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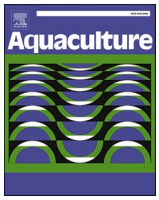

Exploring the occurrence of DNA-fragmentation in Arctic charr sperm (*Salvelinus alpinus*) and its impact on embryo viability

Khrystyna Kurta [a,*], Christos Palaiokostas [a], Henrik Jeuthe [b]

[a] Department of Animal Biosciences, Swedish University of Agricultural Sciences, 750 07 Uppsala, Sweden
[b] Aquaculture Center North, Åvägen 17, 844 61 Kälarne, Sweden

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ABSTRACT

Sperm from Arctic charr sires can contain high levels of DNA fragmentation. Nevertheless, the frequency of this phenomenon and its impact on embryo viability are not fully known. The current study aimed to further elucidate the extent of DNA fragmentation in Arctic charr sperm through a comprehensive sampling of Swedish hatcheries. Furthermore, associations between sperm DNA fragmentation and subsequent embryo viability were investigated. Arctic charr males ($n = 166$) were sampled from five major Swedish hatcheries, and DNA fragmentation, together with sperm concentration and viability, were recorded. Fragmentation levels were classified as: intact, moderate, severe, and stellar fragmentation. The median DNA fragmentation level (severe to stellar fragmentation) was 32 % (5–50 %; $n = 93$). Moreover, the median sperm concentration was 3.6×10^9 cells/ml (range 0.3–11.5 $\times$ 10^9) and cell viability was 90 % (range 68–96 %). Significant differences in sperm concentration, cell viability, and DNA fragmentation were found among the five hatcheries (Kruskal-Wallis, $p < 0.05$). The effect of sperm concentration on sperm cell viability was significant ($p < 0.001$). A strong Spearman correlation coefficient (Rho = 0.76, $p < 0.05$) was observed for severe or the sum of severe and stellar DNA fragmentation with early embryo mortality. In conclusion, DNA fragmentation levels were notably high in farmed Arctic charr while individual fragmentation levels were clearly associated with the timing of embryo abortions in the offspring. However, the impact of sperm DNA fragmentation on the overall embryo viability during routine hatchery production remains uncertain.

1. Introduction

Sperm DNA fragmentation is an indicator of milt quality in fish, reflecting the integrity of the carried genetic material (Rex et al., 2017). Naturally, DNA integrity in sperm ensures the accurate transmission of genetic information to the next generation after fertilization and is essential for successful reproduction (Kopeika et al., 2004). Damage in DNA as a limiting factor for male fertility in fish has been widely studied in recent years, especially in connection to milt cryopreservation (Figueroa et al., 2020; Sandoval-Vargas et al., 2021). However, DNA in spermatozoa can start to degrade before release by the male (Pérez-Cerezales et al., 2010) and during short-term storage after stripping (Pérez-Cerezales et al., 2009), often as a result of oxidative stress (Cabrita et al., 2014). Studies in rainbow trout have previously shown that fertilization with sperm carrying differing degrees of fragmented DNA preserved good fertilization ability but produced embryos with lower viability (Pérez-Cerezales et al., 2010). In addition, a negative correlation between embryo development and viability and DNA damage was shown in zebrafish (Gosálvez et al., 2014) and sturgeon (Gazo et al., 2022). Furthermore, high levels of sperm DNA damage in Arctic charr and brown trout were associated with elevated skeletal abnormality incidence in the progeny with a subsequent increase in mortality (Devaux et al., 2011). These findings underscore the importance of studying fragmented DNA in gametes due to its contribution to embryo viability and reproductive success.

It should be stressed, that the capacity to repair DNA damage is very limited in mature spermatozoa. Nonetheless, sperm DNA can be repaired together with the maternal DNA in the zygote and during early development (Fernández-Díez et al., 2015). A study on rainbow trout analyzing the reproductive outcome when using sperm carrying varying levels of DNA fragmentation concluded that the embryo has a high capacity to repair sperm DNA damage, estimated to be up to 10 % of chromatin fragmentation (Pérez-Cerezales et al., 2010). Hence, the consequences of sperm DNA fragmentation on embryo viability depend

* Corresponding author.
E-mail address: khrystyna.kurta@slu.se (K. Kurta).

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both on the chromatin fragmentation levels of the sperm and the repair capacity of the oocyte (González-Marín et al., 2012).

The most common method for the assessment of sperm chromatin fragmentation in fish is the comet assay or SCGE (single cell gel electrophoresis; Cabrita et al., 2014). The technique is based on the fact that DNA fragments will migrate in an electrophoresis gel according to size. Non-fragmented DNA of spermatozoa will remain at the head of the cell, while fragmented strands will further migrate away from the head during electrophoresis at increasing length as fragment size increases. The resulting pattern resembles a comet, where the shape and length of the tail reflect the level of chromatin fragmentation. Specific software can then be used to quantify fragmentation in individual sperm and calculate the average fragmentation level in a sample or proportion of cells. Even though this method provides detailed data on fragmentation and works well with fish sperm, it requires specialized laboratory equipment and software. An alternative technique which also relies on differential migration patterns of fragmented DNA strands, but has lower technical requirements, is the sperm chromatin dispersion test (SCD). Commercial kits for sample preparation are available and can be used in a rudimentary laboratory set-up for sampling in 'the field'. To our knowledge, there is software for automated analysis of SCD results for several animals and humans, but not yet for fish. Hence, result interpretation is made using visual inspection under a fluorescence microscope or custom image analysis software, which may constitute comparisons with previous studies difficult. However, evaluation studies of this method on tench (*Tinca tinca*) aligned well with parallel results obtained using the comet assay (López-Fernández et al., 2009).

In the case of the Arctic charr (*Salvelinus alpinus*), it has been shown that impaired oocyte quality is a major constricting factor in hatchery production in Sweden, and that thermal stress in the broodstock is most likely the most critical cause (Jeuthe et al., 2013). Moreover, previous studies on Arctic charr broodstock in Sweden have also shown that the majority of non-viable eggs are lost due to embryonic mortality rather than failed fertilization (Jeuthe et al., 2019). Part of this mortality can be connected to paternal factors (Jeuthe et al., 2019). Notably, high levels of DNA fragmentation have been found in sperm from Arctic charr males using the SCD-method (Jeuthe et al., 2022). As such, DNA fragmentation is likely to be one of the limiting factors for embryo viability following artificial reproduction in this species. However, no direct connection between those two parameters has been found so far under routine hatchery conditions.

The aim of this study was to map the extent of the potentially detrimental issue of DNA fragmentation in Arctic charr sperm across the entire Swedish hatchery industry and, secondly, to test for connections between levels of DNA fragmentation and subsequent embryo viability.

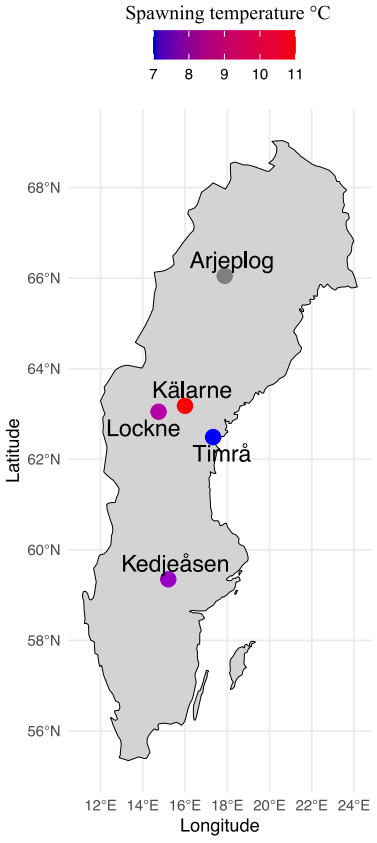
2. Materials and methods

2.1. Ethical statement

This study was performed in accordance with the Swedish legislation described in the Animal Welfare Act 2018:1192 (ethics permit: 5.2.18–09859/2019).

2.2. Hatcheries and broodstock background

The five largest Swedish Arctic charr hatcheries at the time of the study were involved. The hatcheries are located in Kedjeåsen in Örebro county, Timrå in Västernorrland county, Kälarne and Lockne in Jämtland county, and in Arjeplog in Norrbotten county (Fig. 1). The hatchery in Kälarne is the main facility for the Swedish breeding program of Arctic charr and their broodstock has been used in several



Location	Water supply	Temperature, °C			Rearing units	Feed	Sampling date	Hatching rates (%)
		summer	spawning	winter				
Kedjeåsen	ground	8	8	8	Concrete tanks outdoor	Biomar	01.10	70
Timrå	ground	8	7	6	Concrete tanks outdoor	Skretting	02.10	50
Kälarne	lake	14	2-11	2	Concrete tanks indoor + ponds outdoor	Skretting	09.10-22.10	47
Lockne	ground/ creek	8.5	6-8.5	6-7	Plastic tanks indoor	Skretting	09.10	n/a
Arjeplog	lake	n/a	n/a	n/a	Concrete tanks indoor + outdoor	Skretting	29.10	n/a

Fig. 1. Geographic location and specifications for the Swedish hatcheries included in the study are depicted in the form of coloured dots. N/A indicates missing data. The colour annotation shows the upper spawning temperature at every hatchery, the grey colour indicates missing data.

previous studies of reproduction and gamete quality (Jeuthe et al., 2019, 2022; Kurta et al., 2023). All involved facilities reared their broodstock in land-based flow-through systems. Although water recirculation (RAS, recirculation aquaculture system) is applied for earlier life stages in Kedjeåsen. The facilities in Kälarne and Arjeplog use lake water, Timrå, Lockne, and Kedjeåsen use ground water, while in Lockne water from a creek is mixed in with the ground water during seasons when this is available. These differences in water supply result in different temperature conditions over the year in those facilities. The detailed information on each hatchery is given in Fig. 1.

The broodstock in all hatcheries except Kedjeåsen belonged to the selectively bred strain of Arctic charr (trademarked as Arctic superior) from the Swedish breeding program. The involved sires from the breeding program were of the 8th generation, year classes 2013–2015, that during the study were 4–6 years old. They were fed with Skretting vitalis feed specifically formulated for Arctic charr broodstock. However, Kedjeåsen used their own broodstock, sharing a partial common ancestry with the Arctic superior (Henrik Jeuthe, personal communication). The broodstock at Kedjeåsen were fed with Arctic charr broodstock feed supplied by Biomar.

2.3. Milt collection and analyses

Milt was collected from 166 sires in the five locations during peak reproductive season (Fig. 1). 93 samples were used for DNA fragmentation analysis, while all 166 were analyzed for sperm concentration and viability. Milt collection was achieved through gentle massage of the abdomen, after the sires were anaesthetized (Tricaine mesylate), rinsed in clean water, and dried off around the genital papillae.

Milt quality was assessed through measurements of concentration and cell viability using the automated cell counter NucleoCounter SP-100™ (Chemometec, Allerod, Denmark) and DNA fragmentation using the SCD method (Halomax HT-TT40, Halotech DNA, Madrid, Spain). Concentration measurements and preparation of Halomax slides were performed on-site at the different hatcheries immediately after sample collection. Concentration was measured using the pre-set programs for total cell count on samples diluted 1:1000 with lysis buffer (20 µl sample and 20 ml buffer; Reagent S-100, Chemometec, Allerod, Denmark). In the case of the viability analysis samples were diluted in isotonic PBS (Chemometec) according to the manufacturers protocol. Analyses were conducted using the setting for bull semen, in line with recommendations from the manufacturer and our previous studies (Jeuthe et al., 2019, 2022; Kurta et al., 2023).

2.4. DNA fragmentation analysis

Preparation of the milt samples for DNA fragmentation analysis was done in accordance with the guidelines to Halomax kit (HT-TT40, Halotech DNA; Madrid, Spain) also published in López-Fernández et al. (2009). Briefly, PBS was used for the dilution of the samples. The dilution factors were calculated from the relevant NucleoCounter recordings, resulting in 20–30 million cells per ml for each Halomax slide. The processed samples were later stained with SYBR® Green (Merck, Darmstadt, Germany) and visually assessed with a fluorescence microscope. A total of 200 cells were classified per slide. Fragmentation levels were determined based on four predefined categories as described previously (López-Fernández et al., 2009): intact (SCD1, compact cells with no clear halo), moderate fragmentation (SCD2, halo width smaller or equal to nucleus diameter), severe fragmentation (SCD3, halos clearly wider than the remaining nuclei but still with clearly defines outer edges), and stellar fragmentation (SCD4, halos were fading with little to no remaining intact nuclei) as shown in Fig. 2. The proportion of sperm with moderate to severe DNA fragmentation is represented as the sum of SCD2 to SCD4 (SCD2–4) divided by the total number of cells. Similarly, the proportion with severe to stellar DNA fragmentation is represented as the sum of SCD3 and SCD4 (SCD3–4) divided by the total number of cells.

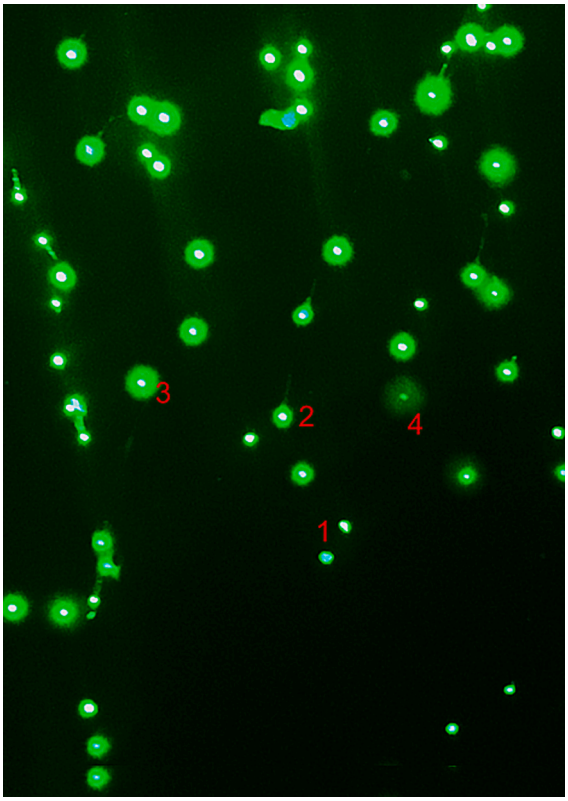


Fig. 2. Fragmentation level categories: (1) SCD1 – no fragmentation (intact); (2) SCD2 – moderate fragmentation; (3) SCD3 – severe fragmentation; and (4) SCD4 – stellar fragmentation.

2.5. Fertilization trial

In addition to sampling at the different hatcheries, nine of the milt samples from Kälarne were selected at random to be used in a fertilization trial. Milt from the nine sires was used to fertilize small aliquots of eggs from one dam ($n = 584 \pm 18$ each), thereby eliminating any confounding female factors in the comparison of individual sperm quality. Individually adjusted milt volumes (46–176 µl) containing 400 million cells were added to each egg aliquot, giving an equal sperm to egg ratio of approximately 680,000 sperm per egg. Fertilization was done using the dry method where milt was added to the "dry" egg batch and gently mixed. Next, water (the same as in the rearing tank) was added to the fertilization vessel, maintaining approximately 2:1 water to egg volume ratio. The fertilized eggs were incubated in separate stainless steel mesh baskets placed in a communal hatching tray at a constant temperature of 6 °C. During incubation, dead eggs were collected and fixed in FAA-solution (Formalin–acetic acid–alcohol) separately for each egg aliquot. The fixed samples were later examined using a low-magnification microscope to determine mortality rates at different stages of embryonic development. More specifically, the embryonic stages included early development (unfertilized-cleavage-blastula-gastrula), somite stage, eyed stage, and point of hatching. Unfertilized eggs could not be distinguished from early abortions with enough certainty to form a separate category.

2.6. Statistical analysis

All statistical analyses were performed using the R programming language v.4.3.2 (R Core Team, 2019). The data were tested for differences between sampling locations with regard to DNA fragmentation

levels, sperm concentration and viability using the Wilcoxon test for pairwise comparisons and the Kruskal-Wallis test to account for the multiple comparisons. These tests were selected based on the criteria for normality, which were assessed using the Shapiro-Wilk test presented in Supplementary Table 1.

A linear mixed-effects model was employed to evaluate the relationship between sperm concentration, sperm viability, and DNA fragmentation, while accounting for variability between hatcheries. The model was implemented using the "lme4" package in R (Bates et al., 2015) including a random effect (hatchery location) into the model. The model was specified as follows:

$$\text{Response Variable} \sim \text{Predictor} + (1|\text{Hatchery}).$$

Additionally, the studied sperm quality parameters were tested for correlations using the Spearman's rank-order metric. Finally, a linear regression model was used to assess the relationship between DNA fragmentation levels and embryo mortality from the fertilization trial. The model was fit using the "lm()" function in R. In addition, the associations between these parameters were assessed using Spearman's rank-order correlation. A p-value below 0.05 was considered statistically significant.

3. Results

3.1. Sperm quality at different hatcheries

Sperm concentration in milt sampled from Arctic charr males across the five hatcheries ranged from 0.34×10^9 to 11.46×10^9 cells/ml with a median of 3.60×10^9. Cell viability ranged from 68 % to 96 %, with a median of 90 %. Among the hatcheries, the highest sperm concentration was observed in milt samples from Arjeplog, while samples from Timrå had the lowest concentration. The highest sperm cell viability was observed in samples from Kedjeåsen and Lockne. DNA integrity (SCD1) varied widely (range: 7–55 %, median: 28 %), and the proportion of sperm with intact DNA was the highest in Lockne. DNA fragmentation was generally large, with the highest median (76 %) for moderate to stellar fragmentation in samples from Arjeplog. Timrå and Kedjeåsen broodstocks showed the highest proportion (39 %) of combined severe to stellar fragmentation (Table 1).

Statistical comparisons revealed significant differences in sperm parameters across the studied hatcheries (Fig. 3). Sperm concentrations differed significantly between samples from Arjeplog and Timrå, as well as between Kälarne and Arjeplog (Wilcoxon test, $p < 0.01$). Cell viability also varied significantly across the five hatcheries (Wilcoxon test, $p < 0.05$). Notably, the proportion of sperm with intact DNA was significantly different among Kälarne, Arjeplog, Kedjeåsen, and Lockne ($p < 0.05$). Moderate DNA fragmentation levels varied significantly across all five locations ($p < 0.05$). The proportion of sperm with severe or severe to stellar DNA fragmentation exhibited significantly ($p < 0.05$) different mean values across samples from the studied locations. Finally, moderate to stellar DNA fragmentation also varied significantly ($p < 0.05$).

3.2. Relationship between sperm quality and DNA fragmentation

A linear mixed-effects model was used to assess the relationship between sperm concentration, sperm viability, and DNA fragmentation, accounting for variability across hatcheries. The mixed effects model showed that the effect of sperm concentration on sperm cell viability was significant ($\beta = 0.34$, $p < 0.001$). Additionally, weak positive correlations were found between the two parameters (Spearman Rho: 0.24, $p = 0.002$). A significant effect of sperm concentration was observed on the proportion of sperm with moderate (SCD2, $\beta \approx 0.64$, $p < 0.05$) and severe ($\beta \approx -1.5$, $p < 0.001$) DNA fragmentation (SCD3 and the sum of SCD3 and SCD4). Furthermore, a positive Spearman correlation was found between concentration and moderate fragmentation (Rho: 0.32, $p < 0.05$). On the other hand, a negative Spearman correlation was observed between sperm concentration and severe fragmentation (Rho: 0.31, $p < 0.05$). Finally, no significant effects or Spearman correlations were found between sperm viability and DNA fragmentation levels (Table 2).

3.3. Relationship between DNA fragmentation and embryo mortality

Mortality and hatching rates in the egg aliquots fertilized with sperm from nine different sires are presented in Fig. 4. One egg aliquot had zero survival and was excluded from further analyses, as it was likely the result of failed fertilization. Early mortality (unfertilized-cleavage-blastula-gastrula) ranged from 31 % to 90 %, somite mortality was between 9 % and 17 %, and mortality at the eyed stage ranged from 8 % to 17 %. Hatching rates ranged from zero to 37 %.

Spearman correlation analyses showed that early mortality (sum of unfertilized and aborted before the somite stage) had a positive correlation with higher levels of DNA fragmentation (Table 3). A linear regression analysis revealed a significant positive effect ($\beta = 0.02$, $p < 0.05$) of severe (SCD3) and severe to stellar DNA fragmentation (SCD3–4) on the early embryo mortality (Fig. 5). Strong negative correlations were found between DNA fragmentation levels with somite (SCD2–4, $p < 0.05$) and eyed (SCD3, SCD3–4, $p < 0.08$) mortality. However, a linear regression analysis estimated that the effect size of DNA fragmentation on mortality at these stages was zero. In addition, somite and eyed mortality were highly negatively correlated with the early mortality, with coefficients of −0.95 and − 0.89 ($p = 0.002$), respectively. Strong and negative Spearman correlations of −0.69 at a lower significance level of $p < 0.06$ were estimated between DNA fragmentation (SCD3, SCD3–4) and survival to the eyed stage. However,

Table 1
Sperm quality parameters shown as median (minimum – maximum value) measured in milt samples collected at five Arctic charr hatcheries in Sweden.

	Sperm Chromatin Dispersion							NucleoCounter		
Location	Sample size	SCD1	SCD2	SCD3	SCD4	Sum SCD2–4	Sum SCD3–4	Sample size	Concentration (10^9/ml)	Cell viability (%)
Arjeplog	16	24 (7–50)	45 (39–52)	29 (5–46)	0.5 (0.0–10)	76 (50–93)	29 (5–46)	48	4.96 (1.12–11.46)	89 (87–95)
Kedjeåsen	16	29 (19–46)	34 (28–44)	39 (16–47)	1 (0–4)	72 (54–81)	39 (17–48)	16	4.48 (1.18–7.22)	92 (88–94)
Kälarne	29	28 (18–54)	39 (29–55)	29 (15–42)	1 (0–3)	73 (47–82)	31 (17–43)	70	3.59 (0.65–8.68)	90 (68–96)
Lockne	16	39 (15–55)	37 (24–48)	23 (19–42)	1 (0.5–7)	60 (45–85)	25 (19–44)	16	3.09 (1.34–10.17)	92 (88–93)
Timrå	16	27 (19–49)	32 (16–43)	36 (23–50)	1.0 (0–8)	73 (52–81)	39 (26–50)	16	2.38 (0.34–8.01)	91 (87–93)
Total	93	28 (7–55)	38 (16–55)	31 (5–50)	1.0 (0–10)	73 (45–93)	32 (5–50)	166	3.60 (0.34–11.46)	90 (68–96)

DNA fragmentation abbreviations: SCD1 intact, SCD2 moderate fragmentation, SCD3 severe fragmentation, SCD4 stellar fragmentation, Sum of SCD2–4 moderate to stellar, and sum of SCD3–4 severe to stellar DNA fragmentation.

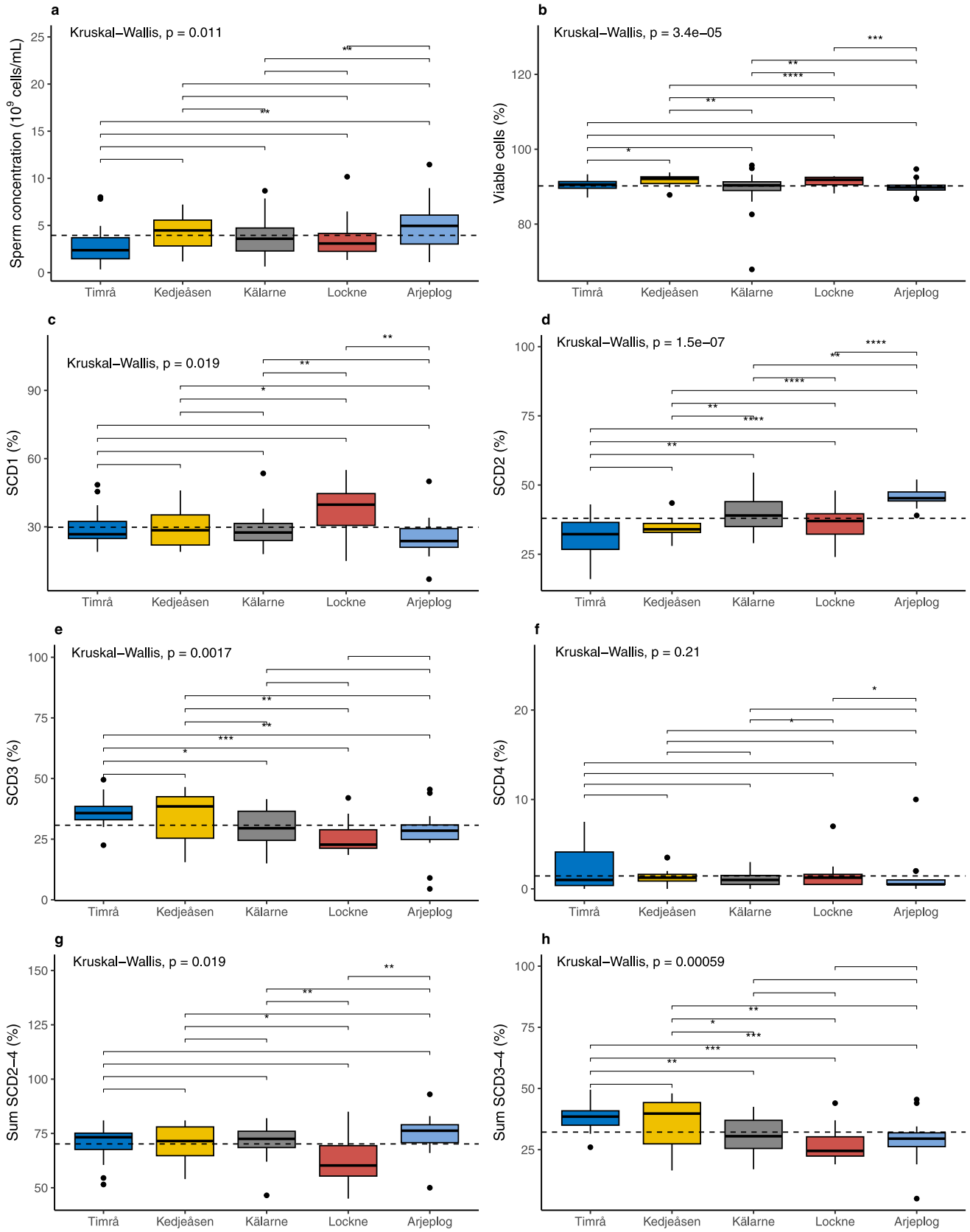


Fig. 3. Sperm quality parameters measured in milt collected from Arctic charr sires at five different hatcheries in Sweden. Sperm concentrations (a) and cell viability (b) were measured using a NucleoCounter® SP-100™. The dashed line indicates the median values for the entire group. DNA fragmentation (c-h) was analyzed using the SCD-method (Halomax® for freshwater fish). Significance levels are annotated as $***p < 0.001$, $**p < 0.01$, $*p < 0.05$, determined using the Wilcoxon test for pairwise comparisons. DNA fragmentation abbreviations: SCD1 intact, SCD2 moderate fragmentation, SCD3 severe fragmentation, SCD4 stellar fragmentation, Sum SCD2–4 moderate to stellar, and sum of SCD3–4 severe to stellar DNA fragmentation.

Table 2
Linear mixed-effects model results for sperm concentration, sperm cell viability and DNA fragmentation with corresponding Spearman rank correlation coefficients (Rho).

Predictor	Response variable	Effect size	SE	*t*-value	*P*-value	Rho	Rho P-value
Concentration	Cell viability	0.34	0.09	3.68	0.00	0.240	0.002
Concentration	SCD1	0.67	0.43	1.58	0.12	0.046	0.661
Concentration	SCD2	0.64	0.29	2.16	0.03	0.323	0.002
Concentration	SCD3	−1.52	0.39	−3.89	0.00	−0.306	0.003
Concentration	SCD4	0.12	0.08	1.51	0.13	0.044	0.677
Concentration	Sum SCD2–4	−0.67	0.43	−1.58	0.12	−0.046	0.661
Concentration	Sum SCD3–4	−1.37	0.38	−3.59	0.00	−0.314	0.002
Cell viability	SCD1	−0.24	0.61	−0.39	0.70	0.019	0.857
Cell viability	SCD2	0.35	0.42	0.83	0.41	−0.11	0.292
Cell viability	SCD3	0.06	0.59	0.10	0.92	−0.016	0.875
Cell viability	SCD4	−0.06	0.11	−0.53	0.60	0.091	0.383
Cell viability	Sum SCD2–4	0.24	0.61	0.39	0.70	−0.02	0.86
Cell viability	Sum SCD3–4	0.00	0.57	0.00	1.00	−0.0005	0.995

Each cell contains the model estimate, standard error (SE), t-value, p-value, Rho and Rho p-value. DNA fragmentation abbreviations: SCD1 intact, SCD2 moderate fragmentation, SCD3 severe fragmentation, SCD4 stellar fragmentation, sum SCD2–4 moderate to stellar, and sum SCD3–4 severe to stellar DNA fragmentation.

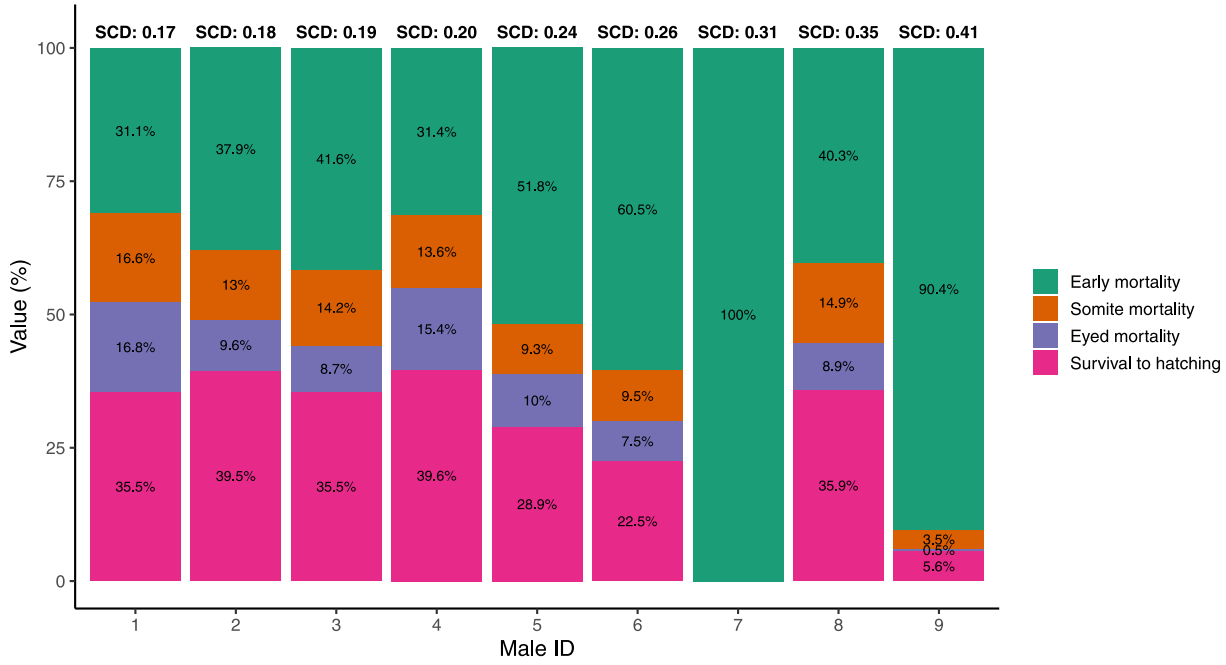


Fig. 4. Mortality and hatching rates in egg aliquots from the same Arctic charr female, fertilized with sperm from nine different males. Each bar represents one egg aliquot fertilized with sperm from one male. Bars are ordered according to increasing level of the sum of severe and stelar DNA fragmentation (sum SCD3–4).

Table 3
Spearman correlation coefficients (Rho) between DNA fragmentation levels in individual sperm samples and embryo viability in fertilized eggs from one Arctic charr female.

Parameter	SCD1	SCD2	SCD3	SCD4	SCD3–4	SCD2–4
Early mortality	−0.66	0.00	0.76*	−0.33	0.76*	0.66
Somite mortality	0.95**	−0.42	−0.55	0.44	−0.55	−0.95**
Eyed mortality	0.39	0.11	−0.69′	−0.01	−0.69’	−0.39
Survival to eyed stage	0.56	0.13	−0.69′	0.21	−0.69’	−0.56
Survival to hatching	0.39	0.42	−0.48	0.19	−0.48	−0.39

Significance levels are annotated as ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, $p <$ 0.06′. DNA fragmentation abbreviations: SCD1 intact, SCD2 moderate fragmentation, SCD3 severe fragmentation, SCD4 stellar fragmentation, sum SCD2–4 moderate to stellar, and sum SCD3–4 severe to stellar DNA fragmentation.

a low effect size ($\beta = -0.01$, $p < 0.05$) was estimated between the two using a linear model (Fig. 4). Overall, DNA fragmentation showed moderate negative correlations with hatching rates (Table 3). However, these correlation coefficients were not statistically significant ($p > 0.05$).

4. Discussion

We observed high levels of DNA fragmentation in the milt samples, consistent with our previous findings from the Kälarne hatchery (Jeuthe et al., 2022). However, it is important to note that the fragmentation level categories differed between the two studies. The earlier study (Jeuthe et al., 2022) classified sperm into two categories: fragmented and non-fragmented based on the defined halo size cut-off (López-Fernández et al., 2009), which limits direct comparison of values. In contrast, the present study used more easily reproducible category definitions (see Materials and methods section). Given the absence of a standardized protocol for quantifying DNA fragmentation in salmonid sperm using the SCD method, we aimed to determine which fragmentation levels are most relevant for fertility assessments.

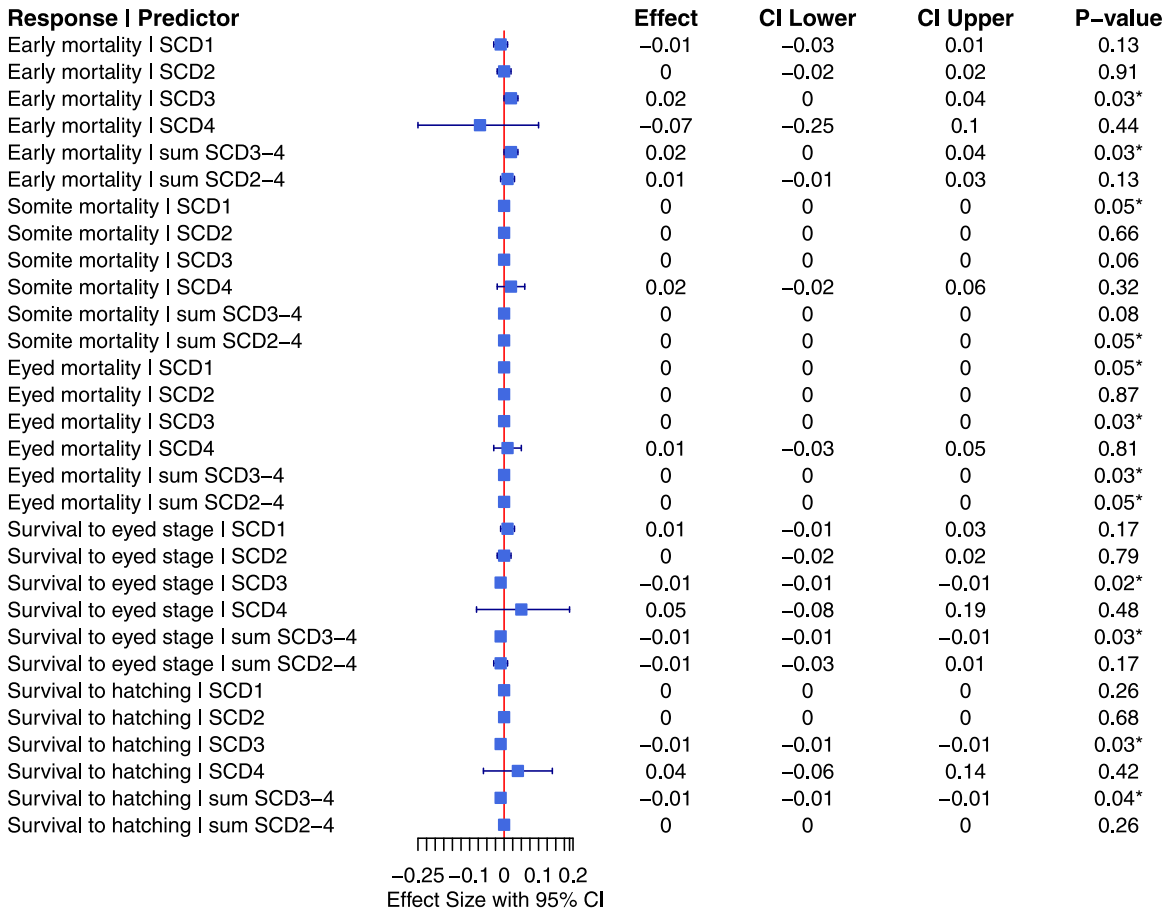


Fig. 5. Effect sizes, confidence intervals, and *p*-values estimated with linear regression for DNA fragmentation levels in individual sperm samples ($n = 9$) and viability in fertilized eggs from one Arctic charr female. Significance levels are annotated as $*p < 0.05$. DNA fragmentation abbreviations: SCD1 intact, SCD2 moderate fragmentation, SCD3 severe fragmentation, SCD4 stellar fragmentation, sum SCD2–4 moderate to stellar, and sum SCD3–4 severe to stellar DNA fragmentation.

Even though it can be challenging to compare SCD results across different species, it is worth mentioning that López-Fernández et al. (2009) reported that the sperm of individual male tench (*Tinca tinca*) exhibited 0–20 % fragmented chromatin. In this context, the fragmentation levels we found here in Arctic charr sperm seem unnaturally high. In the future, a comparison between hatchery broodstock and wild, healthy stocks of Arctic charr would be useful for further interpretation of these findings.

Notably, in our previous study (Jeuthe et al., 2022) a median fragmentation level of 67 % (range: 24–86 %) was reported, while in the current study fragmentation levels of 32 % (range: 5–50 %) for severe to stellar (SCD3–4) and 73 % (range: 45–93 %) for moderate to severe (SCD2–4) fragmentation were found. It should be stressed that storage times from collection to sample preparation differed between those studies. In the current study, samples were prepared for SCD analysis (fixed in gel on microscope slides) immediately after collection and concentration measurements, i.e., within an hour of stripping. In contrast, previously, the samples were transported for four hours to a laboratory before preparation. It is likely that short term storage affects DNA fragmentation level in sperm, as it was previously shown in rainbow trout *Oncorhynchus mykiss* sperm (Pérez-Cerezales et al., 2009).

Our comparison between hatcheries revealed some differences in sperm quality parameters. However, it is particularly difficult to pinpoint the causative factors behind those differences as too many known and unknown factors differ between the facilities. More importantly, though, we saw that the SCD levels, previously found in Arctic charr sperm, are not observed only in the main hatchery of the Arctic charr breeding program in Kälarne but are generally found among most Swedish Arctic charr broodstock. The appearance of this pattern of generally high chromatin fragmentation levels among Arctic charr hatcheries also means that we cannot infer whether it is the baseline for the species or an actual reproductive issue resulting from captive breeding. A comparison of SCD results with males from an undisturbed natural Arctic charr population would be useful, as well as SCD results from other closely related farmed salmonids.

Data from the fertilization trial showed a significant positive correlation between DNA fragmentation levels in the individual sperm samples and early mortality (sum of unfertilized and mortality rates before the somite stage) which was also supported through fitting a linear model. Similarly, a high negative correlation between embryo viability and sperm DNA fragmentation was previously reported in zebrafish *Danio rerio* ($r = -0.56$, Gosálvez et al., 2014), and in sterlet *Acipenser ruthenus* ($r = -0.73$; Gazo et al., 2022), suggesting that sperm with high DNA fragmentation negatively impacts embryo viability after fertilization.

Additionally, DNA fragmentation had a low but statistically significant negative effect on embryo survival at the eyeing stage, where Spearman correlations were negative. However, some unexpected relationships between sperm DNA fragmentation and subsequent embryo survival were also found. Correlations were negative between DNA-fragmentation levels and mortality rates after the formation of somites and eyeing, although the estimated effect size was equal to zero. Our interpretation of this is that DNA fragmentation in the fertilizing sperm mostly affects the embryonic development at a very early stage, which is in line with previous reports (Fernández-Díez et al., 2015; Pérez-Cerezales et al., 2010; Wu et al., 2011). As an example, in an egg batch of

suboptimal quality, a certain proportion of the eggs will die due to poor egg quality (maternal factor). If these eggs are fertilized with high quality sperm, the timing of abortions is independent of paternal factors. However, when eggs are fertilized with inferior sperm (DNA fragmentation levels superseding the repair capacity of the oocyte), the majority of the low-quality eggs will die at an early stage. This would explain why early and late mortality rates showed an opposing correlation with DNA fragmentation levels in the fertilizing sperm. This was also seen in the very strong negative correlation between early and late mortality rates. Hence, in egg batches fertilized with good sperm, more eggs survive the early developmental stage but are then aborted at a 'normal' rate. Since egg survival generally appeared to be negatively associated with DNA fragmentation levels, we conclude that DNA fragmentation likely accounts for a moderate portion of the variation in eying and hatching rates observed in the fertilization trial (R^2 = 0.5, Supplementary Table 2). It is also plausible that DNA fragmentation contributes to the observed variation in egg survival rates under routine conditions in Arctic charr hatcheries.

Unfortunately, we were unable to distinguish between failed fertilization and early embryo mortality in the eggs collected from the fertilization trial, which limits the certainty of our conclusions about the relationship between sperm DNA fragmentation and embryo viability. However, previous studies on the same Arctic charr strain indicate that embryo mortality contributes far more to egg loss than failed fertilization (Jeuthe et al., 2022). Thus, we expect fertilization rates to be generally high. Despite this, some portion of the observed early mortality likely reflects failed fertilization, as indicated by the small but significant effect size of severe DNA fragmentation on early mortality. Variability in fertilization rates may have obscured a stronger true relationship between DNA fragmentation and early embryo mortality. Alternatively, individual differences in fertilization capacity, possibly tied to sperm quality traits co-varying with DNA damage, could play a substantial role. If so, the observed negative association between DNA fragmentation and embryo viability (e.g., SCD3 or SCD3–4) may primarily reflect failed fertilization, either directly or as a consequence of DNA damage.

In general, sperm with severe DNA fragmentation (SCD3) appeared to be the strongest predictor of embryo mortality. When we used SCD3 as the cut-off for sperm fragmentation, the correlation with embryo survival was slightly stronger. Furthermore, the effect was statistically significant when a linear regression model was fitted. The above was not the case when SCD2 was used as the threshold. In addition, stellar fragmentation (SCD4) showed no correlation with embryo survival. Previous studies have shown that fragmented DNA from the father to some extent, can be repaired in the oocyte during the first cell cleavages in the closely related rainbow trout (Pérez-Cerezales et al., 2010). This could explain a stronger relationship between egg survival and proportions of cells with severe (rather than moderate) fragmentation, as moderate fragmentation may have been repaired in the zygote. Regarding the cells with stellar fragmentation, one could speculate that these sperm are in a generally bad shape and unable to fertilize any oocytes and hence have no direct effect on the embryo survival.

Furthermore, we detected a weak but significant positive association between sperm concentration and cell viability, suggesting that higher sperm concentrations may contribute to a modest increase in cell viability. Furthermore, a weak negative association was found for sperm concentration with severe DNA fragmentation, implying that an increase in sperm concentration may be linked to a lower proportion of cells exhibiting severe DNA fragmentation. In our previous study using a lower resolution method for DNA damage analysis (Jeuthe et al., 2022), we did not detect a significant correlation between DNA fragmentation and sperm concentration in Arctic charr. However, studies in human spermatozoa showed a highly significant negative correlation between sperm concentration and DNA damage (comet assay, $r = -0.52$, Irvine et al., 2013).

Overall, no significant association was detected between DNA fragmentation and sperm cell viability (Table 2). Studies in zebrafish using the SCD method for sperm DNA damage and the fluorescence sperm cell counter for sperm viability reported the same results (Cattelan and Gasparini, 2021). In contrast, in our previous study using flow cytometry for viability assessment, we found a weak correlation (r = 0.30) between the two variables (Jeuthe et al., 2022). The fact that we did not find the effect of DNA fragmentation on sperm cell viability in the present study raises the question of whether the accuracy of the NucleoCounter for measurements of cell viability is sufficient to reveal such a relationship among the current sperm population. Morrell et al. (2010) reported a strong correlation between sperm concentration in stallion semen measured with the NucleoCounter versus measurements from a Bürker counting chamber. However, cell viability measurements from the NucleoCounter showed some inconsistencies compared to a parallel assessment using flow cytometry, although the two sets of results did show significant correlation. Morrell et al. (2010) concluded that the NucleoCounter is a useful tool for measuring sperm cell viability, though the exact values obtained may be underestimated and influenced by factors such as the choice of milt extender. Additionally, potential sample handling factors during analysis, may also have contributed to discrepancies between methods and should be considered when interpreting results. Based on our results, we conclude that while sperm viability estimates using the NucleoCounter are sufficiently accurate for internal comparisons, absolute values should not be directly compared with those from other studies using different methods.

5. Conclusions

DNA fragmentation levels were particularly high in the sperm of farmed Arctic charr sampled across Sweden. Notably, the observed DNA fragmentation levels were clearly associated with the timing of embryo abortions. However, the impact of sperm DNA fragmentation on the overall embryo viability during routine hatchery production remains uncertain.

CRediT authorship contribution statement

Khrystyna Kurta: Writing – review & editing, Visualization, Formal analysis, Data curation. **Christos Palaiokostas:** Writing – review & editing, Investigation. **Henrik Jeuthe:** Writing – original draft, Formal analysis, Data curation, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2025.742682.

Data availability

Data will be made available on request.

References

Bates, D., Mächler, M., Bolker, B.M., Walker, S.C., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67 (1). https://doi.org/10.18637/jss.v067.i01.

Cabrita, E., Martínez-Páramo, S., Gavaia, P.J., Riesco, M.F., Valcarce, D.G., Sarasquete, C., Herráez, M.P., Robles, V., 2014. Factors enhancing fish sperm quality and emerging tools for sperm analysis. Aquaculture 432, 389–401. https://doi.org/10.1016/J.AQUACULTURE.2014.04.034.

Cattelan, S., Gasparini, C., 2021. Male sperm storage impairs sperm quality in the zebrafish. Sci. Rep. 11 (1), 1–8. https://doi.org/10.1038/s41598-021-94976-x.

Devaux, A., Fiat, L., Gillet, C., Bony, S., 2011. Reproduction impairment following paternal genotoxin exposure in brown trout (*Salmo trutta*) and Arctic charr (*Salvelinus alpinus*). Aquat. Toxicol. 101 (2), 405–411. https://doi.org/10.1016/J.AQUATOX.2010.11.017.

Fernández-Díez, C., González-Rojo, S., Montfort, J., Le Cam, A., Bobe, J., Robles, V., Pérez-Cerezales, S., Herráez, M.P., 2015. Inhibition of zygotic DNA repair: transcriptome analysis of the offspring in trout (*Oncorhynchus mykiss*). Reproduction 149 (1), 101–111. https://doi.org/10.1530/REP-14-0382.

Figueroa, E., Lee-Estévez, M., Valdebenito, I., Farías, J.G., Romero, J., 2020. Potential biomarkers of DNA quality in cryopreserved fish sperm: impact on gene expression and embryonic development. Rev. Aquac. 12 (1), 382–391. https://doi.org/10.1111/RAQ.12323.

Gazo, I., Naraine, R., Lebeda, I., Tomčala, A., Dietrich, M., Franěk, R., Pšenička, M., Šindelka, R., 2022. Transcriptome and proteome analyses reveal stage-specific DNA damage response in embryos of sturgeon (*Acipenser ruthenus*). Int. J. Mol. Sci. 23 (12), 6392. https://doi.org/10.3390/IJMS23126392/S1.

González-Marín, C., Gosálvez, J., Roy, R., 2012. Types, causes, detection and repair of DNA fragmentation in animal and human sperm cells. Int. J. Mol. Sci. 13 (11), 14026–14052. https://doi.org/10.3390/IJMS131114026.

Gosálvez, J., López-Fernández, C., Hermoso, A., Fernández, J.L., Kjelland, M.E., 2014. Sperm DNA fragmentation in zebrafish (*Danio rerio*) and its impact on fertility and embryo viability — implications for fisheries and aquaculture. Aquaculture 433, 173–182. https://doi.org/10.1016/J.AQUACULTURE.2014.05.036.

Irvine, D.S., Twigg, J.P., Gordon, E.L., Fulton, N., Milne, P.A., Aitken, R.J., 2013. DNA integrity in human spermatozoa: relationships with semen quality. J. Androl. 21 (1), 33–44. https://doi.org/10.1002/j.1939-4640.2000.tb03273.x.

Jeuthe, H., Brännäs, E., Nilsson, J., 2013. Effects of egg size, maternal age and temperature on egg, viability of farmed Arctic charr. Aquaculture 408–409, 70–77. https://doi.org/10.1016/j.aquaculture.2013.05.034.

Jeuthe, H., Schmitz, M., Brännäs, E., 2019. Evaluation of gamete quality indicators for Arctic charr *Salvelinus alpinus*. Aquaculture 504, 446–453. https://doi.org/10.1016/j.aquaculture.2019.02.024.

Jeuthe, H., Palaiokostas, C., Johannisson, A., 2022. DNA fragmentation and membrane integrity in sperm of farmed Arctic charr (*Salvelinus alpinus*). Aquaculture 547, 737537. https://doi.org/10.1016/J.AQUACULTURE.2021.737537.

Kopeika, J., Kopeika, E., Zhang, T., Rawson, D.M., Holt, W.V., 2004. Effect of DNA repair inhibitor (3-aminobenzamide) on genetic stability of loach (*Misgurnus fossilis*) embryos derived from cryopreserved sperm. Theriogenology 61 (9), 1661–1673. https://doi.org/10.1016/J.THERIOGENOLOGY.2003.09.010.

Kurta, K., Jeuthe, H., Naboulsi, R., de Koning, D.J., Palaiokostas, C., 2023. Seasonal and age-related changes in sperm quality of farmed arctic charr (*Salvelinus alpinus*). BMC Genomics 24 (1), 1–17. https://doi.org/10.1186/S12864-023-09614-9/FIGURES/7.

López-Fernández, C., Gage, M.J.G., Arroyo, F., Gosálbez, A., Larrán, A.M., Fernández, J. L., Gosálvez, J., 2009. Rapid rates of sperm DNA damage after activation in tench (*Tinca tinca*: Teleostei, Cyprinidae) measured using a sperm chromatin dispersion test. Reproduction (Cambridge, England) 138 (2), 257–266. https://doi.org/10.1530/REP-09-0105.

Morrell, J.M., Johannisson, A., Juntilla, L., Rytty, K., Bäckgren, L., Dalin, A.M., Rodriguez-Martinez, H., 2010. Stallion sperm viability, as measured by the Nucleocounter SP-100, is affected by extender and enhanced by single layer centrifugation. Vet. Med. Int. 2010. https://doi.org/10.4061/2010/659862.

Pérez-Cerezales, S., Martínez-Páramo, S., Cabrita, E., Martínez-Pastor, F., de Paz, P., Herráez, M.P., 2009. Evaluation of oxidative DNA damage promoted by storage in sperm from sex-reversed rainbow trout. Theriogenology 71 (4), 605–613. https://doi.org/10.1016/J.THERIOGENOLOGY.2008.09.057.

Pérez-Cerezales, S., Martínez-Páramo, S., Beirão, J., Herráez, M.P., 2010. Fertilization capacity with rainbow trout DNA-damaged sperm and embryo developmental success. Reproduction 139 (6), 989–997. https://doi.org/10.1530/REP-10-0037.

R Core Team, 2019. R: A Language and Environment for Statistical Computing. Available at: *https://www.r-project.org/*.

Rex, A.S., Aagaard, J., Fedder, J., 2017. DNA fragmentation in spermatozoa: a historical review. Andrology 5 (4), 622. https://doi.org/10.1111/ANDR.12381.

Sandoval-Vargas, L., Silva Jiménez, M., Risopatrón González, J., Villalobos, E.F., Cabrita, E., Valdebenito Isler, I., 2021. Oxidative stress and use of antioxidants in fish semen cryopreservation. Rev. Aquac. 13 (1), 365–387. https://doi.org/10.1111/RAQ.12479.

Wu, S.F., Zhang, H., Cairns, B.R., 2011. Genes for embryo development are packaged in blocks of multivalent chromatin in zebrafish sperm. Genome Res. 21 (4), 578–589. https://doi.org/10.1101/GR.113167.110.