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Proteomic identification of quality factors for oocytes in the Pacific oyster *Crassostrea gigas*

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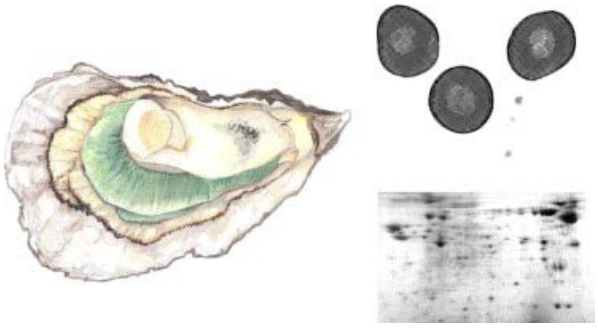
Abstract:

We used a 2-DE proteomic approach to identify abundant proteins linked to oocyte quality in the Pacific oyster *Crassostrea gigas*, an economically important bivalve. Oocyte quality of 14 females was estimated by recording fertilisation and early developmental success until D-larval stage under controlled conditions. Proteins that were differentially expressed between females showing high or low oocyte quality were identified by nano-liquid chromatography tandem mass spectrometry. Twelve up-accumulated spots associated with low quality oocytes revealed 10 distinct proteins, including vitellogenin — breakdown products and metabolic enzymes. Eight up-accumulated spots from high quality oocytes revealed 6 distinct proteins, including chaperone molecules and cell-cycle control proteins. This is the first proteomic study dedicated to oocytes in *C. gigas*. Our results improve current knowledge about protein factors associated with oocyte quality in this species, and our understanding of the proteomic processes involved in their developmental competence.

Graphical abstract



Highlights

► 14 Pacific oyster females were fertilised *in vitro* by a common pool of spermatozoa. ► Individual variability of D-larva yields was due to the intrinsic quality of oocytes. ► Two-dimensional proteomics identified protein factors for oocyte quality.

Abbreviations

MS, mass spectrometry; HPLC, high performance liquid chromatography; 2-DE, two-dimensional electrophoresis.

Keywords: Marine bivalves; *Crassostrea gigas*; Oocyte quality; Proteomics

1. Introduction

Most marine bivalve production in Europe still relies on the collection of wild seed, but global change has led to an increasingly unpredictable seed supply. The case of the Pacific oyster in France illustrates this problem (http://wwz.ifremer.fr/velyger). Bivalve hatcheries have thus grown up to complement the wild seed supply for the shellfish industry, and also offering new opportunities for genetic improvement [1]. Today, Pacific oyster is one of the most important bivalve species produced by artificial reproduction [2], but reproductive success and profitability of hatcheries remain inconsistent. This is partly due to variability of oocyte quality [3], which is defined as the potential of oocytes to produce a viable progeny [4]. Previous studies on Pacific oyster have assessed oocyte quality by diameter [5] or total lipid content [6]. Novel approaches, such as two-dimensional proteomics, could become powerful tool to study oocyte quality in bivalves, as in other species such as vertebrates and sea bass [7].
A fully grown oocyte accumulates large amounts of maternal messenger RNA and proteins [8]. Early embryonic development, before the major wave of embryonic genome activation, is believed to be dependent on these maternal factors, essential for fertilisation, first cleavage and embryonic genome activation [9]. Many studies demonstrate that maternal history modulates the expression and function of proteins in oocytes in a way that will impact offspring metabolism and survival [10] [11]. For example, the direct modulation of oocyte signalling pathways using pharmacological compounds or hormones can affect fertilisation success and developmental potential [12] [13]. In *Dicentrarchus labrax*, recent studies have indicated the contribution of some oocyte-specific proteins to the regulation of egg quality [7] [14].
To find out which maternal protein factors could be related to oocyte quality in the Pacific oyster Crassostrea gigas, we conducted a proteomic study using two-dimensional electrophoresis (2-DE). The 2-DE methodology was chosen to identify main changes in abundant and soluble oocyte proteins without a priori. This methodology does not allow to obtain a comprehensive description of the whole oocyte proteome. Recent developments in genomic resources for Pacific oyster [15] allowed us to identify proteins according to their MS/MS spectra obtained by tandem mass spectrometry.

2. Material and methods

2.1. Biological material

Twenty two-year-old oysters (mean weight ± SD = 58.7 ± 10.5 g) were collected in February from the Aber Benoît Estuary (Finistère, France) and transferred to the nearby Ifremer' facilities in Argenton. In order that the oysters achieved gonad maturation under controlled conditions, they were fed two microalgae, Isochrysis galbana Tahiti clone and Chaetoceros gracilis, at a ratio of 2:100 (dry mass algae to fry mass oyster) and maintained in 600-L tanks of 1-µm-filtered sea water at 17°C, with continuous sea water renewal [16]. After a conditioning period of 2 months, the oysters were sacrificed and a 2-3 mm cross section of the visceral mass was made in front of the pericardic region of each individual for later histological examination of the gonadic tissue. The left section of each gonad was rapidly rinsed in sea water at 20 °C and oocytes were immediately collected by stripping after scarification and filtration at 20 µm to remove cell debris [17]. The oocytes were then incubated in 2 L of filtered sea water and counted under light microscope.

2.2. Oocyte diameter measurement

For each female, a 50 µl sample of oocytes was collected with a 200 µl pipette tip and deposited in a 24 well micro-plate (Nunc™) filled up with 1 µm-filtered seawater at 20°C.

Immediately after collection, oocyte pictures were taken using a camera (SONY 3 CCD Color Video Camera) connected to a microscope (Leica, DMIL, x10 objective). Oocyte diameter was measured using the Image J software (n=30 oocytes / female).

2.3. Oocyte fertilization and D-larva yield

Oocytes were incubated less than 1 hour in sea water at 20°C before their fertilisation. Fertilisation followed a standard protocol as described in [17]. Briefly, for each female, triplicates of 25,000 collected oocytes were fertilised *in vitro* by a common mixed pool of spermatozoa collected from 3 males, using a non-limiting 800 sperm to egg ratio. To assess the percentage of oocytes of each female that was successfully fertilised and developed through the early embryo stages, the D-larva yields were quantified under a light microscope 48 h after fertilisation.

2.4. Total protein extraction from oocytes

For total protein extraction, $2 \times 10^{\wedge 6}$ oocytes were sampled by filtration at 20 µm into a 50 ml Falcon tube and collected by gentle centrifugation at 1000 rpm for 2 min at 4 °C. The conservation of oocyte morphology was checked under a light microscope. To solubilize proteins, 5 ml lysis buffer (150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5 % Igepal, 1 tablet of complete EDTA free protease inhibitor cocktail in 25 ml of buffer, phosphatase inhibitor cocktail III) were added. Total proteins were then extracted as described by [18]. Briefly, total protein lysates were obtained after homogenization with an Ultra-Turrax and soft centrifugation at 3,000 g for 1 h at 4 °C for elimination of lipids. The lipid layer was in the upper phase and was easily eliminated by pipeting. The interphase (around 4,5 ml) which contained the proteins was collected and the small pellet of cell debris was left. The interphase was then centrifugated at 10,000 g for 45 min at 4 °C to ensure total lipid depletion. The interphase (around 4 ml) was collected, aliquoted and stored at - 80 °C. For each lysate, the total protein content was quantified using a DC protein assay (Bio-Rad, Hercules, CA, USA) with 96-well micro-plates (NuncTM) in a micro-plate reader (Bio-Tek® SynergyTM HT) and KC4 v3 software to compare results with a calibration curve of standard proteins (Bovine Serum Albumin) provided with the DC protein assay kit.

2.5. Two-dimensional electrophoresis

Based on histological analyses, we selected 14 females in stage 3 of gonadic maturation (ripe oocytes as described in [19]) for protein analysis. For each female and oocyte sample, an equal amount (500 µg) of total protein lysate was precipitated and desalted to limit the presence of non-protein impurities such as salts, buffers, or remanent lipids in the samples before IsoElectroFocusing (IEF). Precipitation and desalting were performed by adding 2 volumes of 20 % TCA on ice for 20 min. The samples were then centrifuged at 12,000 g for 15 min and the pellet washed 5 times with 80 % acetone in 0.05 M Tris-HCl pH 8. Proteins were resuspended in a rehydrating solution (DeStreak, GE Healthcare) containing 1 % Immobilized pH Gradient ampholytes (IPG) and placed at room temperature for 1 h before IEF. A pool of 6 oocyte samples was used as an internal control of experimental repeatability. The IEF dimension was conducted on a Bio-Rad Protean IEF Cell system™ (Bio-Rad, Hercules, CA, USA). Total proteins were loaded onto each strip (Immobiline DryStrip pH 3-10, 11 cm, GE Healthcare), and passive rehydratation allowed at room temperature overnight using mineral oil to prevent sample evaporation. The IEF was carried out at 20 °C in four steps: a calibration step at 100 V constant voltage for 3 h, an active hydration step at 300 V constant voltage for 1 h, a continuous increase in voltage up to 8000 V over 8 h to start migration, and a migration step at 8000 V constant voltage for 11 hours. Before the second dimension, the IPG strip was rehydrated for 15 min in a solution for saturation containing 6 M urea, 2 % SDS, 30 % glycerol and 1 % DTT in 0.05 M Tris-HCl pH 8.8. The strip was then further equilibrated for 15 min in a similar buffer, in which DTT was

replaced with 2.5 % iodoacetamide to alkylate the proteins. For the second dimension of the 2-D electrophoresis, 8-16 % gradient SDS-PAGE precast gels (Bio-Rad, Hercules, CA, USA) were used, run on a Criterion system (Bio-Rad, Hercules, CA, USA). The gels were loaded with broad range SDS-PAGE molecular weight markers. Migration was realized at 200 V constant voltage for 1 h. After 2-DE, protein spots were stained by Coomassie blue coloration (PlusOne Coomassie Tablets, PhastGel Blue R-350, GE Healthcare) and washed in 7 % acetic acid, 30 % methanol solution. For image analysis, 16-bit level images of the gels were captured in tagged image file format (TIFF) and analysed using Progenesis™ SameSpots v1.5 software (Nonlinear Dynamics, Newcastle upon Tyne, UK). A composite image of the 14 gels (one for each studied individual) was generated, spots were detected automatically and all spot volumes were normalized using built-in algorithms. After automatic spot detection, spot boundaries were manually edited and artefacts on the images removed. Normalized spot intensities were obtained for each spot on the images. A comparison was made between the spots on the two groups of 7 gels (high *versus* low quality oocytes).

2.6. In-gel digestion for mass spectrometry

Twenty Coomassie blue-stained spots of interest were excised from the SDS-PAGE and processed for trypsin digestion. Excised spots were washed twice with milliQ water and de-stained for 15 min in 100 mM NH_4HCO_3 and 50 % acetonitrile (ACN), then dehydrated in 100 % ACN. They were re-hydrated in 100 mM NH_4HCO_3 and again dehydrated in 100 % ACN. Excised spots were air-dried and in-gel digestion was performed overnight at 37 °C with modified trypsin (Promega, Charbonnières-les-Bains, France) at 12.5 ng/µL in 70 µL of 50 mM NH_4HCO_3. The resulting tryptic peptides were extracted from the gel spots by sequential incubation in ACN / water / formic acid, 70:30:0.1 (v/v/v), then 100 % ACN and, finally, ACN / water / formic acid, 70:30:0.1 (v/v/v). The tryptic digests were then concentrated by vacuum centrifugation to reach a final volume of 30 µL.

3. Nano LC-MS/MS analysis

LC-MS/MS analyses were performed on a nano-HPLC system (Ultimate 3000, Dionex, Jouy-en-Josas, France) coupled on-line with an Esquire HCT Ultra PTM Discovery mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nanoflow electrospray ionisation source and an ion trap analyzer. Tryptic digests were concentrated by injecting 22 µL volumes into a Dionex C18 PepMap300 trapping reverse phase pre-column (5 µm, 300 Å / 5 mm x 300µm inner diameter). Peptides were then passed onto a Dionex C18 PepMap100 reverse phase nano-column (3 µm, 100 Å / 150 µm x 75 µm inner diameter) and eluted with a gradient of 2 % to 90 % ACN in 0.05 % formic acid for 52 min at a flow rate of 250 nL/min. The mass spectrometer was operated in positive ionization mode with an analytical scan range of m/z 150-2500 Th. Ion accumulation time was automatically set with an ion charge control (ICC) target of 90 000 to avoid space charge effects. EsquireControl™ software (Bruker Daltonics) automatically alternated MS and MS/MS acquisitions in an exclusion dynamic mode (the seven most intense ions were selected and excluded from further selection for a duration of 0.25 min). MS/MS data were acquired using a 4 *m*/*z* unit ion isolation window and a 1.2 V fragmentation amplitude.

3.1. Databases searches

MS/MS data files were used to query Gigasdatabase, a database containing 82,312 contigs (**Database ID**: http://public-contigbrowser.sigenae.org:9090/Crassostrea_gigas/, [15]), using the Mascot algorithm (Mascot server v2.2; http://www.matrixscience.com). Search parameters were as follow: one missed cleavage allowed, carbamidomethylation of cysteins set as fixed modification, methionine oxidation set as variable modification, 0.25 Da

precursor tolerance and 0.5 Da fragment tolerance. Peptide identifications were accepted if the individual ion Mascot scores were above the identity threshold (an ion score f $-10 \times \log(p)$ where p is the probability that the observed match is a random event). In case of ambiguous assignments (one compound fitted to more than one peptide), peptides were accepted based on the peptide score, meaning that the peptide sequence with the highest score was accepted and it did not matter if the protein was contained in the list. The matching of identified peptides to proteins was performed with the ProteinExtractor algorithm [20], so that every protein reported was identified by at least one peptide with a significant ion Mascot score (above the identity threshold), that could not be mapped to a higher-ranking protein already in the result list. This means that the protein lists provided contain only those proteins and protein variants that could be distinguished directly by MS/MS. Homology searches obtained from the Gigasdatabase were validated or sometimes modified using xblasting in NCBI. This one by one annotation of the selected ESTs therefore improved their annotation compared to the automatic annotation done for the Gigasdatabase contigs, probably due to some false stop codons coming from sequencing errors and/or from mutations known to be very abundant in C. gigas [21]. For every protein reported in the identification list, a combined protein score (metascore) was calculated from the peptide scores using the ProteinExtractor algorithm. The calculated peptide mass and pI of the translated ESTs were evaluated on the EXPASY website (http://web.expasy.org/compute_pi/) [22].

3.2. Statistical analysis

Results were expressed as mean ± standard deviation (SD). Percentages were square-root transformed and statistical analyses were performed using Student's test at a significance level of $p = 0.05$. For 2-DE pattern analyses, three biological replicates for each female and two analytical replicates for each protein sample were compared between high quality and low quality oocytes. Differences in spot abundance were statistically evaluated after normalisation ($p < 0.05$), using the Progenesis Stats tool from the Progenesis SameSpots© software package (Prodigy ©).

4. Results and discussion

4.1. D-Larva-D yield

When the histological examination of the gonads showed female oysters to be in the process of oocyte resorption [19], these individuals were excluded from all analyses. As a result, 14 females at stage 3 of gonadic maturation (i.e. mature oocytes with apparent nuclei and nucleoli) were selected for our study. A high variability between individuals was observed for D-larva development, ranging from 1 to 58 % (Fig. 1). Because the environmental conditions and experimental procedures were kept uniform between females, this variation was most likely to be due to the intrinsic quality of oocytes of each female. The mean D-larva yield observed in this study is close to values reported for a standardised experimental fertilisation protocol established for Pacific oyster oocytes [17]. Such individual variability is commonly observed in molluscs [3] [23] as well as in fish [24]. The females were split into two groups, each containing 7 individuals, between which there is a significant difference for early developmental success of fertilised oocytes ($p < 0.001$): a high quality group (52 % ± 5 as mean ± SD) and a low quality group (11 % ± 9 as mean ± SD). No statistical difference was obtained in the oocyte diameter between high quality group (77.7µm ± 10.1 as mean ± SD) and low quality group (71.1 µm ± 4.5 as mean ± SD).

4.1.1. Proteomic analysis of differentially expressed proteins

Spots analysed were strongly detectable using classical Coomassie staining of standard 2-D gels. Fig. 2A and B illustrates gels of high and low quality females, respectively. A comparison was made between 7 proteome maps from high quality females and 7 proteome maps from low quality females. Gels were obtained from triplicates of biological sample for each female and were done in duplicates as recommended in Sinha and Chattopadhyay (2011) [25]. The analysis of 2-DE pattern revealed 106 resolved spots corresponding to abundant soluble proteins extracted from oocytes. Among them, only 20 spots presented significant oocyte quality-dependent changes (Fig.3), showing a statistically significant down ($n = 12$) or up-accumulation ($n = 8$) in high quality oocytes. These differentially expressed spots ($n = 20$; $p < 0.05$) were excised and subsequently digested with trypsin. All selected spots ($n = 20$) produced peptides suitable for identification using nano-liquid chromatography coupled tandem mass spectrometry with significant hits (100 % identification success). Table 1 shows the list of the 20 spots identified at a significant level of confidence by MS/MS which corresponded to 16 distinct proteins. The resolution of protein separation obtained using classical 2-D proteomic methodology led sometimes to the identification of several proteins in the same spot (Table 1). Average fold change, represented by increase in spot intensity, varied from 1.1 to 1.8 for low quality, and from 1.1 to 1.9 for high quality oocytes (see supplementary data). In this study, we did not obtain any *de novo* spots typical of high or low quality.

This work is the first 2-DE proteome study dedicated to oocytes of Pacific oyster. Proteomic approach with unsequenced organisms remains a challenge but, with 82,312 EST contigs currently available for *C. gigas* [15], we were able to identify proteins on the basis of mass spectrometry results. Similar proteomic studies were recently published for two other oyster species in which genomic information is not as abundant: to study proteome from hemolymph of Sydney rock oyster *Saccostrea glomerata* and proteome from shell and mantle of the Pearl oyster *Pinctada margaritifera* [26] [27]. The abundant maternal proteins that are easily detectable through proteomics might have a variety of important functions during oocyte maturation and early development [28]. Here we analysed the oocyte proteins stained with Coomassie blue that identified abundant proteins, such as vitellogenins, but made it difficult to identify proteins expressed at low levels [29] [30]. Although we did not provide a comprehensive description of the whole proteome changes related to oocyte quality, based on the differing nature of the proteins identified in the low and high quality oocytes, we can discuss the possible physiological meaning of their variation.

4.2. Up-accumulated proteins in low quality oocytes

Twelve spots up-accumulated in low quality oocytes corresponded to ten distinct proteins (Table 1). Among them, five vitellogenin (Vg) protein members were identified that corresponded to Vg-breakdown products, likely related to oocyte ageing. We also identified four enzymes, suggesting the influence of intracellular metabolism on oocyte quality and subsequent embryonic development. The last, protein we identified was one involved in cytoskeletal dynamics.

4.2.1. Yolk protein vitellogenins

Vertebrate and invertebrate Vg proteins (homodimers of 250 – 600 kDa) are large phospholipoglycoprotein precursors that are cleaved to generate yolk storage proteins traditionally regarded as the energy reserve for nourishment of the developing embryos [31]. In Pacific oyster, Vg are internalized by developing oocytes and proteolytically cleaved during the blastula stage to generate vitellin, which is later used as a nutrient source by developing embryo [32] [33].

The oyster protein vitellogenin Vg (Genbank accession # AB084783; [34]), with a predicted molecular weight of 179.2 kDa and pI of 9.2, was isolated from several up-accumulated spots from low quality oocytes. It had an apparent molecular weight around 200 kDa with pI of 9.2, as expected (Table 1; spots # 2 and 3) but showed different results in the other spots: 20, 50 and 90 kDa (Table 1, spots # 1; 8 and 4) with pI of 6.2, 7.0, or 10, respectively. These molecular weights suggest that Vg had been cleaved into small parts and that these spots were cleavage products of the whole Vg protein of 179.2 kDa. This idea is supported by *in silico* analysis of Vg using the FindPept tool at EXPASY (http://web.expasy.org/compute_pi/) [22], which predicted this size of Vg peptides, and the fact that spots and predicted peptides both contained the unique matching peptides for Vg in our MS/MS identification. In the stink bug *Plautia crossota stali*, the cleavage of a Vg protein of 140 kDa was demonstrated in oocytes by bands of 23, 37, 50, 70 and 90 kDa in a western – blot, identified using anti-Vg antibody [35]. This breakdown of Vg-containing yolk granules was obtained when degeneration of mature vitellogenic oocytes was induced by starvation, indicating that Vg was targeted by proteases when oocytes began resorption [35] [36]. In our study, females showing clear patterns of resorption in a histological examination were excluded from our proteomic analysis, but we can not ascertain whether biochemical mechanisms involved in oocyte resorption could have begun in low quality oocytes. Further studies should be necessary to address this question by comparison of proteome between low quality and resorbed oocytes.

However, we can hypothesized that low quality of oocytes in Pacific oyster involved the same mechanisms observed for oocyte ageing in other species. Indeed, in rainbow trout, Vg-breakdown products were accumulated during ageing of post-ovulatory oocytes, which can last 2 to 3 weeks, and oocyte ageing was associated with a progressive egg-viability decrease [37]. A proteomic analysis of the coelomic fluid showed that post-ovulatory ageing of oocytes increased the amount of Vg fragments in the coelomic fluid and could indicate egg-quality defects [37]. These Vg fragments came from the leakage of some oocyte components and might be due to increased proteolytic activities in poor-quality oocytes; these might include cathepsin D activity, as suggested in sea bream [38]. Oocyte protease activities might thus play a major role in oocyte quality.

Moreover, electrophoretic mobility shifts in 2-DE gels revealed that Vg protein might also contain post-translational modifications, as demonstrated in oocytes of *Mya arenaria,* rainbow trout, and Green bug, *Plautia crossota stali* [39] [37] [36]. For the four other Vg members up-accumulated in low quality oocytes – three vitellogenin precursors and Vitellogenin-2 – post-translational modifications were suggested since these were identified in spots with a higher molecular weight that the one predicted (Table 1; spots # 4; 5; 6 and 7). Indeed, Vg members have potential sites for lipidation, glycosylation and phosphorylation, and post-translational modifications can alter the biochemical properties and function of Vg in oocytes, as demonstrated in the clam *Mya arenaria* [39]. In fact, hyperphosphorylation led to particular chemical composition of Vg that changed Vg-binding properties and induced dysfunction of Vg in the oocyte and developing embryo [39].

4.2.2. Up-accumulation of metabolic enzymes

In our study, Isocitrate DeHydrogenase (IDH) was up-accumulated in low quality oocytes (Table 1, spots # 8 and 9) and could reflect a particular type of redox metabolism. IDH is known to be involved in energy homeostasis, which is crucial for fertility in mouse oocytes [40]. In a comparison of two lines of Pacific oysters selected to be resistante (R) or susceptible (S) to summer mortality (for further details, see [41]), the mRNA encoding IDH was over-expressed in the gonad of resistant oysters and was related to their greater capacity to detoxify reactive oxygen species (ROS) [42]. ROS are known to be deleterious as they modify biological molecules, including lipids, proteins and nucleic acids, inducing abnormal development and embryonic lethality in mouse [43]. Further studies are necessary

to better understand the biological significance of IDH protein up-accumulation and redox metabolism in oocytes, and how differences in these processes lead to low D-larva yields.
Two enzymes involved in *de novo* purine synthesis were up-accumulated in the low quality oocytes: PhosphoribosylAminoImidazole carboxylase (PAI carboxylase; Table 1, spot # 8) and ATP synthase subunit α (Table 1, spot # 10). Synthesis of purines and phosphoinositides is involved in spontaneous and progesterone-induced nuclear maturation in *Bufo arenarum* oocytes [44], while maturation in mouse oocytes is induced *in vivo* when purine metabolism is altered [45] [46]. These studies point to the critical role of purine metabolism in the maintenance of oocyte maturation.
We also found up-accumulation of a DyP-type peroxidase in low quality oocytes (Table 1, spot # 11). In the sea urchin, such an "ovoperoxidase" is stored within the oocyte and helps to block polyspermy at fertilisation by modifying the surface envelope of the eggs [47] [48]. This peroxidase could be spermicidal [49] and could also lead to the production of a hard fertilisation membrane capable of blocking the entry of additional sperm and/or killing sperm in the vinicity of the fertilised egg. We can hypothesize that the Dyd-type peroxidase up-accumulation we observed in low quality oocytes could have altered the oocyte envelope in oyster, thus affecting fertilisation success.

4.2.3. Up-accumulation of cofilin

Cofilin is a small protein of 15 kDa (Table 1, spot # 12), belonging to the family of actin-depolymerizing factors that changes cytoskeletal dynamics and regulates cell motility, cytokinesis, and calcium signalling pathways [50]. When injected into starfish oocytes, cofilin altered the spatio-temporal pattern of physiologically occurring Ca(2+) signals essential for oocyte maturation and fertilisation [50]. In *Xenopus* oocytes, the protein cofilin is localised in the cytoplasm and its function is regulated at the post-translational level. At fertilisation, dephosphorylated cofilin migrates along the membrane to ensure the subsequent cleavage of blastomeres [51]. Taken together, we can suppose that up-accumulation of cofilin in low quality oocytes of Pacific oyster could indicate an abnormal regulation of cytoskeletal dynamics and Ca(2+) signalling that could lead to early embryo failure.

4.2.4. Up-accumulated proteins in high quality oocytes

Eight spots up-accumulated in the high quality oocytes corresponded to six distinct proteins (Table 1). Among them we identified vitellogenin (with a different size than in low quality oocytes), three chaperone molecules involved in folding and maturation of nascent polypeptides, and two proteins involved in cell-cycle control.

4.2.5. Yolk protein vitellogenins

In high quality oocytes, two Vitellogenin (Genbank accession # AB084783) cleavage products with individual molecular weights of 70 kDa and 40 kDa were up-accumulated (Table 1; spots # 13 and 14). Some protease activities during oogenesis [47] might thus ensure specific Vg breakdown related to the status of oocytes and with consequences for fertilisation and the developmental success [33].

4.2.6. Chaperone molecules

We observed a higher amount of Heat-Shock Cognate protein of 70 kDa (HSC70) in high quality oocytes (Table 1; spots # 14 and 15). These molecular chaperone proteins bind to nascent polypeptides, promote correct protein folding and prevent the aggregation of non-native and misfolded proteins [52] [53]. In mouse, activation of the zygotic genome initiates

an accumulation of embryonic transcripts in the early two-cell embryo that needs heat-shock proteins to ensure the accurate translation and folding of nascent proteins [54]. Interestingly, HSC70 was seen to sustain the block to polyspermy in amphibian oocytes [55] and viability of bovine eggs is related to the amount of constitutive HSC70, which has protective effects against oxidative stress, temperature and pH fluctuation, and exposure to visible light [56]. In Pacific oyster oocytes, the up-accumulation of HSC70 could be related to better folding of proteins in two cell embryos, and better protection of oocytes and early embryos directly exposed to such environmental factors in the sea water.
Cyclophilin, a protein known to catalyze protein folding in cooperation with heat-shock protein [57] [58] was also up-accumulated in high quality oocytes (Table 1, spots # 6; 7 and 8). This is a peptidylprolyl isomerase that belongs to the immunophilin family, and could thus be advantageous for the viability and immune protection of eggs and early embryos of Pacific oyster. Protein Disulfide Isomerase (PDI) is also a chaperone molecule up-accumulated in high quality oocytes (Table 1; spots # 16). PDI catalyzes the rearrangement of disulfide bounds in proteins, particularly Vg [59]. In mouse, PDI is one of the highly abundant proteins localised at the surface of the mature oocyte plasma membrane (oolemma) [29], that is essential for the sperm-egg fusion at fertilisation [60]. In oyster, up-accumulation of PDI could reflect a better capacity of oocytes to produce Vg maturation products and/or control sperm fusion.

4.2.7. Cell-cycle control

14-3-3 ε protein was up-accumulated in high quality oocytes (Table 1; spot # 17). 14-3-3 ε protein belongs to a highly conserved acidic protein family expressed in oocytes [61] [62] that can interact with over 200 target proteins involved in a variety of cellular processes including regulation of cell cycle, metabolism and protein trafficking [63]. Today, little is known about 14-3-3 ε function in oocytes, but it has been suggested that 14-3-3 proteins inhibit G2/M progression to hold the cell in prophase arrest in mammalian and amphibian oocytes [64]. Moreover, in the sea urchin, 14-3-3ε was involved in the stress response elicited by UV-B radiation in early embryo at the gastrula stage by promoting embryo survival [65]. Up-accumulation of 14-3-3ε could thus be related to cell-cycle control in oocytes and better protection of embryos in Pacific oyster.
Proliferative Cell Nuclear Antigen (PCNA) was also up-accumulated in high quality oocytes (Table 1; spot # 17). PCNA is a nuclear protein that helps the processivity of DNA replication and is involved in cellular processes such as cell-cycle control, base-excision repair and apoptotic pathways. In the gonadic tissue of Pacific oyster, PCNA expression was observed to increase during oogenesis [66] [67]. PCNA might be a maternal factor and its accumulation in high quality oocytes could be linked with the processivity of DNA synthesis during early development. In fact, recent and increasing information becomes available on PCNA in the gonad of Pacific oyster whose expression was exclusively restricted to germinal cells [66]. PCNA thus appears as a good candidate to construct an enzyme-linked immunosorbent assay to monitor oocyte quality between females.

5. Conclusions

Females Pacific oysters do not all produce oocytes of equal quality. Using a 2-DE proteomic approach, we identified several proteins that were differentially accumulated according to oocyte quality. Characterization of these proteins provided important insight into the mechanisms of early development in this marine bivalve. Future studies will search for environmental factors and maternal effects that might regulate the amounts of oocyte proteins produced, in order to improve our understanding of their variation under natural or controlled conditions (e.g. in oyster hatcheries).

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Supplementary data

The proteomic identification data (.dat files) were converted and submitted to the EBI (European Bioinformatics Institute: www.ebi.ac.uk/) [68] and are accessible via the PRIDE repository (http://www.ebi.ac.uk/pride/startBrowse.do) under the project name: "Qualityseed" (Accession numbers: 22028-22047), with reviewer access (username: review69532 and password: $aeWKCFw).

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Figures

Figure 1

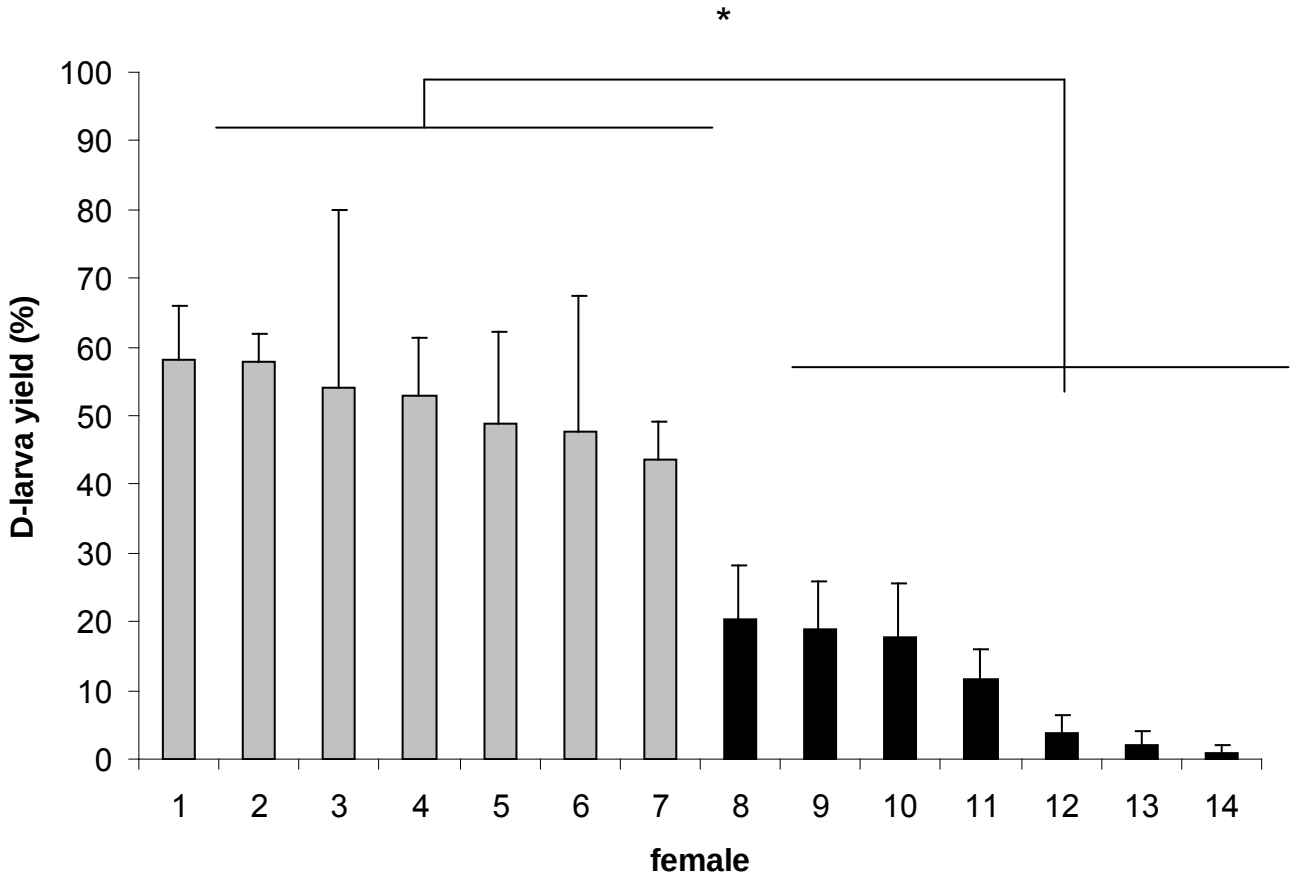

Figure 1: Individual D-larva yield (mean ± SD) obtained from 14 females Pacific oysters *Crassostrea gigas* divided into two groups: high quality (grey bars) and low quality (black bars) oocytes ($p < 0.001$). Results are expressed as the percentage of oocytes that reached D-larval stage 48 hours after fertilisation.

Figure 2

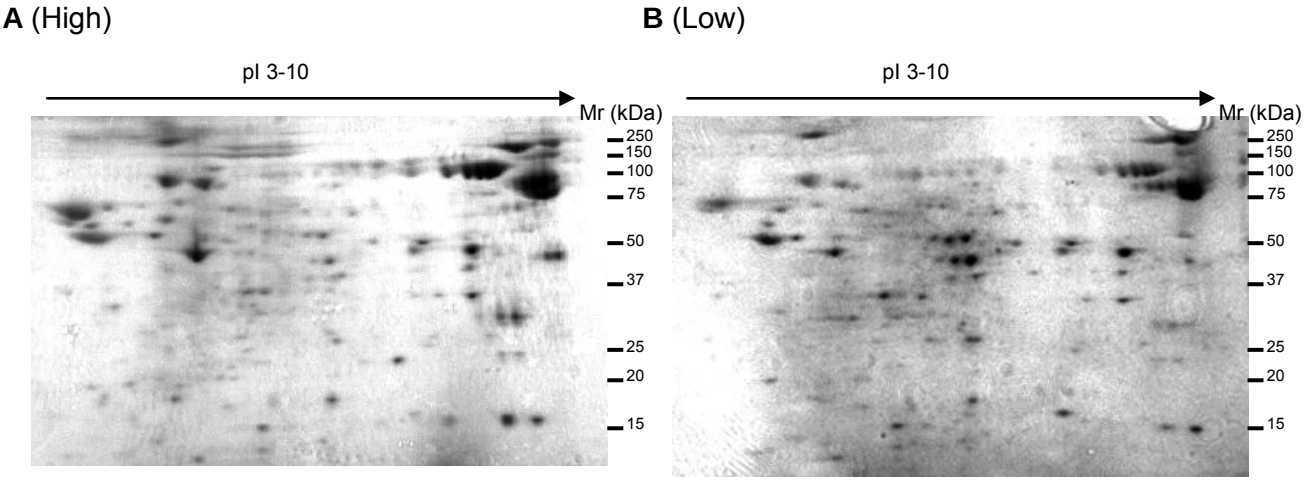

Figure 2: Gels of a 2-DE protein profile of high (A) or low (B) quality oocytes collected from female Pacific oysters. Results were obtained by separating 500 µg of protein samples on a 11 cm pH 3-10 strip followed by SDS-PAGE. The gels were stained by Coomassie blue coloration. Mr: molecular marker.

Figure 3.

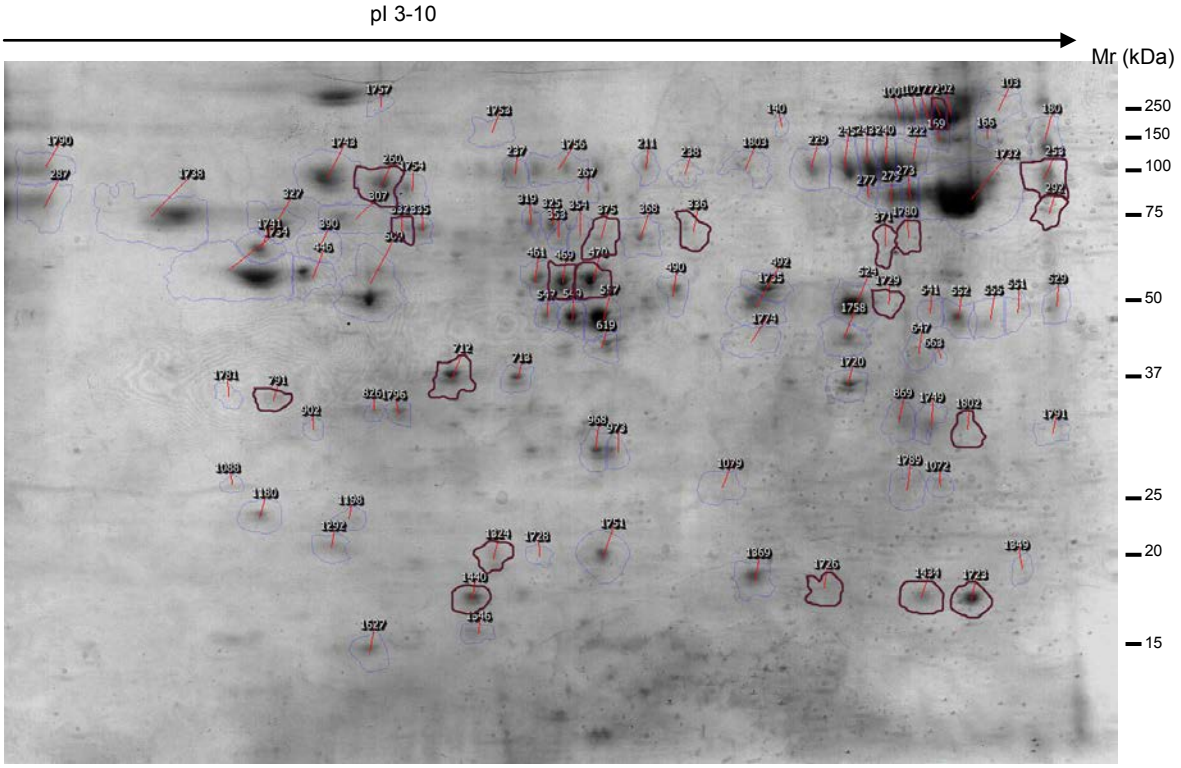

Figure 3: Reference image showing a gel of low quality oocytes. Resolved spots are marked with corresponding spot number and excised spots were encircled in brown. When a spot is identified, the name of the protein is shown in Table 1 and details of the corresponding mass spectrometry data are given in the Supplementary data. Mr: molecular marker.

Table 1. Identified proteins up-accumulated in low or high quality oocytes from Pacific oyster.

serial number	spot number	average fold change	protein annotation [organism]	Genbank accession number	Mr (kDa)/pI theoritical	Mr (kDa)/pI experimental	Sequence coverage (%)	unique peptide matched	score
Up-accumulated spots in low quality oocytes (12)									
1	1324	1.1	Vitellogenin [Crassostrea gigas]	AB084783	179.2 / 9.2	20 / 6.2	2.5	2	176.8
2	1772	1.2	Vitellogenin [Crassostrea gigas]	AB084783	179.2 / 9.2	200 / 9.2	9.9	14	902.8
3	253	1.3	Vitellogenin [Crassostrea gigas]	AB084783	179.2 / 9.2	200 / 10.0	12.1	13	841.1
4	292	1.8	Vitellogenin [Crassostrea gigas]	AB084783	179.2 / 9.2	90 / 10.0	9.4	14	926.3
			Vitellogenin precursor [Crassostrea gigas]	CX069168	25.0 / 9.2	90 / 10.0	12.3	2	143.0
			Vitellogenin-2 [Crassostrea gigas]	CU682295	60.8 / 8.8	90 / 10.0	3.3	2	105.9
5	371	1.2	Vitellogenin-2 [Crassostrea gigas]	CU682295	60.8 / 8.8	70 / 8.8	18.4	9	557.1
			Vitellogenin precursor [Crassostrea gigas]	FP089745	21.9 / 9.7	70 / 8.8	9.0	2	302.2
6	1780	1.8	Vitellogenin precursor [Crassostrea gigas]	CX069168	25.0 / 9.2	70 / 9.0	12.8	2	118.5
			Vitellogenin-2 [Crassostrea gigas]	CU682295	60.8 / 8.8	70 / 9.0	2.0	1	114.7
7	1802	1.3	Vitellogenin precursor [Crassostrea gigas]	FP089757	24.6 / 9.1	25 / 9.5	24.8	6	362.3
			Vitellogenin precursor [Crassostrea gigas]	CX069168	25.0 / 9.2	25 / 9.5	10.6	1	360.8
8	469	1.2	Isocitrate dehydrogenase [Crassostrea gigas]	CK172330	50.3 / 7.9	50 / 7.0	14.4	6	383.1
			Phosphoribosylaminoimidazole carboxylase [Bombyx mori]	AM866034	28.7 / 5.3	50 / 7.0	6.3	3	265.1
			Vitellogenin [Crassostrea gigas]	AB084783	179.2 / 9.2	50 / 7.0	2.5	4	264.2
9	470	1.2	Isocitrate dehydrogenase [Crassostrea gigas]	CK172330	50.3 / 7.9	50 / 7.3	28.8	10	668.7
10	375	1.3	ATP synthase subunit alpha [Bos taurus]	BQ426532	59.8 / 8.5	70 / 7.2	23.0	12	870.7
11	712	1.2	Dyp-type peroxidase [Schistosoma mansoni]	AM858788	41.4 / 8.2	37 / 6.0	7.9	10	688.9
12	1440	1.1	Cofilin [Schizosaccharomyces pombe]	AM854555	15.6 / 5.8	15 / 5.6	57.3	5	364.6
Up-accumulated spots in high quality oocytes (8)									
13	1729	1.3	Vitellogenin [Crassostrea gigas]	AB084783	179.2 / 9.2	40 / 8.8	6.3	12	762.2
14	336	1.1	Vitellogenin [Crassostrea gigas]	AB084783	179.2 / 9.2	70 / 7.3	3.5	6	394.3
			Heat shock 70 kDa protein cognate [Crassostrea gigas]	AF144646	72.0 / 5.38	70 / 7.3	3.4	2	119.5
15	260	1.3	Heat shock 70 kDa protein cognate [Crassostrea gigas]	AF144646	72.0 / 5.38	80 / 5.5	24.4	16	1152.9
16	332	1.1	Disulfide-isomerase [Gallus gallus]	AM854930	55.5 / 5.7	60 / 5.8	15.4	6	432.5
17	791	1.7	14-3-3 protein epsilon [Drosophila melanogaster]	AM854805	29.1 / 4.7	30 / 4.8	26.5	9	565.9
			Proliferating cell nuclear antigen [Macaca fascicularis]	EW779000	28.7 / 4.6	30 / 4.8	37.6	9	531.8
18	1723	1.4	Cyclophilin [Homo sapiens]	FU6OSJA01BDE13	17.5 / 8.7	15 / 9.5	29.2	7	452.2
19	1726	1.9	Cyclophilin [Homo sapiens]	FU6OSJA01BDE13	17.5 / 8.7	16 / 8.0	6.7	2	128.6
20	1434	1.8	Cyclophilin [Homo sapiens]	FU6OSJA01BDE13	17.5 / 8.7	15 / 9.2	20.1	3	193.6