, GenBank accession number of the target gene and PCR product sizes are presented in Table 1. Negative controls were performed by omitting cDNA from the real-time PCR reaction in order to check for genomic DNA contamination.

Yolk protein electrophoresis patterns

Yolk proteins were isolated from preGVBD full-grown oocytes and unfertilized eggs (ovulated oocytes) using a protocol adapted from a previously described method (Shahsavarani et al., 2002). Oocyte chorion was removed using a pair of forceps and the combined yolk and cytoplasmic proteins were placed in a microcentrifuge vial with 2 ml of 50 mmol l^{-1} imidazol and 50 mmol l^{-1} potassium chloride (KCl) ice-cold buffer (pH 8.0). Samples were vortexed to ensure that yolk was completely dissolved in the buffer. Samples were then centrifuged (4°C) for 5 min at 12 000 ***g***. At this step, cytoplasmic proteins were pelleted at the bottom of the tube while yolk proteins remained in the supernatant. Yolk proteins concentration was determined using a Coomassie protein reagent (Interchim, Montluçon, France).

SDS–PAGE was performed using eight preGVBD oocyte samples and eight egg samples. Owing to the very high concentration of yolk proteins, yolk extract samples were

Table 1. *Nucleotide sequence of real-time PCR primers, GenBank accession number, and PCR product size for target genes*

Gene	GenBank acc. no.	Forward sequence	Reverse sequence	Size
rtGR1	Z54210	CCATCGTCAAGCGGGAAGAG	GGAACTCCACGCTAAGGGATTTATTC	150
rtGR2	AY495372	CTCCGCTTTCTCCAGCAGCTA	GTGAGCCACCCCGTAGTGACAG	150
rtMR	AF209873	GAAACAGATGATCCGCGTGGT	TGGATCAGGGTGATTTGGTCCT	87
11β-HSD	AB104415	AAGGGACGCATCGTCACAATCT	AACAGGTTGAGAGCTGCCTTGG	95
EF1α	AF498320	AGCGCAATCAGCCTGAGAGGTA	GCTGGACAAGCTGAAGGCTGAG	160

diluted 100 times to allow better visualization of protein bands. All samples were run on continuous 12% SDS-polyacrylamide gels after loading 0.5 μg of total proteins in each well. Electrophoresis was performed using a SE 250 mini vertical electrophoresis system (Amersham Bioscience, Orsay, France). After electrophoresis, the proteins were visualized by silver staining (Blum et al., 1987).

Statistical analysis

Wet and dry mass *in vivo* data were analyzed by one-way analysis of variance (ANOVA) followed, when significant, by Bonferroni tests to determine differences among days. *In vivo* oocyte water content changes between ovarian stages were analyzed using a Mann–Whitney test. *In vitro* data on WM and DM changes were analyzed by a Kruskal–Wallis ANOVA followed, when significant, by Wilcoxon tests for paired samples to detect differences among hormonal treatments. For mRNA differences between ovarian stages, a Kruskal–Wallis ANOVA was performed and, when significant, followed by a Mann–Whitney test. The level of significance used in all tests was $P<0.05$.

Results

In vivo oocyte hydration

A significant oocyte WM increase was observed between preGVBD and ovulation in all females (13.8–18.9%, mean 16.9%). In addition, a significant WM increase was also observed between preGVBD and maturation, and between maturation and ovulation in all females. In contrast, no further significant WM increase was observed after ovulation (Fig. 1).

A limited but significant oocyte dry mass increase was also observed between preGVBD and ovulation in three females, and between preGVBD and 7 days after ovulation in the fourth female (Fig. 1). On average, DM increase between preGVBD and ovulation represented 3.9% of the initial WM and 8.4% of the initial DM. Finally, a significant oocyte hydration (Fig. 2) was also observed between preGVBD and ovulation (20.8–27.1%, mean 24.7%) and between preGVBD and maturation (10.2–21.6%, mean 17.3%). By contrast, between maturation and ovulation, the observed hydration (mean 7.4%) was just below significance ($P=0.054$).

Yolk gel electrophoresis

Fig. 3 shows a representative SDS-PAGE of yolk proteins originating from preGVBD oocytes and unfertilized eggs. Migration patterns were similar in oocytes and eggs and no different bands could be observed between preGVBD and ovulation. In both samples, major bands were observed at 92, 73 and 18 kDa (Fig. 3).

In vitro response to hormonal stimulation

The effect of hormonal treatments on oocyte maturation and mass was assessed after a 60 h incubation. Negative control (ethanol), cortisol and DOC treatments did not induce any morphological evidence of oocyte maturation. Oocytes incubated with 17,20β-P (MIS, 40 ng ml^{-1}) exhibited a high percentage of GVBD (72% in the first experiment and 87% in the second experiment). The addition of cortisol to the MIS did not increase maturational response (GVBD occurrence). Ovulation never occurred in any hormonal treatment.

No change in oocyte DM was observed during *in vitro* incubation. By contrast, changes in oocyte WM were observed. In experiment 1 (Fig. 4), all cultured oocytes, including the negative control (treated with ethanol vehicle), exhibited a significant WM increase when compared with initial control (initial oocyte mass, WM_0). DOC (5 ng ml^{-1}) led to a 5.9% WM increase, and negative control to a 4.2% increase. However, no significant difference was observed between DOC treatment and negative control. In addition, WM increase observed in negative control and DOC treated samples was significantly lower than WM increase observed for MIS (40 ng ml^{-1}) and cortisol (12 ng ml^{-1}) treatment alone or in combination. Furthermore, MIS (40 ng ml^{-1}) led to a 15.9% WM increase when compared to $_{WM0}$. Cortisol (12 ng ml^{-1}), alone or in combination with MIS (40 ng ml^{-1}) induced a similar WM increase (14.5 and 15.2%, respectively).

In experiment 2 (Fig. 5), cortisol, at all three tested concentrations, led to a significant WM increase when compared with negative control. By contrast, incubations performed with DOC alone did not lead to any significant WM increase. Combination of MIS (40 ng ml^{-1}) and cortisol at 12 and 50 ng ml^{-1}, and MIS (40 ng ml^{-1}) and DOC at 12 ng ml^{-1}, also led to a significant WM increase. For all other treatments, no significant WM increase was observed when compared with negative control. Furthermore, all effective treatments, besides DOC at 50 ng ml^{-1}, led to a WM increase that was not significantly different from MIS-induced WM increase.

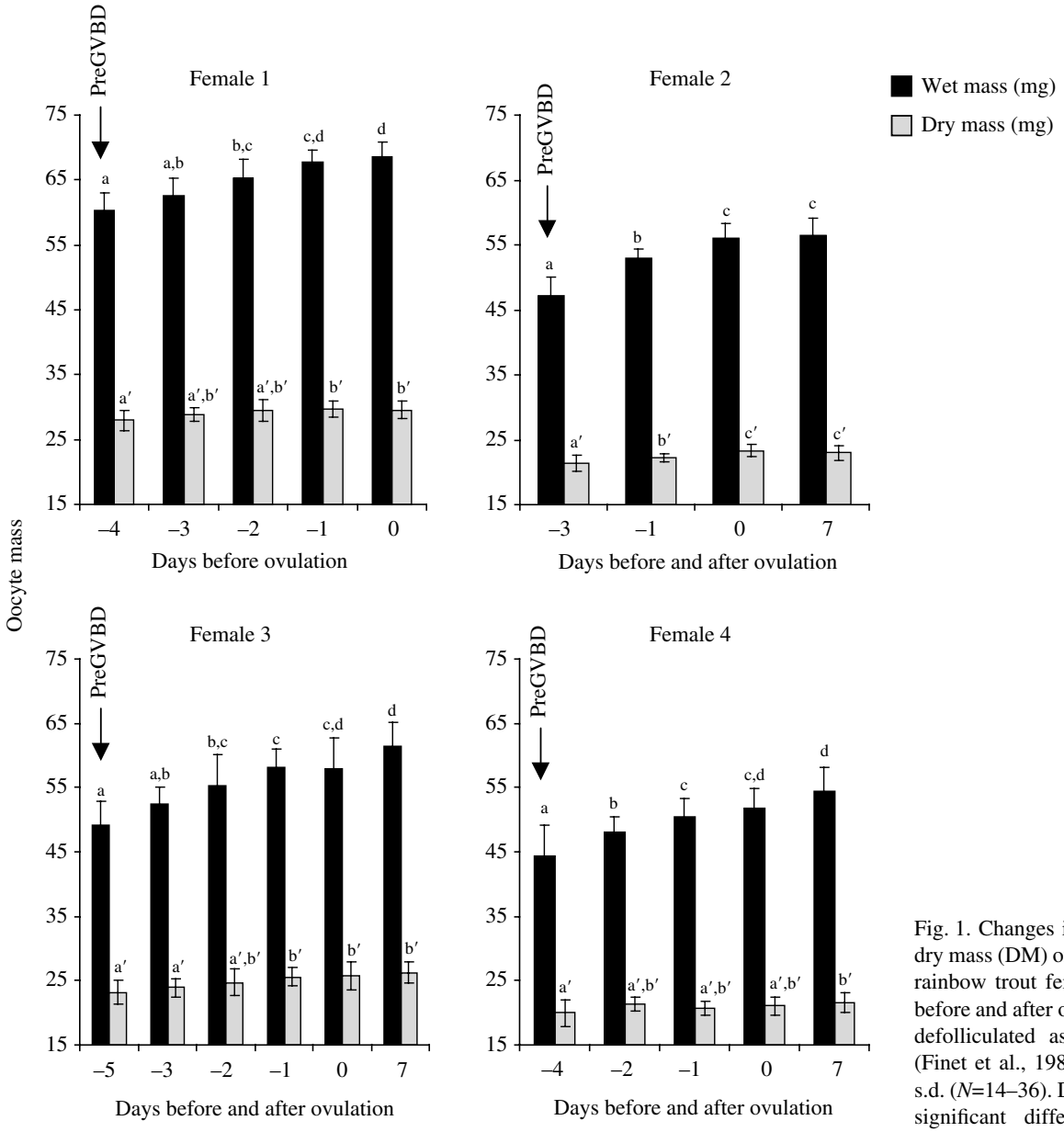

Fig. 1. Changes in wet mass (WM) and dry mass (DM) of oocytes obtained from rainbow trout females at different days before and after ovulation. Oocytes were defolliculated as previously described (Finet et al., 1988). Values are mean ± s.d. (N=14–36). Different letters indicate significant differences between days (P<0.05).

Quantification of glucocorticoid receptors, mineralocorticoid receptor and 11β-hydroxysteroid dehydrogenase mRNA abundance

The mRNA levels of glucocorticoid receptors (rtGR1 and rtGR2), mineralocorticoid receptor (rtMR) and 11β-hydroxysteroid dehydrogenase (11β-HSD) genes were monitored in the ovary at three different stages of oogenesis: late vitellogenesis, prior to GVBD (preGVBD) and during oocyte maturation (Fig. 6). EF1α mRNA, that did not show any stage-dependent change, was used as an internal standard. In addition, standardization using 18S rather than EF1α resulted in no difference in gene expression profiles. RtMR mRNA was barely detected and considered too low for quantitative monitoring (average CT of 30). In contrast, rtGR1, rtGR2 and 11β-HSD mRNAs were measured at much higher levels (average CT of 27, 26 and 23, respectively). During the preovulatory period, there was no significant change in rtGR1 gene expression. In contrast, an 86% increase in rtGR2 mRNA levels was observed between late-vitellogenesis and oocyte maturation (GVBD) (Fig. 6). 11β-HSD mRNA levels did not significantly change between late vitellogenesis and preGVBD, whereas a 200% increase was observed at the time of oocyte maturation (Fig. 6).

Discussion

In the present study, we showed that a significant oocyte hydration (24.7%) occurs during oocyte maturation in rainbow

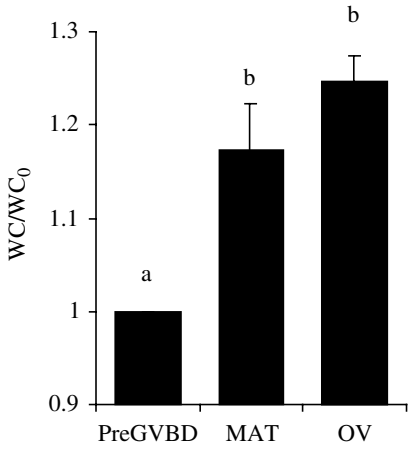

Fig. 2. Oocyte water content (WC)/water content before maturation (WC_0, PreGVBD) ratio of oocytes sampled at three ovarian stages: before oocyte maturation with visible germinal vesicle (PreGVBD), during oocyte maturation (MAT) and after ovulation (OV). Oocytes were defolliculated as previously described (Finet et al., 1988). Values are mean ± s.d. (N=4). Different letters indicate significant differences between stages (P<0.05).

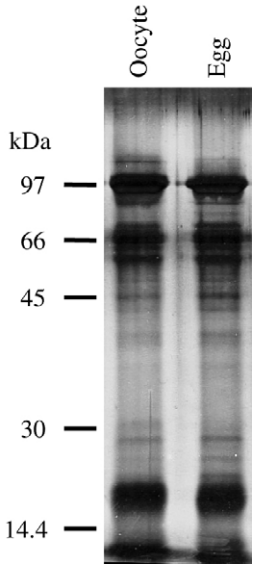

Fig. 3. Representative SDS–PAGE (12%) of yolk proteins from preGVBD oocytes and ovulated eggs of rainbow trout. Each lane had 0.5 mg of yolk proteins originating from three oocytes/eggs. The position of molecular size markers is indicated.

trout. To our knowledge, this is the first report of oocyte hydration in the rainbow trout preovulatory ovary, thus contradicting a previous study that reported no significant oocyte hydration associated with oocyte maturation in rainbow trout and other freshwater species (Craik and Harvey, 1984). In this previous study, however, conclusions were drawn from non-sequential data obtained using a small number of different females, probably too small with respect to the variability of oocyte size between females. In the present study, in order to overcome this problem of individual variability, oocyte mass changes during maturation were monitored by sequential sampling of oocytes from the same females, thus leading to unequivocal conclusions about the reality and the extent of oocyte hydration in rainbow trout. While oocyte hydration in marine species was thoroughly studied, data obtained in freshwater species are scarce. In *Barbus tetrazona* and *Rasbora trilineata* a perceptible increase of oocyte size was observed during oocyte maturation (Greeley et al., 1986) thus suggesting an oocyte hydration. Together with these previous results, the present study strongly suggests that a more limited but significant oocyte hydration also occurs in freshwater species during oocyte maturation. Finally, while the oocyte hydration observed in rainbow trout could be considered as a minor phenomenon in comparison to that in marine species, it can, however, be hypothesized that oocyte hydration occurring immediately prior to ovulation is needed for the completion of ovulation. Indeed, a swelling of the oocyte could mechanically participate in the rupture of follicle walls and the extrusion of the oocyte from its follicle (Pendergrass and Schroeder, 1976). Interestingly, a previous study in common carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*) reported an ovarian

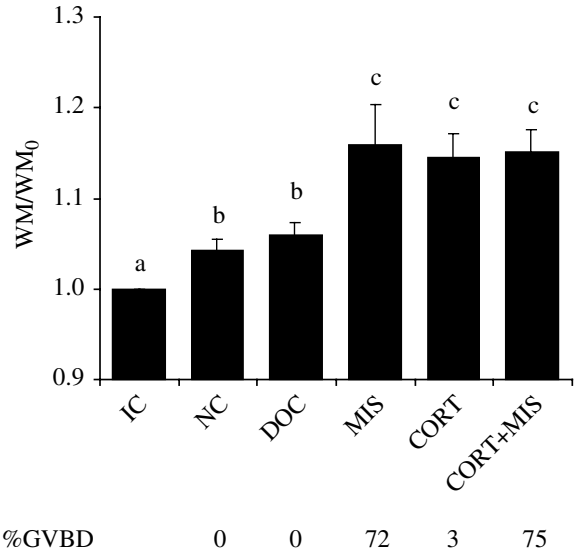

Fig. 4. Oocyte wet mass (WM)/'initial control' wet mass (WM_0) ratio after a 60-h *in vitro* incubation. Follicle-enclosed oocytes were incubated in the presence of different steroids: 11-deoxycorticosterone (DOC, 5 ng ml^{-1}); 17,20β-dihydroxy-4-pregnen-3-one (MIS, 40 ng ml^{-1}); cortisol (12 ng ml^{-1}); cortisol (12 ng ml^{-1}) plus 17,20β-dihydroxy-4-pregnen-3-one (40 ng ml^{-1}). IC, initial control; ethanol vehicle was used as the negative control (NC). Values are mean ± s.d. (N=7). Different letters indicate significant differences between treatments (P<0.05). The percentage of germinal vesicle breakdown (GVBD) after a 60 h incubation is shown below for each group.

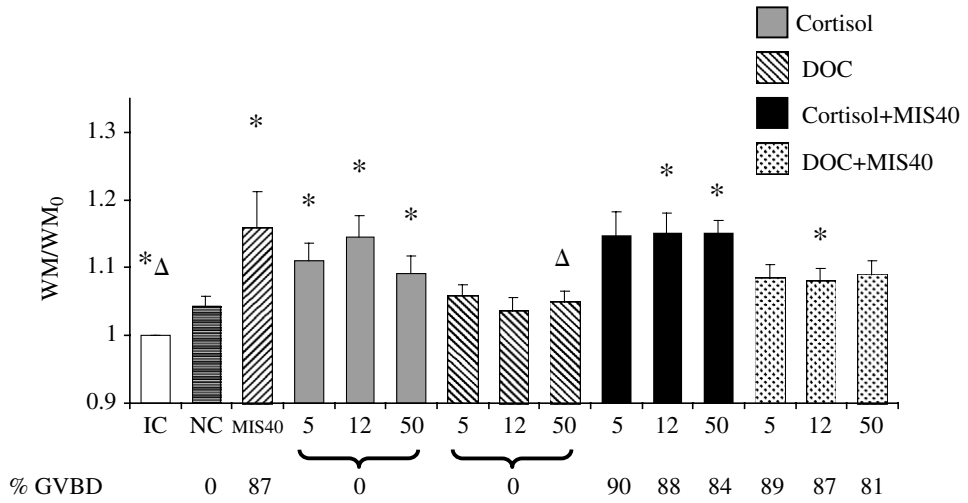

Fig. 5. Oocyte wet mass (WM)/'initial control' (IC) wet mass (WM_0) ratio after a 60 h *in vitro* incubation. Follicle-enclosed oocytes were incubated in the presence of different steroids: 17,20β-dihydroxy-4-pregnen-3-one (MIS40; 40 ng ml^{-1}); cortisol (5, 12 or 50 ng ml^{-1}); 11-deoxycorticosterone (DOC, 5, 12 or 50 ng ml^{-1}); cortisol (5, 12 or 50 ng ml^{-1})+MIS40; DOC (5, 12 or 50 ng ml^{-1})+MIS40. IC, initial control; ethanol vehicle was used as the negative control (NC). Values are means ± s.d. (*N*=5). Asterisks indicate significantly different from NC. Triangles indicate significantly different from MIS40 value ($P<0.05$). The percentage of germinal vesicle breakdown (GVBD) after 60-h incubation is shown below for each group.

hydration during both hormone-induced and natural ovulation (Clemens and Grant, 1964). These authors observed that the ovarian water content was higher in ovulating females than in non ovulating females and concluded that the water increase was a part of the ovulation process. Consistent results were observed in ayu (*Plecoglossus altivelis*), in which an increase of ovarian water content was observed in human chorionic gonadotropin (hCG)-injected fish (Hirose et al., 1974).

Yolk protein proteolysis has previously been linked to oocyte hydration in several species (Greeley et al., 1986; Iwamatsu et al., 1992; Matsubara and Sawano, 1995; Thorsen and Fyhn, 1996). SDS–PAGE revealed the disappearance of a large band of about 100 kDa, presumably lipovitellin, during the hydration process. Concomitantly, new proteins with lower molecular mass appeared (Greeley et al., 1986; Matsubara and Sawano, 1995; Thorsen and Fyhn, 1996). This proteolysis is

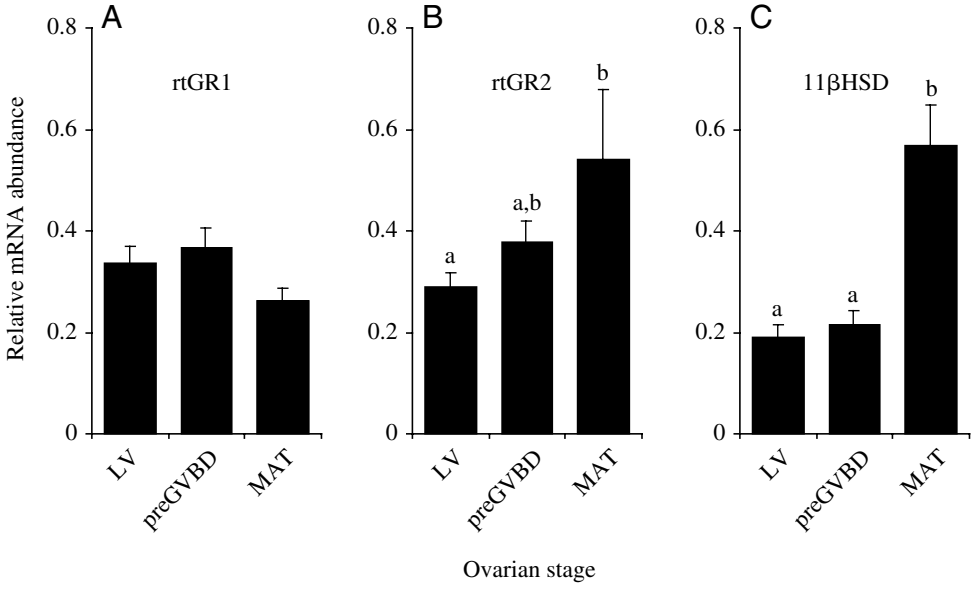

Fig. 6. Abundance of messenger RNA of (A) glucocorticoid receptor 1 (rtGR1), (B) glucocorticoid receptor 2 (rtGR2) and (C) 11β-hydroxysteroid dehydrogenase (11β-HSD) in the whole ovary of rainbow trout females sampled at three stages of ovarian development: late vitellogenesis (LV, 3–4 weeks before expected ovulation, *N*=6), prior to germinal vesicle breakdown (preGVBD, *N*=8) and during oocyte maturation (MAT, *N*=6). Values are mean ± s.e.m. Different letters indicate significant differences between stages ($P<0.05$).

believed to participate in the mechanism of oocyte hydration by increasing the oocyte free amino acids (FAA), thus leading to a transient hyperosmolarity (Finn et al., 2002a; Thorsen and Fyhn, 1996). Indeed, a good correlation was reported between the extent of yolk proteolysis and oocyte hydration (Greeley et al., 1986). However, no or little changes of protein migration patterns were observed between premature oocytes and ovulated eggs in several freshwater species laying demersal eggs (Greeley et al., 1986). In the present study, similar yolk protein electrophoretic patterns were observed between preGVBD oocytes and ovulated eggs. This observation is consistent with existing data on rainbow trout yolk proteins during ovarian development (Tyler, 1993). These observations suggest that no or little proteolysis occurs during oocyte maturation and are therefore in good agreement with prior observations made in other freshwater species laying dermersal eggs (Greeley et al., 1986). It can, therefore, be hypothesized that rainbow trout hydration is not induced by yolk proteolysis. The mechanisms of rainbow trout oocyte hydration thus remain unknown and require specific investigations. In the marine teleost, seabream (*Sparus aurata*) it was recently shown that oocyte hydration is aquaporin mediated (Fabra et al., 2005). Therefore, it is possible that a similar membrane-mediated mechanism also occurs in rainbow trout.

Interestingly, a weak (3.9% of initial wet mass) dry mass increase was also observed during oocyte maturation in three females. This suggests that an uptake of organic matter still occurs, at least until ovulation, in the rainbow trout oocyte. A dry mass increase during *in vivo* hormone-induced maturation was also reported in grey mullet, *Mugil cephalus*, and was linked to an uptake of small organic molecules with high osmotic activity (Watanabe and Kuo, 1986). An uptake of proteins has also been shown during *in vitro* oocyte maturation in *Fundulus heteroclitus* (Selman and Wallace, 1983). Our results in a freshwater species are therefore in agreement with these previous studies in marine teleosts. However, the precise nature of this dry mass increase in rainbow trout remains to be clarified.

In vitro, we observed a significant oocyte hydration but no increase of oocyte dry mass. This is probably not surprising since the incubation medium is devoid of organic compounds, besides minimal concentrations of glucose and Hepes. In addition, a small but significant wet mass increase (4.2%) was observed after 60 h in control oocytes only exposed to ethanol vehicle (negative control). This spontaneous hydration could probably be explained by the continuation of a physiological process already started *in vivo* and uncoupled from oocyte maturation. It is also possible that the oocyte undergoes some adjustment to the *in vitro* conditions leading to a small hydration.

The natural maturation-inducing steroid, 17,20β-P, at a concentration within a physiological range was able to trigger both oocyte maturation (GVBD) and oocyte hydration. We observed a significant mass increase of 15.9%, compared to initial control and 11.6% compared to negative control. Thus, the overall 17,20β-P-induced mass increase obtained after only a 60-hour incubation was similar to oocyte hydration occurring *in vivo* between preGVBD and the mature oocyte (17.3%, Fig. 2). Similar results were also reported in *Fundulus heteroclitus* even though the amplitude of hydration was greater than in trout (Greeley et al., 1991; Wallace et al., 1992). In addition, cortisol within a physiological concentration range (5–12 ng ml^{-1}) was able to induce oocyte hydration without inducing meiosis resumption. Cortisol-induced oocyte hydration was similar to 17,20β-P-induced hydration but no additive effect was observed. In female rainbow trout sampled immediately prior or during oocyte maturation, cortisol plasma levels ranging from 7.7 to 10.5 ng ml^{-1} were reported (Bry, 1985). In contrast, cortisol levels were much higher in ovulated females (25.8 to 30.9 ng ml^{-1}) and remained elevated during the post-ovulatory period (Bry, 1985). In the present study, cortisol at a concentration of 5, 12 or 50 ng ml^{-1} was equally able to induce *in vitro* oocyte hydration. This effect of cortisol on oocyte hydration was unsuspected and is reported here for the first time in any teleost species. As discussed above, it can be hypothesized that oocyte hydration is needed for the completion of the ovulatory process. Interestingly, it was shown that cortisol, added to the incubation medium, facilitated the occurrence of ovulation in medaka follicles (Schroeder and Pendergrass, 1976). In contrast, DOC had no effect on oocyte hydration or oocyte maturation. While no significant difference was observed between effective treatments, the highest effects on oocyte hydration were observed using 17,20β-P alone or in combination with cortisol. Together, these observations suggest that 17,20β-P alone could be responsible for the oocyte hydration observed concomitantly to meiosis resumption in rainbow trout. However, the similarity between cortisol plasma levels and concentrations found to be effective *in vitro* suggests that cortisol could also participate in the *in vivo* process.

In rainbow trout, cortisol binds to different corticosteroid receptors, including glucocorticoid receptors (rtGR1 and rtGR2) and mineralocorticoid receptors (rtMR). In the present study, rtMR was barely detected whereas rtGR1 and rtGR2 were expressed at much higher levels. In addition, there was a significant rise in rtGR2 mRNA levels at the time of meiosis resumption. It was previously shown that cortisol enhances transcriptional activity of rtGR2 in trout at lower concentrations than those required to increase transactivation of the rtGR1 when expressed in COS-7 cells (Bury et al., 2003). Thus, rtGR2 is transcriptionally activated *in vitro* by glucocorticoids at concentrations 10- to 100-fold lower than rtGR1. According to these authors, this suggests that cortisol would preferentially bind to rtGR2 in low or mild stressful situations (such as the preovulatory period) and to rtGR1 in stressful ones (Bury et al., 2003). Together, these observations further strengthen the involvement of cortisol with rtGR2 in periovulatory ovarian functions.

In the present study, high levels of 11β-HSD transcripts were observed in the ovary during the periovulatory period, in agreement with a previous study on 11-βHSD in rainbow trout (Kusakabe et al., 2003). It was suggested that this enzyme

could protect the gonad from negative effects of high cortisol concentrations (Kusakabe et al., 2003). The strong expression of 11β-HSD mRNA in the ovary and the observed 200% increase of its abundance during oocyte maturation suggest a physiological role in this process in agreement with a potential role of cortisol in periovulatory ovarian functions. It was previously reported that 11β-HSD transcript was present in thecal and granulosa cells of mid-vitellogenic and post-ovulatory follicles (Kusakabe et al., 2003). It can therefore be hypothesized that the observed increase of 11β-HSD mRNA abundance occurs in follicular layers. This up-regulation of 11β-HSD occurs prior to the rise in the level of plasma cortisol observed after ovulation (Bry, 1985). As high plasma cortisol levels lead to inhibition of gonadal development (Pankhurst and Van Der Kraak, 2000), it seems logical that this protective effect would occur before cortisol has reached its highest level. Our observations are therefore consistent with existing data.

In addition to the increase of 11β-HSD and rtGR2 mRNA levels reported here, a rise of 11β-hydroxylase mRNA levels, an enzyme that controls cortisol synthesis, was observed in the rainbow trout ovary during oocyte maturation (Bobe et al., 2004). Together with the rise in cortisol plasma levels previously observed in ovulated females, these observations strongly suggest that cortisol plays a role in ovarian functions in the rainbow trout ovary at, or immediately prior to, ovulation. Although it is possible that cortisol participates in the control of oocyte hydration, it is also probably involved in other periovulatory ovarian functions.

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