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Research

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Post-ovulatory ageing and egg quality: A proteomic analysis of rainbow trout coelomic fluid

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Abstract

Background: In fish, oocyte post-ovulatory ageing is associated with egg quality decrease. During this period, eggs are held in the body cavity where they bath in a semi-viscous liquid known as coelomic fluid (CF). CF components are suspected to play a role in maintaining oocyte fertility and developmental competence (egg quality). However, CF proteic composition remains poorly studied. Thus rainbow trout CF proteome was studied during the egg quality decrease associated with oocyte post-ovulatory ageing.

Methods: High resolution two-dimensional gel electrophoresis was used to analyze the proteome of rainbow trout (*Oncorhynchus mykiss*) CF in relationship with the egg quality decrease associated with oocyte post-ovulatory ageing. A first experiment was performed using CF pools originating from 17 females sampled at ovulation as well as 7, 14 and 21 days later. These observations were verified using a second set of CF pools originating from 22 females sampled 5 and 16 days following ovulation.

Results: Approximately 200 protein spots of 10–105 kDa molecular mass and 3–10 pI were detected in CF samples. Several protein spots, while undetected at the time of ovulation, exhibited a progressive and strong accumulation in CF during post-ovulatory ageing. After silver-staining and Matrix-Assisted Laser Desorption Time Of Flight (MALDI-TOF) mass spectrometer analysis, some of these protein spots were identified as lipovitellin II fragments.

Conclusions: These observations suggest that egg protein fragments accumulate in the CF during the post-ovulatory period and could therefore be used to detect egg quality defects associated with oocyte post-ovulatory ageing.

Introduction

In salmonids, ovulated oocytes (eggs) are held in the body cavity where they bath in a semi-viscous fluid known as "ovarian" or "coelomic" fluid (CF). In contrast to most

fish species, salmonids can hold their eggs in the body cavity at 10–12°C for at least a week without loss of developmental competence [1,2]. For longer holding times, post-ovulatory ageing is associated with a significant drop

of egg quality characterized by decreased embryonic survival and increased number of morphological and ploidy abnormalities [1]. However, the relative importance of each of these phenomena is highly variable depending on the females [1]. In vitro, trout ovulated oocytes held 3 days at 12°C retain full developmental competence when stored in coelomic fluid but not in trout artificial medium [3]. It is therefore possible that some coelomic fluid components could play a role in maintaining the oocyte ability to be fertilized and allow subsequent normal development.

Trout coelomic fluid pH is around 8 and contains Na⁺, K⁺ and Ca⁺⁺ ions, glucose, fructose, cholesterol, phospholipids, proteins and free amino acids [4,5]. However, its proteic composition remains poorly documented. Several proteases and anti-proteases of maternal origin were previously identified and an anti-bacterial activity of some of these proteins, the trout ovulatory proteins (TOPs) was also demonstrated [6-9]. In addition, enzyme assays also indicated the presence of phosphate, collagenase, gelatinase and lactate dehydrogenase activities [10]. Although some proteic components are believed to be derived from the blood [11,12], most of the others probably come from the post-ovulatory ovary which is devoid of ovarian cavity and widely open to the coelomic cavity in salmonids.

The purpose of the present study was to analyze the changes in coelomic fluid proteome during oocyte post-ovulatory ageing and tentatively identify proteins that accumulate or, in contrast, disappear simultaneously with the decrease in egg quality usually observed during post-ovulatory ageing. Such accumulation/degradation patterns could thus become useful markers of oocyte quality.

To do so, we combined a high resolution two-dimensional gel electrophoresis (2-D) that separates proteins according to both isoelectric point and molecular weight, and mass spectrometry. Coelomic fluids (CF) and oocyte samples were taken from females after short and long post-ovulatory times in the body cavity. For each sampling time, egg quality was evaluated by fertilizing an oocyte sample from each female and monitoring early development. The accumulation patterns of identified proteins were described using pooled coelomic fluid samples originating from 17 spring-spawning females. We observed that several proteins, while undetected or poorly detected at the time of ovulation, accumulate in the coelomic fluid during post-ovulatory ageing. Some of these proteins were identified as fragments of lipovitellin II, one of the vitellogenin-derived proteins. These observations were then verified using CF pools sampled from 22 autumn-spawning females 5 and 16 days following ovulation.

Materials and methods

Animals

Investigations were conducted according to the guiding principles for the use and care of laboratory animals. Mature male and female rainbow trout were obtained from the INRA-SEDI fish farm (Sizun, France) and held in a recirculated water system at 12°C, under natural photoperiod, in INRA experimental facilities (Rennes, France). Females were checked for ovulation 2 or 3 times a week by manual pressure on the abdomen. For ovulation detection as well as coelomic fluid and gamete collection, fish were lightly anesthetized in 0.05% 2-phenoxyethanol.

Coelomic fluid and gamete collection

Coelomic fluid and eggs were collected from the same females after increasing holding times in the body cavity. Coelomic fluid was carefully pipetted out and spun at 3000 g for 15 min to remove red blood cells and debris, and anti-proteases were added (1 mM ethylene-diamine-tetra-acetic-acid (EDTA), 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSEF), 10 µM leupeptin). The protein content of coelomic fluid was determined using a Coomassie protein assay reagent (Interchim, France). A first experiment was performed using 17 females collected at the time of ovulation (OV) and 7 (D7), 14 (D14) and 21 (D21) days following ovulation. A second experiment was performed using 22 females collected 5 (D5) and 16 (D16) days following ovulation.

Fertilization and developmental success monitoring

For each female and each collection time, an egg batch was fertilized as previously described [1] in order to estimate egg quality. Approximately 250–400 eggs were washed with 10 ml of rinsing saline solution (Ovafish, IMV, France). Sperm originating from 5–10 males was added (10 µl) along with 10 ml of sperm extender (Actifish, IMV), tubes were gently swirled and incubated for 10 minutes. After discarding Actifish, eggs were transferred into plastic incubators and held in the dark at 10°C in a recirculated water system during early development (55 days). The number of embryos reaching eyed-stage, hatching and yolk-sac resorption (YSR) was monitored and the number of alevins exhibiting morphological abnormalities at YSR was measured. The quality of each egg batch was estimated using the number of embryos reaching yolk-sac resorption without exhibiting any obvious morphological abnormality (Living and Normal Embryo Rate, LNER).

Preparation of protein samples

Forty micrograms of each coelomic fluid (CF) sample were pooled for each post-ovulatory ageing time (First experiment: OV, D7, D14 and D21; second experiment: D5 and D16). Coelomic fluids were incubated in a

rehydration buffer: 6 M urea, 2 M Thiourea, 4% CHAPS, 1% Dithiothreitol (DTT), 0.5% Immobilized pH Gradients (IPG) buffer (Amersham Biosciences) and bromophenol blue for 1 hr at room temperature.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

A 2D-PAGE was run for each post-ovulatory ageing time for both experiments. For each gel, forty microgram of CF pools were loaded onto a 18 cm nonlinear immobiline dry strip, pH range 3–10; 180 × 3 × 0,5 mm (Amersham Biosciences) for the first dimension. The second-dimensional run was carried out using a precast ExcelGel XL sodium dodecyl sulphate (SDS)-polyacrylamide 12–14% gradient gel (Amersham Biosciences) with precast anode and cathode buffer strips (Amersham Biosciences). Isoelectric focusing (IEF) was performed using an IPGphor IEF system (Amersham Biosciences) during 18.5 hours with a gradually increasing voltage [13]. Immediately after IEF, the IPG strip was equilibrated for 15 min at room temperature in 20 ml of 50 mM Tris-HCL pH 8.2, 6 M urea, 30% glycerol, 2% SDS and bromophenol blue with 65 mM DTT and later for 10 min with 250 mM iodoacetamide and trace of bromophenol blue. The equilibrated IPG strip was transferred to a horizontal Mutiphor II system at 15°C during 3.5 hours. The electrophoresis conditions were set as previously described [14].

Gel staining and image analysis

For image analysis, 2-D gels were silver-stained as described by [15]. After staining, gels were scanned and then processed for image analysis using 2-D Elite image master software (Amersham Biosciences). Optic density of protein spots was measured using multigaugage software (Fuji Ltd, Tokyo, Japan).

In gel digestion and protein identification by mass spectrometry

Proteins resolved by 2D-PAGE were silver-stained using a method compatible with in-gel digestion of proteins mass spectrometry identification [13,16]. Silver-stained protein spots of interest were excised from 2-D gels and then processed for trypsin digestion as described previously [13]. The eluted peptides were subsequently analyzed by mass fingerprinting in a Matrix-assisted laser desorption time of flight (MALDI-TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) processed using the MoverZ software (ProteoMetrics, NY) and internally calibrated using trypsin auto-digestion peptides.

Protein identification by mass spectrometry was performed as previously described [17]. Monoisotopic masses of tryptic peptides observed in the MALDI-MS spectra were used to query National Centre for Biotechnology Information (NCBI) and SWISS-PROT sequences

databases using MASCOT search program <http://www.matrixscience.com>[18].

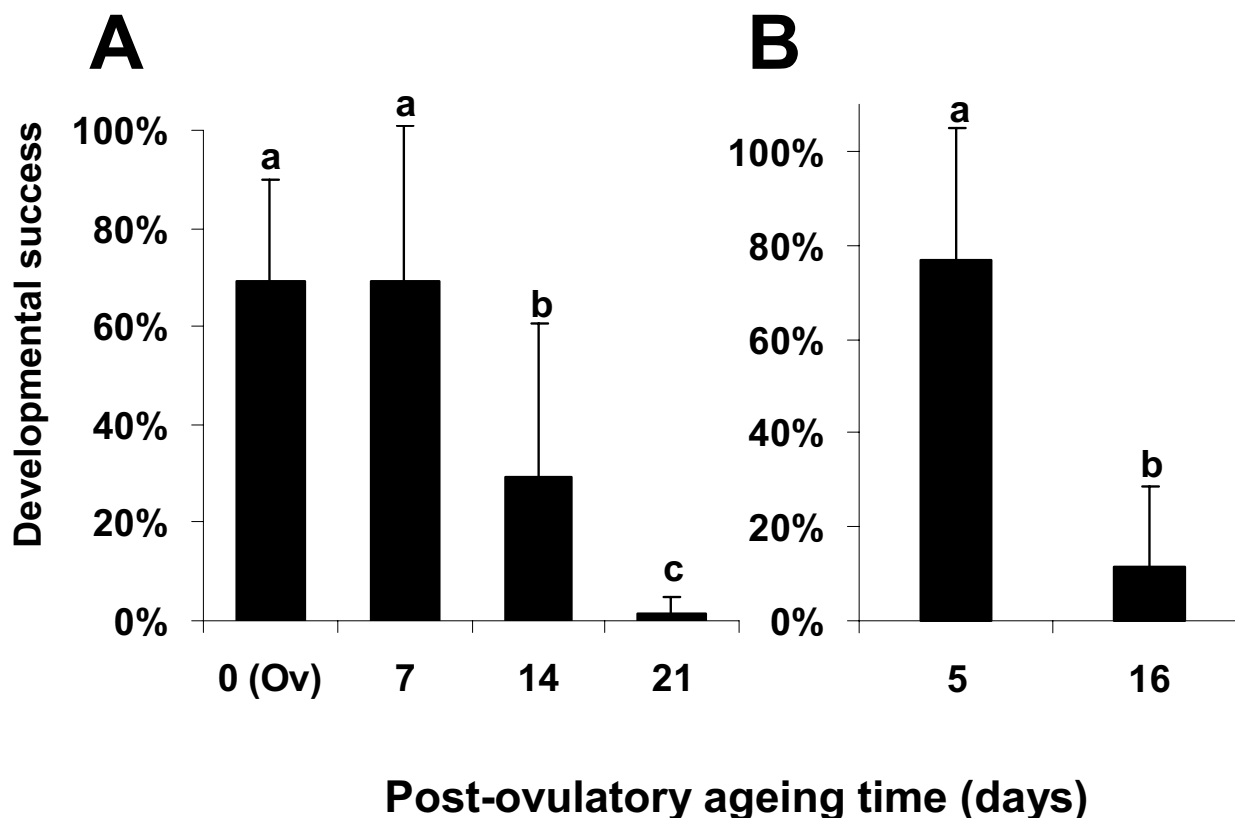
Statistical analysis

Egg quality differences observed between post-ovulatory ageing times were analyzed using paired Student t-tests.

Results and Discussion

The post-ovulatory ageing of ovulated oocytes in abdominal cavity of salmonids is associated with a progressive egg viability decrease that may last 2–3 weeks [1,19]. It was also shown that post-ovulatory ageing is associated with an increased occurrence of morphological abnormalities [1]. In the present study, the number of alevins reaching yolk-sac resorption without exhibiting any obvious morphological abnormality (Living an Normal Embryo Rate, LNER) was used to estimate egg quality for all female at each post-ovulatory sampling time. Indeed, it was shown that only monitoring the embryonic survival was not sufficient to fully estimate the developmental competence of ovulated oocytes [1]. In both experiments a strong and significant decrease in developmental competence was observed for holding times longer than 5 (experiment 2) or 7 (experiment 1) days (Figure 1). Thus, LNER decreased from 70 to 30% between 7 and 14 days post-ovulation (experiment 1) and from 76 to 14% between 5 and 16 days post-ovulation (experiment 2). These observations are in total agreement with previous studies performed on rainbow trout and other salmonids at 12°C [1,2].

From the 2D electrophoresis analysis, approximately 200 protein spots were observed in coelomic fluid samples (Figure 2). Observed molecular mass ranged from 10 to 105 kDa while *PI* ranged from 3 to 10. In experiment 1, the number of detected spots progressively increased during post-ovulatory ageing (Figure 3). Indeed, about 20 spots appeared between ovulation and 21 days post-ovulation. More specifically, 8 protein spots were characterized by a strong accumulation during post-ovulatory ageing (Figure 3). Their abundance exhibited a 260% increase between 7 and 21 days post-ovulation (Figure 3E). Similar observations were made in a second experiment using CF pools originating from 22 females sampled 5 and 16 days following ovulation (Figure 4). For this second experiment the measured abundance exhibited a 500% increase between 5 and 16 days post-ovulation (Figure 4C). Together, these observations show that post-ovulatory ageing is associated with the apparition of several proteins or protein fragments in coelomic fluid. The strong accumulation of some of these spots is consistent with the increase of CF protein concentration reported in the literature [20].

**Figure 1**

Effect of post-ovulatory oocyte ageing on egg developmental success Developmental success was estimated using the percentage of alevins reaching yolk-sac resorption without exhibiting any obvious morphological abnormality (LNER, Living and Normal Embryo Rate). Bars represent the means \pm standard deviations. On each graph, bars sharing the same letter are not significantly different at $p < 0.05$. Experiment 1 was performed using 17 females (A) and experiment 2 using 22 females (B). Post-ovulatory ageing times are indicated for both experiments.

In order to identify the protein spots accumulating in CF during post-ovulatory ageing, excision from 2-D gels and in-gel trypsin digestion were performed. This was performed on the 8 protein spots exhibiting a strong accumulation during post-ovulatory ageing as well as on 14 other spots exhibiting a putative differential expression during post-ovulatory ageing. From the 22 analyzed spots, only 15 protein spots produced peptide products suitable for database search (Table 1). Eleven out of these 15 were identified and are described below while 4 protein spots could not be identified by the Mascot program.

1) Six proteins spots were identified as fragments of *O. mykiss* vitellogenin (Vtg) and more specifically to the lipovitellin II domain [21]. Whereas these fragments could

hardly be detected at the time of ovulation (Figure 3A), they appeared 5–7 days later (Figure 3B), and their abundance progressively increased during the post-ovulatory period (Figure 3C,3D and Figure 4A,4B). Fish vitellogenin is a high molecular weight glyco-phospho-lipoprotein (440–660 kDa in rainbow trout *O. mykiss*) (according to [22]). It is known to be synthesized by the liver cells of mature females under estrogenic control (see [23] for review). It is secreted into the blood-stream from where it is taken up by developing oocytes and cleaved into yolk proteins [24]. These are mainly known as lipovitellin I and II, phosvitin and β' -component [25]. Our observations suggest that vitellogenin fragments originating from the ovulated oocytes held in the body cavity accumulate in CF during post-ovulatory ageing. This would suggest

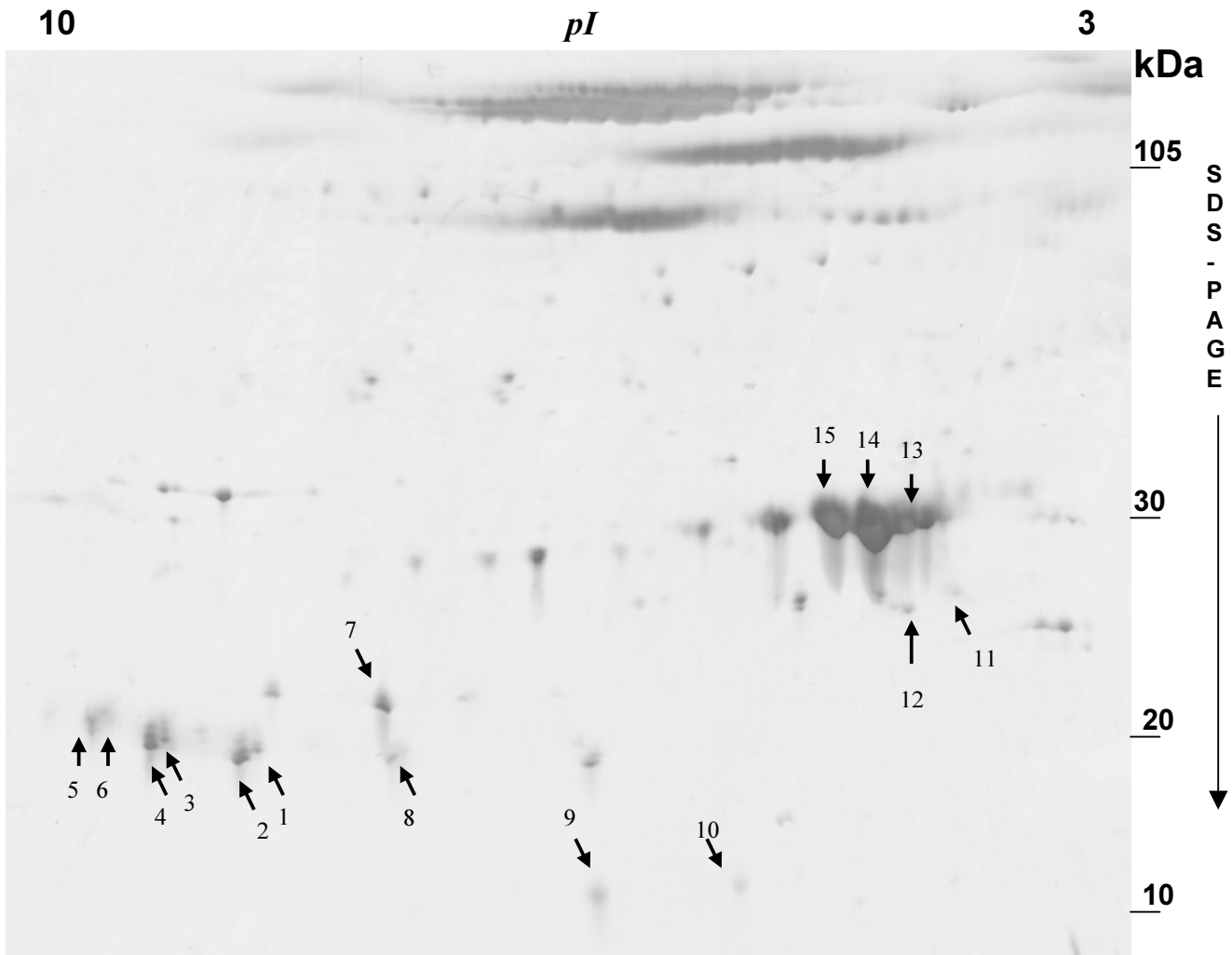


Figure 2
Two-dimensional gel of rainbow trout coelomic fluid Silver stained 2D-PAGE of coelomic fluid 21 days (D21) after ovulation. Proteins (40 µg) were loaded. Protein spots marked with arrows were excised from 2D gels for MALDI-TOF analysis (the numbering of spots corresponds to table 1).

that post-ovulatory ageing is associated with a leakage of some of the oocyte components into the CF. In normal living oocytes, yolk degradation is tightly controlled by specific protease inhibitors associated with yolk proteins. In contrast, proteolytic activities can be expected to increase in poor-quality eggs as shown in sea bass; *Dicentrarchus labrax*, in which the amount of cathepsin D enzymatic activity was found to be much higher in poor-quality eggs than in good-quality eggs. Cathepsin D could thus be considered a possible marker for bad-quality eggs in the sea bass as it has been suggested for the sea bream [26,27]. However, some yolk proteolysis has also been

shown to occur in the preovulatory ovary, during the process of oocyte maturation. The resulting increase in the number of smaller proteins fragments has been hypothesized to favor oocyte hydration that can be particularly important before ovulation in some teleost [28]. The hydration process during oocyte maturation is certainly much lower in rainbow trout than in marine teleosts. However, it cannot be excluded that the protein fragments from oocyte origin that we observe in ageing coelomic fluids are already present as such in just ovulated oocytes. It is therefore possible that their progressive appearance in the coelomic fluid during post-ovulatory time is due to

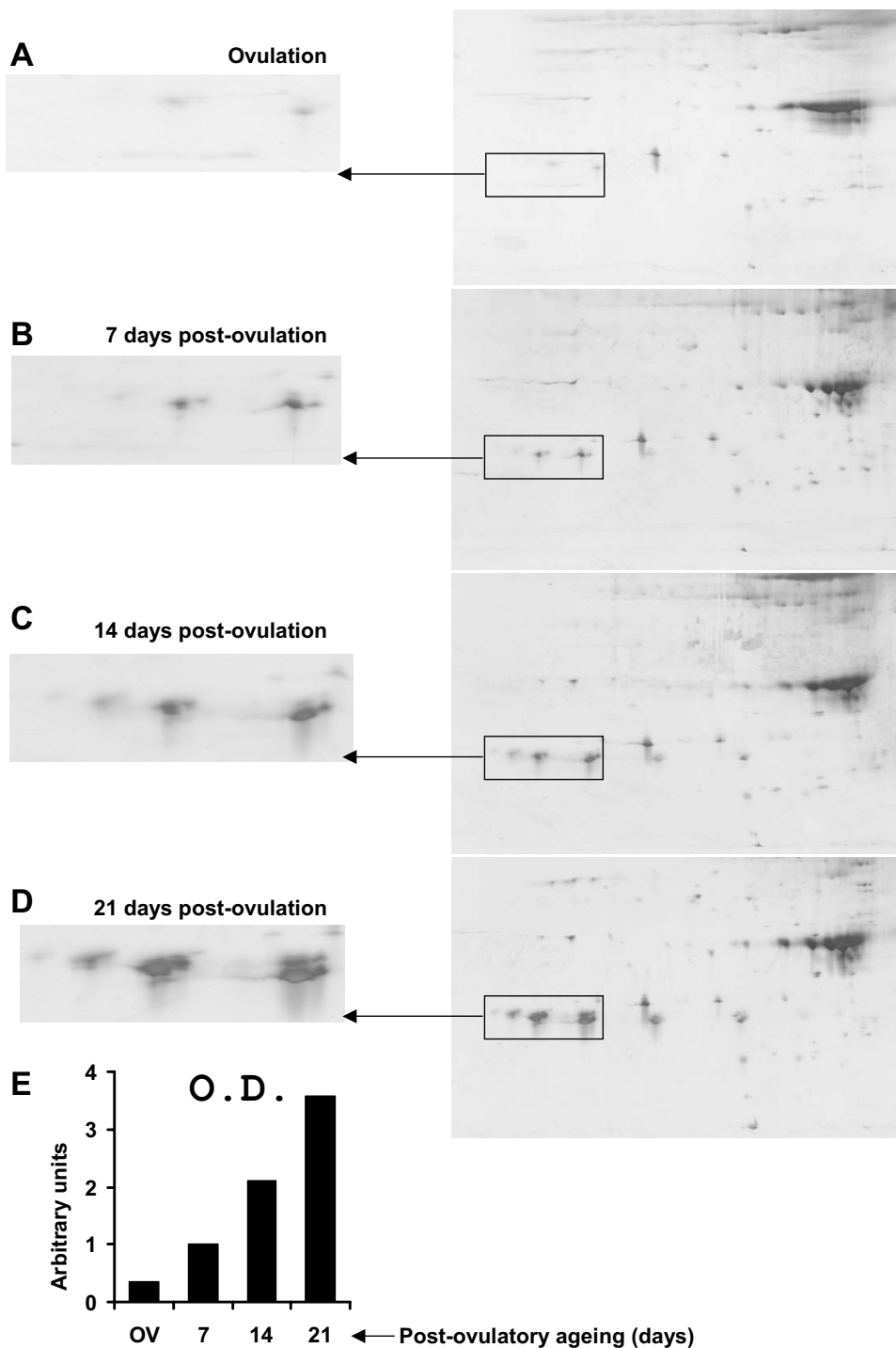


Figure 3
Two-dimensional gel analysis of rainbow trout coelomic fluid during post-ovulatory ageing Two-dimensional gel of rainbow trout coelomic fluid pools originating from 17 females sampled at the time of ovulation (A) and on day 7 (B), day 14 (C) and day 21 (D) after ovulation. Each sample (40 µg) was separated by IEF using a non linear immobilized pH 3–10 gradient for separation in the first dimension combined with SDS-PAGE 12% – 14% gradient gel in the second dimension. Optic density (OD, arbitrary units) of spots shown on the left panel is plotted on the graph (E). OD was arbitrarily set to 1 at 7 days post-ovulation.

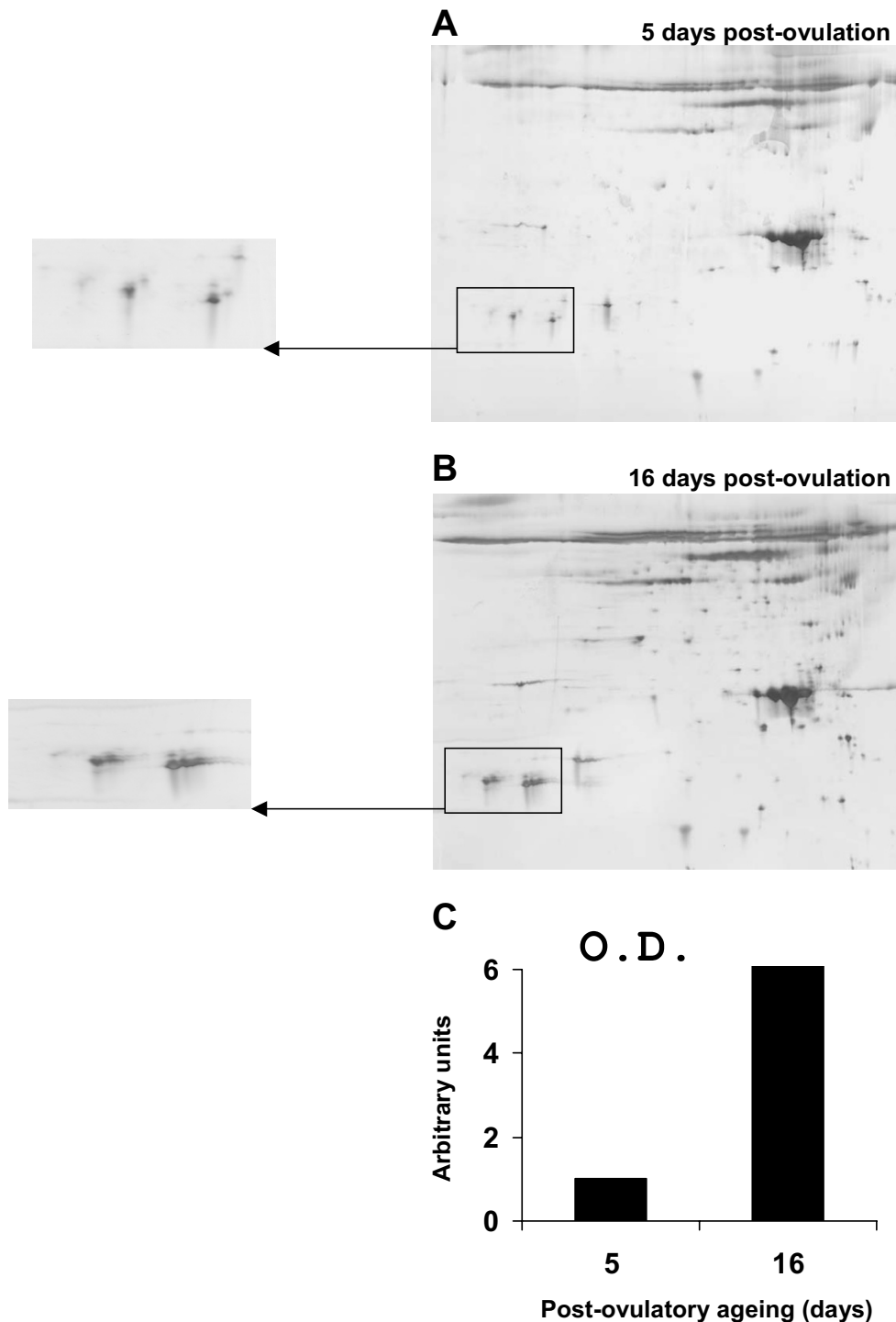


Figure 4

Two-dimensional gel analysis of rainbow trout coelomic fluid at 5 and 16 days post-ovulation Two-dimensional gel of rainbow trout coelomic fluid pools originating from 22 females sampled on day 5 (A) and day 16 (B) after ovulation. Each sample (40 µg) was separated by IEF using a non linear immobilized pH 3–10 gradient for separation in the first dimension combined with SDS-PAGE 12 – 14% gradient gel in the second dimension. Optic density (OD, arbitrary units) of spots shown on the left panel is plotted on the graph (C). OD was arbitrarily set to 1 at 5 days post-ovulation.

Table 1: List of proteins identified in coelomic fluid during post ovulation ageing. The spot identification # corresponds to Fig 2. SwissProt accession numbers and corresponding protein names are shown.

Identification	Protein name	Accession No. (SwissProt)
1	Vitellogenin	AAB02176
2	Vitellogenin	AAB02176
3	Vitellogenin	AAB02176
4	Vitellogenin	AAB02176
5	Vitellogenin	AAB02176
6	Vitellogenin	AAB02176
7	No match	
8	No match	
9	No match	
10	No match	
11	Lectin	AAM21196
12	Lectin	AAM21196
13	Apolipoprotein A I-I	AAB96972
14	Apolipoprotein A I-I	AAB96972
15	Apolipoprotein A I-I	AAB96972

the weakening of the cellular membrane, thus allowing some leakage of small protein fragments as a first evidence of oocyte ageing.

2) Two protein spots were identified as C- type mannose-binding lectin of *O. mykiss*. Lectins are sugar-binding proteins of non-immune origin that agglutinate cells or precipitate glycoconjugates. They are known to be involved in cell defense mechanism [29]. One example is the mannose-binding lectin which can activate the complement system by binding the cell surface glycoproteins of microbes [30]. Lectins inhibit the growth of certain bacterial pathogens of fish [31]. In some fish species, lectins are associated with the content and surrounding membrane of cortical vesicles located within the cytoplasm of maturing oocytes [32,33]. In the present study, lectin spots are detected in CF. It is not clear however whether lectin accumulates or not during post-ovulatory ageing. Our results could suggest that the presence of lectin in CF is due to a leakage from ovulated oocytes. They could also suggest that lectin is secreted by the female into the body cavity to prevent from bacterial infection.

3) Three of the protein spots analyzed were identified as *O. mykiss* apolipoprotein A-I-1. Apolipoprotein A-I-1 is the major protein in plasma high-density lipoprotein and is a potent activator of the lecithin/cholesterol acyl transferase which facilitates the removal of free cholesterol to the liver for excretion [34]. Our results show that this protein is very abundant in coelomic fluids at all times post-ovulation. Interestingly, this protein was also observed in bovine ovarian fluid [35].

Taken together, our observations suggest that cellular leakage occurs from the oocyte to the CF during post-ovulatory ageing. Thus, the higher the proportion of degenerating oocytes present in the body cavity, the higher would be the concentration of some proteins or protein fragments. The accumulation of these proteins or peptides would thus reveal the decreasing overall egg quality of the spawn before it can be detected by direct observations. In addition, the progressive accumulation of some of these proteins in CF would allow their use as markers of oocyte quality defects when the exact post-ovulatory ageing time is unknown. It is also noteworthy that similar observations were made in two different rainbow trout strains (spring spawning and autumn spawning). Indeed the level of these protein fragments in CF could become a useful marker of oocyte quality in rainbow trout and possibly in other salmonids.

In the present study, we observed that the egg quality drop observed during post-ovulatory ageing is associated with the apparition and accumulation of several protein spots in CF. It is believed, however, that CF components are important to maintain ovulated oocyte fertility. Indeed, holding in artificial medium exhibiting pH and osmolarity similar to those of CF leads to a very fast decrease of fertility and developmental competence. Taken together, these observations suggest that the concentration of some CF fluid components could decrease during the egg quality drop observed during oocyte post-ovulatory ageing. However, the proteomic analysis performed here is based on equal loading of total protein quantities on 2-D gels. Therefore, the major increase in the concentration of

some protein fragments (e.g., vitellogenin fragments) could have biased the analysis. Specific studies should be designed in the future to carefully monitor the protein levels of some CF components during post-ovulatory ageing, especially proteins or peptides exhibiting low or moderate expression levels.

Conclusion

In conclusion, we identified several previously unknown components of rainbow trout CF (e.g., lectin) some of them exhibiting a progressive and strong accumulation in CF during post-ovulatory ageing. These results suggest a leakage of some oocyte components into the CF during the post-ovulatory period that could allow the use of these protein fragments as markers of oocyte quality during this period.

Authors' contributions

HR carried out the proteomic analysis and drafted the manuscript. NG participated in the proteomic analysis. CP participated in the proteomic analysis and the design of the study. EB performed the coelomic fluid collection and the estimation of oocyte developmental competence. JB participated in the design of the study, the collection of coelomic fluids and the writing of the manuscript. BJ conceived the study, and participated in its design and coordination.

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