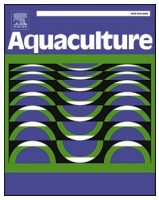
Aquaculture 596 (2025) 741759

Contents lists available at ScienceDirect

Aquaculture

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



Lipid droplet distribution in Atlantic salmon *(Salmo salar L.)* oocytes is related to the fertilisation and developmental outcome

Sonali N. Kaththriarachchi [a,b], Elisabeth Kommisrud [a], Frøydis D. Myromslien [a], Teklu T. Zeremichael [a], Inger S. Grevle [b], João Santana [b], Julia Farkas [c], Bjørn Henrik Hansen [c], Ewa Wielogórska [c], Birgitte Narud [a,*]

[a] *CRESCO, Centre for Embryology and Healthy Development, Department of Biotechnology, Inland Norway University of Applied Sciences, Hamar, Norway*
[b] *Cryogenetics AS, Hamar, Norway*
[c] *SINTEF Oceans, Trondheim, Norway*

ARTICLE INFO

Keywords:
Oocyte quality
Fertilisation
Lipid coalescence
Neutral lipids
Atlantic salmon

ABSTRACT

At present, there is no simple and practical applicable method to determine the quality of Atlantic salmon oocytes upon collection at the fish farm. Investigating oocyte lipid droplet patterns might be a tool for the aquaculture industry to predict oocyte quality in Atlantic salmon. While examining the lipid distribution in oocyte batches from 16 females of Atlantic salmon with a range in fertilisation rate (6–97 %), five categories of lipid droplet patterns were distinguished. In category 1, the lipid droplets were evenly distributed whereas in the remaining categories lipid droplets showed varying degree of coalescence. Categories 2, 3, 4 and 5 were distinguished based on the area of coalesced lipid droplets. The fertilisation rate was observed to be high (≥ 90 %), in females that had more oocytes from category 1, 2 and 3 whereas females with more oocytes from category 4 and 5 showed a low fertilisation rate (<90 %). A similar pattern was revealed between lipid droplet categories and eyed embryo and hatching success. Staining of salmon oocytes further confirmed the observed lipid droplet patterns, and different sizes of lipid droplets were present in almost all oocytes. The study revealed a significant association between lipid droplet category and fertilisation outcome in oocytes of Atlantic salmon that can be a practical tool for predicting oocyte quality.

1. Introduction

An easy and reliable tool to predict the quality of oocytes in fish is lacking in the aquaculture industry, especially for salmonids (Ienaga et al., 2021; Mansour et al., 2007). Oocytes with low quality are not easily detected in eyed egg (eyed embryo) production facilities. Non-viable oocytes turn white but are only detectable after the fertilisation process. Removal of dead eggs from incubators is required, however, labour intensive. Leaving dead eggs with viable eggs in the incubator can cause fungal or bacterial contamination that can spread to other areas in the production facilities with consecutive huge losses (Ciereszko et al., 2009). The quality of oocytes in salmonids has been studied based on biochemical composition of oocytes and ovarian fluid, e.g. pH, protein level, and aspartate aminotransferase enzyme activity in ovarian fluid have been shown to correlate with eyed embryo success (Bahrekazem et al., 2009; Lahnsteiner et al., 1999; Mansour et al., 2008). However, most of these methods are not convenient as a rapid field test in commercial production facilities.

Fish oocytes contain a substantial level of lipids, which is needed for embryonic and larval fish growth. The amount and distribution of lipids have been correlated with larval fish survival in different teleost species (Johnson, 2009). In Eurasian perch, categorization of egg-ribbons based on the degree of oil droplet fragmentation in oocytes at different maturation stages, has been shown as a useful tool in commercial fish production (Żarski et al., 2011). Currently, there is no suitable way to distinguish between high and low quality oocytes in Atlantic salmon based on visual appearance except when they are over-ripe (post-ovulatory ageing). Distribution of lipid droplets in oocytes has been studied in several salmonid species such as brown trout *(Salmo trutta fario)*, Arctic charr (*Salvelinus alpinus*) and rainbow trout (*Oncohynchus mykiss*) (Ciereszko et al., 2009; Mansour et al., 2008; Mansour et al., 2007). In these studies, different patterns of lipid droplet distribution

* Corresponding author.
E-mail address: birgitte.narud@inn.no (B. Narud).

https://doi.org/10.1016/j.aquaculture.2024.741759
Received 15 April 2024; Received in revised form 24 September 2024; Accepted 11 October 2024
Available online 12 October 2024

and sizes have been demonstrated associated to the quality of oocytes. In brown trout, oocytes were classified into four categories where category 1 consisted of evenly spread lipid droplets. Category 2 had some droplets starting to coalesce and category 3 had one or more coalesced areas. Lastly, in category 4 the majority of lipid droplets were coalesced (Mansour et al., 2007). A similar study was conducted on Arctic charr *(Salvelinus alpinus)* where three different patterns of lipid distributions and their possible relation to oocyte quality were investigated (Mansour et al., 2008). Both studies show that oocytes with evenly distributed lipid droplets give higher fertilisation rates than oocytes with coalesced lipid droplets (Mansour et al., 2008).

To our knowledge, there has been no investigations on lipid droplet distribution in Atlantic salmon oocytes so far. It is thus an urgent need to establish practical tools or methods to predict oocyte quality in salmon and thereby increase the productivity in the salmon farm industry. The aim of the current study was to investigate lipid droplet patterns concerning distribution in the oocytes and possible association with fertilisation and subsequent embryo development.

2. Materials and methods

2.1. Collection, selection, and processing of gametes

Gametes were collected at an Atlantic broodstock facility in September (milt) and October (oocytes) 2022, for the subsequent fertilisation trials.

2.1.1. Oocytes

Oocytes in ovarian fluid from 100 mature females were collected on a single day and stored at 4 °C until the next day. The oocytes were obtained from females euthanized with an overdose of benzocaine (Benzoak vet., ACD Pharmaceuticals AS, Leknes, Norway), and were removed by excision from the abdominal cavity. Oocytes from females not giving enough ovarian fluid to cover the oocytes were mixed with 0.9 % saline to keep the oocytes moist. Upon collection, aliquots of the oocytes were fertilised with fresh milt from different males, as part of the egg (eyed embryo) production at the facility. The fertilisation rates were available the next day, and based on these, oocytes from 16 females were selected for further analyses. From these 16 females, oocytes from 8 females were selected with fertilisation rates >90 % (high quality) and oocytes from 8 other were selected with fertilisation rates between 85 and 0 % (low quality). The selected oocyte batches ($n = 16$) in ovarian fluid were all further split into two batches and stored at 0–4 °C in cell culture flasks with filter caps (Sarstedt, Nümbrecht, Germany). On the same day, one batch of oocytes was transported in a Styrofoam box to SINTEF Sealab, Trondheim, for fertilisation trials. The other batch was transported to Cryogenetics AS, Hamar for physiological and biochemical analysis.

2.1.2. Milt

Milt from one male was collected in September 2022 and sent to Cryogenetics AS for cryopreservation. Cryopreserved milt from one single male was used throughout the study to minimise the male variation. Initial quality analysis of frozen-thawed milt from the male was done by observing motility of spermatozoa upon activation by AquaBoost activator® under a bright field microscope. Further, sperm motility of the frozen-thawed milt was analysed by CASA as described for Atlantic salmon milt (Narud et al., 2023), with minor modifications, and revealed a total motility of 86 %. Briefly, milt was diluted with a non-activating agent to a working concentration of 7.83×10^8 sperm cells/mL. The milt was assessed by aspirating 2.7 μL of AquaBoost activator® by using BioHit electrical automatic pipette (Sartorius Corporate, Niedersachsen, Germany) followed by an airgap and aspirating 0.4 μL of diluted milt. The sample with activator was loaded to a Leja® 20 μm 4-chamber microscope slide (Leja products, Nieuw-Vennep, the Netherlands). A minimum of four microscopic fields with at least 800 cells were analysed and the mean of these microscopic fields was calculated for each sample. The camera settings were 200 frame rate (fps), and the software settings were configured according to Atlantic salmon fish species. The sperm cells were identified by the sperm head area of 10–50 μm^2. Total motility was based on VCL (curvilinear velocity), and progressive motility based on STR (straightness) such that; static <17 μm/s, VCL < 80 μm/s for slow-medium, VCL > 120 μm/s for rapid motile and STR > 45 % defined progressive motility. Further, sperm quality was assessed by fertilisation giving 94 % fertilisation rate with the cryopreserved milt.

2.2. Fertilisation trials

Upon arrival at SINTEF Sealab, oocytes were kept in cold condition (4 °C) until the next morning. Oocytes from each female were fertilised with cryopreserved milt at a sperm to egg ratio of 2×10^6: 1. The oocytes were mixed with milt and activated by 50 mL AquaBoost® activator. After incubation for 2 min the eggs were rinsed with 0.9 % saline water to remove excess spermatozoa and ovarian fluid. The fertilisation performed at SINTEF was conducted as two separate trials where trial 1 was performed to obtain initial fertilisation rates and trial 2 to obtain eyed embryo and hatching success for the included females. In trial 1, approximately 100 fertilised eggs were transferred to a separate incubator system (one female per incubator) with water (10 L/min, 6.75 °C) that were ultra filtrated and UV-irradiated, until the next day (24 h). The fertilised eggs were fixed with AquaBoost Quattro® and the fertilisation rate (8–16 cell stage) was assessed for all 16 females. In trial 2, the remaining eggs from each female were divided into 21 sub batches (approximately 24 eggs per sub batch) and transferred to incubators as previously described by Hansen et al. (2022). Briefly, the incubators were made of 50 mL Falcon® tubes (Fisher scientific, Massachusetts, USA) customised with a 120 μm plankton mesh (SEFAR NITEX) fixed at the top and bottom of the tubes. Each incubator was supplied with tap water incubated in a large open reservoir (3 m^3) at the correct temperature (6.35 °C). This reservoir fed each incubator (containing 24 eggs) with fresh tap water at a constant flow rate of 15 mL/min until 79 days post fertilisation (dpf). Eggs were followed from the time of incubation until hatching and dead eggs (white eggs) were picked out every week to minimise contamination.

2.3. Lipid droplet distribution in oocytes

Oocytes were picked randomly from each female and imaged under a MZ1500 stereoscopic zoom microscope with the 1×- lens (High Resolution plan Apo 1×) coupled with 10× eyepieces (Nikon Instruments Inc., New York, USA). The Nikon NIS-Elements Research (version 3) software was used to capture images. The images were taken from two angles by first capturing the coalesced lipid area, if there was any, and then turning the oocyte 180° for capturing a second image. This was repeated for 20 oocytes per female. During the imaging process, oocytes were submerged in phosphate buffered saline (PBS) to maintain the moisture in the sample.

Based on the distribution of lipid droplets, five categories of oocytes were classified. Category 1 (C1): evenly distributed lipid droplets throughout the oocyte; category 2 (C2): lipid coalescence in a smaller sized area; category 3 (C3): lipid coalescence in a slightly larger area than in C2; category 4 (C4): lipid coalescence covering a larger area; category 5 (C5): almost all lipid droplets coalesced also known as overripe eggs. The area size of the lipid coalescence was measured using free hand tool in Image J software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA) which is a free, open-source image processing program. All the images were adjusted to the scales (1DIV = 0.01 mm) and processed.

In addition, the pattern of the lipid distribution in the oocytes were further confirmed by BODIPY™ 493/503 staining. BODIPY™ is a fluorescence stain applied to trace neutral lipids and other non-polar lipids.

Three oocytes from each female were stained with 1 μg/mL BODIPY™ 493/503 (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene) (Invitrogen by Thermo Fisher scientific, Massachusetts, USA) in PBS to determine the lipid distribution. The oocytes in each sample were stained for 30 min at room temperature and during staining the sample was gently shaken using a rotary shaker (IKA® MS 3 digital, IKA®-Werke GmbH & Co, Staufen, Germany). Thereafter, the oocytes were rinsed with PBS to remove excess BODIPY™. A negative control of non-stained oocytes was analysed prior to the analysis to establish the threshold of BODIPY non-stained oocytes. The oocytes were evaluated by using a Nikon ECLIPSE Ti-U fluorescence microscope (Nikon Corporation, Tokyo, Japan) equipped with brightfield, phase contrast and fluorescence optics. BODIPY™ staining was visualised by using a FITC filter (465–495 nm excitation wavelength and 515–555 nm emission wavelength). The Nikon NIS-Elements Research (version 3.00) software was used to capture images of the stained oocytes.

2.4. Statistical analysis

Statistical analyses were performed using Microsoft Office Excel (Microsoft, Redmond, WA, USA) and GraphPad Prism 10.2.1 (395) for windows (GraphPad Prism Software, La Jolla, CA, USA). The five categories were further subgrouped into "high quality" (HQ) and "low quality" (LQ) where the percentage of categories 1, 2 and 3 were included in subgroup HQ and category 4 and 5 in subgroup LQ. Student's t-test was used to compare the two subgroups. Significance level was set to 0.05 in all statistical analyses.

3. Results

3.1. Evaluation of fertilisation rates and success of embryo development

The fertilisation trial 1 performed at SINTEF confirmed that the collected oocyte batches had fertilisation rates ranging between 0 and 100 % (Table 1). Oocytes from 9 females (Female ID 1–8 and 15) showed fertilisation rates ≥90 % (high quality) and 7 (Female ID 9–14 and 16) with fertilisation rates <90 % (low quality). In trial 2, the subsequent eyed embryo success and hatching success for female ID 15 were observed to be low despite the high fertilisation rate. Also, female ID 12 showed a substantial decrease in eyed embryo success ending up with 0 % hatching success even though it had a fertilisation rate of 77 %. Overall, the hatching success varied among the 16 females.

Table 1
Fertilisation rates, eyed-embryo and hatching success of the oocytes from 16 selected Atlantic salmon (*Salmo salar L*) females.

Female ID	Fertilisation rate (%)	Eyed-embryo success (%)	Hatching success (%)	Oocyte quality based on fertilisation rate
1	97	96	94	High
2	98	91	94	High
3	91	88	84	High
4	95	89	86	High
5	90	83	82	High
6	94	93	89	High
7	97	82	77	High
8	93	96	94	High
9	6	0.0	0	Low
10	72	52	33	Low
11	41	21	12	Low
12	77	4	0	Low
13	67	74	65	Low
14	66	52	45	Low
15	92	77	49	High
16	74	77	76	Low

3.2. Investigation of lipid droplet categories

In C1, lipid droplets were distributed evenly (Fig. 1A) while C2 had one or two small lipid globules starting to coalesce in a smaller area (Fig. 1B) with average area size and standard deviation of 0.34 mm^2 ± 0.15. In C3, more lipid droplets had started coalescing (Fig. 1C) and had spread into a greater area with an average area size of 1.0 mm^2 ± 0.25. C4 had the largest areas with coalesced lipid droplets (Fig. 1D, E) which emphasized the area with darker orange colour with an average area size of 2.43 mm^2 ± 0.76. In C5, the lipid droplets were almost all coalesced (Fig. 1F) and formed one large lipid globule with an average area size of 8.53 mm^2 ± 2.38, which in hatcheries are known as over-ripe. Average area size of an oocyte is approximately 12 mm^2 ($n = 50$).

The results presented in Fig. 2C show that the area size of the lipid coalescence in oocytes gradually increased from C2 (0.34mm^2) to C5 (8.5 mm^2).

The majority of the oocyte batches consisted of at least four lipid droplet categories and the number of oocytes for each category varied between the females (Fig. 2A). Category 5 was observed only in five females along with other categories.

After subgrouping the lipid droplets as shown in Fig. 2B, two females (fish ID 2 and 8) had oocytes only in subgroup HQ whereas one female (fish ID 10) had more than 95 % of the oocytes in subgroup LQ.

3.3. Relationship between lipid droplet distribution and fertilisation outcome

A comparative analysis was conducted between subgroups HQ and LQ (Fig. 3A), when the number of oocytes (%) in HQ were above or equal to 74 % ($p < 0.0001$). Further, the difference between HQ and LQ was assessed (Fig. 3B) when the number of oocytes (%) in HQ were less than 74 % ($p < 0.05$). The results indicate that when the number of oocytes in subgroup HQ (category 1, 2, 3) is greater or equal to 74 %, the number of oocytes in LQ (category 4 and 5) decreases (Fig. 3).

For investigation of association between lipid droplet distribution and fertilisation outcome, females were sorted based on the distribution of oocytes within the HQ subgroup. Ten of the females had oocytes with higher or equal to 74 % (range 74–100 %) from subgroup HQ and six females had oocytes with less than 74 % (range 5–64 %) from HQ.

According to the results presented in Fig. 4A, a higher fertilisation rate (≥90 %) was obtained when there were more oocytes (≥74 %) from subgroup HQ present in the oocyte batch. The fertilisation rate was significantly lower (<78 %) when less oocytes (≤64 %) from subgroup HQ ≥ 74 % were present in the oocyte batch.

A similar pattern is shown in Fig. 4B and C between subgroups and subsequent eyed embryo success and hatching success, which resulted in a higher eyed embryo success (>70 %) and hatching success (>65 %) in the presence of more oocytes from subgroup HQ ≥ 74 %.

3.4. Fluorescence staining

The fluorescence intensity appeared strong and clear in lipid droplets (Fig. 5D and E), and the beginning of lipid droplet coalescence is clearly visible as shown in Fig. 5E. The over-ripe oocytes showed a blurry fluorescence signal (Fig. 5F), and the lipid droplets (Fig. 5C), were not clearly visible at all. In addition, fluorescence images (Fig. 5D-E) showed how the fluorescence intensity varies between different sizes of lipid droplets.

4. Discussion

Lipid droplets are found in almost all cells contributing to various cellular functions. Mainly, lipid droplets provide lipid storage for energy generation, membrane synthesis and protein degradation (Walther and Farese Jr., 2012). During teleost oocyte growth and differentiation, the fatty acid profile in oocytes changes rapidly (Reading

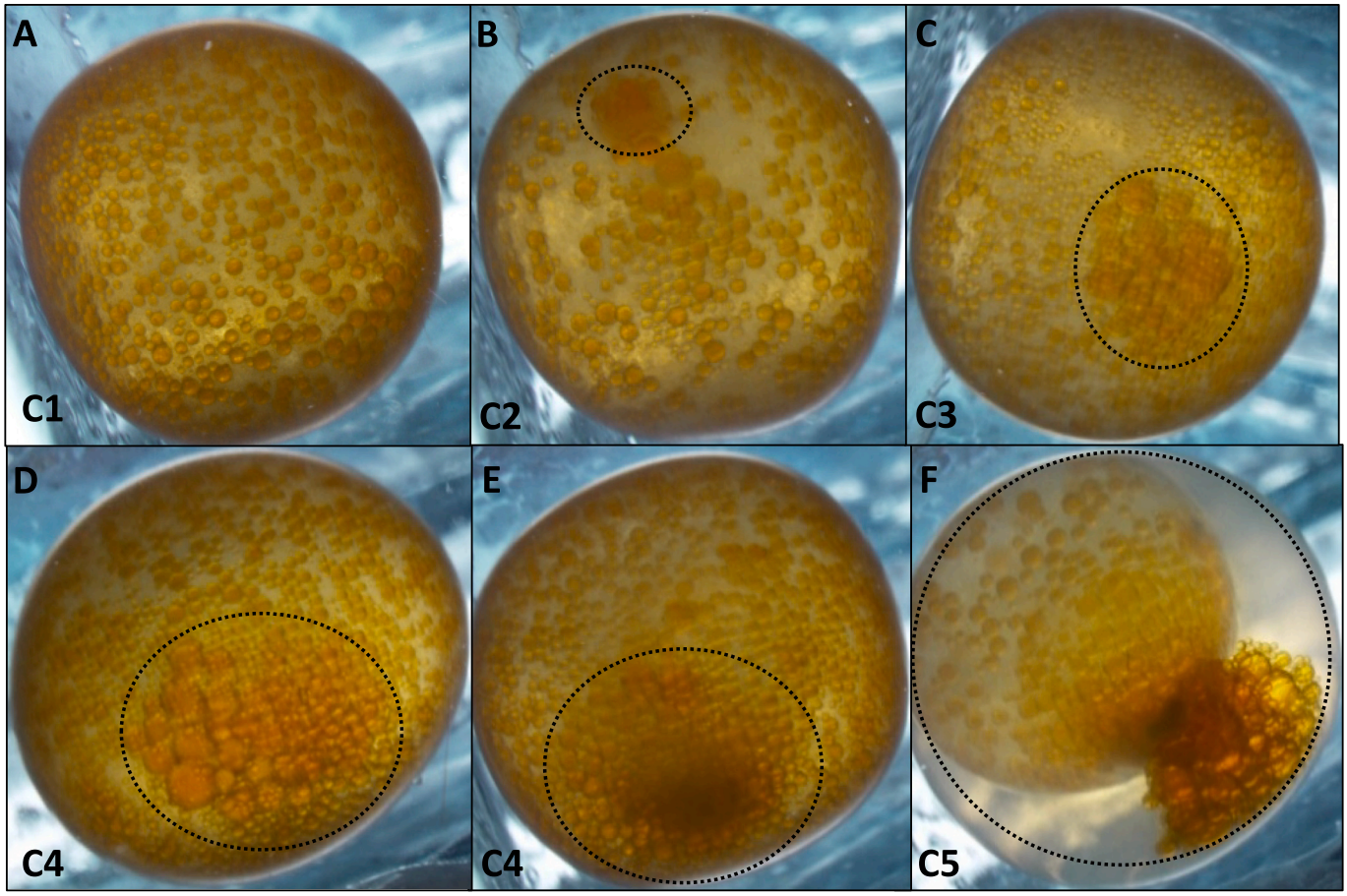


Fig. 1. Categories of lipid droplet distribution in Atlantic salmon (*Salmo salar L.*) oocytes under stereo microscope, with the 1×- lens (High Resolution (HR) plan Apo 1×) coupled with 10× eyepieces (A) Category 1 (C1): evenly distributed lipid droplets throughout an oocyte; (B) category 2 (C2): lipid coalescence in a smaller area size; (C) category 3 (C3): lipid coalescence in a slightly larger area than in category 2; (D,E) category 4 (C4): lipid coalescence is spread to a greater area; (F) category 5 (C5): almost all lipid droplets are coalesced also known as over-ripe eggs. The dashed circled areas indicate the lipid coalesced areas in each category.

et al., 2018). Teleost fish oocytes consist of a significant amount of lipids that are essential for embryonic and larval growth (Johnson, 2009). In some species, such as the Eurasian perch, oocyte quality evaluation before fertilisation has been shown useful in commercial production (Żarski et al., 2011).

The present study revealed that the lipid distribution in Atlantic salmon oocytes might be a marker of oocyte quality. The lipid distribution patterns in categories 1, 3, 4 and 5 presented in this study have been investigated previously in studies for rainbow trout (Ciereszko et al., 2009), brown trout (Mansour et al., 2007) and Arctic char (Mansour et al., 2008). However, classifying lipid distribution patterns by an objective criteria is missing in most of the previous research studies, except for brown trout (Mansour et al., 2007) where lipid droplet patterns were classified based on the diameter of the coalescence. In the current study, the oocytes with lipid coalescence were however classified based on the area size of the coalesced lipid droplet distribution, and furthermore an additional category (C2) consisting of lipid droplet coalescence in a smaller area size was introduced. Category 2 contributed considerably to predict oocyte quality and constituted together with categories 1 and 3 subgroup HQ which consisted of oocytes with superior quality compared to subgroup LQ. This new approach providing more information may represent an improvement in oocyte quality evaluation and give added value in comparison with the previous mentioned studies.

Most of the oocyte batches from the 16 females contained at least three categories of lipid droplet distribution, and the number of categories varied according to fertilisation rates. The females that had majority of oocytes in categories 1, 2 and 3 were observed to have high fertilisation rates, while lower fertilisation rates were seen in females with more oocytes in categories 4 and 5. The results indicate that oocyte batches with more oocytes (≥74 %) belonging to subgroup HQ showed a higher fertilisation rate, eyed-embryo rate and hatching success, whereas low number of oocytes (<74 %) from subgroup HQ showed a reversed effect. This is contradictory to the conclusion from Ciereszko et al. (2009) who found a lack of consistency in the relationship between oocyte lipid droplet distribution and fertility outcomes in rainbow trout. However, lipid droplet distribution was restricted to three categories and relatively high and homogeneous fertility rates were obtained, which might have influenced their results.

A few of the females in our study had fertilisation rates, eyed embryo and hatching success not corresponding totally with the sub-grouping performed based on oocyte lipid droplet patterns. Female ID 12 had an apparently high fertilisation rate, but the embryos did not survive until hatching. This result was consistent throughout seven incubator replicates and might be due to an intrinsic abnormal cell division, preventing the eggs in developing to hatching. Female 15 was observed with a low hatching success (49 %) even though most of the oocytes of this female were categorized as high quality. The decrease in hatching success could be due to several factors, including genetic variation and utilisation of energy during the embryo development (Callet et al., 2022; Riddle and Hu, 2021). Female 16 gave a high eyed embryo success (77 %) and hatching success (76 %) even though 56 % of the oocytes were categorized as low quality. However, this female only had oocytes within categories 2, 3 and 4, which might have influenced the fertility

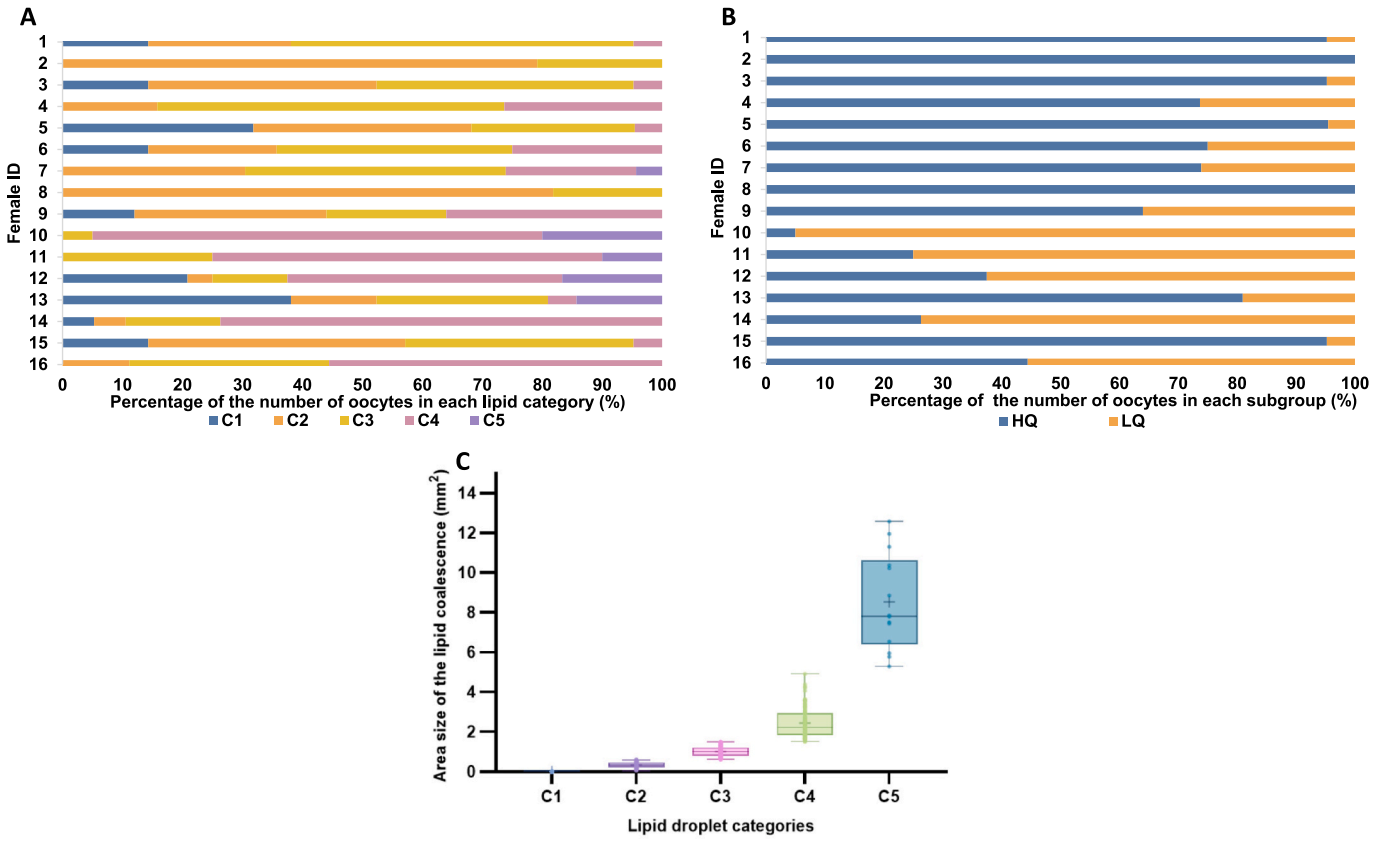


Fig. 2. Categories and subgroups based on the lipid distribution in oocytes ($n = 20$) from 16 females of Atlantic salmon (*Salmo salar L*). (A) Percentage of oocytes in each lipid category (C1-C5) per female; (B) Percentage of oocytes present in sub-grouped lipid categories (High quality (HQ): percentage of categories 1, 2 and 3; Low quality (LQ): percentage of categories 4 and 5) for each female; (C) Box and whisker plots presenting measured lipid coalesced area sizes in each category with individual data points. The whiskers represent the range of the data i.e. minimum and maximum and + indicates the median in each category.

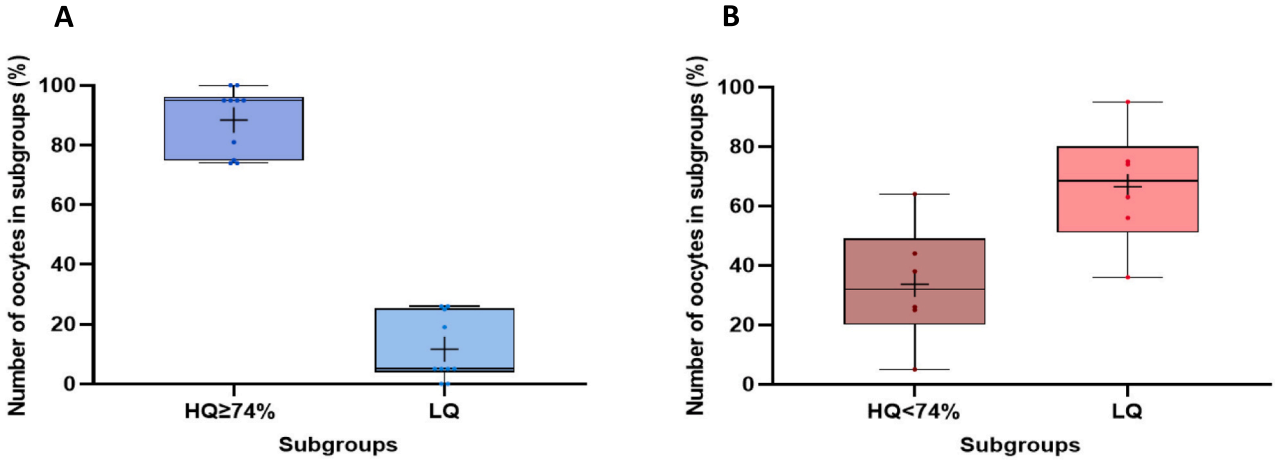


Fig. 3. Box and whisker plots presenting differences in subgroups with lipid distribution patterns in Atlantic salmon (*Salmo salar L*). (A) Difference between the subgroups HQ and LQ at HQ $\geq$ 74 % ($p < 0.0001$); (B) Difference between the subgroup HQ and LQ at HQ < 74 % ($p < 0.05$). The whiskers represent the range of the data i.e. minimum and maximum and + indicates the median in each subgroup. HQ $\geq$ 74 % - more or equal than 74 % of oocytes from categories 1, 2, 3; LQ-Low quality (oocytes percentage of categories 4,5).

outcome.

The results of the present study show that dividing the study population into subgroups could be suitable for studying oocyte quality and fertility outcome in Atlantic salmon. According to our findings, we can assume that a high eyed embryo success (>70 %) may be achieved when more than 74 % of the oocytes are classified as having lipid droplet distributions of categories 1, 2 and 3 (subgroup HQ $\geq$ 74 %). Since the end product of egg production facilities is eyed embryos, this information would benefit effective egg production planning. Furthermore, the present study confirms that there is a significant correlation between subgroups and fertilisation outcome. This is in agreement with Mansour et al. (2007) where differences in lipid droplet distribution in oocytes of

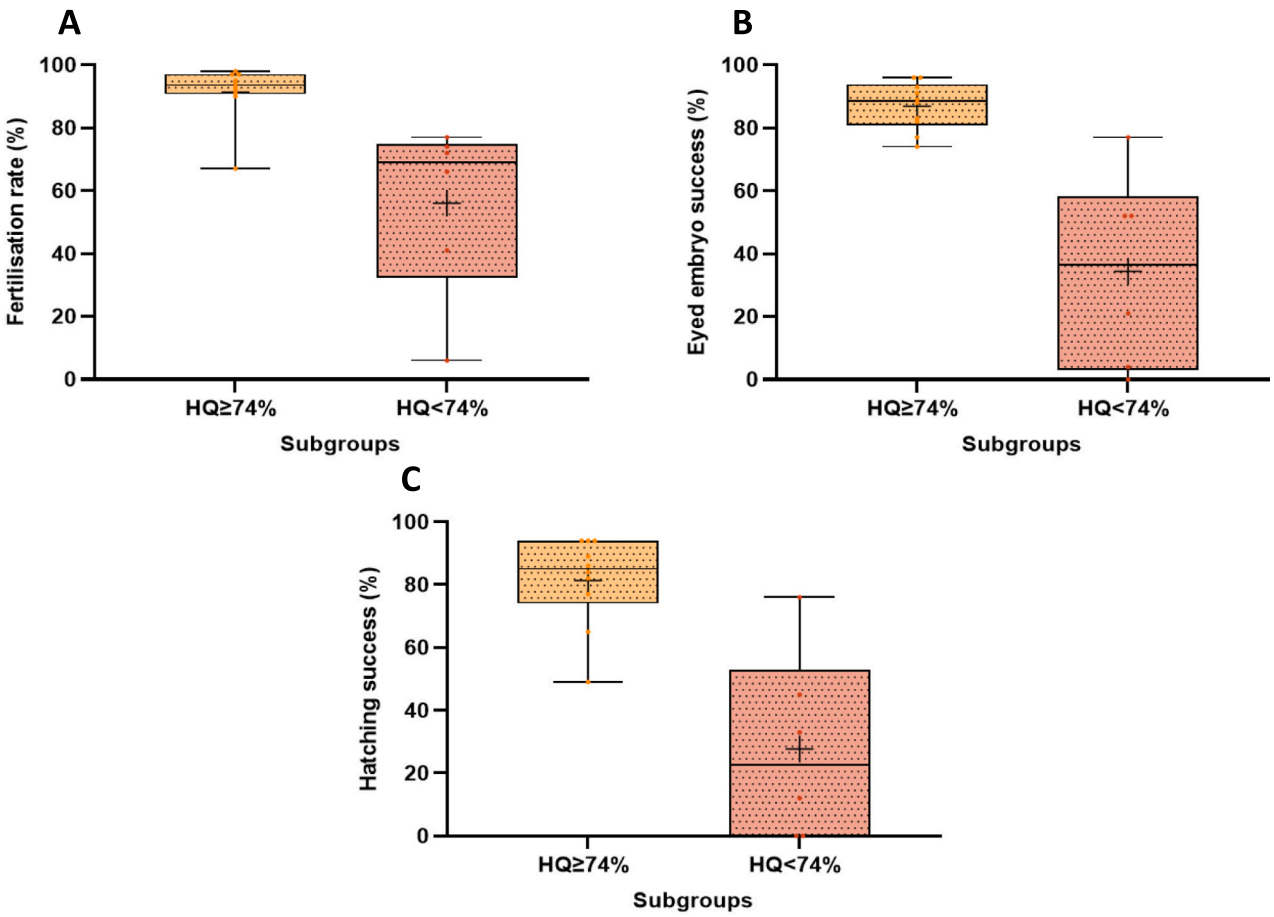


Fig. 4. Box and whisker plots presenting differences in subgroups with lipid distribution patterns based on fertilisation outcome in Atlantic salmon (*Salmo salar L*). (A) Difference between the subgroup HQ ≥ 74 % and HQ < 74 % calculated based on the fertilisation rate ($p = 0.025$) (B) Difference between the subgroup HQ ≥ 74 % and HQ < 74 % calculated based on the eyed embryo rate ($p = 0.008$); (C) Difference between the subgroup HQ ≥ 74 % and HQ < 74 % calculated based on the hatching success ($p = 0.005$). The whiskers represent the range of the data i.e. minimum and maximum and + indicates the median in each subgroup. HQ ≥ 74 % - more or equal than 74 % of oocytes from categories 1, 2, 3; HQ < 74 % - Less than 74 % of oocytes from categories 1, 2, 3).

brown trout were found to distinguish high and low quality oocytes. Usually, in eyed embryo production facilities category 5 (over-ripe) is already known for low quality and visually noticeable in oocyte batches upon collection, but tracing oocytes based on other categories might facilitate sustainable production.

To the best of our knowledge, there is no previous research focused on detecting lipid droplets by staining with BODIPY in Atlantic salmon oocytes. BODIPY™ 493/503 is a known neutral lipid dye that has been used previously to detect lipid droplets mainly in mammalian oocytes (Dunning et al., 2014). However, visualising a large area of the oocyte in salmon was not possible under the fluorescence microscope due to the large size. We observed, however, a clear difference in oocytes with evenly distributed lipid droplets and areas with coalesced lipid droplets by the method applied. A similar study (Mansour et al., 2007) was conducted in oocytes of brown trout (*Salmo trutta fario*) stained with neutral oil red to examine the nature of lipid distribution. They observed the difference between the oocytes with evenly distributed lipid droplets and coalesced. In the present study, the weak signal in completely coalesced lipid area in overripe oocyte showed the absence of neutral lipids. These results are in agreement with a recent study (Clarkson et al., 2024) conducted on overripe oocytes that showed a decrease in total neutral lipid content.

Interestingly, we observed lipid droplets with several different sizes in all oocytes. The larger sized lipid droplets did not show any fluorescence signal whereas smaller ones showed a stronger fluorescence signal. This indicates that larger lipid droplets are not neutral lipids, given that BODIPY™ 493/503 stains only neutral lipids, and suggests that there are polar lipids other than neutral lipids present as lipid droplets in the oocyte. This is in agreement with a literature review by Olzmann and Carvalho (2019) stating that the number, size and composition of lipid droplets can vary broadly between different cells or within the same cell. The reason behind the lipid coalescence is not clear and have not yet been fully understood. According to Mansour et al. (2007), the lipid droplets are in the form of suspension in the yolk protein and changes in physical properties in the yolk can influence the induction of lipid coalescence. These yolk lipids could be present as discrete oil droplets or as lipids emulsified with yolk proteins (Johnson, 2009).

Generally, lipid droplets are mostly composed of neutral lipids e.g. triglycerides and sterol esters and bound by a monolayer of surface phospholipids and proteins (Olzmann and Carvalho, 2019). However, coalescence of lipid droplets can reduce the phase boundary area that leads to release of energy of the emulsion. Presence of surface proteins or surfactants help to avoid coalescence and maintain lipid droplets intact by lowering surface tension (Walther and Farese Jr., 2012). Therefore, it is plausible that deviations in surface proteins or surfactants could be the cause of the lipid coalescence seen in Atlantic salmon oocytes in this study. In the present study, we observed that coalesced lipid droplets in oocytes classified into subgroup LQ (categories 4 and 5) were frequently relocated in the ooplasm during handling and analysis whereas this was not observed in oocytes from subgroup HQ (categories 1, 2 and 3). This is in accordance with a previous study by Mansour et al. (2007) where

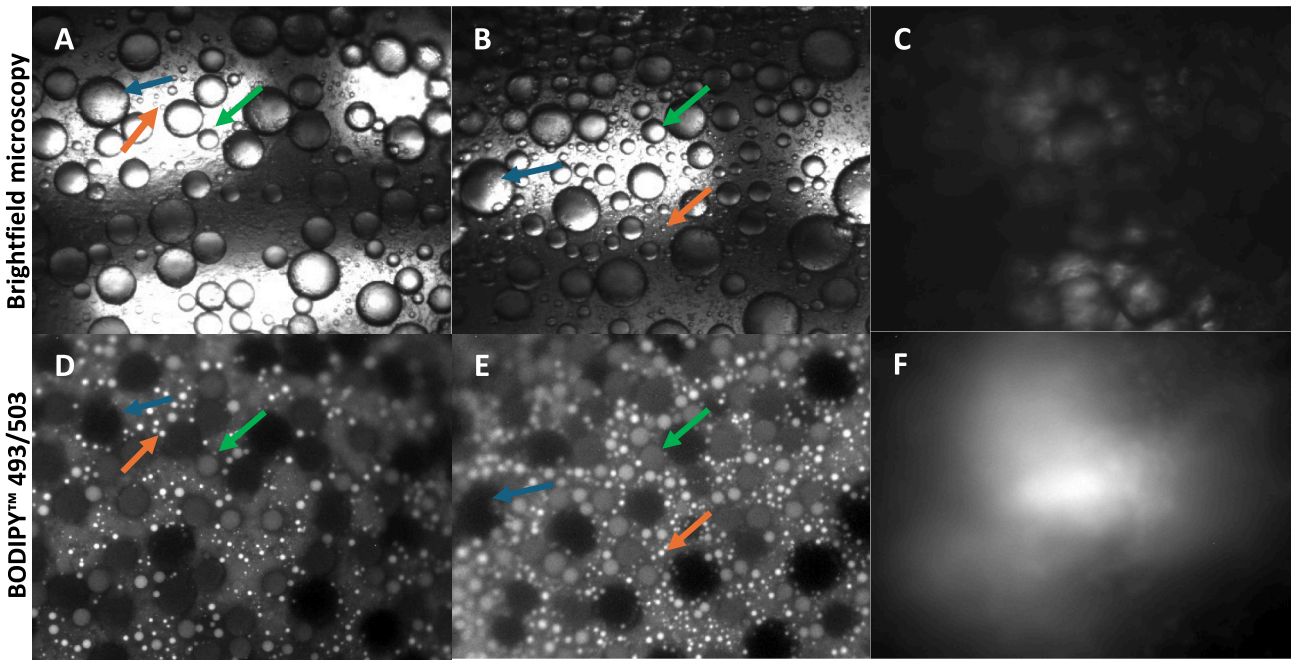


Fig. 5. Microscopy evaluation of lipid droplets in oocytes of Atlantic salmon (*Salmo salar L*). Oocytes were stained with 1 μg/mL BODIPY™ 493/503 for fluorescence microscopy analysis. (A-C) Brightfield microscopy images of lipid droplets; (D-F) Fluorescence images of BODIPY stained neutral lipids shown as white (red arrow) and light grey small circles (green arrow). The non stained appeared in large black circles (blue arrow). (A, D) images of a selected area in an oocyte showing evenly distribution of lipid droplets; (B, E) are images showing an area with very closely positioned lipid droplets (beginning of coalescence) in an oocyte; (C, F) are images showing an area of an overripe oocyte. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

coalesced lipid droplets in oocytes of brown trout migrated towards the upper pole during oocyte handling. The oocytes that had evenly distributed lipid droplets remained in the same position regardless of the oocyte movement.

Most egg producing companies apply vaccination schedules against infectious diseases on larvae prior to smoltification. The oocytes used in the present study were provided by one commercial supplier who received their broodstock from two other suppliers based on the genetics of interest. It appeared that the two broodstocks received different vaccines prior to smoltification and it was later discovered that one of the vaccines might have had a negative effect on reproduction (personal communication). In this study oocytes from seven fish, selected based on the initial low fertilisation rates originated from the broodstock exposed to the suspicious vaccine. However, information obtained retrospectively confirmed that oocyte batches from other females in the same broodstock provided good fertilisation rates. Oocyte quality can be influenced by broodstock management and environmental factors in the aquaculture industry (Bobe, 2015). In the current study the oocytes from females with low fertilisation may have been affected by artificially induced factors like vaccines as well as genetic factors.

In conclusion, the results from the present study show that the categories of lipid droplet distributions hereby applied are predictive of fertilisation outcomes in Atlantic salmon. The content of lipid droplets in mature oocytes, as well as the distribution and aggregation thereof, may be a determinant of the quality of oocytes. Our findings might facilitate developing a practical and cost-effective method to predict oocyte quality for improved production efficiency in Atlantic salmon oocytes upon collection.

Funding

Financial support was received from Research Council of Norway (grant number 329151).

CRediT authorship contribution statement

Sonali N. Kaththriarachchi: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Elisabeth Kommisrud:** Writing – review & editing, Project administration, Conceptualization. **Frøydis D. Myromslien:** Writing – review & editing, Conceptualization. **Teklu T. Zeremichael:** Writing – review & editing, Data curation. **Inger S. Grevle:** Writing – review & editing, Data curation, Conceptualization. **João Santana:** Writing – review & editing, Data curation. **Julia Farkas:** Writing – review & editing, Data curation. **Bjørn Henrik Hansen:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Ewa Wielogórska:** Writing – review & editing, Formal analysis. **Birgitte Narud:** Writing – review & editing, 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

Data will be made available on request.

References

Bahrekazem, M., Matinfar, A., Soltani, M., Abtahi, B., Pusti, I., Samarin, A., 2009. The relation between egg viability, selected aspects of egg and ovarian fluid composition and time of stripping in endangered Caspian Brown trout (*Salmo trutta* caspius). J. Fish. Aquat. Sci. 4, 306–315.

Bobe, J., 2015. Egg quality in fish: present and future challenges. Anim. Front. 5, 66–72.

Callet, T., Cardona, E., Turonnet, N., Maunas, P., Larroquet, L., Surget, A., Corraze, G., Panserat, S., Marandel, L., 2022. Alteration of eggs biochemical composition and progeny survival by maternal high carbohydrate nutrition in a teleost fish. Sci. Rep. 12, 16726.

Ciereszko, A., Wojtczak, M., Dietrich, G.J., Kuźmiński, H., Dobosz, S., 2009. A lack of consistent relationship between distribution of lipid droplets and egg quality in hatchery-raised rainbow trout, *Oncorhynchus mykiss*. Aquaculture 289, 150–153.

Clarkson, M., Taylor, J.F., Mota-Velasco, J.C., Hamilton, A., Albalat, A., Morro, B., Mullen, W., Latosinska, A., Chalmers, L., Vera, L.M., Migaud, H., 2024. The impact of post-ovulatory ageing on the development of diploid and triploid Atlantic salmon (*Salmo salar* L.). Aquaculture 582, 740305.

Dunning, K.R., Russell, D.L., Robker, R.L., 2014. Lipids and oocyte developmental competence: the role of fatty acids and β-oxidation. Reproduction 148, R15–R27.

Hansen, B.H., Kjørsvik, E., Malzahn, A.M., Sarno, A., Kulild, O.M., Farkas, J., Nordtug, T., Rye, R., Kvæstad, B., Lein, I., Hagemann, A., 2022. Ova lipid profiling and egg quality in wild and captive lumpfish, *Cyclopterus lumpus* (Linnaeus, 1758). Aquaculture 560, 738556.

Ienaga, N., Higuchi, K., Takashi, T., Gen, K., Tsuda, K., Terayama, K., 2021. Vision-based egg quality prediction in Pacific bluefin tuna (Thunnus orientalis) by deep neural network. Sci. Rep. 11, 6.

Johnson, R.B., 2009. Lipid deposition in oocytes of teleost fish during secondary oocyte growth. Rev. Fish. Sci. 17, 78–89.

Lahnsteiner, F., Weismann, T., Patzner, R.A., 1999. Physiological and biochemical parameters for egg quality determination in lake trout, *Salmo trutta* lacustris. Fish Physiol. Biochem. 20, 375–388.

Mansour, N., Lahnsteiner, F., Patzner, R.A., 2007. Distribution of lipid droplets is an indicator for egg quality in brown trout, *Salmo trutta* fario. Aquaculture 273, 744–747.

Mansour, N., Lahnsteiner, F., McNiven, M.A., Richardson, G.F., 2008. Morphological characterization of Arctic char, *Salvelinus alpinus*, eggs subjected to rapid post-ovulatory aging at 7 °C. Aquaculture 279, 204–208.

Narud, B., Khezri, A., Zeremichael, T., Eriksen, A.-L., Grevle, I., Nordborg, A., Klinkenberg, G., Wilson, R., Kommisrud, E., 2023. Sperm quality parameters, fertilizing potential, metabolites, and DNA methylation in cold-stored and cryopreserved milt from Atlantic salmon (*Salmo salar* L.). Front. Genet. 14.

Olzmann, J.A., Carvalho, P., 2019. Dynamics and functions of lipid droplets. Nat. Rev. Mol. Cell Biol. 20, 137–155.

Reading, B.J., Andersen, L.K., Ryu, Y.-W., Mushirobira, Y., Todo, T., Hiramatsu, N., 2018. Oogenesis and egg quality in finfish: yolk formation and other factors influencing female fertility. Fishes 3, 45.

Riddle, M.R., Hu, C.-K., 2021. Fish models for investigating nutritional regulation of embryonic development. Dev. Biol. 476, 101–111.

Walther, T.C., Farese Jr., R.V., 2012. Lipid droplets and cellular lipid metabolism. Annu. Rev. Biochem. 81, 687–714.

Żarski, D., Palińska, K., Targońska, K., Bokor, Z., Kotrik, L., Krejszeff, S., Kupren, K., Horváth, Á., Urbányi, B., Kucharczyk, D., 2011. Oocyte quality indicators in Eurasian perch, *Perca fluviatilis* L., during reproduction under controlled conditions. Aquaculture 313, 84–91.