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DNA fragmentation and membrane integrity in sperm of farmed Arctic charr (*Salvelinus alpinus*)

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ABSTRACT

Despite the relatively long history of captive breeding, the Arctic charr still exhibits a generally low, but highly variable reproductive performance in aquaculture. A recent publication exposed potential paternal factors influencing the reproductive outcome of Arctic charr broodstock from the Swedish breeding program. Interestingly, the paternal factor appeared to be more closely connected to embryo survival than to fertilisation rates. This lead to speculations on whether e.g. chromatin related issues, potentially related to oxidative stress could be involved. In order to investigate this hypothesis the present study assessed the levels of DNA fragmentation, using the SCD-method, and membrane integrity, using flow cytometry, in sperm of farmed Arctic charr. Moreover, the existence of associations was tested between DNA fragmentation and membrane integrity in individual semen samples and viability of their resulting progeny. We found high levels of DNA fragmentation in sperm from the Arctic charr sires, ranging from 24% to 86% with a median of 67%. Membrane integrity values were high, with individual levels of 93.1% to 99.6% viable sperm cells, median 98.8%. DNA fragmentation and membrane integrity values were moderately correlated ($r = 0.304$, $p < 0.05$). Fertilisation rates and proportions of eyed eggs showed substantial individual variation and were correlated ($r = 0.497$, $p < 0.05$). However, large differences between proportion of eyed eggs and fertilisation rate, median 52% and 81.6% respectively, highlight that the main loss occurred due to embryo mortality rather than failed fertilisation. No correlation was found between either DNA fragmentation or membrane integrity and the resulting reproductive outcome (fertilisation rate and eyed eggs) of the individual Arctic charr sires. Overall, our study identified very high levels of DNA fragmentation, which could influence the fertility of the broodstock in question and thereby be a mitigating mechanism involved in the low reproductive success most often observed in farmed Arctic charr. Further exploration of this relationship would be needed, though.

1. Introduction

The Arctic charr (*Salvelinus alpinus*) is an important fish species for freshwater aquaculture in the Nordic countries. In Sweden it has been farmed and selectively bred for food production purposes since the early 1980's (Eriksson et al., 2010). Despite the relatively long history of captive breeding, the species still exhibits a generally low but highly variable reproductive performance in aquaculture. Poor egg quality is a well-known issue in Arctic charr hatcheries (Jobling et al., 1998) and is, at least in part, caused by inadequate rearing conditions (e.g. temperature) (Jeuthe et al., 2015) which disturb the process of oocyte maturation and ovulation (King et al., 2003). One study on Arctic charr broodstock showed rapid changes in sperm quality over generations as an effect of captive selective breeding (Kekäläinen et al., 2013).

Historically, most of the effort to unravel fertility issues in fish have been focused on maternal factors, motivated by the higher energy investments involved in female gamete development. During the last decades however, there has been an increased research focus on the sensitivity and complexity of male reproduction in fish and the importance of good quality sperm, not only to achieve fertilisation but also for embryonic development (Cabrita et al., 2014; Herráez et al., 2017).

The mitigating mechanisms are not yet fully mapped out, but great progress has been made in recent years. For instance, we know now that epigenetic factors in the gametes resulting from e.g., environmental

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conditioning of the broodfish may be transferred to the offspring, both from the sire and the dam (Gavery and Roberts, 2017). A study on steelhead (*Oncorhynchus mykiss*) showed that methylation patterns in germ cells of first time hatchery reared fish differed from wild conspecifics (Gavery et al., 2018). Furthermore, haploid selection has shown to play an important role for offspring fitness in Zebra fish (*Danio rerio*) (Alavioon et al., 2017) where fertilisation with long-lived sperm resulted in offspring with both lower embryonic mortality and, once sexually mature, higher fertility. Interestingly spermatozoa with damaged DNA or RNA can fertilise oocytes, but result in early termination of the zygote (Aitken et al., 2012).

DNA fragmentation in sperm is often a result of oxidative stress (Aitken et al., 2012). Cell membranes of fish sperm are also particularly sensitive to oxidative stress as they contain high proportions of polyunsaturated fatty acids (PUFA)(Cabrita et al., 2014). Reactive oxygen species (ROS) cause damage to both cell membranes and DNA in fish sperm and have been proposed as one of the main causes of impairment in connection to cryopreservation (Sandoval-Vargas et al., 2021). There are several different methods for assessment of both DNA fragmentation in sperm (Cabrita et al., 2014). The most common method used for fish is the Comet assay, followed by the TUNNEL assay and sperm chromatin structure assay (SCSA®). Another method less commonly applied on fish sperm is the sperm chromatin dispersion test (SCD). This method has been used for tench (*Tinca tinca*) and showed good correlation with results of parallel evaluation using the Comet assay (López-Fernández et al., 2009). The advantages of the SCD compared to aforementioned alternatives are the low requirements of specialised equipment and uncomplicated sample preparation and analysis (Cabrita et al., 2014). The downside is that it requires species-specific adaptations and results from SCD on fish of different species are scarce. To our knowledge, there are no previous publications on Arctic charr or salmonids for that matter to aid analysis and result interpretation.

Recently, there have been indications that paternal factors influence the reproductive outcome of the Arctic charr broodstock of the Swedish breeding program (Jeuthe et al., 2019). The results from this previous study were not clear regarding the mediating mechanisms, but much of the variation seen in survival to the eyed stage could be linked to the individual sires rather than the dams. Interestingly, the paternal factor appeared to be more closely connected to embryo survival rather than fertilisation rates. This lead to speculations on whether epigenetic or chromatin related issues, potentially associated with oxidative stress, could be involved. In order to investigate this hypothesis the present study assessed the levels of DNA fragmentation and membrane integrity in sperm of Arctic charr sires from the Swedish breeding program. Moreover, the existence of associations was tested between these potential indicators of oxidative stress of individual sires and the embryonic viability of their offspring.

2. Materials and methods

2.1. Animals

The study involved 43 sires and 43 dams from the 8th generation of the Swedish Arctic charr breeding program. All individuals were five years old, hatched in 2013. The hatchery is located in central Sweden, Kälarne in Jämtland County, where the fish have been reared in indoor tanks with a flow-through water system, from egg hatching to sexual maturity. The broodstock reached sexual maturity at two or three years of age and were used for fry production once per year since then. At the current age of five, dams are considered to be at their prime of reproduction. Yet they show large individual variation in egg quality (Jeuthe et al., 2013). On the other hand, the sires pass their prime a year or two earlier with sperm production-quality reducing thereafter.

2.2. Sampling

Semen sampling was performed during routine production of full sibling families within the breeding program. The reproductive period of the current group of fish extended from 10 October to 13 November 2018, with weekly examination and gamete stripping. Sampling was done at three out of six occasions on 43 out of the 73 sires used during this season (Table 1). On each occasion, samples were collected over a period of maximum two hours, from the first to last sire. Collected semen samples were stored in a cooling box at approximately 5 °C and transported to the laboratory in Uppsala, a four-hour drive from the hatchery. Hence, the laboratory analyses commenced four to six hours after collection.

2.3. Sperm analyses

2.3.1. Sperm concentration and cell membrane integrity

Sperm concentrations in the individual semen samples were measured prior to further analyses (NucleoCounter® SP-100™, Chemometec, Allerod, Denmark). Semen samples were diluted 1:1000 with lysis buffer (20 μL sample and 20 mL buffer; Reagent S-100, Chemometec, Allerod, Denmark). Analyses were performed using the setting for bull semen, as recommended by the manufacturer.

Membrane integrity was analysed using the LIVE/DEAD™ sperm viability kit (L7011, Thermofisher Scientific, Waltham, MA,USA. Initially, the samples were diluted 1:100 with PBS. Thereafter 300 μL of the obtained solution were stained with 1 μL SYBR-14, diluted 1:50 in PBS and 3 μL Propidium Iodide. Samples were analysed using a FACSVerse flow cytometer (BDBiosciences, Franklin Lakes, NJ, USA). Forward and side scatter, as well as green and red fluorescence were collected. After gating on single spermatozoa in the scatter-plot, spermatozoa were classified as membrane-intact, membrane-damaged or membrane-ruptured based on green and red fluorescence. The percentage of membrane-intact was used for further calculations.

2.3.2. DNA fragmentation

Sperm DNA fragmentation was analysed through SCD using the Halomax® kit for freshwater fish (HT-TT40, Halotech DNA; Madrid, Spain) in accordance with the product instructions (also presented in (López-Fernández et al., 2009)). The processed samples were stained with SYBR® Green and visually assessed in a fluorescence microscope. A cut-off halo size was determined based on (López-Fernández et al., 2009), as presented in Fig. 1, where cells with a larger halo were considered to suffer significant DNA fragmentation. 300 sperm cells were examined in each sample, except in six samples with low sperm concentrations where only 100 or 200 cells were examined. The proportion of these cells displaying a halo larger than the cut-off size denote the fragmentation level in percent.

2.4. Fertilisation and offspring viability

Sperm from each sire was used to fertilise eggs from one individual dam using dry fertilisation. After stripping, the eggs were rinsed in $NaHCO_3$-solution (13.7 g/L). Semen was added to the “dry” egg batch

Table 1
Overview of the reproductive period and sampling of the Arctic charr sires involved in the study. In all, 73 sires were used for fry production N_{tot} and 43 of them ($N_{sampled}$) were sampled for sperm analyses.

Date	N_{tot}	$N_{sampled}$
2018-10-10	12	0
2018-10-16	27	13
2018-10-23	21	20
2018-10-30	10	10
2018-11-05	2	0
2018-11-13	1	0

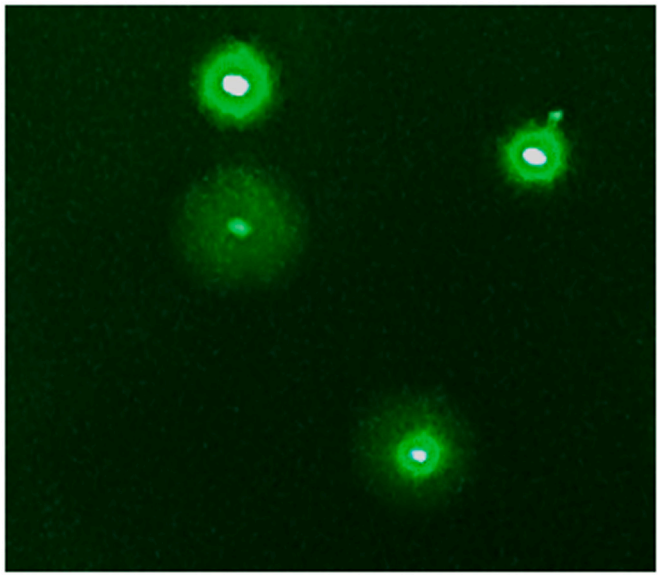

Fig. 1. DNA fragmentation levels in sperm from Arctic charr were assessed using the SCD-method, where a cut-off value was set based on a previous publication on tench [13]. The two upper cells in the image were considered non-fragmented, while the lower two cells were considered fragmented.

and gently mixed. Then, water (same as in the rearing tank) was added to the fertilisation vessel (approximately 2:1 water to egg volume ratio). Semen volume was not measured, but only ejaculates large enough to result in sperm to egg ratios well above previous estimations of critical limits for salmonids were used (Billard, 1975). Excess semen was rinsed away a few minutes after fertilisation and the eggs were left in the fertilisation vessel with clean water for swelling, before moved to the hatchery.

Fertilisation success was estimated by visual examination of 50 eggs from each of 32 individual egg batches (13, 9, and 10 per sampling occasion). The eggs were collected from the hatching trays 24 h after fertilisation. At this time embryonic development had reached the eight blastomere stage. The collected eggs were immersed in Formalin–acetic acid–alcohol (FAA) fixative solution and studied under a low magnification microscope. Eggs with visible cell divisions were counted as fertilised while eggs without visible cell cleavage were considered unfertilised. As soon as the developing embryos had reached the eyed stage (eyes are pigmented and clearly visual by the naked eye), live and dead eggs were sorted and counted using a sorting machine. The proportion of live eggs at this stage was used as an indicator of offspring viability.

2.5. Statistics

All statistical analyses were performed using Minitab 17 Statistical Software. For analyses requiring natural distribution, proportion data variables including values of zero or one (fertilisation and eyed eggs) were arcsine transformed using the following equation:

$$Y = arcsine\sqrt{p}$$

where p is the proportion of fertilisation success or eyed staged embryos. Proportion data variables not containing values of zero or one (DNA fragmentation and cell viability) were logit transformed using the following equation

$$logit(p) = log\left(\frac{p}{1-p}\right).$$

Since sample preparation and analysis was delayed due to the long transport to the laboratory, DNA fragmentation was tested for correlation with relative storage time (ordinal variable based on sampling sequence) using Spearman's rank-order correlation in an attempt to identify potential effects of storage time on DNA fragmentation.

3. Results

3.1. Sperm concentration and cell membrane integrity

Sperm concentrations ranged from 0.552×10^9 to 13.5×10^9 cells per mL, median 3.46×10^9 (Fig. 2a). No significant change in sperm concentration over the collection period was found (Pearson's correlation $r = 0.22$ $p > 0.05$, Fig. 3a). Sperm cell viability (membrane integrity) ranged from 93.1% to 99.6% for the individual sires, with a median value of 98.8% (Fig. 2b). No change in mean viability over the collection period was evident (Pearson's correlation on logit-transformed values $r = 0.09$ $p > 0.05$, Fig. 3b). The highest individual proportions of dead or dying sperm cells were found at the last two sampling dates (seven sires with values >3%), but no significant difference in variance could be detected between sampling dates, Levene's test = 1.10, $p > 0.05$ (logit-transformed values, Fig. 3b).

3.2. DNA fragmentation

The proportion of sperm cells with fragmented DNA in semen from individual sires ranged from 24% to 86%, with a median value of 67% (Fig. 2c). DNA Fragmentation levels increased over time during the collection period (three sampling occasions, each one week apart, Fig. 3c), Pearson's correlation $r = 0.35$ $p < 0.05$ (logit-transformed values). Sample preparation and analyses at the laboratory started four to six hours after collection depending on sampling time at the farm. No effects of storage time on fragmentation levels could be detected; i.e., correlation analysis of relative storage time (sampling sequence) and DNA fragmentation resulted in Spearman's rho-values 0.20, 0.15, −0.52 and $p > 0.05$ for the three sampling occasions respectively. A weak but significant positive correlation was found between the DNA fragmentation levels and the proportions of non-viable cells (sum of dead or dying), Pearson's correlation $r = 0.304$, $p < 0.05$ (logit-transformed values; Fig. 4). No significant correlation was found between DNA fragmentation and sperm concentration, Pearson's correlation $r = 0.275$, $p > 0.05$.

3.3. Fertilisation and offspring viability

Fertilisation rates varied greatly between individual sire/dam crossings, ranging from 8.2% to 98.4%, median 81.6% (Fig. 5a). The proportion of eyed eggs ranged from zero to 100%, median 52% (Fig. 5b). Fertilisation rate and proportion of eyed eggs were correlated, Pearson's correlation $r = 0.497$, $p < 0.05$ (arcsine values). No change in either fertilisation rate or proportion of eyed eggs could be seen over the collection period (Pearson's correlation on logit-transformed values $p > 0.05$, Fig. 6). No correlation was found between any of the sperm quality parameters – concentration, cell viability or DNA fragmentation level – and fertilisation rate or eyed success.

4. Discussion

4.1. DNA fragmentation

We found high levels of DNA fragmentation in sperm from the Arctic charr sires, ranging from 24% to 86% with a median of 67%, using the SCD-method. As far as we know, there are no previous publications with results from DNA fragmentation measurements using the SCD-method on sperm from Arctic charr, or any salmonid fish species for that matter. López-Fernández et al. (2009) presented fragmentation levels of 0.3–22% in fresh non-activated sperm from tench. They also showed a rapid increase of fragmentation levels in activated sperm, up to 45–90%

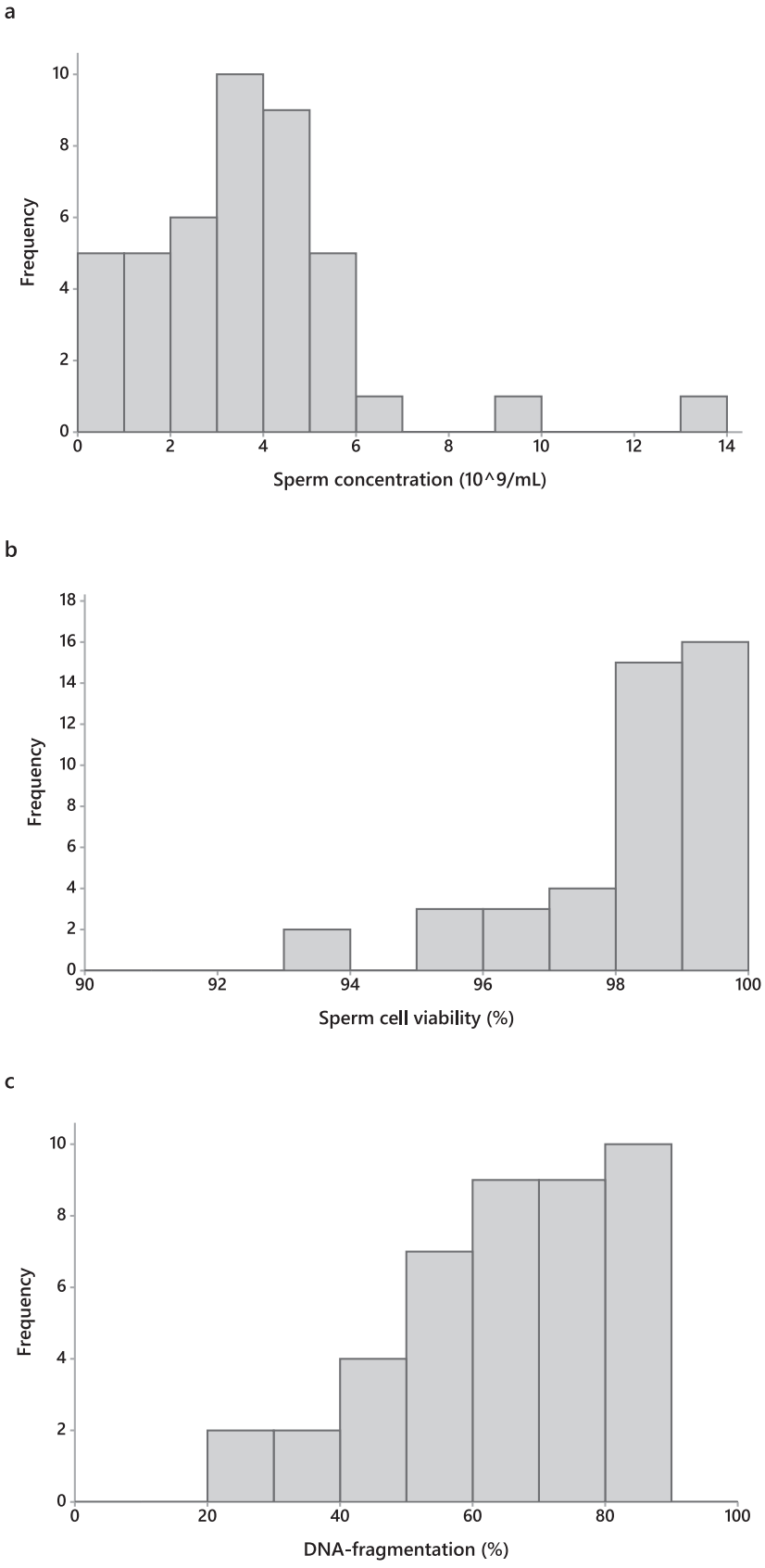

Fig. 2. Sperm quality parameters measured in semen collected from Arctic charr sires ($n = 43$). Sperm concentrations were measured using a NucleoCounter® SP-100™; median value for the entire group was 3.46 × 10^9 cells/mL. Proportion of non-viable cells (b) was measured through membrane integrity using flow cytometry; median value 98.8%. DNA fragmentation (c) was analysed using the SCD-method (Halomax® for freshwater fish); median value 67%.

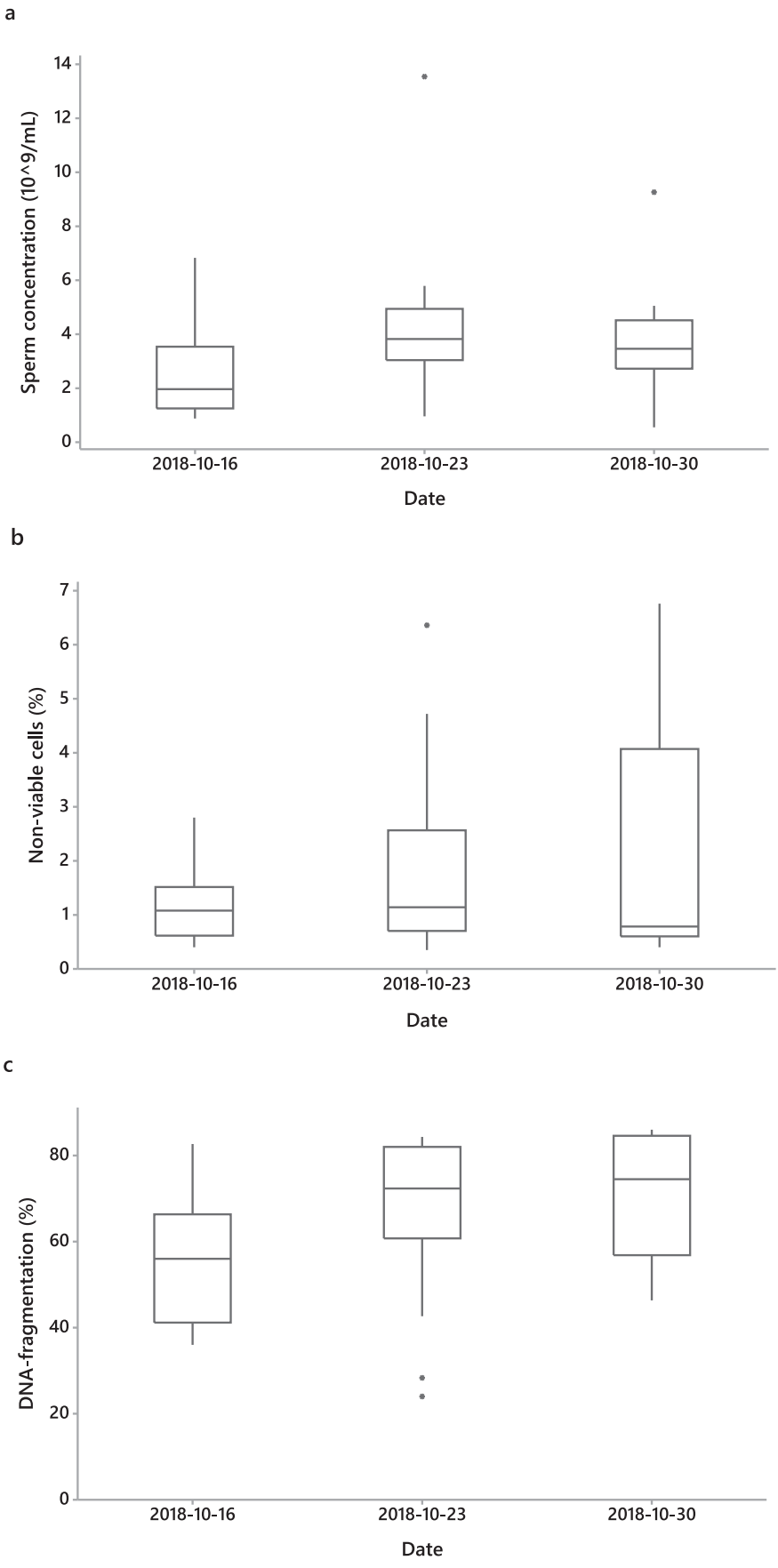

Fig. 3. Sperm concentration (a), proportion of non-viable cells (b), and DNA fragmentation (c), in semen collected from Arctic charr sires on three occasions ($N = 13$, 20 and 10 respectively) during the reproductive season. Boxplots show median, interquartile range, range, and outliers. No significant changes in sperm concentration or cell viability were detected over the studied period, $r = 0.22$ $p > 0.05$ and $r = 0.09$ p > 0.05 respectively, while DNA fragmentation levels increased over the studied period, $r = 0.35$ $p < 0.05$.

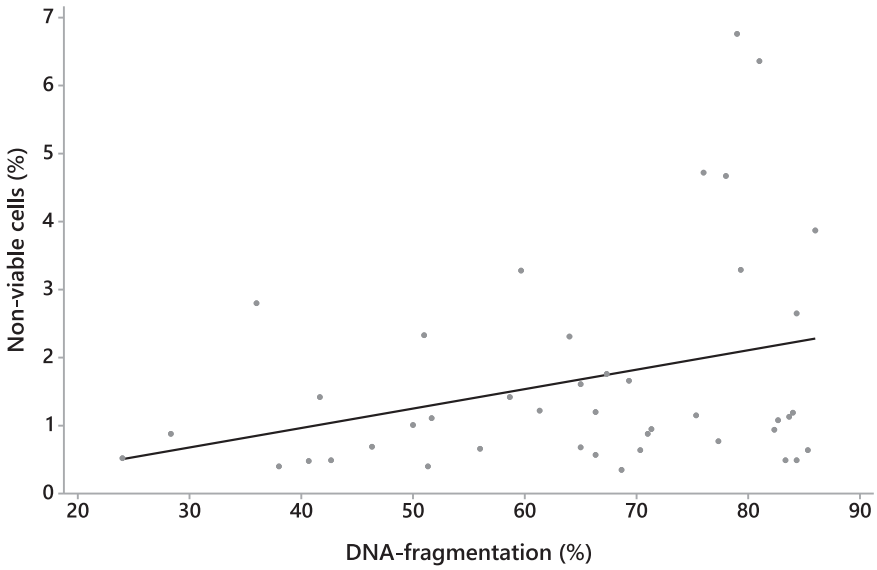

Fig. 4. DNA-fragmentation levels and proportion of non-viable sperm cells (damaged cell membranes) in Arctic charr sperm. The line shows the corresponding regression line for a significant positive correlation found between the DNA fragmentation levels and the proportions of non-viable cells (sum of dead or dying), Pearson's correlation $r = 0.304$, $p < 0.05$ (logit-transformed values).

at 15 °C, 60 min post-activation. Furthermore, Gosálvez et al. (2014) presented baseline fragmentations levels of 1–7% in Zebrafish sperm using the SCD-method.

Due to logistical reasons, our samples were stored on ice (4 °C) for 4–6 h before processing at the laboratory. The samples were stored as raw semen, with no added extender. Both in research and during routing breeding work at fish hatcheries storage time from sampling to fertilisation and/or analyses may vary. In the present study processing of the sperm samples was delayed both due to a prolonged sampling procedure during mating of individual dams and sires of the selective breeding program, and also due to a lengthy transport to the laboratory. The question did arise, whether the high DNA fragmentation values attained in this study, in part, were results of storage before analysis. By exploring the effects of sampling sequence on the individual SCD-results we made an effort to reveal any effects of storage time on DNA fragmentation. We did not find any consistent or significant correlation between the two. I.e., correlations were positive on two sampling occasions and negative on one occasion, all with $p > 0.05$. In Zebrafish, DNA fragmentation has been shown to progress quite rapidly during storage in an extender at 15 °C, resulting in significant degradation within 15 min of storage (Gosálvez et al., 2014). On the other hand, in the case of sturgeon (*Acipenser baerii* and *A gueldenstaedtii.*) semen could be stored at 4 °C for two or three days (respectively) without any significant change in DNA-damage (Shaliutina et al., 2013). A study on rainbow trout, more closely related to our species, reported that DNA fragmentation (Comet assay) increased from 6.1% ± 0.34 in fresh (2 h after collection) to 15% ± 0.1 after two days of storage at 4 °C (Pérez-Cerezales et al., 2009). Based on this information it seems unlikely that our storage times of 4–6 h before analyses affected the SCD-results severely. However, we cannot rule any such effects. Nevertheless, our measurement resulted in very high levels of DNA fragmentation. If accurate, they are likely to influence fertility of the broodstock in question and thereby be a mitigating mechanism involved in the low reproductive success most often observed in farmed Arctic charr. Therefore, further exploration of this is needed. Firstly, more extensive mapping of DNA fragmentation in Arctic charr sperm need to be done, including comparison with other broodstock as well as wild populations, to determine whether high fragmentation levels is a common issue. In addition, the effects of short term storage of Arctic charr semen on DNA fragmentation also need further investigation.

On a more general note regarding assessment of DNA fragmentation in fish sperm, and the use of the SCD-method in particular, there are many factors affecting the results as well as their interpretation. As previously stated, measurements of fragmentation levels using the SCD-method are based on a predetermined cut-off point regarding halo size, where cells with larger halos are considered to suffer significant DNA fragmentation. However, as the DNA fragmentation is a continuous process, the cut-off point is rather arbitrary. To our knowledge, there are no studies evaluating different cut-off points for fish sperm and their relevance for fertility in fish. Choosing a precise and biologically relevant cut-off point is crucial for general interpretation of the results as well as for repeatability and comparison between studies. Hence, further development and standardisation of the visual/image analysis of SCD in fish would be beneficial. Some of the CASA-systems available on the market today have software for assessment of DNA fragmentation using SCD, but to date, they cannot be used for fish.

4.2. Membrane integrity

Sperm cell viability, measured as membrane integrity using flow cytometry, was high among the Arctic charr sires, 93.1% to 99.6% median 98.8%. This can be compared to previously published values for fresh semen from brook trout (*Salvelinus fontinalis*) 85.3% ± 10.6 (Lahnsteiner et al., 2011), Atlantic salmon (*Salmo salar*) 90.5% ± 3.9 (Figueroa et al., 2016), and from rainbow trout (*O. mykiss*) 99% (De Baulny et al., 1997). The high viability values found among the current Arctic charr sires could indicate that their sperm are not subjected to any severe issues of oxidative stress. Reactive oxygen species (ROS) can cause damage to both membranes and DNA in the spermatozoa (Cabrita et al., 2014). The high levels of DNA fragmentation could be expected to be accompanied by low viability due to damages cell membranes. The viability levels we observed in our study are, as previously mentioned, in line with base line values from studies on related species. However, we did see a weak correlation between levels of DNA- and membrane-damage.

4.3. Changes over sampling period

An increase of DNA fragmentation levels was observed over the sampling period. At first glance, there also seemed to be a decreasing

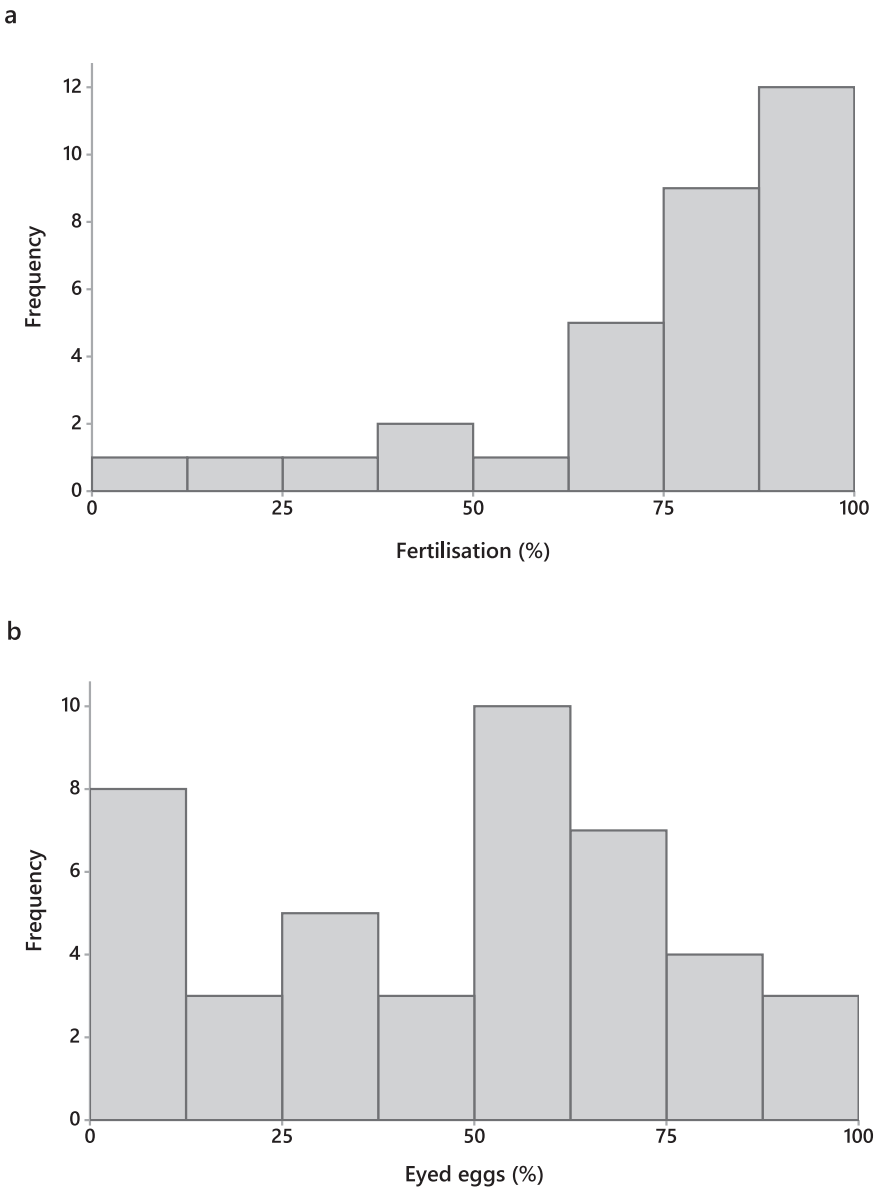

Fig. 5. Distribution of fertilisation rates (a) and proportions of eyed eggs (b) in individual egg batches fertilised using one sire and one dam each ($N = 32$ for fertilisation rates and $N = 43$ for eyed eggs).

trend in sperm cell viability, or at least an increasing variability. However, this was not statistically supported. Sperm quality measured through traditional indicators like motility, concentration, volume etc. often changes over the reproductive season (Bobe and Labbé, 2010). However, there is low consistency in the literature regarding the pattern of this change in salmonids. There are reports on increasing, decreasing and dome-shaped patterns of these sperm quality parameters over the season (Hajirezaee et al., 2010; Johnson et al., 2013; Piironen, 1985). We found no equivalent data for DNA fragmentation or membrane integrity. Although, a study on rainbow trout reported on clear alterations in lipids and proteins over the course of the spawning season (Shaliutina-Kolešová et al., 2018) which was suggested to have resulted from increased oxidative stress towards the end of the season. Another study on rainbow trout also showed that the total antioxidant capacity (TAC) in seminal plasma decreased during the spawning season (İnanan et al., 2016). Together, these earlier findings could explain the trends we see in our data. Understanding the changes in all relevant sperm quality parameters over the course of the reproductive season is important for a successful and resource efficient hatchery production. Collection of semen was done on three occasions, one week apart, in our study. This covered the majority of the period of artificial fertilisation. However, no collection was done during the first week (10 sires) and last two weeks (3 sires). As such, seasonal pattern is yet another aspect that needs to be studied in connection to the rather young research area of (non-genetic) paternal influence on offspring viability.

4.4. Fertilisation and embryo viability

We observed a large variation in both fertilisation rates and survival to the eyed stage between the sibling groups of our study. This is quite typical of the reproductive issues we see in farmed Arctic charr. The low hatching rates are due to substantial individual variation rather than a generally low survival. More specifically, the proportion of eyed eggs in our study ranged from zero to 100%. It should be noted however, that these values are based on actual counts of eyed eggs, but estimated numbers of stripped eggs from volumetric measurements. Hence, the values are not exact.

Fertilisation rate and proportion of eyed eggs were correlated.

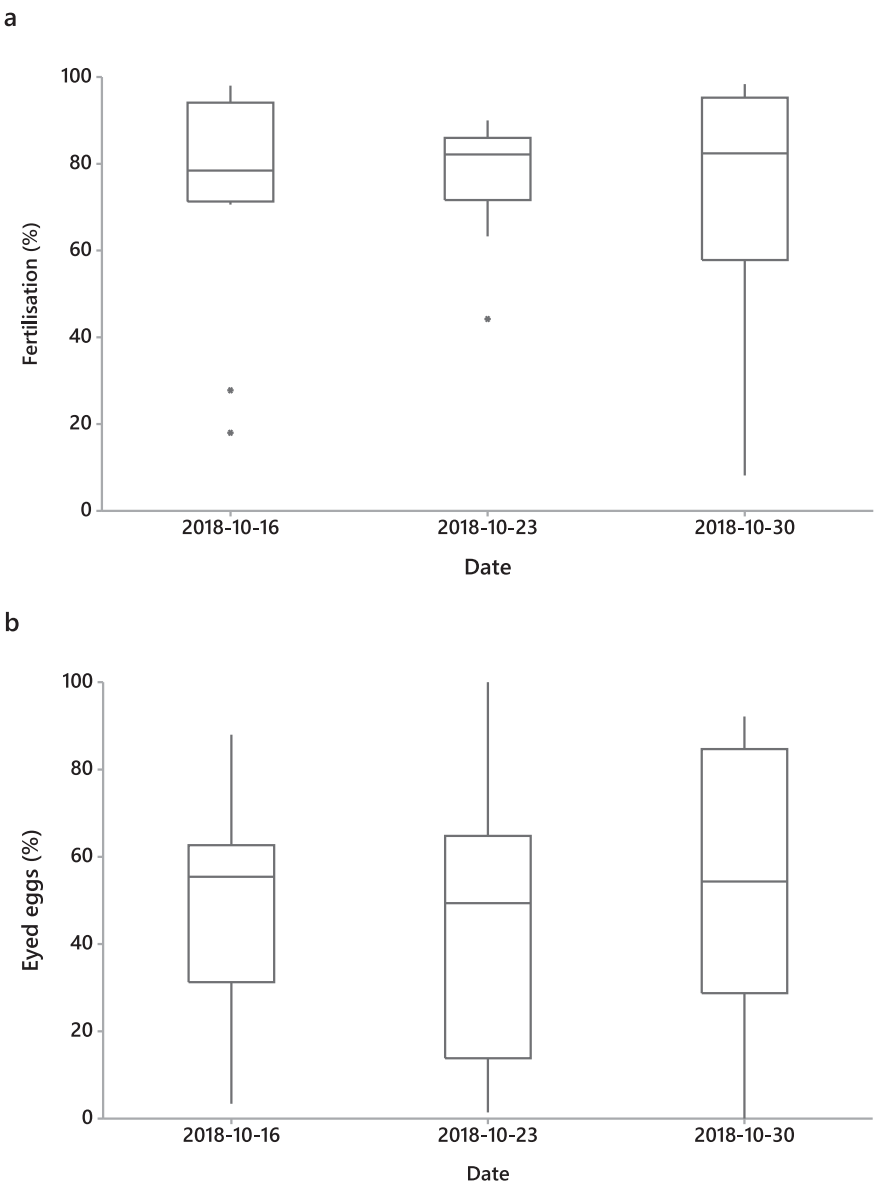

Fig. 6. Fertilisation rates (a) and proportions of eyed eggs (b) in Arctic char eggs collected on three occasions ($N = 13$, 9 and 10 for fertilisation rate and N = 13, 20 and 10 for eyed eggs respectively) during the reproductive season. Boxplots show median, interquartile range, range, and outliers. No change over the sampling period was detected.

However, the large difference between proportion of eyed eggs and fertilisation rate highlights the fact that the main loss occurs due to embryo mortality in fertilised eggs rather than failed fertilisation. Our results are in concurrence with a previous publication of the fertility of this particular strain of Arctic charr (Jeuthe et al., 2019). Results of this previous study by Jeuthe et al. (2019) indicated a paternal factor connected to the variation of progeny embryonic mortality. There was a relationship between embryonic survival and paternal hormone levels as well as sperm swimming characteristics, but the actual mechanism remained unclear. One theory was that this mechanism could be DNA fragmentation, as there are few other causes to embryonic mortality that can be linked to sperm quality (Cabrita et al., 2014; Herráez et al., 2017). Although there was great variation in both DNA fragmentation levels between the different sires, and egg survival between the resulting sibling groups in the current study, we were unable to find any connection between the two variables. As previously mentioned, storage time of the sperm samples may have been a confounding factor. A previous study on the effects of storage time on DNA fragmentation in zebrafish sperm revealed a relatively stable degradation process, i.e. the internal ranking of individual samples regarding fragmentation level were quite constant between repeated measurements over the incubation period (Gosálvez et al., 2014). Therefore, it should still have been possible to connect sperm DNA fragmentation with resulting egg survival rates, if there was such a relationship within the current group of study subjects, despite the long storage time. Another factor that could influence the relationship between DNA fragmentation levels and subsequent embryo survival is the ability of oocytes to repair moderate damages in the paternal DNA during early cell cleavage. This has been shown in the closely related rainbow trout (Pérez-Cerezales et al., 2010). Notably, a perhaps more important confounding factor of the current, somewhat crude experimental setup is egg quality. Since our aim was to assess the influence of sperm quality under "real world" hatchery conditions, sampling was done during routine breeding where fertilisations were performed using one female and one male at the time. Therefore, all sperm samples were used to fertilise eggs from different females. Individual variation in egg quality between dams is extensive (Jeuthe et al., 2013) and may have over-shadowed any paternal factors. In the future a more refined experiment where several males are used to

fertilise aliquots of the same egg batch (individual or pooled) should be performed.

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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