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Abstract

Successful *in vitro* fertilization in aquaculture is highly dependent on the method used to handle or treat the eggs and milt. As such it is of paramount importance that timely evaluation of the methods is conducted to enhance hatchery productivity and reduce the cost of production. In this study, three methods of handling eggs of Arctic charr in Sweden were evaluated, with the objective of ascertaining the importance of retaining the ovarian fluid in the egg batch. Further, activation of fish sperms with a commercial activator, ActiFish^{TM}, was tested to compare the fertilization rates, in a bid to overcome the current low egg fertilization and hatching rates among farmed Arctic charr. Variation of the volumetric amount of the ovarian fluid did not yield dissimilar fertilization rates. As such, tempering with the volume of the ovarian fluid under the current study did not affect the performance of the sperms and consequent fertilization rates. Further, no differential fertilization rates were recorded for the sperm extender and freshwater. However, positive relationships were recorded for fertilization rate and sperm velocity (VCL). The study contends that high and successful fertilization rates are likely to be obtained with or without the ovarian fluids under *in-vitro* fertilization of Arctic charr eggs.

Key words: *Arctic charr, Ovarian fluid, Sperm motility, fertilization rate, VCL*

List of Acronyms

CASA	– Computer assisted sperm analysis
OF	– Ovarian fluid
VAP	– Average path velocity
VCL	– Curvilinear velocity
VSL	– Straight line velocity

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1.0 Introduction

Poor availability of high-quality feed ingredients (alternatives to fishmeal), low production of viable seeds (fingerings) and lack of capital (mainly in 3rd world countries) are some of the challenges hindering the sustainability of global aquaculture industry (FAO, 2018). Similarly, some of these factors affect the sustainability of the Arctic charr (*Salvelinus alpinus* L.) production in Sweden. Alternative protein sources, a shortened production cycle through selective breeding and the development of an adaptive feeding management model are some of the responsive measures identified for the Swedish Arctic charr production (Carlberg, 2016). However, the main challenge in the production chain lies in the success of fertilization rates, hatchability and survival of alevins. From the thousands of eggs produced by a female per fecund, only a small fraction of the eggs survives to the alevin stage, leaving numerous questions over the reliability of the present production cycle. This has sparked interest among researchers to strictly investigate causality of poor fertility and low survival of eggs in the breeding of Arctic charr. To improve the performance of commercially farmed Arctic charr in Sweden, there is need to review hatchery protocols and ascertain the level at which under performance challenges occur. One way to do this is by reevaluating the fertilization process, which is a core element in successful alevins production.

The Swedish Arctic charr broodstock (Arctic superior) on which this study was based has undergone over 30 years of successful selective breeding; resulting in a more improved and stable strain, now in the eighth generation (Carlberg *et al*., 2018; Nilsson *et al*., 2010). Despite the success in the selective breeding program, the efficacy of alevins production remain questionable due to low fertilization rates and egg hatchability. This has been attributed to the fact that fertilization and hatching rate were not included as selection parameters in the initial stages of the breeding program in the early 1980s (Brännäs *et al*., 2008). However, for the past decade or so, attention has been drawn to this issue. Extensive work has been done in reviewing the sustainability of farming Arctic charr (Carlberg *et al*, 2015 & 2016; Brännäs *et al*., 2011) and evaluation of factors affecting the reproductive processes; including physiological limitations (maternal age and egg size), physicochemical effects (temperature, stress) and hormonal balance (Jeuthe *et al*., 2013 & 2015; Jeuthe, 2015).

Regardless of the growing enthusiasm to improve the reproductive success of the Arctic superior, vital micro-parameters have also been neglected or have not so far been extensively studied. One such parameter is the assessment of the role the ovarian fluid (OF) plays in the initial success of the reproductive process. Arguably, this is an important aspect that may have been contributing to the low hatchery success rates in the breeding of Artic charr and considering that *in vitro* fertilizations are performed. In many species, ovarian fluid is known to regulate and maintain sperm quality (Galvano *et al*., 2013). OF forms a considerable volumetric component of the egg batch mass and is rich in nutrients (Rosengrave, 2008). OF play vital roles of protecting gametes, facilitate fertilization, and act as mediators of post-mating sexual selection (Cardozo *et al*., 2018; Galvano *et al*., 2013; Urbach *et al*., 2005).

According to Brännäs *et al*., (2011) Salmonid ovarian fluid is about 10-30% of the total egg batch volume and has been found to greatly influence reproductive success in these

species. For instance, Butts *et al*., (2012) reported that ovarian fluid not only enhanced sperm velocity in lake trout (*Salvelinus namaycush*), but it also acts as a recognition system to select sperm based on genetic relatedness. Further, Makiguchi *et al*., (2016) demonstrated that ovarian fluid differentially affects sperm performance between small parr males and large dominant anadromous males in Masu salmon (*Oncorhynchus masou*). It has further been established that ovarian fluid in Arctic charr prolongs sperm mobility, velocity, and may be linked to cryptic mate selection (Urbach *et al*., 2005; Brännäs *et al*., 2011). Doubling the concentration of ovarian fluid in Arctic Charr has been reported to increases sperm longevity, motility and linearity in movement (Turner & Montgomerie, 2002).

On the contrary, ovarian fluid has been reported to mediate temporal sperm decline in guppies (*Poecilia reticulata*) (Gasparini & Evans, 2013). Their results revealed that the temporal decline in sperm viability was significantly reduced in the presence of OF compared to a saline control. Beirão *et al*., (2014) demonstrated that despite wild males of Atlantic cod (*Gadus morhua*) actively courting farmed females, they were unable to hybridize, because their ovarian fluid quality inhibited fertilization success; largely owing to the nutritional deficiencies of farmed individuals. Generally, ovarian fluids have been known to influence fertility success based on preferred phenotypes. Under natural conditions, female ovarian fluid likely increases the paternity of the preferred parental male phenotype, as these males release fewer but faster sperm (Alonzo *et al*., 2016).

These characteristics make the ovarian fluid a worthwhile component of the fertilization process and requires attention. Conversely, the higher volumetric ratio of the ovarian fluid in a given fecund, may have serious implications on the fertilization process. The reasoning behind this that, in nature, the swimming ability of Arctic charr sperms have been found to be low in relation to egg circumference. Thus, to insure fertilization, males must shed sperm in the immediate vicinity of the ova where the ovarian-fluid concentration is likely to be high (Kime *et al*., 2001; Urbach *et al*., 2005). Further, Urbach *et al*., (2005) contended that sperm velocity of Arctic charr in water is positively correlated with paternity, such that males with faster sperm fertilized more eggs than males with slower sperm; emphasizing the importance of sperm velocity in terms of fertilization. It has also been recommended that future studies should investigate how OF impacts fertility as it has been suggested that sperm released closer to an egg will be exposed to higher concentrations of OF and possibly gain a fertilization advantage (Galvano *et al*., 2013; Turner & Montgomerie, 2002). Therefore, under *in vitro* fertilization, the ratio and intensity of the ovarian fluid in the egg batch must be well balance in order to achieve high egg-sperm interactions.

The scientific outcome that OF promotes and enhances sperm performance of Arctic charr has not been fully utilized to enhance hatchery success rates. Additionally, very few studies, if any, have experimentally linked sperm performance in ovarian media to fertilization rates. Under current production regimes, hatchery manages are forced to either wash off the OF in Sodium bicarbonate ($NaHCO_3$) or sieve/ strain it prior to proceeding with the fertilization process. This makes it easier to fertilize the eggs and reduces clogging of the oocytes with the OF once water is introduced. The choice of the

method is dependent on the discretion of the hatchery manager and vary from one place to another (Andersson T. pers. Coms).

The egg batches are completely washed off the OF in sodium bicarbonate to disinfect the eggs and allow them not to swell before milt is introduced. This increases amphoteric properties, promotes postembryonic growth and improve hatching of the eggs (Avdesh *et al*., 2012; Brännäs *et al*., 2011; Sawant *et al*., 1992). On the contrary, the egg batches are strained off the OF to reduce the amount of the fluid for easy fertilization and avoid the fluid clogging around the ova. OF from different females exhibit an array of thickness intensity depending on the physiology, fitness and age of the fish (Lahnsteiner, 2000). Very thick OF is assumed to hinder effective and complete fertilization process. As a result, different success rates in the fertility of the eggs may be recorded depending on the method used and competency of the hatchery managers.

This study, however, tried to establish a clear link and understanding between two practical methods, considering the importance of retaining the OF in the fertilization process. In fish, the identification of computer parameters that are strongly correlated with sperm quality and fertility is still pending (Aberkane & Iguer-ouada, 2016). Currently, potential sperms fertilizing eggs in fish is assessed using results from computer-assisted sperm analysis (CASA), but these results do not accurately predict fertility rates that will be obtained with a semen sample or test subject (Amann & Waberski, 2014; Urbach *et al*., 2005; Kime *et al*., 2001). However, this computer method has popularity in breeding programs because it provides the closest understanding of the viability/quality of semen and possible fertilization rates. Amann & Waberski, (2014) postulated that when carefully validated, current CASA systems provide information important for quality assurance of semen planned for marketing and understanding of the diversity of sperm responses to changes in the micro-environment in research. Especially regarding the effect of different preservation methods (cryopreservation) on sperm motility has been extensively researched (Richardson *et al*, 2011); otherwise patchy information exists on the influence of the OF on the fertilization rates.

The current study was based on the hypothesis that reduced OF will enhance sperm performance and egg fertilization rate. It was hypothesized that sperms activated in the presence of just enough OF would have better motility, speed and high fertilization success. This was investigated by running fertilization trials on eggs with variant amounts of ovarian fluids and linking the success rates to sperm motility ratios under different ovarian media. Further, a commercial sperm activator/extender was used to compare fertilization rate with the spermatozoa activated only with freshwater.

2.0 Materials and methods

2.1. Experimental setup

The study was conducted at the Vattenbrukscentrum Norr AB (VBCN), between October and November, 2018; coinciding with the breeding season of Artic charr. The VBCN is a fish hatchery and aquaculture research centre situated in Kälarne, Sweden, and is together with SLU responsible for the national breeding programmes for Arctic charr and Rainbow trout.

Nine mature and ovulating Artic charr females (F1 – F9) were randomly selected by scooping them from a rearing tank one at a time. This reduced the selection bias and all females used were of the same age-class to avoid superiority in egg size (Jeuthe *et al*., 2013). The females were then placed overnight in an oxygenated holding tank and stripped the morning of the following day. Prior to stripping, the fish were anaesthetised in MS-222 (at 50ppm) for 3 to 4 minutes. Stripping was done by gently massaging the female abdomen towards the vent and eggs were collected in a clean dry beaker.

Two males were also selected and placed in a separate tank as described for the females. After anesthetization, male ejaculates (milt) were collected by first wiping dry the ventral of the fish and stripped by gently massaging the abdomen towards the vent; thereby allowing the milt to run down into a dry beaker. A portion of the sperm was preserved for motility analyses in a test tube, kept under iced-water (at approximately 4°C). Two males were used to suffice sperm requirements for fertilizations and motility analysis; seven females were fertilized with sperms of the first male (M1) and the second male (M2) was used to fertilize the remaining two females. A 9.0mL plastic spoon was used to scoop and distribute the eggs from the holding jar and handled as per treatment. Each scoop consisted of 90 eggs on average.

Two treatments were setup, whereas, the fertilization protocol was adapted from Brännäs *et al*., (2011) with minor alterations to suit the experiment. 1) *Reduced* – the scooped eggs were placed on a tea strainer and the OF was allowed to drain off into a dry beaker. The strainer was gently shaken or bitten on the sides to increase the flow of the fluid and after 30 – 40 seconds, the eggs were placed in an incubation jar and fertilized. The strained OF fluid for each female was siphoned from the beaker and store in Eppendorf tubes for sperm motility analysis. 2) *Washed* – the preceding procedure was repeated, but this time eggs on the tea strainer were rinsed in 1.0% of $NaHCO_3$ solution for an equal duration and placed in the incubation jar and fertilized. *Untempered* (*Control*) – the eggs where scooped from the holding jar, directly placed in the incubation jar and fertilized. Immediately, two to three drops of milt were introduced to the incubation jar and thoroughly mixed using a feather. Thereafter, fertilization was done by introducing freshwater to activate the sperms (Fig. 1). These steps were done simultaneously for each female and replicates. Further, eggs for testing the commercial sperm activator (ActiFish^{TM}; IMV-Technologies) were treated as for treatment two (washed) except that this time the sperms were activated with ActiFish solution (1.0mL of milt for every 10mL of ActiFish) Then freshwater was added, and the eggs rinses carefully for incubation.

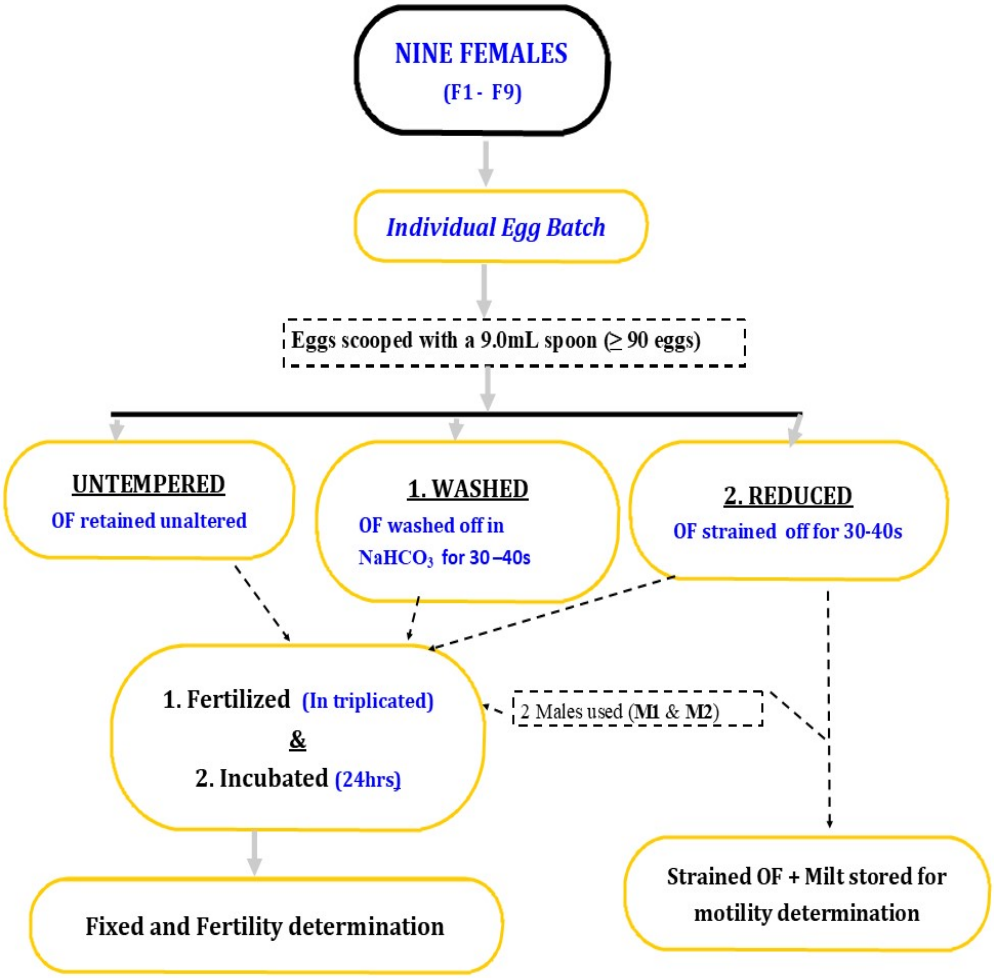

Fig. 1. *Schematic flow diagram of the experimental setup*

Each incubation jar was slowly shaken to achieve even water-milt-egg mixture and left to stand for about 60 minutes. After this time, fertilization had taken place and the egg shell hardened. This first water was decanted (extra milt clog was rinsed off and broken eggs taken out) and another set of freshwater was introduced to the jar to stand for the entire incubation period. The eggs were left to incubate for approximately 24hrs (Fig. 2); by this time embryogenesis had commenced and resulted in eight to sixteen blastomeres. Thereafter, water was decanted and the eggs where fixed for fertility analysis (Refer to detailed description in the coming section). All the treatments were run in triplicates with a lag time of two (2) minutes between each replicate.

***Fig. 2**. Experimental setup of the incubation jars left on the floor of the hatchery (maintained at approx. 5 – 7⁰C) for a period of 24hrs from the time of fertilization.*

2.2 Analytical procedures

2.2.1 Sperm motility analysis

With a corresponding egg fertilization for Treatment 1 and the Control (*Reduced & Untempered*), a 2.0µL of semen sample was activated in an Eppendorf tube (with 50µL OF and 1.0mL freshwater) and a 10µL subsample was directly deposited (4 – 5 seconds lag time) on a glass slide (Leja; 4 chambers, 20 micron) mounted on the stage of the microscope. Sperm activation for Treatment 2 (*Washed*) was done only with freshwater, while for the sperm activator, ActiFish^{TM} solution was used (1.0mL of ActiFish, 50µL OF and 9.0mL freshwater). Motility of the activated spermatozoa was determined in duplicate for each semen-OF, semen-freshwater and semen-ActiFish samples, and videos recorded at the interface of the solutions with a duration 35-45seconds from activation under a microscope (NIKON Optiphot – plan 20(04)-160/0.17) at X200 magnification.

The videos were later analyzed for motility characteristic using a computer-assisted sperm analysis CASA software (ImageJ CASA plug-in application, ij1.52-win-java8). A one second image analysis was done in triplicate of post activation at intervals of 10, 15 and 20 seconds, respectively. All images were processed in TIF files. The movements we see in the samples are due to the liquid flow of filling the chamber. When the camber is full and the liquid flow stops, the sperm cells have gained momentum and continue to drift and thus video images were analyzed only 10 seconds post activation and a setting of above 25.0µms^{-1} for velocity curvilinear (VCL) was used to track motile sperms. Other sperm kinetic parameters, threshold and stepwise settings were done as displayed in Fig. 3., below. The scale setting were changed and adapted to our camera and microscope

specification (such as pixel, frames and microns), whereas, other parameters in the plugin were maintained as recommended by Wilson-Leedy & Ingermann, (2007) The plugin was set to output mean motion characteristics of all motile sperm in each image per execution; thereafter, results were imported into a spreadsheet and labelled accordingly.

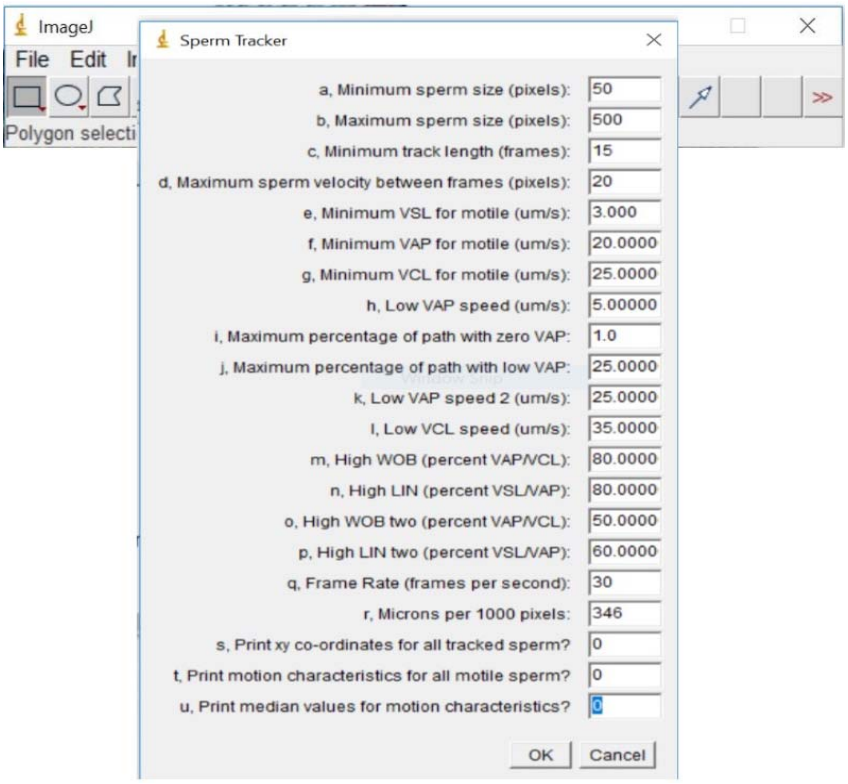

Fig. 3. *Dialog box for CASA plugin within ImageJ showing the sperm parameters used (adapted from Wilson-Leedy & Ingermann, 2007)*

2.2.2 Determination egg fertilization rate

After a 24-hour incubation period, water was decanted from the incubation jars and eggs were fixed in a solution (300mL of ethanol, 50mL of acetic acid and 150 mL 37% formaldehyde) for microscopic embryonic development determination. The solution was specifically adapted for the experiment and due to the presence of acetic acid, the embryonic section of the ova remained unaltered for the entire examination period. Just enough solution to have the eggs submerged was used. Prior to microscopic determination, all the eggs were removed from the fixative, placed on a petri dish with freshwater and observed under a light microscope (NIKON type 4), at X10 magnification. Fertilization rate of eggs was calculated as the percentage of the fertilized eggs and the total number of eggs incubated, as stated in the equation below. This was done for each replicate and the total number was pooled to estimate the fertility of the treatments and individual females.

$$\textbf{Fertilization rate}\ (\%) = \frac{No.\ fertilized\ eggs}{Total\ No.of\ incubated\ eggs}\ x\ 100$$

2.3. Data analysis

Eggs from female eight (F8) were indeterminate after incubation (ova were completely white and blastomere not visible) and thus were excluded from all fertilization analysis. Eggs that could not display a distinct blastomere (unfertilised, dead or broken) were all enumerated as unfertilised. To compare results for egg fertilization rates and sperm performance, kinematics of spermatozoa was restricted to only curvilinear velocity (VCL – in micrometres/second) as a parameter to best describe sperm performance across the treatments. This parameter was chosen because of the curved trajectories of fish spermatozoa in an aqueous media.

Results for fertilization rates and motility were first tested for normal distribution using a univariate procedure. Analysis of variance (ANOVA) was performed to compare fertilization rates across the treatments and females. A paired t-test was used to analyse motility parameters of the two males across at the three-time intervals; 10, 15 and 20 seconds. A Turkey Post Hoc tests was employed were differences occurred and P-value <0.05 was considered statistically significant and presented in the text. Where possible, results are reported as means and standard error (Mean ± SE) or otherwise states as mean values. A Pearson correlation analysis was performed to compare motility parameters and fertilization rates between the three-time intervals and the reduced treatment (as the best representative of sperm velocity in CASA. All Statistical analyses were performed using JMP Pro 13 (SAS Institute Inc.) and graphs were generated in Microsoft Excel.

3.0 Results

3.1 Fertilization rates

A total of 7,713 eggs were enumerated for fertilization rate among the treatments; reduced, washed and untempered (control). These eggs do not include the ones for female eight (F8) that were exluded in the analysis as indeterminate. Analysis for the fertilization rates showed now significant differences across all three treatments; reduced OF, washed OF and the untempered (ANOVA, F=0.28, p> 0.05, Fig. 4).

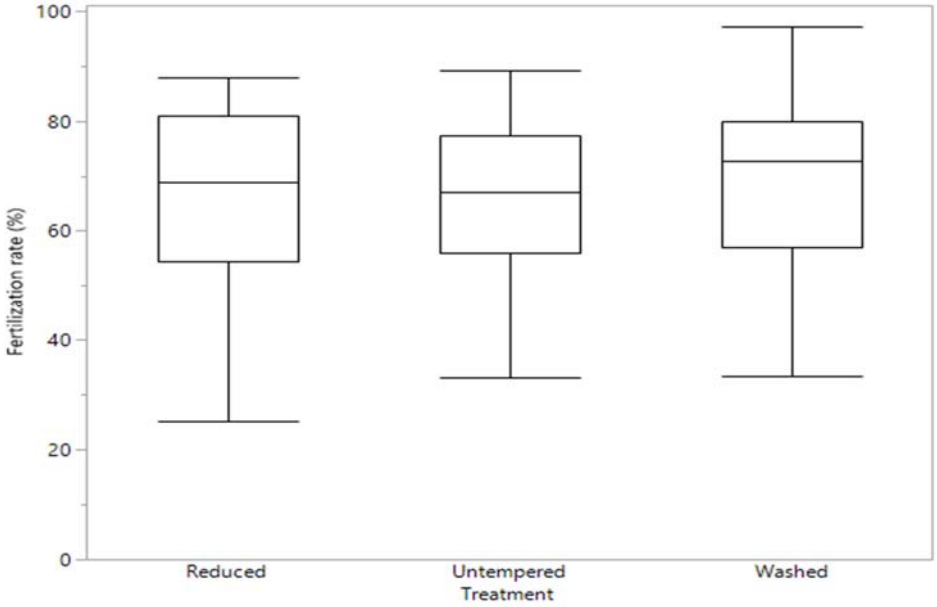

Fig. 4. *Box plot for fertilization rates among the treatments, data pooled for all the replicates and the horizontal line indicates the median.*

Further, there was no siginificant difference (*t*-value=2.32, *p*> 0.05) between the eggs fertilized with sperms activated with a commercial fish sperm activator (ActiFish) and that activated with only with freshwater (Fig. 5). However, the eggs from the ActiFish treatment had distinct divisions across the blastomere compared to the other treatments, thus making it easier to distinguish the fertilised eggs from the unfertilised ones in this treatment. The rest of the treatments had similar array in the divison of the cells on the blastomere.

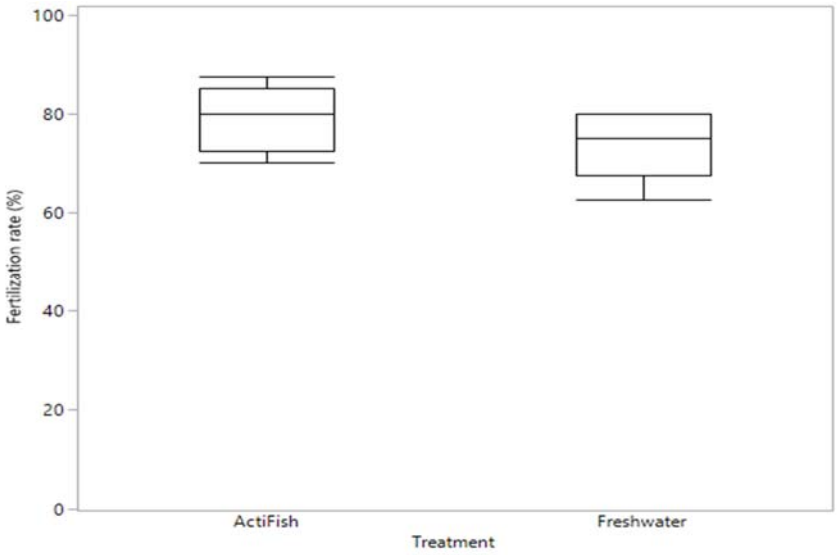

Fig. 5. *Box plot for fertilization rates between sperms activated with Acticfish and freshwater; data is pooled for all the replicates and the horizontal line indicates the median*

Fertilization rates among individual females showed a significant difference (ANOVA, F=4.15, *p*< 0.05, Fig. 6). TheTukey Post Hoc test revealed that most of the females were undistinguishable in the fertilization rates except for female two (F2; 83.3 ± 4.6%) which was significantly higher than female three (F3; 54.9 ± 4.6%) and female five (F5; 60.0 ± 4.6%).

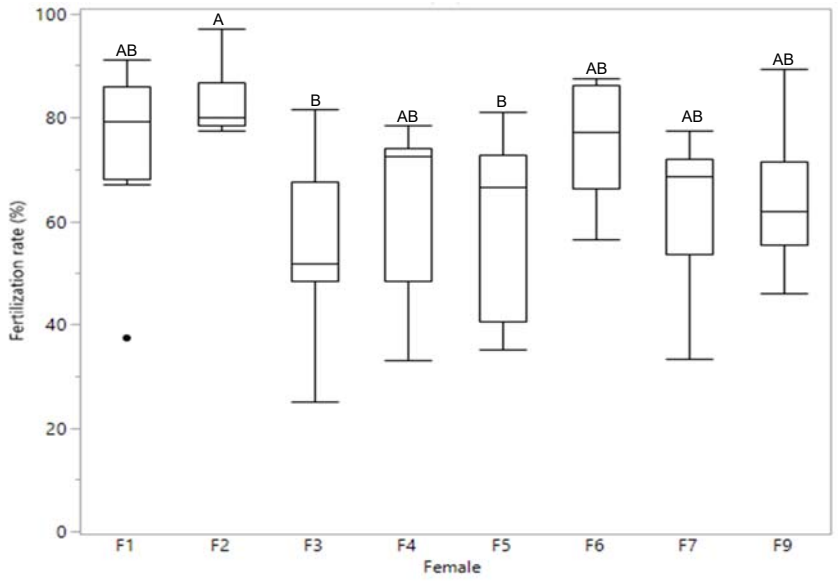

Fig. 6*. Box plot for the individual female fertilization rates; different letters denote significant differences (P<0.05), the dot represents outliers and the horizontal line indicate the median.*

3.2 Sperm motility

As the analysis of sperm motility was restricted to VCL (µm/s), the result of the paired t-test showed a significant difference between the three-time intervals ($p< 0.05$, Table. 1); with the velocity being the greatest at the initial time of measurement (10s) and lowest at 20s. Further, the velocity decreases sharply between the 10th and the 15th second compared to between the 15th and the 20th second where it was gradual in all females.

Table 1. *Comparison of spermatozoa VCL (µm/s) of a paired t-test at the three post-activation time intervals for male one (M1) and male two (M2) in different OF, water and ActiFish. MD – mean difference, SD – Standard deviation and SE – Standard error.*

Male One – M1								
Intervals	Mean	SD	MD	SD of MD	SE of MD	95% CI	*t*-value	*p*-value
10s	100.73	6.87	27.02	12.83	3.11	20.42 – 33.61	8.68	<0.001
15s	73.71	10.97						
15s	73.71	10.97	8.04	14.84	3.50	0.66 – 15.42	2.30	<0.05
20s	65.8	7.82						
Male Two – M2								
10s	94.03	22.64	24.38	18.11	4.15	15.65 – 33.11	5.87	<0.001
15s	69.64	15.15						
15s	69.64	15.51	10.46	7.38	1.57	7.19 – 13.73	6.65	<0.001
20s	57.66	13.31						

On average, VCL of spermatozoa varied depending on the female OF and aqueous media subjected to. Male one (M1, Fig. 7a) had consistent spermatozoa speed in most of the female OF and in water across all the intervals; except in females six (F6 – brown line, Fig. 6a) that had its speed higher average velocity at the 15th second, whereas, the lowest average VCL was recorded in female eight (F8 – purple line, Fig. 6a) at the same interval. In Acticfish solution, M1 behaved differently; the spermatozoa had its velocity increased from the 15th to the 20th second after decelerating from the 10th second (orange line, Fig. 6a).

However, varied average velocities were observed for male two (M2, Fig. 6b), with the highest speed being in water (blue-dotted line) and ActiFish solution (orange line). Again, female eight (F8 – purple line, Fig. 6b) had the lowest sperm velocity across all intervals (<60µm/s); indicating that the fertilization success of M2 in this female are minimal or may not occur at all. It should also be noted that F8 displayed an unusual motility characteristic in M2; where the velocity increased from the 10th second to the 15th second and then declined thereafter towards the 20th second.

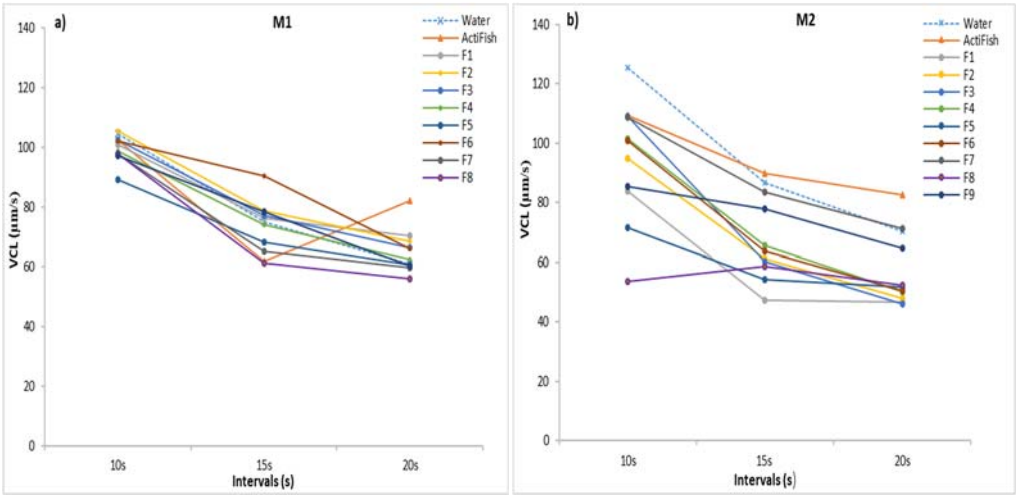

Fig. 7. *Comparison of the variation of VCL (µm/s) for the two males (M1 & M2) in different female OF media, water (blue-dotted line) and ActiFish. Each line represents mean VCL of the males in different activation media.*

3.3 Sperm motility and fertilization rates

Since eggs from the eighth female were not included in the evaluation of fertilization rates, it meant that only one fertilization rate for male two (M2) was obtained. Therefore, the comparison of sperm motility and fertilization rates for female nine (F9) is the motility parameter from male two (M2). Further, since only a single value of fertilization rate was obtained for this male, no comparisons were made with the first male. Generally, female OF that exhibited higher motility values had corresponding higher fertility rates. For instance, females F1, F2 and F6 had relatively higher fertilization rates (>70%) that matched with their high average motility values (>75µm/s). The same is true for the rest of the females that had lower average motility rates (Fig. 7 and Fig 6a).

Additinally, high fertility rates were recorded between and close to the fifteenth interval (15s), whereas, no fertility rate was recorded close to the tweentieth interval (20s, Fig. 8). As such, successful fertilization may have happened close to, or just after the 15th second.

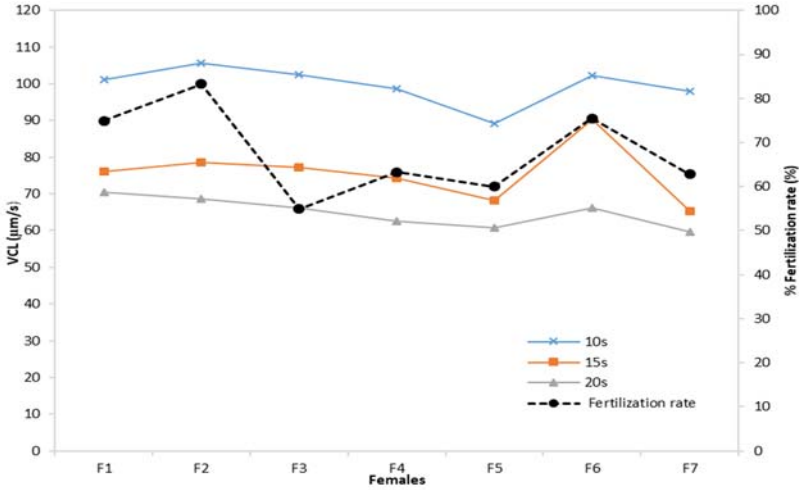

Fig. 8*. Comparison between of average VCL of M1 in OF of and average fertilization rates of the seven females (F1 – F7).*

The Pearson correlation coefficient revealed positive relationships of mean fertilization rate and average VCL of the reduced OF treatment at all intervals, for the seven females (F1 - F7). The 10th and 20th second had similar and slightly higher correlation (r= 0.706, *p*= 0.076, r= 0.672, *p*= 0.098; Fig. 9, a & c) compared to the 15th second (r= 0.599, *p*= 0.155 Fig. 9b). The correlations are not significant (*p*> 0.05), however a positive relationship is still likely to be there between amounts of OF and fertilization rates.

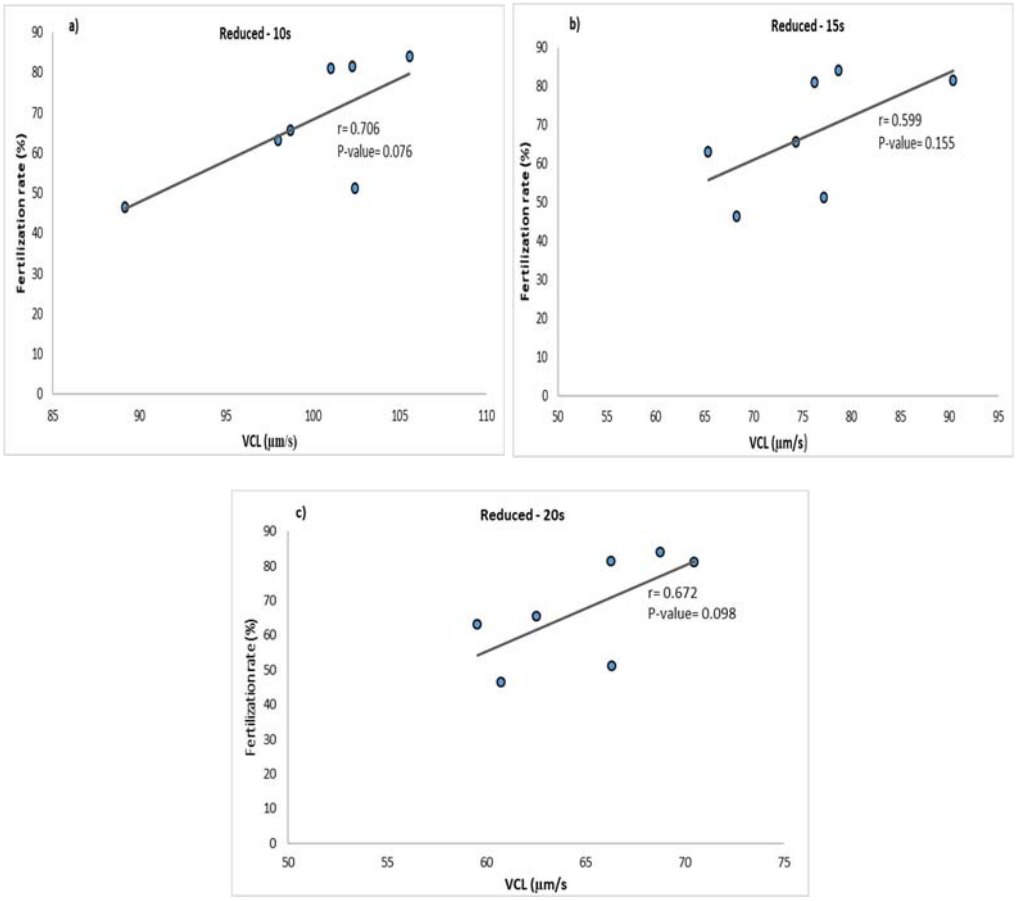

Fig. 9*. Correlation of the fertilization rates and average VCL with respect to time intervals of the seven females (F1 – F7)*

4.0 Discussion

Variation of the volumetric amount of the OF did not yield dissimilar fertilization rates, as the results obtained were not significant. On average, more than 50% fertilization rates were obtained for all the treatments (Fig. 4). As such, tempering with the volume of the OF under the current study did not affect the performance of the sperms and consequent fertilization rates. Even though the efficacy of the OF has been reported to influence sperm motility parameters and fertilization success (Urbach *et al*, 2005; Turner & Montgomerie, 2002), it remains questionable, as per my results, whether retaining the fluid in the fertilization process might skew the results. Unlike in natural state where eggs are laid on a given crate by the female and the males are required to shade their sperms in the immediate vicinity, and potentially faster swimming sperms aided by the OF may encounter the oocytes (Kime *et al*., 2001; Urbach *et al*, 2005), this may not be the case in the hatchery setup. With *in-vitro* fertilization, the distribution of the sperms by thorough mixing of the eggs with the milt prior to the activation of the spermatozoa, somewhat overrules the importance of the OF in the entire fertilization process. Because by mixing, sperms are already placed in the immediate vicinity with the micropyle and OF may not hugely account for the fertilization success. But, probably to the cryptic selection and directional attraction of the sperm to the micropyle.

Moreover, because of successfully selective breeding process that has been done on the Arctic superior (Carlberg *et al*., 2018), it could be that there is a genetic relatedness among the broodstock in use. Thus, the fertilization rate may be high and the same, regardless of the method used to treat the eggs. As such, the OF may not cryptically discriminate the sperms that are attracted on the micropyle. But the quality of the sperms again remains a factor in this regard. However, this study does not contend that variation in the fertilization rates among females (Fig. 6) is due to cryptic discrimination of the sperms by the OF. As also alluded to by Urbach *et al*., (2005) demonstrating directional or non-directional cryptic female choice through OF requires the eggs to be differentially fertilized by different sperm and sperm velocity to be related to fertilization success. This could not be done because only one male (M1) was used to fertilise the seven females on whose fertilization rate is reported. On the other hand, even though my results indicate some similarity in pattern between fertilization rate and velocity (Fig. 8), its still inclusive to assert to this hypothesis.

Furthermore, it is common knowledge that individual females may achieve varied fertilization success with different egg treatment methods depending on individual egg and reproductive fitness of the broodstock. Under the current study, it's unlikely to ascertain as to whether the recorded variation in fertilization rates among the females (Fig. 6) were due to the effect of the OF on sperm motility or individual females' eggs or reproductive fitness. Nevertheless, it was clearly demonstrated that the velocity of the sperms of both males seemed to be dependent on the male-to-female interactions in the ovarian media (Fig. 7; a & b), suggesting that the ovarian fluid differentially affected the speed of the sperms. The consistency in the curvilinear velocity of M1 (Fig. 7a) in all the females indicate that the sperms had a higher chance of meeting the oocytes and thus, may account for successful fertilization. The viscosity of the OF is greatly determined by its ionic content (Lahnsteiner *et al*., 1995) and the motility of the spermatozoa may to a larger extent be influenced by this. Since a common diet is administered to both males

and females, the likelihood that the osmolarity of OF might be favorable from one male to another is high. But mostly depending on the sperm quality of a male. Further, cryptic mate selection by OF in fish has been reported to be more pronounced between interpopulation breeding (Beirão *et al*., 2015). This is not the case on the current study, as broodstock used where from the same age class and population. Thus, it may help to explain the uniformity in the fertilization rates across the treatments.

Notably, the motility of M2 in F8 (Fig 7b) presented an unusual pattern, where the velocity increased from the initial time of measurement to the 15th second and later decelerated. Such a motion pattern could also account for the indeterminate nature of the eggs of female eight (F8) and thus no fertilization occurred, as the sperms had less momentum in the critical time. Conversely, this may otherwise show that OF had increased spermatozoa motility and longevity in that space of time. It is however hard to tell. Generally, the velocity of the sperms exhibited a gradual deceleration in their velocity (Table 1). On average both males (M1 & M2) had their velocity decreased from over 90µm/s at the 10th second to almost half the speed at the 20th second. These observations agree with Kime *et al*., (2001) that motility of salmonids is short. Further, some females OF showed to support increase in velocity of spermatozoa (Fig. 7). This differential effect of OF on spermatozoa speed has a bearing on paternity or successful mating. Urbach *et al*., (2005) suggested that OF might not only serve females to insure fertilization but support increasing their fitness benefits through adaptive postcopulatory mate choice. As such, a male with desireable phynotypes is likely to have consistently high spermatozoa speed in most females. From this, it can be noted that M1 may have a better mating opportunity than M2, due to the consistency of velocity of the former in all females (Fig. 7a).

Additionally, sperms activated with a commercial activator did not differentially influence the fertilization process (Fig. 5), even though the velocity of the sperms was as expected increased (Fig. 7a). However, through this method, the determination of fertilization rates could be easily ascertained owing to the clear and distinct cell divisions on the blastomere. This also led to the low variance in this treatment (Fig. 5) compared to others. Regarding sperm competition in Arctic charr, males with faster sperm may fertilize more eggs than males with slower sperm (Urbach *et al*., 2005). The results of this study however contradict with this earlier hypothesis as fertilization rates among the treatments did not differ significantly ($p>0.05$, Fig. 5). With knowledge that Arctic charr dominant males are unable to maintain high sperm velocity even among the small fraction of the fastest cells and that sperm number has much higher impact on the fertilization success (Vaz Serrano et al., 2006; Torvald, 2010), the speed of the sperm may not hugely account for the fertilization success. Therefore, the number of sperm cells meeting the oocyte maybe the major determinant of fertilization success.

The current study used curvilinear velocity (VCL, µm/s – point to point velocity) as the sperm kinematic parameter to best describe the performance of the spermatozoa in the ovarian media and consequent fertilization success. VCL includes all points of the sperm head position from the recording and represents the best available description of the movement activity (Boryshpolets *et al*., 2013). Although not clearly ascertained, VCL has been argued for as the most reliable indicator of fertility in fish, as opposed to straight line velocity (VSL). This is because in fish, the trajectory of sperm is generally more

curved than in mammals and fish sperm can move three-dimensionally in the aqueous medium (Kime *et al*., 2001). The intervals of motility analyzed were further restricted to between 10s and 20s; as this period provides the most useful data through CASA analysis (*ibid,* Table 1). Unlike in mammalian reproduction where VSL has been ascertained as the ideal indicator of fertility rate, it's still unclear on the appropriate parameter to use in fish, because among major studies that have been done, no threshold has been provided (Rurangwa et al. 2004; Viveiros *et al*., 2010; Aberkane *et al.* 2016).

In this regard, Viveiros *et al*., (2010) reported the highest positive correlations (r = 0.8) between VCL and fertilization rates in streaked prochilod (*Prochilodus lineatus*) sperms, compared to other parameters (VSL and VAP). This correlation is also corroborated by Gallego *et al*., (2013) on pufferfish. Their results, however, are the highest and strongest correlation of fertilization rate and VCL in fish that I could find among the literature reviewed. On the other hand, lower and weaker correlations (r= 0.16) were reported by Aberkane *et al*., (2016) for barbel (*Barbus barbus callensis*). In this study, I recorded positive correlation between VCL and fertilization rates (r= 0.7, Fig. 9) that are more similar as those reported for other freshwater and marine fish species (Viveiros *et al*., 2010; Gallego *et al*., 2013). This may be because of the experimental setup and how the eggs were treated; otherwise, these results offer an initial indulgence on the direction of fertility rates regarding the three methods of fertilizing eggs of Arctic charr.

No conclusive results on the relationship between presence of OF, sperm motility and fertilisation success were found in the present study. Again, no studies of similar setup were found to understand the fertilization rates of Arctic charr eggs and sperm motility. I was able to compare results only with other fish species and not any report was found in the salmonid family. The results indicate both positive effects from the activator on fertilisation rate and a positive relationship between VCL and fertilisation rate. The relationships are not quite significant, but may be valid, especially that it's the first time an attempt on connecting VCL and fertilization rate in Arctic charr was made. On average recorded VCL were within the range for domesticated species, offering considerably high possible fertilization rates. This outcome offers a directional understanding of the relationship of VCL and fertilization rates. However, due to the small sample size of the eggs used in relation to sperm motility parameter, it's difficult to precisely quantify this under actual production regimes yet.

Conversely, CASA parameters have not been precisely reported to predict sperm motility and fertility rate but have been used to assess the quality of the sperms. Even though some of the studies (Viveiros *et al*., 2010) have strongly advocated to have a clear threshold on how fertilization rates links to kinetic parameters of fish sperm (VCL, VAP, VSL and BCF), results by many aquaculturists are neutral over the subject (Rurangwa *et al*., 2004; Turner & Montgomerie, 2002). I used VCL to try and predict fertilization rate and the results are herein positive. This is an indication that VCL may be the appropriate parameter to understand fertilization rates in Arctic charr, as also reported for other fish species. By considering the characteristic of the spermatozoa in aqueous medium (curvature motion), its however not clearly if the sperm attains directional motion once near the micropyle. Paternal-maternal gametic interaction has been shown to be directional near the micropyle and is influenced by the ionic (Ca^{2+}) concentration in OF (Litvak & Trippel, 1998; Beirão *et al*., 2015). Clearly, how this influences the speed of

the sperm attracted to the micropyle remains unresolved. Therefore, the general motility characteristic of the spermatozoa at the moment is the best parameter to explain fertilization rates.

It seems we must rely on the progressive nature of the sperms as the ultimate measure of successful fertilization; considering that males with fast spermatozoa are the best to use. To this effect, conflicting results have been reported and Rurangwa *et al*., (2004) claims that due to the subjective nature of the assessment method used (CASA, percentage progression, etc), the experimental results cannot be effectively used in production regimes. But can be used to validate the quality of the sperms. This is because the duration of motility of fish sperms is very short (<30s in salmonids; Kime *et al*., 2001) and various factors such as viscosity of the OF media, cryptic selection base on genetic relatedness and method used to fertilize the eggs might influence the results. Otherwise, alternative sperm assessment methods to CASA plugin, such as the cooling devices (Caldeira & Soler, 2018) can be tested on Arctic charr to validate sperm quality prior to fertilization. Once the motility quality of the spermatozoa is known before fertilization (exposing milt to different OF), it will be easier to assess micro-parameters affecting the fertilization rates in this species.

5.0 Conclusion

By way of concluding, the study investigated the effect of varying the amount of ovarian fluid by washing, sieving and untempering with the OF on the fertilization rate of Arctic charr. These are the three methods commonly used for *in-vitro* fertilization of Arctic charr eggs. The results are only preliminary and indicate that the use of any of these methods might just be as effective. Sperm competition is a factor in Arctic charr mating patterns and as such, the positive relationship of sperm speed and fertilization rate must be investigated further. VCL may still be considered as the parameter to best describe relationships of gametic motion and successful fertilization in Arctic charr. But more robust research is needed to clearly ascertain this, especially comparing methods described here on hatching rates and alevin fitness. Only then can conclusive advice be offered for production purposes. There is need then also to focus on ascertaining other premises, such as determining that sperm number and integrity are the major factors affecting fertilization rates and not sperm velocity per se.

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7.0 References

Aberkane B. & Iguer-ouada M. (2016). Sperm motility parameters of *Barbus barbus callensis* throughout the reproduction season: Computer aided semen analysis and gametes motility duration. Iranian Journal of Fisheries Sciences 15(4): 1556 -1567.

Alonzo, SH., Stiver, KA. & Marsh-Rollo, SE. (2016). Ovarian fluid allows directional cryptic female choice despite external fertilization. Nature communications, **7**: 12452.

Amann RP. & Waberski D. (2014). Computer-assisted sperm analysis (CASA): Capabilities and potential developments. Theriogenology **81**: 5 -17

Avdesh, A., Chen, M., Martin-Iverson, MT., Mondal, A., Ong, D., Rainey-Smith, S., Taddei, K., Lardelli, M., Groth, DM., Verdile, G. & Martins, R.N. (2012). Regular Care and Maintenance of a Zebrafish (*Danio rerio*) Laboratory: An Introduction. J. Vis. Exp. (**69**), e4196.

Beirão, J. Purchase, CF. Wringe BF. and Fleming, IA. (2015). Inter-population ovarian fluid variation differentially modulates sperm motility in Atlantic cod Gadus morhua. Journal of Fish Biology **87**; 54 –68

Beirão, J., Purchase, C., Wringe, B. & Fleming, I. (2014). Wild Atlantic cod sperm motility is negatively affected by ovarian fluid of farmed females. Aquacult Environ Interact **5**: 61-70.

Boryshpolets, S. Kowalski, RK. Dietrich, GJ., Dzyuba, B. & Ciereszko A. (2013). Different computer-assisted sperm analysis (CASA) systems highly influence sperm motility parameters. Theriogenology **80**; 758–765.

Brännäs E., Nilsson, C. Andersson T. & Pickova J. (2008). The influence of fatty acid composition in broodstock nutrition and of temperature during hatching on muscle development and growth in Arctic charr fingerling. Arch. Tierz., Dummerstorf **51** Special Issue, 37.

Brännäs, E., Larsson, S., Sæther, BS., Siikavuopio, S., Thorarensen, H., & Sigurgeirsson, Ó. & Jeuthe, H. (2011). Arctic charr farming; production of juveniles – a manual. Available at: https://pub.epsilon.slu.se/8468/1/Brannas_E_etal_111125.pdf, retrieved on 2019.01.24.

Butts Ian AE. Johnson K., Wilson CC. & Pitcher TE. (2012). Ovarian fluid enhances sperm velocity based on relatedness in lake trout, (Salvelinus namaycush). Theriogenology **78**: 2105-2109.

Caldeira, C., Soler, C. (2018). Fish Sperm Assessment Using Software and Cooling Devices. J. Vis. Exp. (**137**), e56823

Cardozo G. & Pilastro A. (2018). Female nutritional condition affects ovarian fluid quality in guppies. Biol. Lett. **14**: 01-22.

Carlberg, H. (2016). Sustainable farming of Arctic charr *Salvelinus alpinus*. Doctoral thesis, SLU, Umeä. 978-91-576-8667-

Carlberg, H., Cheng, K., Lundh, T. & Brännäs, E. (2015). Using self-selection to evaluate the acceptance of a new diet formulation by farmed fish. Appl Anim Behav Sci. **171**: 226-232.

Carlberg, H., Nilsson J., Brännäs, E. & Alanärä, A. (2018). An evaluation of 30 years of selective breeding in the Arctic charr (*Salvelinus alpinus* L.) and its implications for feeding management. Aquaculture **495**: 428-434.

FAO. 2018. *The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals.* Rome. Licence: CC BY-NC-SA 3.0 IGO.

Gallego V., Pérez L., Asturiano, J.F. Yoshida M. (2013). Relationship between spermatozoa motility parameters, sperm/egg ratio, and fertilization and hatching rates in pufferfish (*Takifugu niphobles*). Aquaculture **416-417**: 238–243.

Galvano P.M., Johnson, K., Wilson C.C., Trevor E. Pitcher a, Butts Ian AE. (2013). Ovarian fluid influences sperm performance in lake trout, (*Salvelinus namaycush*). Reproductive biology **13**: 172-175

Gasparini C. & Evans JP. (2013). Ovarian Fluid Mediates the Temporal Decline in Sperm Viability in a Fish with Sperm Storage. PLoS ONE **8**(5): e64431.

Jeuthe, H. (2015). Reproductive Performance of Farmed Arctic Charr Doctoral thesis, SLU, Umeå. 978-91-576-8259-8.

Jeuthe, H., Brännäs, E. & Nilsson, J. 2015. Thermal stress in Arctic charr Salvelinus alpinus broodstock: a 28-year case study. Journal of Fish Biology **83**: 1139-1152.

Jeuthe, H., Brännäs, E., & Nilsson, J. (2013). Effects of egg size, maternal age and temperature on egg, viability of farmed Arctic charr. Aquaculture. s **408–409**: 70–77.

Kime, D.E, Van Look K.J.W., McAllister, B.G., Huyskens. G., Rurangwa, E & Ollevier F. (2001). Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comp Biochem Physiol C Toxicol Pharmacol **130**:425–433.

Lahnsteiner, F. (2000). Morphological, physiological and biochemical parameters characterizing the over-ripening of rainbow trout eggs. Fish Physiol Biochem **23**:107–118.

Lahnsteiner, F., Weismann, T. & Patzner RA. (1995). Composition of the ovarian fluid in 4 salmonid species: *Oncorhynchus mykiss, Salmo trutta flacustris, Saivelinus alpinus* and *Hucho hucho*. Reprod Nutr Dev **35**; 465 -474

Litvak, M.K., Trippel, E.A., (1998). Sperm motility patterns of Atlantic cod *ŽGadus morhua*. in relation to salinity: effects of ovarian fluid and egg presence. Can. J. Fish. Aquat. Sci. **55**; 1871-1877.

Makiguchi Y., Torao, M., Kojima, T. & Pitcher TE. (2016). Reproductive investment patterns and comparison of sperm quality in the presence and absence of ovarian fluid in alternative reproductive tactics of masu salmon, Oncorhynchus masou. Theriogenology 86: 2189-2193.

Nilsson, J., Brännäs, E. & Eriksson, L.O. (2010). The Swedish Arctic charr breeding programme, Hydrobiologia **650**: 275 -282.

Richardson, GF. McNiven MA. & Mansour N. (2011). Effect of methanol concentration and thaw rate on the viability and fertility of cryopreserved Arctic charr, *Salvelinus alpinus* (L.), spermatozoa. Aquaculture Research, **42**: 1096-1100.

Rosengrave, P., Taylor, H., Montgomerie, R., & Metcalf, V., McBride, K., & Gemmell, N. (2008). Chemical composition of seminal and ovarian fluids of Chinook salmon (*Oncorhynchus tshawytscha*) and their effects on sperm motility traits. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology. **152**: 123-129.

Rurangwa, E., Kime DE., Ollevier F., Nash JP. (2004). The measurement of sperm motility and factors affecting sperm quality in cultured fish, Aquaculture **234**: 1–28.

Sawant NH. And Belsare SG. (1992). Effect of sodium bicarbonate on hatching and on postembryonic growth of *Brachydanio Rerio*, *Cyprinus Carpio*, *Labeo Rohita*, *Cirrhinus Mrigala* And *Catla Catla*. Journal of the Indian Fisheries Association **22**: 89-91.

Torvald, G. (2010). Sperm competition in the external fertilizer Arctic charr: playing by sperm quality, sperm quantity or gametes release synchrony? Nord University.

Turner, E. & Montgomerie, R. (2002). Ovarian fluid enhances sperm movement in Arctic charr. Journal of Fish Biology **60**: 1570-9.

Urbach, D., Folstad, I. & Rudolfsen G. (2005). Effects of ovarian fluid on sperm velocity in Arctic charr (*Salvelinus alpinus*). Behav Ecol Sociobiol **57**:438–444.

Vaz Serrano, J., Folstad, I., Rudolfsen, G. and Figenschou, L. (2006). Do the fastest sperm within an ejaculate swim faster in subordinate than in dominant males of Arctic char? *Canadian Journal of Zoology*, **84**(7): 1019-102

Wilson-Leedy, JG. & Ingermann RL. (2007). Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology **67**: 661-72.

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