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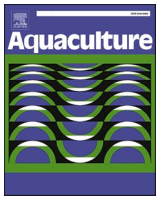
Aquaculture 593 (2024) 741270

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



Early postovulatory aging reveals the first proteomic markers of egg quality in pikeperch

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ARTICLE INFO

Keywords:
Fish
Egg quality
Postovulatory aging
Reproduction
Proteomics

ABSTRACT

This study explored the molecular mechanisms underlying egg quality deterioration due to *in vivo* postovulatory aging in pikeperch (*Sander lucioperca* L.), a key species in aquaculture. We employed tandem mass tag (TMT) peptide labeling coupled with LC–MS/MS quantitative proteomics to analyze eggs collected at various post-ovulation intervals. Our research revealed four distinct proteomic markers (Gins4, Atrx, DnaJB14, and Mrpl10) that are differentially expressed in response to early aging, shedding light on their potential roles in DNA replication, chromatin organization, protein folding, and mitochondrial function. The study confirmed that eggs maintain morphological integrity up to 5 h postovulation but exhibit compromised fertilization capacity, underscoring the importance of timely egg utilization in aquaculture practices. These findings enhance the understanding of egg aging at the molecular level, offering insights for improving reproductive success and larval quality in pikeperch aquaculture. The data are available *via* ProteomeXchange with the identifier PXD048349.

1. Introduction

Egg quality is widely acknowledged as one of the most critical factors influencing the reproductive performance of both wild and cultured fish stocks and has further direct and relevant effects on recruitment success and aquaculture effectiveness (Żarski et al., 2021). Therefore, it is of utmost importance to comprehend the processes that influence egg quality. This understanding will enable the development of appropriate measures for both ecological and commercial purposes. High-quality eggs can undergo successful fertilization and develop into viable embryos (Bobe and Labbé, 2010). Reduced egg quality is characterized by reduced activation capacity, fertilization failure, decreased embryo hatchability, and increased larval mortality or deformity (Żarski et al., 2012a, 2012b, 2021). In general, in fish, the quality of eggs is strongly influenced by various factors, both extrinsic (*e.g.*, environment, husbandry practice, broodstock diet) and intrinsic (*e.g.*, maternal transcripts and proteins stored in eggs; *in vivo* aging), and some of these factors have been linked to specific embryonic mortalities and malformations (Bonnet et al., 2007).

One of the most specific intrinsic factors influencing egg quality in various fish species (Samarin et al., 2016, 2019a, 2019b) is so-called postovulatory *in vivo* aging (in which eggs are retained following ovulation in the body cavity or in the ovary—depending on the species—and are exposed to processes leading to atresia). At first, molecular structures and mechanisms responsible for fertilization are affected, and next – whenever prolonged – to atresia, where eggs are degenerated to be further reabsorbed by the fish body. Considering that this process is a specific challenge test for eggs and that the loss of quality can be precisely controlled by the duration of retention in the ovary/body cavity (Samarin et al., 2019b), this approach constitutes an excellent research strategy for revealing the processes involved in shaping the developmental competence of eggs. However, prolonged postovulatory aging is known to induce severe morphological and biochemical alterations in eggs, commonly referred to as "overripening". These changes are closely

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https://doi.org/10.1016/j.aquaculture.2024.741270
Received 11 January 2024; Received in revised form 7 June 2024; Accepted 21 June 2024
Available online 23 June 2024

associated with reduced fertilization rates, compromised embryo quality, and the occurrence of abnormalities in offspring (Samarin et al., 2019b). Most related research has focused primarily on investigating the consequences of excessive postovulatory aging on the developmental competence of eggs (Aegerter et al., 2005; Rime et al., 2004), and explorations of the specific molecular mechanisms impacted during the early phases of egg aging are lacking. Therefore, evaluating the age-induced molecular mechanisms that alter intracellular egg homeostasis and impair the subsequent development of eggs while concurrently preserving the structural integrity of the cellular components of eggs without observable signs of atresia (*i.e.*, decomposition of the structural integrity of cells leading to observable deterioration of the eggs) enables the identification of novel quality-dependent molecules and related biological processes. It is commonly known that aging is a species-specific process and begins after hours or days post ovulation (Aegerter and Jalabert, 2004; Nomura et al., 2013). Therefore, post-ovulatory aging must be studied individually for each fish species. This is particularly critical for pikeperch (*Sander lucioperca* L.), an economically important freshwater fish species intensively cultured in closed recirculating aquaculture systems (RASs), which are entirely artificial environments under human control. However, RAS has adverse effects on pikeperch reproductive physiology, primarily associated with unpredictable and highly variable egg quality (Policar et al., 2019; Żarski et al., 2020).

To date, assessments of fish egg quality have been conducted using various approaches, including evaluating the morphological characteristics of eggs, analyzing biochemical parameters, measuring fertilization rates, assessing hatching success, and monitoring the rates of embryo and larval malformations. In the case of pikeperch, distinguishing high-quality from low-quality eggs can be performed by microscopic assessment of the intensity of the cortical reaction and fragmentation of the oil droplet (Żarski et al., 2012a, 2012b). In subsequent studies on pikeperch egg quality, the integration of transcriptomic and proteomic analyses enabled to gain new insights into the mechanisms underlying egg quality and allowed for comprehensive characterization of the molecular composition of eggs (Żarski et al., 2020, 2021). However, a molecular portrait of high- and low-quality eggs has yet to be drawn. This can be achieved by molecular characterization of eggs of various qualities obtained following a wide spectrum of zootechnical strategies that can have deterioratory effects on the developmental competence of the eggs. Importantly, different types of molecules, such as poorly studied proteome, should be considered.

In our previous studies we explored protein markers associated with high-quality pikeperch eggs using gel-based proteomics, specifically fluorescence gel electrophoresis 2D-DIGE (Nynca et al., 2020), to study domesticated and wild pikeperch stocks. However, this method had several drawbacks, including low sensitivity, poor reproducibility, underrepresentation of low-abundance proteins, and overlapping proteins in a single spot (Haynes and Yates, 2000). To address these issues, we switched to a gel-free approach using high-throughput mass spectrometry (LC-MS/MS), which allowed a more comprehensive analysis of pikeperch egg proteins. This change led to the identification of 1450 proteins, forming the first complete protein inventory for pikeperch eggs (Nynca et al., 2022; Żarski et al., 2021). However, this extensive profiling found no significant differences in protein abundance between high- and low-quality eggs, suggesting that the proteome might not directly relate to egg quality (Żarski et al., 2021). This contradicted other studies where protein abundance had been linked to egg quality (Yilmaz et al., 2017). The lack of association might result from sample preparation methods lacking fractionation, or the insufficient sensitivity of the mass spectrometry technique used. To improve our approach, in this study we adopted the FASP sample preparation method, yielding high-purity peptides free from contaminants that could affect mass spectrometry results. We also introduced peptide fractionation before analysis, which is crucial for successful protein sequencing (Wiśniewski, 2017). Additionally, we used a more sensitive proteomic technique with tandem mass tags (TMT) for isobaric labeling, which allows for more accurate quantification and enables the multiplexing of multiple samples in a single experiment, reducing variability and increasing precision (Chen et al., 2021). These enhancements paved the way for a more detailed investigation into the protein markers of fish egg quality.

The objective of this study was to elucidate the molecular mechanisms contributing to developmental failures and to identify predictive proteomic markers associated with the loss of quality of eggs induced by the *in vivo* aging process in pikeperch. It has already been reported that *in vivo* aging in pond-grown fish, prepared for spontaneous reproduction (where males and females were expected to spawn spontaneously) in specifically prepared tanks (with nest-like bottom) reduces the egg viability only after 12 h at 15 °C (Samarin et al., 2015). However, our first trials showed that this period is too long during the fully controlled pikeperch reproduction (including hand-stripping of the eggs and *in vitro* fertilization). Therefore, we performed first the initial experiments to identify the most suitable period of time of egg *in vivo* aging (prolonged retaining of the eggs inside the ovaries after ovulation) in pikeperch. Secondly, we employed TMT peptide labeling coupled with LC–MS/MS to perform the main experiment focused on the proteomic comparison of eggs collected 1 h (1 hPO, control group) and 5 h post-ovulation (5 hPO, aged group). The 5-h time point was deliberately chosen based on results from initial experiments.

2. Materials and methods

2.1. Ethics

All procedures involving the handling of animals, including reproductive processes, were conducted in accordance with European and national regulations pertaining to animal welfare and ethical considerations in animal experimentation. Whenever appropriate, the procedures were approved by the Local Ethical Committee in Olsztyn, Poland (Number 30–2021).

2.2. Initial experiments

In order to address the research question methodically appropriately we have performed two initial experiments only to determine the most appropriate moment of egg sampling. In other words, we have undertaken initial experiments to follow the *in vivo* egg aging in pikeperch in order to determine the most suitable period for molecular investigation of this phenomenon. To achieve this, we introduced several criteria which we were following while identifying symptoms of egg aging, which included highly reduced cortical reaction [*i.e.* chorion of the eggs after water activation did not cause significant alteration of the eggs shape; for details see (Żarski et al., 2012b; Fig. 1)], preservation of egg structural integrity [hereinafter referred to as “internal damages”, being obvious sign of commencement of atresia (Fig. 2)], and a significant reduction in the fertilization rate.

2.2.1. Initial experiment 1 – Evaluation of maximum aging period of pikeperch eggs in domesticated stock

In this experiment, we used domesticated pikeperch broodstock grown in a recirculating aquaculture system with controllable photothermal conditions. The fish were subjected to an optimal photothermal program for pikeperch (Dietrich et al., 2021) and controlled reproduction (Żarski et al., 2019). Briefly, $n = 3$ pikeperch females were kept at 12 °C with 14 h of light day and hormonally induced (500 IU of human chorionic gonadotropin per kg of body weight, injected intraperitoneally) and further subjected for evaluation of their oocyte maturation stage as described (Żarski et al., 2012a). When they reached maturation stage 6 (after the germinal vesicle breakdown but before ovulation), the females were sutured with surgical thread to prevent egg loss (Żarski et al., 2015, 2017). The fish were monitored every 6 h for ovulation signs; each time, the suture was gently loosened to check for

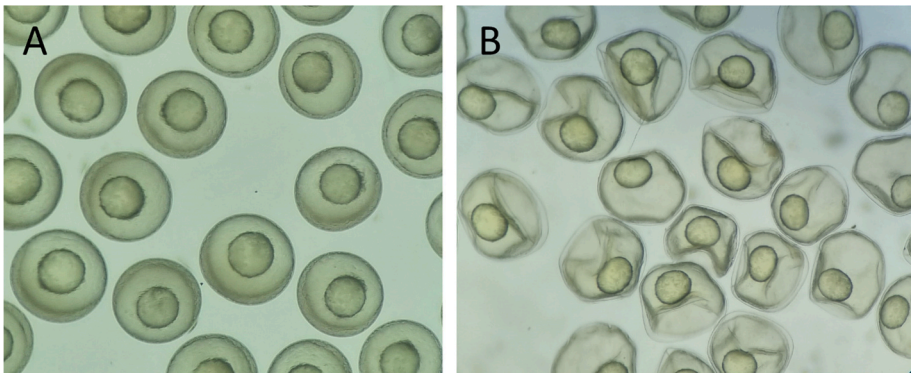


Fig. 1. Cortical reaction as a phenotypic indicator of egg quality in pikeperch. A: Phenotype of eggs of low quality (not exhibiting cortical reaction 5 min after water activation); B: Phenotype of eggs of good quality (exhibiting cortical reaction leading to temporary egg malformation 5 min post activation). For details see (Żarski et al., 2012b).

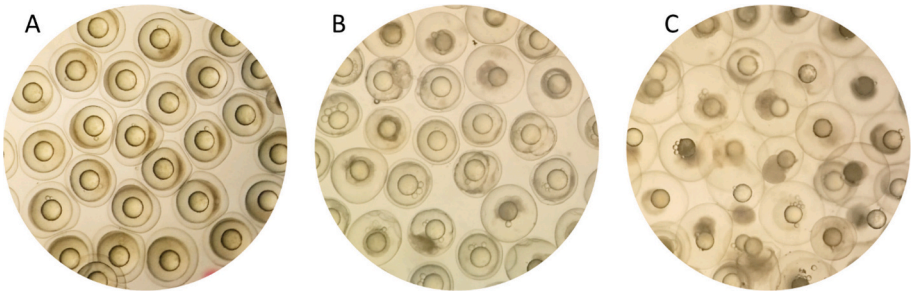


Fig. 2. Phenotype of eggs of pikeperch 30 min after water activation. A: normally developing eggs with animal pole formed; B: eggs with significant (>50%) phenotypic alterations and internal damages in the eggs; C: eggs with severe alterations exhibited by internal deteriorations of all the cells.

ripeness and possibility of stripping the eggs. Once ovulation began, the females were checked every 1–2 h to determine the optimal time for egg collection. When the eggs flowed freely under gentle abdominal pressure, a portion (about 10 g) was taken, and the genital papilla was re-sutured to allow the fish to recover from anesthesia in well-aerated freshwater. Additional egg samples were collected at 1, 2, 4, 6, and 8 h post ovulation (every 2 h, not every 1 h, to avoid excessive stress on the fish from hourly handling), with the latter showing signs of atresia and egg resorption. As most appropriate time for molecularly profile aged eggs we considered the following criteria: the eggs should have exhibited reduced cortical reaction (where the egg's chorion does not significantly alter its shape), still preserved internal egg structure (so that proteomic profiling would not be biased by apparent degradation of internal cellular structures), and a significant reduction of their developmental competence should be recorded.

Egg developmental competence has been estimated with a standard fertilization assay, as described by Żarski et al. (2019). Briefly, sperm samples from two randomly selected males (from a stock of 20) were collected using a catheter (Sarosiek et al., 2016). Collected pooled sperm has been used for *in vitro* fertilization. For this purpose, we added 5 ml of hatchery water to a glass Petri dish (5 cm in diameter), and next simultaneously about 200 eggs with 25 µl of pooled sperm. The eggs and sperm were mixed for about one min and left for 5 min at 15 °C, during which the eggs adhered to the glass what facilitated the entire incubation operation. Excess sperm was removed by replacing the water, and the dishes were then placed in plastic beakers in a temperature-controlled water bath at 15 °C. Water was renewed twice daily, and fertilization was done in triplicate for each time point and for each of the three females. After 6 h, the developing eggs were counted under a stereomicroscope to determine the fertilization rate (FR). The eggs were returned to the respective incubators until they hatched. After the larvae hatched, their number was estimated by direct counting, which allowed us to determine the hatching rate (HR). This rate was calculated based on the initial number of eggs. Next, the percentage of larvae with apparent morphological alterations was estimated to determine the larval deformity rate (DR = number of larvae showing deformity/the total number of larvae hatched) (Roche et al., 2018; Żarski et al., 2019). For each female, the eggs were fertilized and monitored for all the zootechnical parameters in triplicate. All manipulations with fish were performed under anesthesia with tricaine methanesulfonate (MS-222) at a dose of 150 mg/L. Eggs from initial experiment 1 were not taken for any molecular analysis.

2.2.2. *Initial experiment 2 – Evaluation of maximum aging period of pikeperch eggs in pond-grown stock*

After conducting the first initial experiment, where small portions of eggs every 1–2 h were collected, we were concerned that frequent handling might affect the aging process or our observations (considerably different from the ones of Samarin et al. (2015), who observed reduction in egg quality not earlier that after 12 h) could be biased due to the use of domesticated broodstock. To address this, we repeated the experiment using pond-reared broodstock, similarly to Samarin et al. (2015). We used females ($n = 6$) from the Research Center for Fisheries and Aquaculture (HAKI) at the Hungarian University of Agriculture and Life Sciences (MATE). These fish were prepared for reproduction following Ljubobratović et al. (2021). We applied the same protocols, including the photo-thermal regime, hormonal stimulation, egg collection, fertilization, and incubation, as in initial experiment 1. Additionally, after each egg collection, we took a subsample of around 300 eggs to conduct a cortical reaction test (to check for chorion malformation 5 min after activation). We also examined the internal damage rate by counting damaged eggs 30 min after water activation. Unfortunately, incubation failed due to technical issues, so we relied on cortical reaction rate and internal damage rate for evaluation from this experiment. Eggs from initial experiment 2 were not taken for any molecular analysis.

2.3. Main experiment – Proteomic analysis of eggs stored for 1 and 5 hPO

Initial experiments showed that aging process is progressing exactly in the same manner regardless the stock used. However, we became concerned that using domesticated or pond-grown pikeperch can create a bias stemming from reduced egg quality of cultured stocks (being typical problem in farmed pikeperch; Policar et al., 2019). Therefore, for the main study, wild-origin pikeperch spawners were used (from Tałty Lake, NE Poland), which were caught using gillnets during the spawning season (April). All the fish were transported on the boat in a holding tank filled with water from the lake. After delivery to the hatchery (located right on the shore of the "Mikołajki" Fish Farm), the fish were placed in the RAS, enabling automatic control of the temperature. The fish were held at 12 °C. Males ($n = 6$) were already mature, as the sperm could be obtained following gentle pressure on the abdomen. The sperm of each male, which was characterized by at least an 80% motility rate (confirmed subjectively under a light microscope at ×400 magnification), were pooled and used for fertilization. Immediately after being caught, the females were checked for maturity based on the classification of *in vivo* catheterized oocytes (as described by Żarski et al., 2012a). For further procedures, only females with oocyte stage III were used (with migrating germinal vesicles and the commencement of oil droplet formation; Żarski et al., 2012a). Next, the females (n = 6) were injected with hCG. After 3 days, the fish were again catheterized to verify their maturity. Whenever the female reached the VI stage of maturity (oocyte after the germinal vesicle broke down and with a single large lipid droplet), the genital papilla was sutured. Next, the fish were placed back in the rearing tank and checked every 2–6 h (depending on the estimated time of ovulation) to determine whether the eggs were strippable. After ovulation was confirmed (eggs could be hand stripped with gentle pressure on the abdomens), the fish were sutured, and the first portion of the eggs (approximately 10 g) was removed and placed in a dry plastic container 1 h later. After the first portion was collected, the genital papilla was sutured again, and the remaining eggs were removed 4 h later.

A subportion of the collected eggs (at either 1 or 5 h post ovulation) was immediately snap frozen in liquid nitrogen for further molecular analysis, and the remaining eggs were used for evaluation of cortical reactions and internal damage inspection as well as for fertilization assay, as was described in initial experiment (see section 2.2.1).

2.3.1. Sample preparation

Eggs were homogenized on ice in 30 mM Tris, 7 M urea, 2 M thiourea, and 4% CHAPS, pH 8.0, and subsequently sonicated. Following a 1-h incubation on ice, the samples were centrifuged at 14,000 ×*g* for 15 min at 4 °C.

2.3.2. TMT peptide labeling coupled with liquid chromatography–mass spectrometry

2.3.1.1. Protein digestion and reversed-phase peptide fractionation. The samples were prepared following a FASP protocol with slight adaptations (Wiśniewski, 2017). Initially, proteins suspended in a solution containing 4 M urea and 100 mM triethylammonium bicarbonate buffer (TEAB, pH 8.5) were reduced by incubating for 1 h at 37 °C with 10 mM tris(2-carboxyethyl)phosphine (TCEP). Subsequently, the samples were transferred to Vivacon 30 kDa molecular weight cutoff filters (Sartorius Stedim, Germany), centrifuged at 14,500 ×*g* for 30 min, and washed with 100 µl of an 8 M urea solution in 200 mM TEAB (pH 8.5). Cysteines were blocked by a 15-min incubation with 20 mM *S*-methylmethanethiosulfonate (MMTS) at room temperature. The digestion process occurred overnight with 4 µg of trypsin at 37 °C in 100 µl of 200 mM TEAB. Peptides were eluted from the Vivacon filters *via* washes with 200 mM TEAB and 500 mM NaCl solution and subsequently vacuum-dried and reconstituted in 120 µl of 200 mM TEAB buffer. To determine peptide concentrations, a Pierce™ Quantitative Colorimetric Peptide Assay (Thermo Fisher Scientific, Rockford, IL, USA) was used. Each peptide sample, consisting of 60 micrograms in 60 µl of buffer, was labeled with TMTpro 18-plex (Thermo Fisher Scientific) in 20 µl of acetonitrile (ACN) for 1 h. The reaction was quenched by adding 8 µl of 5% hydroxylamine. Labeling efficiency was assessed using the Evosep One/Exploris 480 system, as described in the mass spectrometry section. The labeled peptides were combined, vacuum-dried, and desalted using four 30 mg Oasis HLB columns (Waters, Milford, MA, USA) for each sample set. The samples were loaded onto columns and washed with 1 ml of 0.1% formic acid (FA) and 3% ACN. Peptides were eluted from the columns by 0.1% FA and 90% ACN. The resulting aliquots were dried and then resuspended in a solution consisting of 2% ACN in 10 mM ammonium hydroxide.

The labeled peptides were fractionated using high-pH reverse-phase chromatography following the procedure detailed in (Ziemlińska et al., 2021). The separation process was conducted on an XBridge Peptide BEH C18 column (4.6 × 250 mm, 130 Å, 5 µm; Waters) at a flow rate of 0.8 ml/min over 27 min utilizing a Waters Acquity UPLC H-class system. The mobile phases employed were composed of water (A), acetonitrile (B), and a 100 mM ammonium hydroxide solution (C), with the concentration of ammonium hydroxide held constant at 10 mM throughout the gradient. The elution profile of the peptides was continuously monitored at 214 nm using a UV detector. For each TMTpro set, a total of twenty-five fractions were meticulously collected into deep-well 96-well plates and subsequently subjected to vacuum drying. The peptides were reconstituted in 100 µl of a solution containing 0.1% FA.

2.3.2. Mass spectrometry

The peptide fractions were analyzed using an LC–MS system consisting of an Evosep One (Evosep Biosystems) coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific) through a Flex nanoESI source (Thermo Fisher Scientific). To prepare the samples for analysis, we followed the manufacturer's protocol for Evotips C18 trap columns (Evosep Biosystems; (Jancewicz et al., 2021)). In brief, the Evotips were activated through a 1-min centrifugation at 600 *g* using 25 µl of Evosep solvent B (comprising 0.1% FA in ACN), followed by a 2-min incubation in 2-propanol. Afterward, they were equilibrated with 25 µl of solvent A (containing 0.1% FA in water), and 10 µl of each sample solution was loaded onto the C18 tip. Subsequently, the peptides were washed with 50 µl and covered with 300 µl of solvent A. Gradient separation was executed at a flow rate of 500 nl min^{-1} over a 44-min gradient (30 samples per day) on an EV1106 analytical column (Dr. Maisch C18 AQ, 1.9 µm beads, 150 µm ID, 15 cm long; Evosep Biosystems). The spectra were acquired in positive mode employing a data-dependent method with the following parameters: (i) MS1 resolution: 60,000, with a normalized AGC target of 100%, a maximum injection time of 50 ms, and a scan range of 375 to 1575 *m/z*; (ii) MS2 resolution: 45,000, with a normalized AGC target of 200%, a maximum injection time of 120 ms, and an isolation window of 0.7 m/z; (iii) dynamic exclusion: 30 s, with an allowed mass tolerance of ±10 ppm, a precursor intensity threshold of 5e4, and a precursor fit threshold of 70%; (iv) fragmentation in HCD mode with a normalized collision energy of 30%; and (v) a spray voltage set at 2.1 kV, a funnel RF level of 40, and a heated capillary temperature of 275 °C. The cycle time was set to 2 s.

2.3.3. Data analysis

Offline recalibration, along with peptide and protein identification, was carried out using the MaxQuant/Andromeda software suite (version 2.0.1.0) (Tyanova et al., 2016), employing the *Sander* NCBI database (version 2021.07.30). The search encompassed tryptic peptides, with methylthio (C) as a fixed modification and oxidation (M) as a variable modification. Reporter MS2 quantification was configured to generate values for quantitative analysis, and TMTpro 18-plex correction factors were set according to the manufacturer's specifications. To ensure the reliability of the results, a reverse database was used for target/decoy statistical validation, with the peptide and protein false discovery rate

(FDR) set at 0.01. Subsequently, the identified protein groups, along with their quantitative data, were subjected to further analysis in Perseus (version 1.6.15.0). Hits originating from the reverse database, proteins solely identified by site, and contaminants were excluded from the analysis. Additionally, proteins with fewer than three values in each biological group were filtered out. Reporter intensities were measured per channel on computed loading factors, and the resulting normalized intensities were log2-transformed. Missing values were considered to indicate a normal distribution (width 0.3, downshift 1.8). Subsequently, paired *t*-tests were conducted to compare expression changes between the groups with permutation-based FDRs. The significance threshold for the resulting q value was set at 0.05. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium *via* the PRIDE.

2.3.4. Functional analysis of proteomic data

The UniProt accession numbers for all identified proteins were mapped against a reference *Homo sapiens* dataset. Gene Ontology (GO) analyses were also conducted using the ShinyGO v.0.80 online platform (http://bioinformatics.sdstate.edu/go80/, Ge et al., 2018), focusing on biological processes as the primary targets. The first GO analysis was performed for both proteome datasets of pikeperch eggs: (i) obtained in this study by TMT labeling and (ii) provided by (Żarski et al., 2021) using a label-free approach to identify shared and unique higher-level GO categories (100 categories for each dataset). Both datasets (obtained by TMT and label-free) underwent an identical analytical process. Subsequently, enrichment analysis was carried out to identify 500 significantly enriched biological process terms (FDR < 0.05) in the proteome dataset obtained in this study. An analysis of protein–protein interactions among differentially abundant proteins between the control group and aged group was carried out using the STRING database v.12 (https://string-db.org/). This resource provides information about functional relationships between proteins, enabling predictions regarding the functional consequences of alterations in protein abundance.

3. Results

3.1. Initial experiment 1 – Evaluation of maximum aging period of pikeperch eggs in domesticated stock

It has been observed, that eggs lost their fertilizing capacity only after 6 h post ovulation, but very high standard deviations has been still observed. The hatching rate has been significantly reduced, however, already after 4 h post ovulation. And certainly, there were very low egg quality parameters recorded after 8 h of *in vivo* aging. Interestingly, we paid attention to the time 0 (at certified ovulation time) and 1 h later, when the fertilization rate was higher at the time of 1 h. Considerable larval deformity rate was observed only after 8 h, so this parameter has been excluded as a suitable for our study (see Fig. 3).

3.2. Initial experiment 2 – Evaluation of maximum aging period of pikeperch eggs in pond-grown stock

We found that cortical reaction has been highly variable at the time when the ovulation has been certified as compared to the time of 1 h post fertilization. A very sharp reduction in cortical reaction rate has been noticed between 4 and 6 h post ovulation. The internal damages were sporadic in the batches of eggs stored *in vivo* for at least 4 h, after which significant increment of this parameter has been recorded (Fig. 4).

Based on the observation during these two initial experiments we have designated for our main experiment time points of 1 h (where higher consistence in terms of egg quality were recorded) and 5 h after ovulation. The latter one was chosen because we observed still a significant cortical reaction and high fertilization rate up to 4 h post-ovulation, which suggested that early egg aging did not occur yet significantly. However, at 6 h post ovulation we already observed internal damage and inconsistent embryo development what indicated that any molecular analysis could be biased due to already well noticeable excessive degradation of cellular structures.

3.3. Main experiment – Proteomic analysis of eggs stored for 1 and 5 hPO

3.3.1. Assessment of indices of the biological quality of eggs

The control group of eggs collected at 1 hPO was characterized by 100% cortical reaction, and no internal damages were detected;

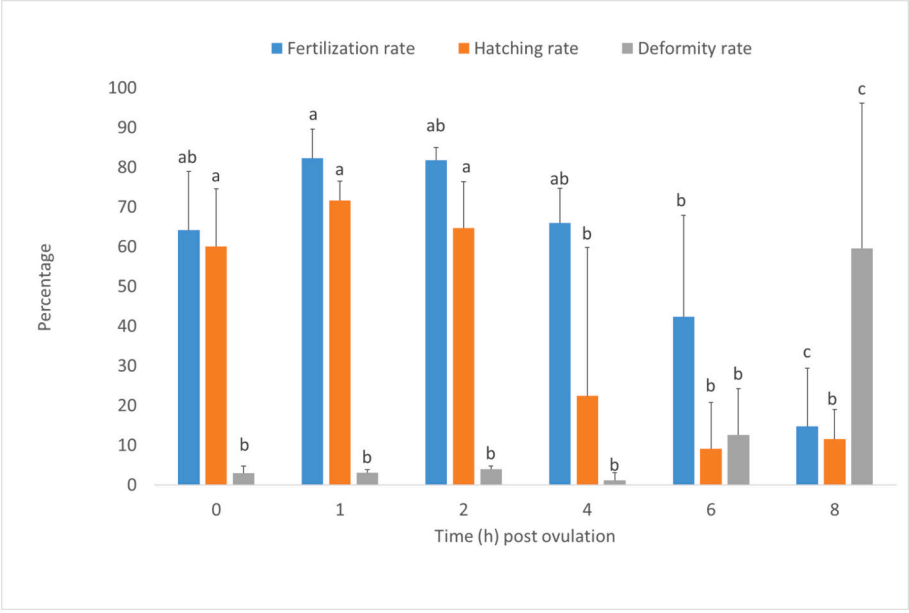


Fig. 3. The effect of *in vivo* aging time on egg quality parameters in pikeperch ($n = 3$). Different letters indicate statistical differences (one-way ANOVA; $P < 0.05$) within the same parameter and between different time points post ovulation. Eggs from initial experiment 1 were not taken for any molecular analysis.

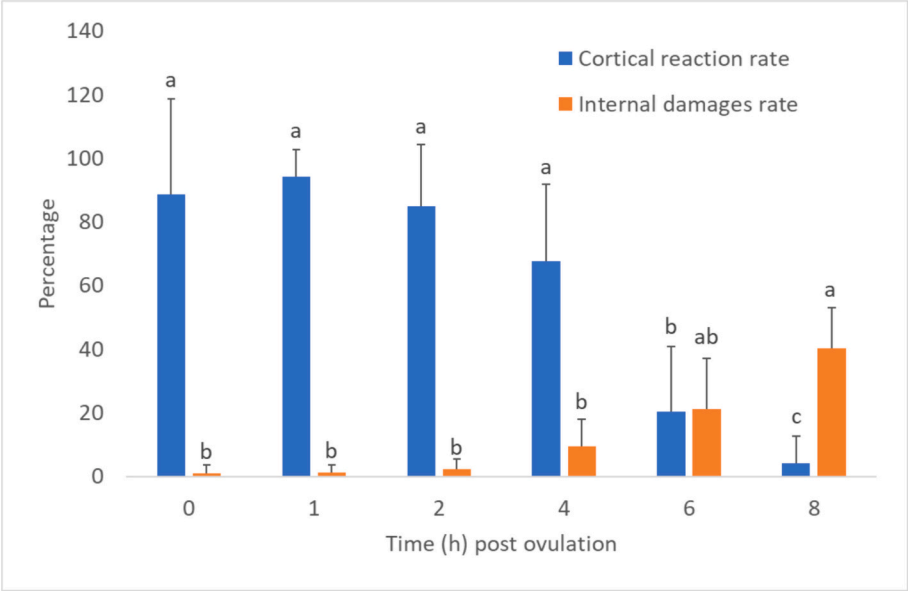


Fig. 4. The effect of *in vivo* aging on cortical reaction and incidence of internal damages in pikeperch eggs ($n = 6$). Different letters indicate statistical differences (one-way ANOVA; $P < 0.05$) within the same parameter and between different time points post ovulation. Eggs from initial experiment 2 were not taken for any molecular analysis.

however, the eggs collected 5 hPO were characterized by very low cortical reaction (13.4 ± 5.5%, Fig. 5), but minor (4.8 ± 1.5%, Fig. 5) internal damages were still detected. Such results confirmed the suitability of the choice of 5 h time point for studying molecular consequences of early egg aging in pikeperch in the conditions applied in our study. The developmental competence of these eggs was significantly reduced after 5 h of *in vivo* storage ($p < 0.01$) for all the measured parameters (FR, HR, and DR; see Fig. 5). The fertilization rates were approximately half of those in the group, and their survival rates were only approximately one-third of the rates observed in the group. Additionally, >6% of the hatched larvae in the aged group exhibited developmental deformities, whereas fewer than 2% of the larvae in the control group exhibited deformities.

3.3.2. Proteomic characterization of eggs

The implementation of TMT labeling in pikeperch egg analysis has significantly improved the number of proteins identified. The application of TMT peptide labeling combined with LC–MS/MS allowed the identification of a total of 4226 proteins (Supplementary File 1). Compared to the previous identification of 1450 proteins obtained through label-free spectrometry (Żarski et al., 2021), an additional 2828 proteins were identified for pikeperch eggs (Supplementary File 1). Of the 4226 proteins identified in this study, 4143 had human homologs and were subsequently selected for further analysis *via* GO.

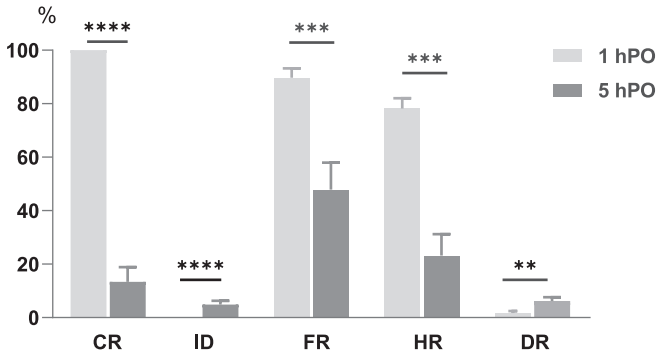


Fig. 5. The results of main experiment evaluating the cortical reaction and incidence of internal damages, and developmental competence of eggs were categorized into a control group (1 hPO) and an aged group (5 hPO; n = 6 for each group). CR – cortical reaction, ID - internal damages, FR – fertilization rate, HR – hatching rate; DR – deformity rate, PO - post ovulation. Asterisks indicate that the data between the groups were significantly different ($p < 0.01$). Eggs from this experiment were taken for further molecular analysis.

3.3.3. Functional analysis of the proteome dataset

The proteome dataset was organized into 100 higher GO biological process categories, as detailed in Supplementary File 2. According to the analysis of these 100 categories, the most prominent were 26 linked to the immune system and response to stimulus, 9 connected to various reproductive processes, and 8 specifically associated with development (Supplementary File 3). Moreover, we compared the higher-level GO biological categories with the same analysis conducted for proteins that were previously identified in pikeperch eggs (Żarski et al., 2021). The 94 high-level GO biological processes were shared by both datasets, indicating a conspecific overlap, whereas 6 were unique to our study's protein identification, and 6 were specific to protein identification *via* the label-free approach (Żarski et al., 2021) (Fig. 6).

Moreover, the top 500 GO terms were identified for our proteomic dataset (false discovery rate [FDR] < 0.05; Supplementary File 4). The most enriched processes were clustered into functional categories related to the cell cycle, gene expression and translation, RNA processing, metabolism, energy production, protein processing, and cellular component biogenesis (Fig. 7).

3.3.4. Differences in the proteomic profiles between the control and aged groups

We identified four differentially abundant proteins (DAPs; FDR < 0.05) between the control and aged groups, including two upregulated proteins, DNA replication complex GINS protein SLD5 (GINS4) and transcriptional regulator ATRX isoform X2 (ATRX), as well as two downregulated proteins dnaJ homolog subfamily B member 14 (DNAJB14) and 39S ribosomal protein L10 (MRPL10) in the aged group in comparison to control.

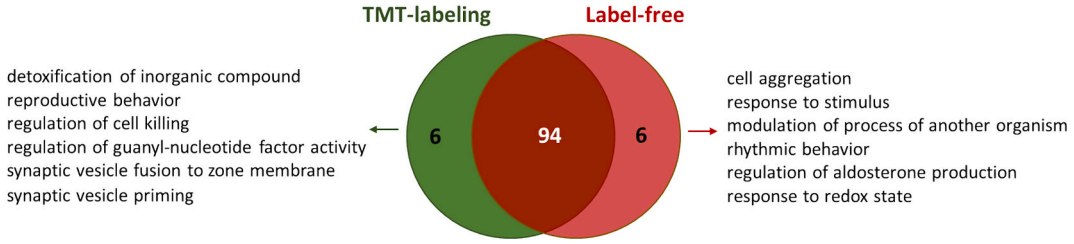


Fig. 6. The Venn diagram illustrates the number of high-level GO biological processes specific to the proteome dataset obtained by TMT labeling of pikeperch eggs, exclusive to the proteome dataset obtained by label-free MS (Żarski et al., 2021), and shared by both proteome datasets.

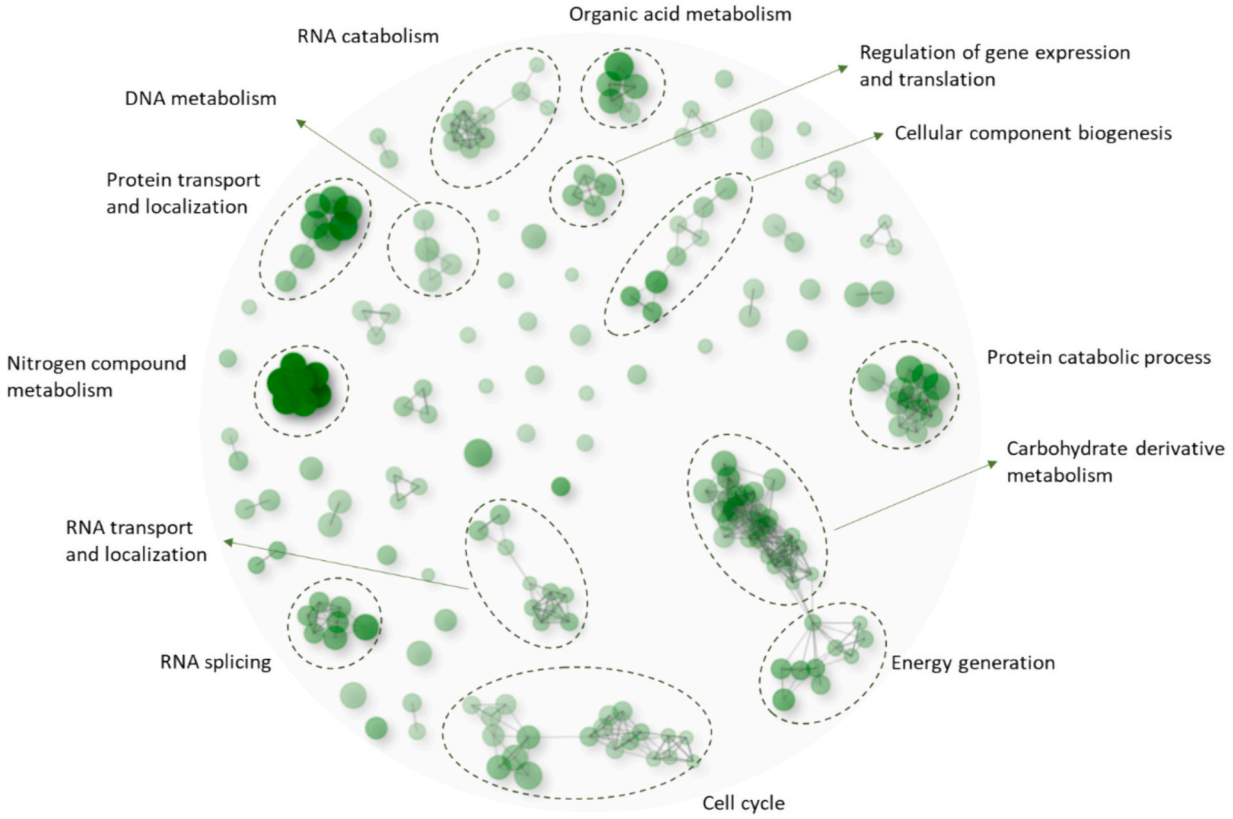


Fig. 7. Clustering of the 500 most enriched biological processes obtained from the functional enrichment analysis (FDR < 0.05) of all proteins identified in our study. For more comprehensive information, please refer to Supplementary File 4.

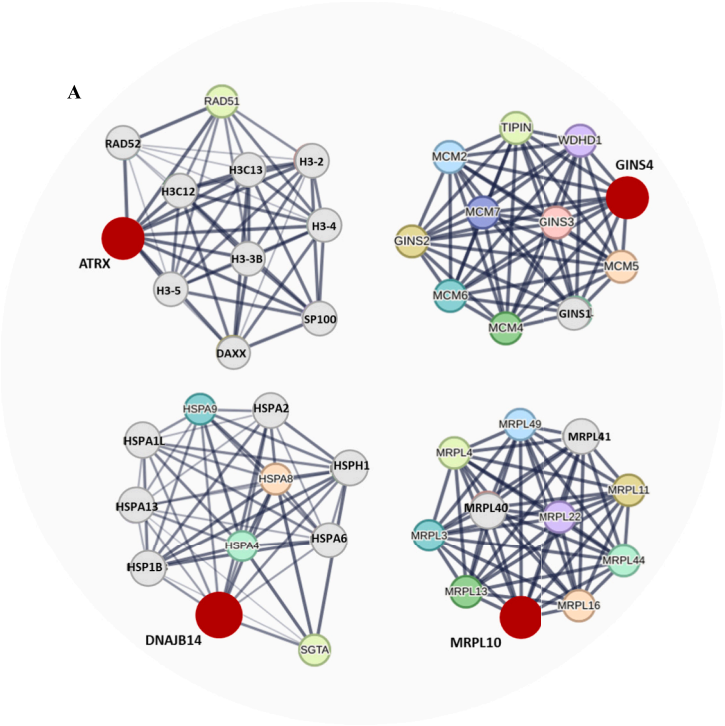
3.3.5. Protein–protein interaction analysis of differentially abundant proteins (DAPs) between the control and aged groups

We investigated the functional protein association network for the proteins ATRX, GINS4, MRPL10, and DNAJB14 to gain a deeper understanding of their roles in egg aging utilizing the STRING database. These proteins were analyzed separately due to the absence of protein–protein interactions. ATRX mainly associates with histones and proteins that participate in DNA safeguarding through protein–DNA complex assembly and telomere and chromosome organization (Fig. 8a-b). Among the proteins in this network, only RAD51 was identified in our study (Supplementary File 1, Fig. 8a). The results revealed that GINS4 interacts with ten proteins, nine of which were also identified in this study (Supplementary File 1, Fig. 8a). This interaction primarily occurs with components of complexes responsible for DNA replication initiation, such as GINS proteins (GINS1, GINS2, and GINS3), and DNA replication licensing factors (MCM2, MCM4, MCM5, MCM6, and MCM7). Additionally, these proteins contribute to DNA integrity and repair (Fig. 8b).

In turn, DNAJB14 was involved in a network comprising diverse chaperones, among which HSPA4, HSPA8, HSPA9, and SGTA were present in our protein dataset (Supplementary File 1, Fig. 8a). These proteins were shown to participate in protein folding and the response to stress (Fig. 8b). Finally, the functional protein association network related to MRPL10 included connections with various mitochondrial ribosomal proteins engaged in mitochondrial translation (MRPL3, MRPL4, MRPL11, MRPL13, MRPL16, MRPL20, MRPL40, MRPL41, MRPL44, and MRPL49; Fig. 8b), most of which were detected in our dataset (Supplementary File 1, Fig. 8a).

4. Discussion

Our research presents a comprehensive protein profile of pikeperch eggs, shedding light on the molecular mechanisms affected during the early stages of egg aging. This period of 5 h of *in vivo* storage after ovulation is characterized by the absence of apparent signs of atresia in the eggs, despite leading to subsequent developmental impairment. Four new predictive proteomic markers (Atrx, Gins4, DnaJB14, and Mrpl10) associated with the decline in egg quality resulting from the early aging



B

Protein	GO-term	Biological process	Enrichment FDR
ATRX	GO:0065004	Protein-DNA complex assembly	9.74e-10
	GO:0006334	Nucleosome assembly	1.72e-07
	GO:0051276	Chromosome organization	1.33e-05
	GO:0032200	Telomere organization	0.00090
	GO:0000730	DNA recombinase assembly	0.0055
	GO:0072711	Cellular response to hydroxyurea	0.0203
GINS4	GO:0006268	DNA unwinding involved in DNA replication	1.41e-20
	GO:0090329	Regulation of DNA-templated DNA replication	1.41e-20
	GO:0006261	DNA-templated DNA replication	1.53e-20
	GO:0000727	Double-strand break repair via break-induced replication	1.82e-16
	GO:0006270	DNA replication initiation	1.20e-11
	GO:0006281	DNA repair	1.77e-08
DNAJB14	GO:0051085	Chaperone cofactor-dependent protein refolding	1.90e-19
	GO:0006457	Protein folding	8.75e-16
	GO:0006986	Response to unfolded protein	2.33e-15
	GO:0042026	Protein refolding	9.46e-15
	GO:0035967	Cellular response to topologically incorrect protein	1.64e-13
	GO:0006950	Response to stress	4.69e-06
MRPL10	GO:0032543	Mitochondrial translation	5.55e-21

Fig. 8. Functional analysis using STRING v.12.0: Protein–protein interaction analysis of four differentially abundant proteins (ATRX, GINS4, DNAJB14, and MRPL10; red nodes) between the control and aged groups. Nodes with different colors represent proteins identified within this research (Supplementary File 2), while gray nodes depict proteins that were not identified in this study. B - The most enriched biological processes assigned to ATRX, GINS4, DNAJB14, and MRPL10. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

process in pikeperch were identified. Furthermore, by employing an exceptionally sensitive TMT-labeling method, we expanded the pool of identified proteins in pikeperch eggs to a total of 4226. To date, this has established the most complex protein dataset available, not only for pikeperch eggs but also among percid fish.

The time-dependent deterioration in egg quality resulting from postovulatory *in vivo* aging presents a significant challenge in aquaculture, as egg collection may be delayed due to unforeseen factors such as increased workloads or unavailability of sperm. In pikeperch, however, this is related to the need to close the genital papilla to prevent spontaneous egg release, which is a major constraint in controlled reproduction (Samarin et al., 2015; Żarski et al., 2015). Such closure of the genital papilla reduces excessive handling (and stress that is connected with that) of fish; thus, identifying the appropriate time for egg collection is necessary, and this approach is widely practiced on fish farms. However, this also creates the risk of inducing egg aging and lowered reproductive performance. In this study, we employed a commercially applicable technique to investigate how *in vivo* storage affects the quality of pikeperch eggs, aiming to pinpoint a reliable time point at which early aging of eggs begins. This period is crucial for safely collecting eggs and aligning with the intervals between each checkpoint for the manual stripping of viable eggs.

Our findings suggest that the early aging of eggs occurs approximately 5 h post ovulation, during which the eggs still maintain a seemingly high morphological quality with preserved cellular integrity and no observable signs of atresia; however, this period of time results in compromised fertilization capacity, leading to lowered developmental competence. This finding is in contrast with the results of Samarin et al. (2015), who observed a reduction in quality only after 12 h following certified ovulation. However, in their study, these authors used different techniques—spontaneous nest spawning (where pairs of pikeperch are left to spawn spontaneously under semicontrolled conditions)—which could limit stress and thus prolong the effective retention of eggs inside the ovary. However, the involvement of stress and thus the verification of this hypothesis require further study. Therefore, as a practical recommendation, for fertilization trials, we advise utilizing only those eggs that have been stored *in vivo* for up to 4 h after ovulation at 12 °C. Consequently, closure of the genital papilla (performed not earlier than 24 h prior to expected ovulation) and checking for ripeness in females not less often than every 4 h prevent a decrease in fertilization success, hatching rate, and occurrence of larval deformities stemming from hatchery practices.

A greater number of proteins in the dataset enhances the effectiveness of functional enrichment analysis, ultimately increasing the likelihood of identifying the most relevant biological processes associated with the biological mechanism under investigation. In our study, we significantly expanded the previously identified list of proteins in pikeperch eggs (Żarski et al., 2021) by identifying an additional 2828 proteins (Supplementary File 1). This is the largest ever study of the percid egg proteome thus far and the largest set of identified proteins, which allows for reliable inferences about the biological function of these proteins. When comparing the higher-level GO classification between our protein dataset and a smaller dataset obtained using the LC–MS/MS approach (Żarski et al., 2021), we found that both sets made comparable contributions to the top-level GO profile (refer to Fig. 6). In addition, both groups were associated with essential processes related to the immune system and responses to stimuli, development, and reproductive processes (Żarski et al., 2021) (Supplementary File 3), which strengthens and confirms the proposed role of egg proteins as important components of crucial biological processes supporting early embryonic development in fish (Nynca et al., 2022; Reading et al., 2018; Yilmaz et al., 2017). Moreover, in light of the concordance in functional analysis results between proteins identified through TMT labeling and those identified through the label-free approach, it can be postulated that, to achieve general insight into the functional roles of proteins within a biological sample, a cost-effective label-free analysis method is recommended. Nevertheless, to discern subtle fluctuations in protein abundance across samples subjected to varying conditions, the

implementation of advanced and highly sensitive techniques, such as TMT labeling, becomes indispensable.

Quantitative proteomic methods rely on the ability to detect subtle changes in protein abundance when comparing an altered state to a control or reference condition. Remarkably, in the context of pikeperch eggs, a total of 4226 proteins were identified, out of which only four proteins exhibited differential abundance (FDR < 0.05) between the control and aged groups. These subtle variations suggest the remarkable stability and resistance of the egg proteome to early aging. This stability potentially enables resource conservation to meet the high demands of developing embryos. It can be postulated that this protein stability is a result of the pivotal role these proteins play in early embryonic development, as well as the high energy cost associated with protein production, as suggested by Zhang et al. (2023) in the context of mouse oocytes. Eggs remain translationally inactive (which our study indirectly confirms) and, consequently, long-lasting rely on preexisting protein stockpiles, which are synthesized and stored during oocyte growth, to support fertilization, reprogramming, and early embryo development prior to zygotic genome activation (Lubzens et al., 2017). This finding underscores the critical importance of protein stability in eggs. Any deficiencies or imbalances in specific compounds within the eggs may hinder the successful development of a viable embryo. Furthermore, the transition of oocytes into fully matured eggs, the largest cells in an organism, is associated with a >300-fold increase in oocyte volume and total RNA content, along with a 38-fold increase in the rate of absolute protein synthesis (Chen et al., 2023). This substantial energy demand for oocyte development underscores the potential energy-efficient nature of protein stabilization. The synthesis of amino acids and the assembly of polypeptides can consume up to 50% of the ATP reserves in rapidly growing cells, as noted by Warner (1999). In conclusion, the minor fluctuations in protein abundance observed in pikeperch eggs after 5 h of *in vivo* storage can be attributed to the exceptional stability of the proteome. This stable proteome plays a crucial role in conserving resources to meet the demanding requirements of rapidly developing embryos in the future. The observed decline in the developmental competence of pikeperch eggs following postovulatory *in vivo* aging could therefore be linked to alterations in other maternally stored molecules, such as transcripts, as postulated earlier (Bizuayehu et al., 2019; Samarin et al., 2019b).

Specifically, two proteins, Gins4 and Atrx, were upregulated, while two proteins, Dnajb14 and Mrlp10, were downregulated in the aged group. Since it is widely accepted that during the window from fully grown oocytes to early embryos, eggs are translationally silent, the observed changes in protein abundance can be explained by protein degradation resulting from a decrease in abundance or protein exchange with the cells surrounding the oocyte (*e.g.*, *via* external vesicles) to increase abundance. It can be assumed that changes in protein abundance can be associated with alterations in protein stability. Posttranslational modifications (PTMs) have been implicated in regulating protein stability (Lee et al., 2023). The key PTM influencing protein stability is ubiquitination, which occurs through the ubiquitin–proteasomeproteasome system. However, TMT labeling was applied to monitor the relative abundance of egg proteins between the control and aged groups, but it was not used to determine the absolute quantity of differentially expressed proteins. Relative quantification involves determining protein quantitative ratios or relative changes by comparing the amounts of individual proteins or entire proteomes across various samples. On the other hand, absolute quantification offers information about the absolute quantity or concentration of a protein in a given sample (Rozanova et al., 2021). Therefore, in future studies aiming to accurately determine the absolute amount or concentration of specific proteins within a single egg, absolute quantification methods will be essential.

The process of egg aging after ovulation triggers a plethora of morphological, biochemical, molecular and epigenetic changes that compromise egg quality and pose a risk to the successful formation and development of embryos. Therefore, identifying predictive markers of these impairments, which could help in fine-tuning commercial reproductive protocols or monitoring certain reproductive impairments in wild stocks, is highly important. To date, limited studies have been conducted to investigate the cellular and molecular changes in fish eggs in response to postovulatory aging, and most of those studies have focused on the effect of aging on transcriptome dynamics (Bizuayehu et al., 2019; Samarin et al., 2019b). In our study, we identified four potential protein markers of early aging in pikeperch eggs. These proteins are involved in processes such as replication initiation and DNA integrity and repair (GINS4, Varga et al., 2020), correct telomere and chromosome organization (ATRX, Aguilera and López-Contreras, 2023), protein folding and stress response (DNAJB14, Sopha et al., 2012), and mitochondrial translation (MRPL10, Li et al., 2016). Chromatin remodeling proteins are recognized for safeguarding genomic integrity through the control of chromatin structure alterations (Liu et al., 2012). The changes in Atrx and Gins4 abundance in pikeperch eggs could reflect issues associated with chromosome organization evoked by early aging. This finding is in agreement with previous studies indicating that postovulatory aging initiates early chromosome separation, dispersion, and decondensation, resulting in chromosome clustering and chromatid separation in eggs and potentially leading to genomic instability that could lead to epigenetic alterations in mammalian offspring (Prasad et al., 2015; Liang et al., 2008; Miao et al., 2009). Furthermore, ATRX, a known epigenetic regulator of chromatin remodeling factor (Aguilera and López-Contreras, 2023), is recognized as a potential biomarker for assessing oocyte quality and developmental potential in more advanced vertebrates (O'Shea et al., 2012, 2017), as revealed in our study.

Maintaining proteome balance in ovulated eggs is crucial, and heat shock proteins (HSPs), including DNAJ (HSP 40), are vital for proper protein assembly and folding during stress. HSP deficiency contributes to aging (Peinado-Ruiz et al., 2022), impairing protein repair and causing misfolding (Calabrese et al., 2014). In particular, HSP70 has been recognized as a promising tissue biomarker indicative of the age and longevity of mice (de Toda et al., 2016). The role of DnaJs in oogenesis and ovary development has been indicated in fish (Feng et al., 2019). Moreover, studies in the European clam (*Ruditapes decussatus*) and the Pacific oyster (*Crassostrea gigas*) have shown that higher expression of DnaJ homologs is associated with good-quality oocytes (Corporeau et al., 2012; de Sousa et al., 2015). These findings suggested that these proteins may play a protective role in oocytes and early embryos that are directly exposed to temperature fluctuations, pH changes, and oxidative stress (Corporeau et al., 2012). Previous mammalian studies have consistently indicated an increase in reactive oxygen species (ROS) and consequential oxidative stress in oocytes during postovulatory aging (Lord and John Aitken, 2013). In the context of pikeperch, (Schaefer et al., 2016) also suggested the involvement of oxidative stress during postovulatory aging of eggs. However, contrasting results emerged from goldfish studies suggesting that oxidative injury is not a major factor in *in vitro oocyte* aging (Samarin et al., 2019a). In our investigation, which focused on postovulatory *in vivo* storage, we found a lower abundance of DnaJ homologs in eggs. This finding suggested that cellular protein homeostasis in stored pikeperch eggs is disrupted, likely following oxidative stress. This disruption impairs protein stabilization, proper protein folding, and the removal of misfolded proteins.

Mitochondria provide the energy necessary for oocyte maturation, fertilization, and embryo formation through oxidative phosphorylation. Unlike sperm mitochondria, oocyte mitochondria are exclusively inherited from the mother, underscoring their pivotal role in maternal contributions to embryonic development. The aging process that occurs after ovulation significantly affects the function of mitochondria, resulting in various mitochondrial defects in aged oocytes (Zhang et al., 2022). These defects include mitochondrial swelling, reduced ATP production, decreased metabolic activity, a diminished number of mitochondria, and impaired repair systems for mitochondrial DNA. All

of these factors have been identified as contributors to the decreased quality of aged oocytes (Miao et al., 2009; Zhang et al., 2022). In our research, we observed the effects of early postovulatory aging on the mitochondrial ribosomal protein Mrpl10 in pikeperch eggs *in vivo*. This protein is a critical component required for proper mitochondrial function and the synthesis of respiratory complex proteins (Li et al., 2016). Previously, the MRPL10 gene was shown to be associated with oocyte developmental competence in mice and cattle (Biase, 2017). Our results confirm the pivotal role of mitochondria, along with their dysfunctions, in shaping oocyte quality and determining the potential for embryo development in fish. Early postovulatory aging of pikeperch oocytes is assumed to be associated with mitochondrial dysfunction, which in turn affects impaired embryo development.

In summary, our results suggest that the recommended post-ovulatory stripping timeframe in pikeperch, which ensures high fertilization capacity and developmental competence, should be limited to within 4 h post ovulation. After this period, eggs appear to maintain the structural integrity of cellular components without evident signs of atresia, but subsequent development of eggs is significantly impaired. We found that the egg proteome is remarkably stable, and early post-ovulatory storage has a limited impact on the protein profile of eggs. Nevertheless, our findings reveal that the storage period leads to marked changes in the abundance of four key proteins, underscoring the critical role of the proteome in resource conservation, a vital strategy for fulfilling the intensive needs of rapidly developing embryos in subsequent stages. These genes are involved in the regulation of key stages of gene expression, such as replication (Gins4, Atrx), translation (Mrpl10), and posttranslational modifications (DnaJB14). This finding underscores the influence of early postovulatory aging on various aspects of gene expression. Our findings highlight that prolonged *in vivo* storage of ovulated eggs can be a substantial contributing factor to genomic and chromosomal instability, disruption of proteostasis, and mitochondrial dysfunction, ultimately impairing the subsequent development of eggs.

Funding

This work was funded by the National Science Center, Poland (NCN) [grant number 2016/22/M/NZ9/00590].

CRediT authorship contribution statement

Joanna Nynca: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Julien Bobe:** Writing – review & editing, Supervision. **Jarosław Król:** Writing – review & editing. **Uroš Ljubobratović:** Writing – review & editing, Resources. **Katarzyna Palińska-Żarska:** Writing – review & editing. **Agata Malinowska:** Investigation, Formal analysis. **Bianka Świderska:** Investigation, Formal analysis. **Daniel Żarski:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Submission details to ProteomeXchange via the PRIDE: Project accession: PXD048349 Project DOI: 10.6019/PXD048349 Reviewer account details: Username: reviewer_pxd048349@ebi.ac.uk Password: nxQcbCb2

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.741270.

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