Evaluative studies on artificial fertilisation of Arctic charr and rainbow trout

Arctaqua project 2022

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Co-funded by the European Union

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Introduction

Egg production constitutes a common bottleneck in Arctic charr (*Salvelinus alpinus*) farming (Olk et al., 2019). The proportion of eggs that successfully reach the eyed stage and hatching is often low compared to other farmed salmonids (Jeuthe et al., 2016). A study involving the Swedish Arctic charr breeding program has indicated that the majority of egg losses in Arctic charr occur due to embryonic mortality (Jeuthe et al., 2019). However, a substantial part of the eggs was also lost due to failed fertilisation. In the aforementioned study, 12% (median) of the stripped eggs remained unfertilised, while 28% were lost due to mortality prior to the eyed stage. Generally, most of embryo mortality is likely due to poor egg quality, but within the Arctic charr breeding program, indications of paternal factors associated with embryo mortality rates have been observed (Brännäs et al., 2011; Jeuthe et al., 2019). These issues, both maternal and paternal, are likely results of suboptimal conditions during maturation and spawning of the parent fish, e.g. high water temperatures (Jeuthe et al., 2015, 2013). However, these issues aside, when a viable oocyte remains unfertilised during artificial reproduction the problem lies either in the quality of the sperm or in the environmental conditions. In either of these two cases the result will be that sperm are unable to reach the micropyle of every egg within the short activation window that they have. In conclusion, based on empirical observations, there seems to be room for improvement regarding fertilisation protocols for Arctic charr.

This report is based on small scale studies on fertilisation procedures at Aquaculture Centre North (Vattenbrukscentrum Norr AB; hereafter VBCN) by SLU researchers and students as well as VBCN staff during the natural reproductive seasons of both Arctic charr (Oct-Nov) and rainbow trout (Oncorhynchus mykiss; March-April) from 2018 to 2021. While the main goal was to produce an improved Arctic charr fertilisation protocol, we included studies on rainbow trout to maximise the output achieved within the study period. We made the assumption that results from rainbow trout, at least in part, can be transferred to Arctic charr. However, our findings should be of interest to trout breeders as well.

It is generally a good idea to test the sperm quality before choosing which sires to use for fertilisation, especially when using a single male to fertilise an egg batch, as opposed to pooling semen from several males. However, we reckon that most commercial hatcheries have neither the time nor the equipment to evaluate sperm quality beforehand, they also may not have enough males to be picky on which ones to use. Therefore, we have decided not to include any sperm quality testing in our suggested hatchery protocol. Instead, our focus is on how to treat the eggs and semen during collection and which procedures and chemicals would benefit the fertilisation process. The studies included in this report have evaluated the practice of rinsing vs. retaining ovary fluid, the use of different activation media, and application of artificial sperm selection (fertilisation with long-lived sperm). The endpoints we used to evaluate these parameters included sperm motility, fertilisation rates, eyeing and hatching rates, and offspring development (Table 1). More specifically the findings presented in this report come from six experiments performed on either Arctic charr or rainbow trout as follows:

Arctic charr

1. Effects of ovarian fluid on sperm motility and fertilisation rates
2. Fertilisation and eyed survival after activation in water vs. Actifish
3. Fertilisation rates in different activation media

Rainbow trout

1. Effects of different activation media on sperm motility and fertilisation rates
2. Effects of sperm selection on fertilisation rates and blastomere development
3. Effects of sperm selection on fertilisation rates and embryo survival

Research background

The influence of ovarian fluid on sperm motility and fertilisation success is somewhat unclear, based on the literature. There are e.g. reports of motility enhancing effects in lake trout (Salvelinus namaycush) (Butts et al., 2012) and Arctic charr (Turner and Montgomerie, 2002; Urbach et al., 2005). Interestingly, there are also reports on inter-male variation in spermatozoa's response to ovarian fluid of different females (Makiguchi et al., 2016). In contrast, there are also studies that showed negative effects of ovarian fluid on sperm performance and longevity as well as fertilisation success in e.g. guppies (Poecilia reticulata) (Gasparini and Evans, 2013) and Atlantic cod (Gadus morhua) (Beirão et al., 2014). These characteristics make the ovarian fluid an interesting factor during the fertilisation process. Further evaluations of its effects in routine hatchery conditions are needed.

An additional factor that plays a key role in the decision on whether to retain the ovarian fluid or to rinse the eggs before fertilisation is the overall quality of the egg batch. We have observed that in some cases where untreated eggs were used, the added semen clumped together giving the appearance of sour milk. When this happens we reckon that it is most likely an effect of ruptured oocytes leaking their cellular content, disrupting the spermatozoa's ability to move freely.

When it comes to the choice of activation medium for artificial fertilisation of fish eggs, the cheapest and perceivably most natural option is water. Water from the broodstock tank is available at an arm's length, cost free, and is guaranteed to be the same temperature as the eggs and semen. Water is also the only activation medium available in the fish's natural environment in the wild, so it should work perfectly. However, there have been extensive evaluations of alternative media through many decades, and several chemical solutions have been shown to be superior to water in promoting sperm performance and fertilisation success. Which chemical composition is best differs between fish species, or more so between fishes with different life reproductive strategies and environments. Billard's solution or derivates thereof are commonly recommended as activation media for salmonids. Billard's solution, also called D532, is a saline solution buffered to pH 9 using Tris and glycine (Billard, 1992, 1977). Some alterations to the composition, like e.g. addition of Calcium ions and caffeine, have been evaluated and (in some cases) shown positive effects (Cosson et al., 1989; Valdebenito, 2007). There are also commercial products available on the market. One example, commonly used for salmonids, is Actifish (IMV Technologies), based on D532 and developed together with INRA, France.

A somewhat recent study on zebrafish showed that offspring resulting from fertilisations with long-lived spermatozoa had higher viability (Alavioon et al., 2017). Fertilisation with long-lived sperm was achieved by activating the sperm prior to contact with oocytes, so that the short-lived sperm were spent by the time oocytes were introduced. If this is applicable also in salmonids, it could be a way to maximise embryonic (and later) viability, thereby eliminating any negative paternal influence and utilising the full potential of each oocyte. In extension, one could speculate whether the use of performance enhancing activation media improve the competitiveness of short-lived spermatozoa thereby having negative effect on offspring viability.

The Arctic charr is very sensitive to high temperatures, especially in connection to reproduction. The rearing temperature for broodstock should always be kept below 10-12°C (Olk et al., 2019). The water temperature just prior to and during spawning is optimal around 4-5°C (Jeuthe et al., 2015, 2013), while the maximum incubation temperature to avoid excess mortality and malformations is around 7-8°C. However, during (approximately) the first week of incubation, the water temperature also needs to be minimum 3-4°C to avoid this issues (Jeuthe et al., 2016).

Table 1. Parameters that have been evaluated in the six studies included in this report. Three studies were performed on Arctic charr (AC) and three on rainbow trout (RT). "Delayed" indicates use of premature sperm activation for selection of long-lived spermatozoa.

	Water	Water (delayed)	Ovarian fluid	NaCl	D532	D532 (caffein)	Actifish
CASA (ImageJ)	AC1		AC1				
CASA (SCA)	RT1			RT1	RT1	RT1	RT1
Fertilisation rate	AC1,2,3 RT1,2,3	RT2,3	AC1	AC3 RT1	AC3 RT1	AC3 RT1	AC2,3 RT1,2,3
Survival to eyed stage	AC2 RT3	RT3					AC2 RT3
Hatching	RT3	RT3					
Cell symmetry	RT2	RT2					RT2

Study descriptions and results

Here follow brief descriptions of experimental design, main findings, and some comments for each of the studies.

Arctic charr

1. Effects of ovarian fluid on sperm motility and fertilisation rates

The experiment was carried out at VBCN 31 October to 1 November 2018 by SLU Masters student Lwabanya Mabo. The Master's thesis with complete details can be downloaded from https://stud.epsilon.slu.se/14612/11/lwabanya_m_190620.pdf.

This study was designed to evaluate how handling of eggs and ovarian fluid during stripping affects sperm swimming performance and in extension fertilisation success. In the Arctic charr hatcheries we have visited, either of the following three different methods is used. The ovarian fluid may be completely retained by stripping the eggs straight into the fertilisation vessel. Alternatively, the eggs can be stripped into a strainer and then transferred to the fertilisation vessel with only a small amount of ovarian fluid remaining. As a third option, the strained eggs can be rinsed in a 1.0% $NaHCO_3$ solution, which prevents activation and swelling of the eggs and has a moderate anti-bacterial effect.

In the current experiment ovarian fluid from seven females and semen from two males where collected and used for sperm motility measurements. In addition to ovarian fluid (50 µl per 1 mL water) motility measurements were done using water, saline solution (0.9%) and Actifish. Sperm was activated in Leija counting chambers, filmed through a microscope, and analysed for motility parameters using the CASA-plug in the open source software ImageJ.

Eggs from the same seven females and semen from one of the two males were also used for a fertilisation trial. Small aliquots of appr. 90 eggs from each female were fertilised following the three handling procedures described above – retained ovarian fluid, reduced ovarian fluid and rinsed. Three replicate aliquots were used for each treatment and female. After fertilisation and swelling in water, the eggs were incubated for approximately 24 hours to the eight (to 16) blastomere stage. At this time the eggs were fixed in FAA-solution (three parts 37% formaldehyde, six parts ethanol, and one part acetic acid) and checked for fertilisation.

Motility, measured as curve-linear swimming velocity (VCL), varied substantially for each of the two males in the different activation media (Figure 1). The highest velocities were measured in Actifish.

VCL in ovarian fluid was also significantly different between males (pairwise t-test, t=2,83, p<0,05), despite being very similar in Actifish. Hence, for one of the males, ovarian fluid seemed to have a negative effect on sperm performance. Motility in water versus saline solution were quite similar. However, due to the low number of observations, comparisons between activation media cannot be done with any statistical certainty.

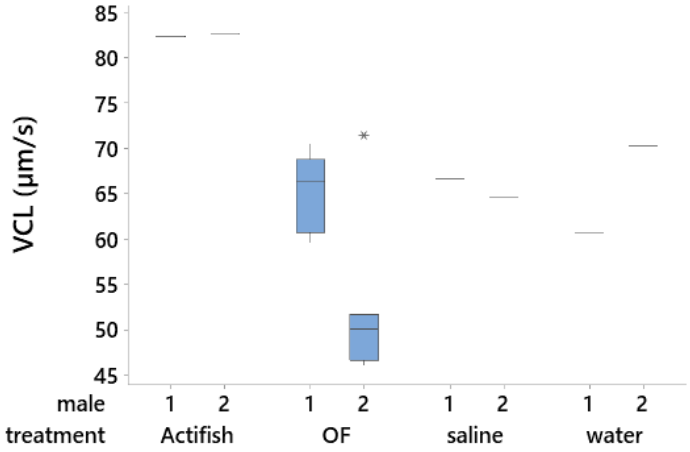

Figure 1. Sperm motility for two individual Arctic charr measured as VCL at 20 s after activation in different activation media: Actifish (commercial product), OF (ovarian fluid from seven different females), saline (0.9% NaCl-solution), and water.

Fertilisation rates were generally rather low, 70% on average, with large overall variation between fertilisation vessels, 25-97%. The variation was seen both within each treatment and for each female (Figure 2). No significant difference in fertilisation rate could be detected between the different treatments (Figure 3). The variation in fertilisation rates were notable but difficult to explain with the current experimental set-up. Differences between females were most likely, in part, a reflection of egg quality. However, there was no consequent difference between treatments among the individual females. Since sperm from only one male was used, it was impossible to discern the males influence on the results. It is plausible that this particular male's sperm responded differently to the ovarian fluid of the different females, thus hiding any general relationship between treatment and fertilisation rate. A factorial design experiment with several males and females would be interesting to do, but wasn't an option at the time of this study due to the increase in work effort required.

When sperm motility in ovarian fluid from the different females was tested for correlation with fertilisation rates for eggs of the respective females, a positive trend could be seen (Figure 4). Correlations coefficients (Pearson) for VCL measured at 10 s after activation and average fertilisation rates were 0.63, 0.71, and 0.10 for retained, reduced, and rinsed respectively.

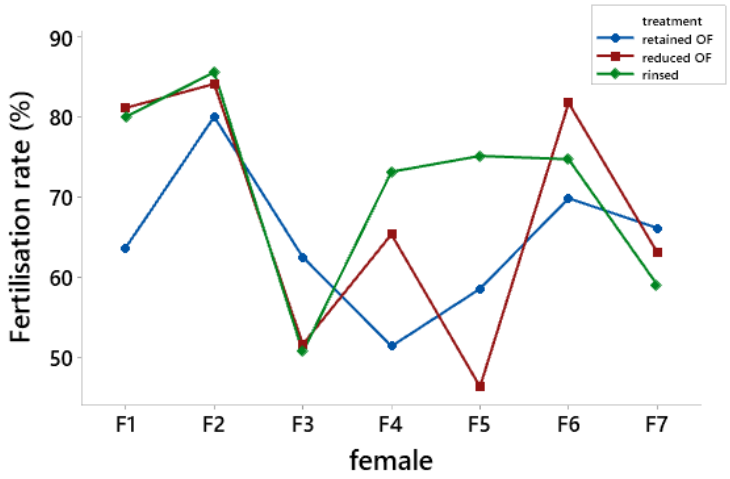

Figure 2. Fertilisation rates shown large variation, both within treatments and females. Each data point represents average values for three replicate fertilisation vessels.

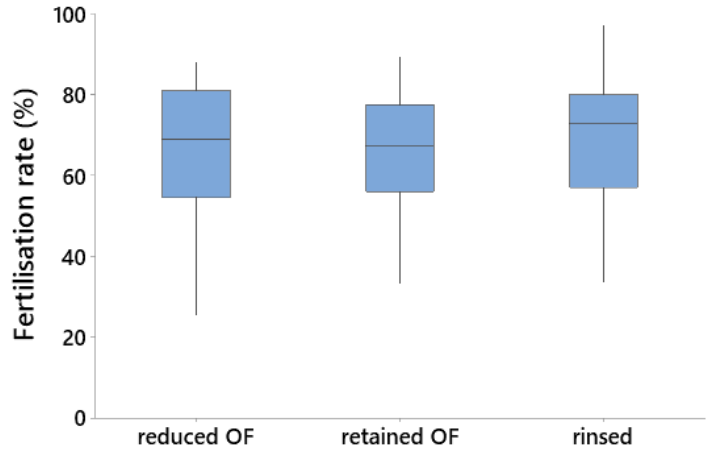

Figure 3. Fertilisation rates in Arctic charr eggs subjected to three different treatments: reduced OF (strained from excess ovarian fluid), retained OF (eggs stripped straight into fertilisation vessel), and rinsed (1.0% $NaHCO_3$ solution). Boxplots present median (horizontal line), 50% of values (box) and max/min values (whiskers). No significant difference was detected between treatments (ANOVA, F=0.28, p> 0.05).

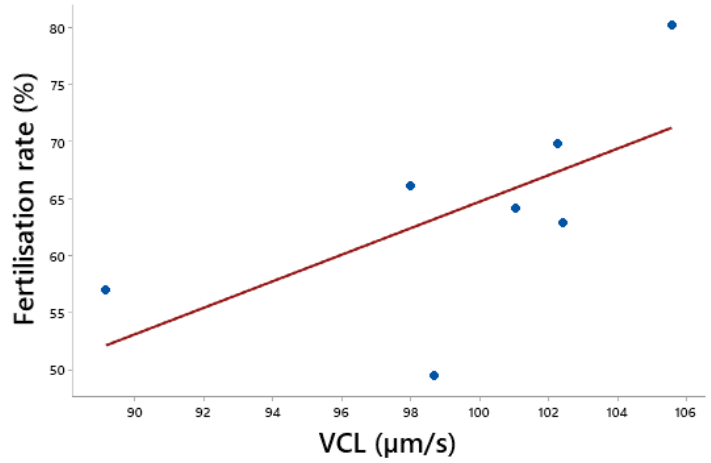

Figure 4. Fertilisation rates for eggs from seven different females fertilised with sperm from the same male showed a positive trend with sperm swimming velocity (VCL) in diluted ovarian fluid from respective females. VCL was measured at 10 s after activation. Fertilisation rates displayed in the graph from eggs with retained ovarian fluid.

It is difficult to make any firm conclusions regarding the best procedure for egg treatment (ovarian fluid retention) based on our findings. Presence of ovarian fluid does not seem to have any generally positive influence on sperm performance compared to other activation media. Therefore, we would rather recommend rinsing the eggs to avoid potential negative effects of e.g., leaked cell content as well as male-specific response to ovarian fluid.

2. Fertilisation and eyed survival after activation in water vs. Actifish

The objective of the current study was to test whether higher fertilisation rates can be achieved in Arctic charr eggs by using the activation medium Actifish instead of water. In extension, if improved fertilisation is achieved, will this also affect the overall outcome of the egg production success.

The experiment was performed in November 2018. Seven females and seven males were used in parallel 1x1 crossings. The eggs from each female were divided into two roughly equal portions, where one portion was fertilised using water as an activator and the other portion was fertilised using Actifish. Both treatments mixing of eggs and semen before adding the activation media. The fertilised eggs were left for a few minutes and then rinsed from remaining semen and activation media. The eggs were then left to harden in clean water and then put in separate (n=14) incubation trays in the hatchery. The number of eggs put in each tray was estimated using the volumetric measurements of the total egg volume and egg size measurements using the Brofeldt method.

The day after fertilisation, subsamples of approximately 40 eggs were collected from each of the incubation trays. The eggs were fixed in FAA and checked for fertilisation under a low magnification microscope. Remaining eggs were left untouched until they reached the eyed stage. Live and dead eggs were sorted in an egg sorting machine and the proportion of intact (live) eggs at this stage was used as the endpoint to measure egg production success.

Median fertilisation rates were 80% for the eggs fertilised in Actifish and 75% in water (mean 78.6% and 73.2% resp.) (Figure 5). Fertilisation rate in Actifish was significantly higher than in water (one-sided paired t-test $T=2.03$ $p<0.05$). Generally, fertilisation rates were quite low, but in line with the previous study (AC1) performed a few weeks before. 2018 was a bad year for Arctic charr egg production, likely as a result of an unusually hot summer this year. The proportions of eyed eggs were 39.0% and 35.6% (median) in eggs fertilised in Actifish and water, respectively (mean 34.3% vs. 30.0%) (Figure 6). Hence, substantial mortality losses occured. In addition, the fertilisation rates were quite even among the families, only one family had an estimated fertilisation rate that was higher in water than in Actifish (Figure 7). Proportions of eyed egg showed more variation. Eggs of one female had zero survival to the eyed stage. Estimation of the original number of eggs using total egg volume is not precise, and adds some uncertainty to the eyed stage results. The sorting machine only provides the number of viable eggs, not the number of discarded eggs. However, based on the fertilisation rate results, there seems to be an advantage to using Actifish rather than water, but it is still unclear whether this advantage makes any lasting difference for the overall egg production success.

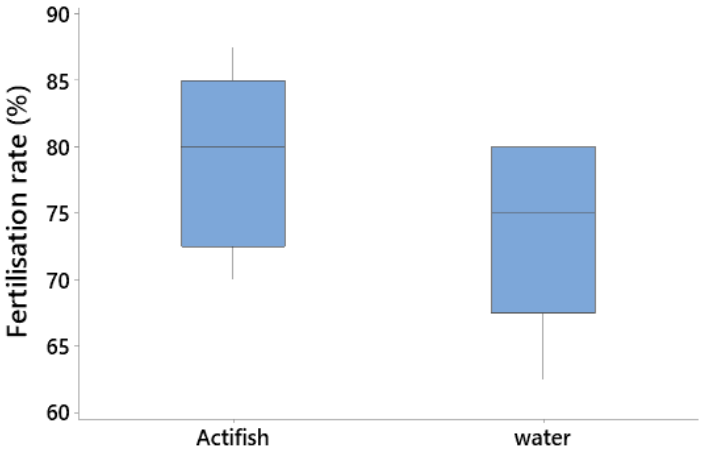

Figure 5. Fertilisation rates of eggs from seven different females, fertilised in either Actifish or water. Fertilisation was determined by examination of subsample of eggs (n=40 each) appr. 24 hours after fertilisation. Boxplots present median (horizontal line), 50% of values (box) and max/min values (whiskers). Fertilisation rates were significantly higher in Actifish (one-sided paired t-test p<0.05).

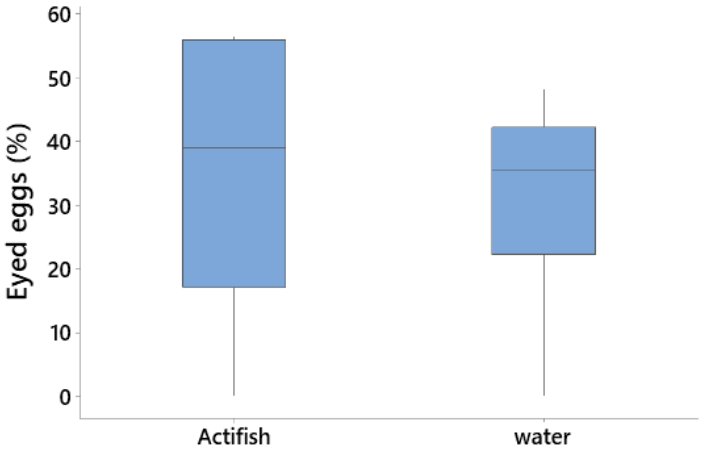

Figure 6. Proportions of eyed eggs from seven different females, fertilised in either Actifish or water. Values were based on counts of viable eggs using an automatic sorter and volumetric measurements of original egg batches. Boxplots present median (horizontal line), 50% of values (box) and max/min values (whiskers). Proportions of eyed eggs were not different between treatments (paired t-test p>0.05).

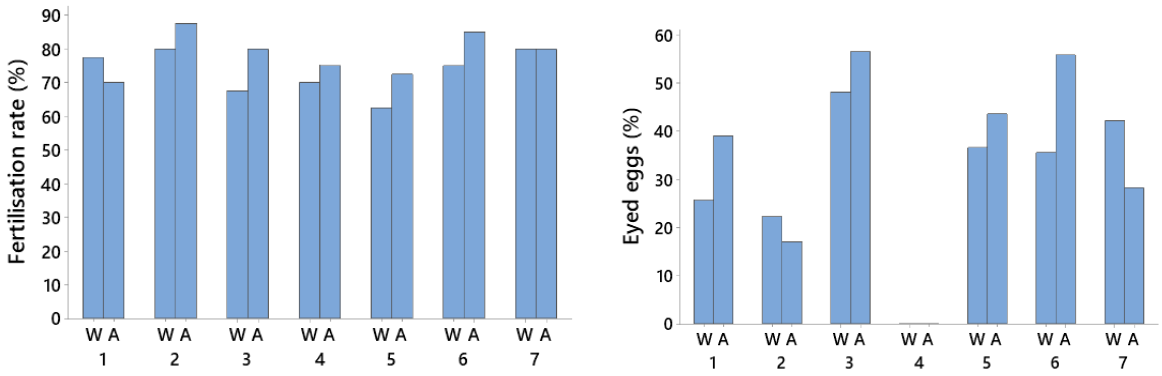

Figure 7. Individual estimates of fertilisation rates and proportions of eyed eggs in Arctic charr eggs fertilised by seven different males in either) water (W) or Actifish (A).

3. Fertilisation rates in different activation media

Previous fertilisation trials with both Arctic charr and rainbow trout have indicated an advantage to using Actifish instead water as the activation medium. Actifish is a commercial product – easy to use with clear instructions. However, it contains antibiotics (gentamicin sulfate), which shouldn't be used unnecessarily due to the risk of promoting antibiotic resistant pathogens. Also, alternative solutions that can be prepared at the hatcheries may be more cost efficient.

The aim of the current experiment was to evaluate fertilisation rates in Arctic charr eggs using five different activation media: water, Actifish, saline solution, Billard's solution (D532), and Billard's solution with addition of caffeine. Composition of the last solution should be close to that of Actifish, but without antibiotics.

Eggs from one female were distributed between 25 plastic cups, ca 50 eggs in each, and fertilised with semen from 5 different males. Eggs in each of the five cups with semen from the same male were fertilised using different activation media. The five activation media consisted of:

- Water (from the rearing tank)
- Saline solution (7.3 g NaCl per L destilled water)
- D532 (20 mM Tris, 30 mM glycine, 125 mM NaCl, 1 mM CaCl2; pH 9.0)
- D532 + caffeine (addition of 10mM caffeine)
- Actifish (Commercial; ultra-pure water, glycine salts, caffeine, biological buffer, antibiotic <0.05%: gentamicin sulfate)

Fertilisations was achieved using the 'wet' method. Activation media were first added to the eggs (50mL), directly followed by addition of semen, and a gentle stir. Eggs were rinsed from excess milt after a few minutes and left to harden in clean water. The eggs were then incubated for ca 40 hours (eight blastomere) in a refrigerator, fixed in FAA and checked for fertilisation success.

Median fertilisation rates ranged from 60.4% in water to 76.6% in Actifish. Mean values ranged from 62.2% in water to 72.7% in saline. No significant differences between treatments were detected. However, if any of the results stand out it would be water (again), with slightly lower success rates. The generally low fertilisation rates achieved in this study may in part be due to use of the 'wet' fertilisation method. This approach was followed to make sure that fertilisation took place with the respective activation media.

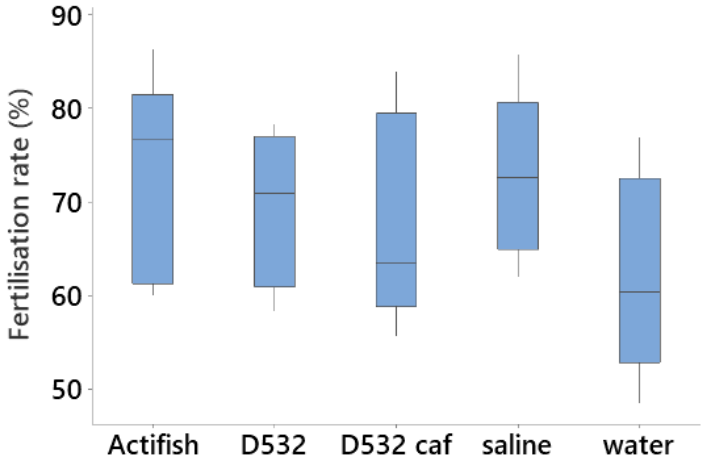

Figure 8. Fertilisation rates of eggs from one female Arctic charr, fertilised with sperm from five different males in either five different activation media. Fertilisation was determined by examination of fixed eggs (n=50 each) appr. 40 hours after fertilisation. Boxplots present median (horizontal line), 50% of values (box) and max/min values (whiskers).

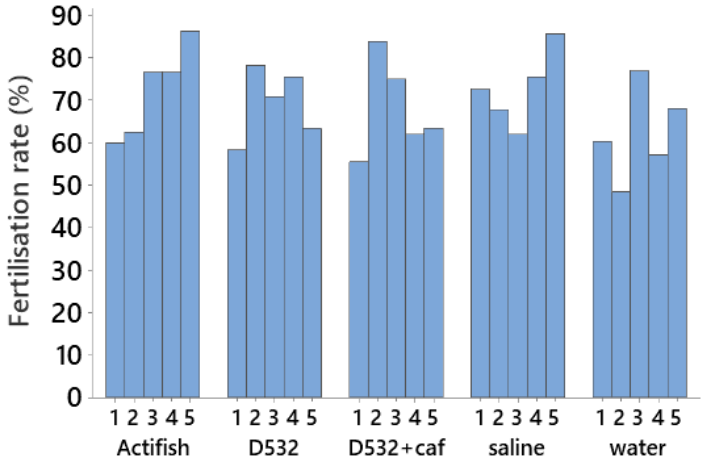

Figure 9. . Individual estimates of fertilisation rates in Arctic charr eggs fertilised by five different males in five different activation media.

Rainbow trout

1. Effects of different activation media on sperm motility and fertilisation rates.

The aim of this study was to evaluate the use of different activation media, including effects on fertilisation rates and sperm swimming performance. In particular, we wanted to find a solution that performs equally good as Actifish, but can be produced in a simple laboratory or even at a hatchery. Tree different solutions were tested, in addition to Actifish and water:

- Saline solution (7.3 g NaCl per L distilled water)
- D532 (20 mM Tris, 30 mM glycine, 125 mM NaCl, 1 mM CaCl2; pH 9.0)
- D532 + caffeine (addition of 10mM caffeine)

Eggs from one female were distributed between 25 plastic cups, ca 50 eggs in each, and fertilised with semen from 5 different males. Eggs in each of the five cups with semen from the same male were fertilised using the different activation media.

Fertilisation was achieved using the 'wet' method. Activation media were first added to the eggs (50mL), directly followed by addition of semen, and a gentle stir. Eggs were rinsed from excess milt after a few minutes and left to harden in clean water. The eggs were then incubated for ca 40 hours (eight blastomere) in a refrigerator, fixed in FAA and checked for fertilisation success.

Sperm swimming performance was analysed using the CASA system (computer assisted sperm analysis) SCA from Microptics.

All tested activation media resulted in high fertilisation rates. Median values were 93% for Actifish and 95% for saline, D532, and D532 + caffeine, but 45% in water (Figure 13). No significant difference in fertilisation rate between the different chemical solutions could be detected. Fertilisation rates in water ranged from zero to 100%, while values from all fertilisation vessels with chemical solutions were above 85%.

The effects of activation media could also be seen in the sperm swimming performance. The small number of replicates prevents any relevant statistical testing, but visualisation of the results indicates a generally lower and steeper decline in swimming speed in water compared to all other media (Figure 14).

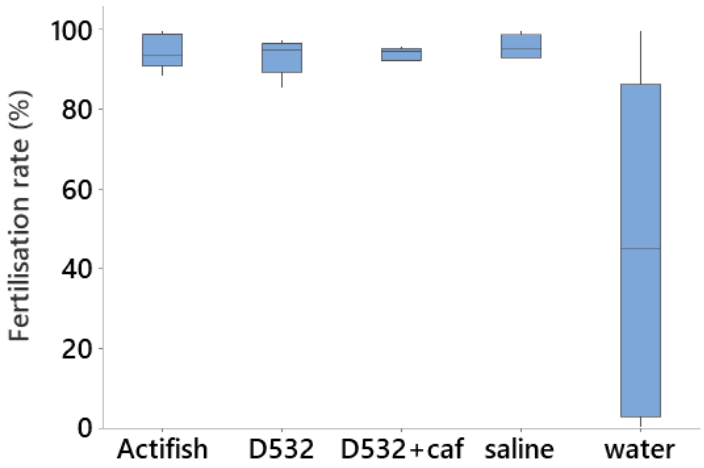

Figure 10. Fertilisation rates of eggs from one female rainbow trout, fertilised with sperm from five different males in either five different activation media. Fertilisation was determined by examination of fixed eggs (n=50 each) appr. 40 hours after fertilisation. Boxplots present median (horizontal line), 50% of values (box) and max/min values (whiskers).

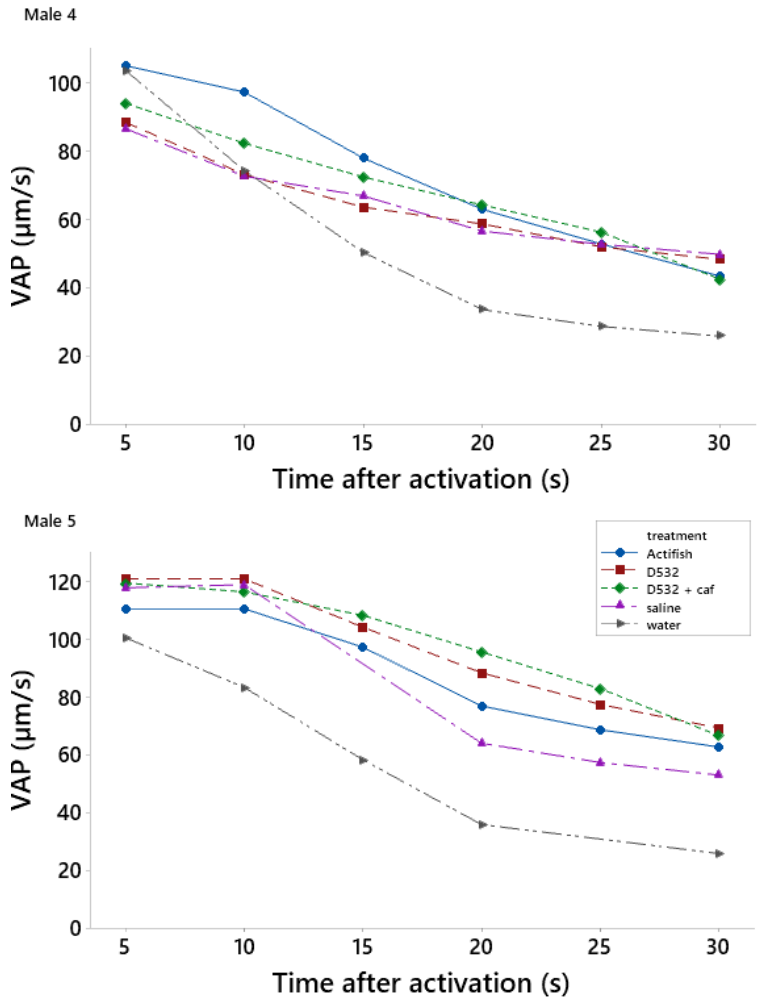

Figure 11. Swimming speed measured as average path velocity (VAP) of sperm from two of the rainbow trout males in the current study. Measurements were done in five different media using the CASA system SCA from Microptics.

2. Effects of sperm selection on fertilisation rates and blastomere development.

Previous studies on zebrafish have shown a positive relationship between sperm longevity and offspring viability. The aim of the current study was to test whether such a relationship can be seen in rainbow trout as well. Due to the slow developmental rate of trout, compared to zebrafish, we chose blastomere symmetry as an early indicator of offspring viability and development. We found the eight-blastomere stage to be optimal for determination of cell symmetry (Figure 10), but the four- and 16-blastomere stages are also quite clear.

We hypothesised that the use of pre-activated sperm would result in higher degree of symmetry in the fertilised embryos compared to activation of sperm in the presence of eggs either with water or Actifish. To test this, eggs from one female were divided between 12 cups and fertilised with sperm from four different males in the following three different treatments.

1. "Normal" fertilisation where eggs and semen were mixed together and activated using water.
2. "Delayed" fertilisation where the sperm were activated in water 30 seconds before being added to the eggs.
3. "Aided" fertilisation using the same method as "normal" but with Actifish as the activation medium instead of water.

We also hypothesised that fertilisation rates would be higher in Actifish than in treatments using water as activation medium.

After rinsing the fertilised eggs, they were left to harden and incubate in a refrigerator for approximately 26 hours. At this point they had reached the four-blastomere stage and were fixed in FAA solution for later examination of fertilisation rates and cleavage symmetry. The experiment was performed in the spring of 2019.

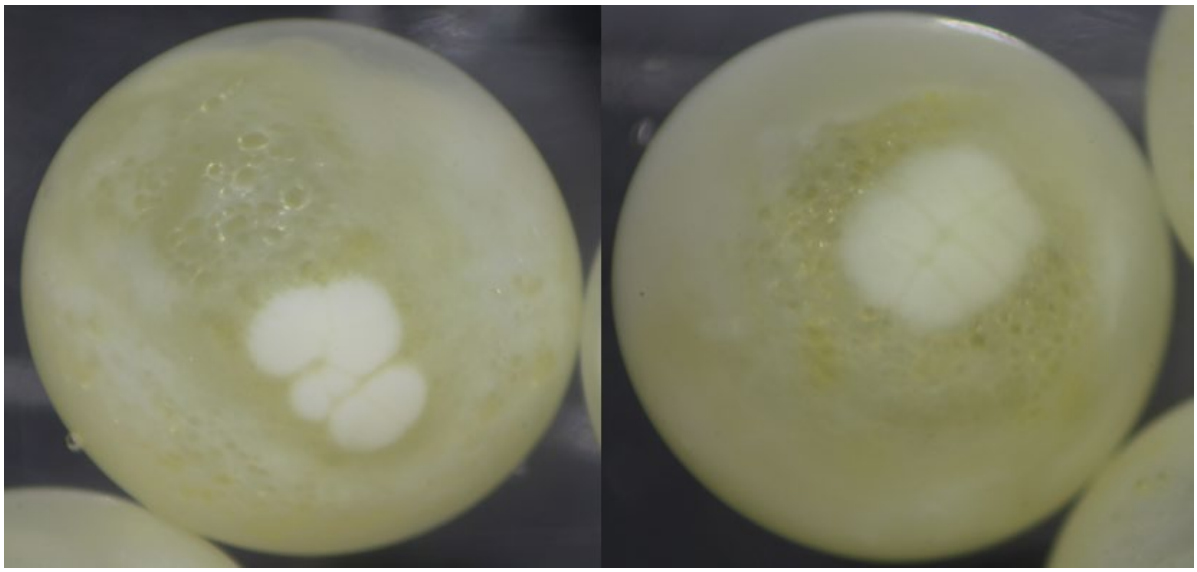

Figure 12. Examples of fertilised eggs at the eight-blastomere stage with symmetric (right) and asymmetric cleavage patterns. Images display eggs from Arctic charr (photo: Henrik Jeuthe).

Generally, eggs and sperm were of good quality. Fertilisation rates ranged from 87% to 100%, median values of 99% in Actifish and 96% in water (normal and delayed) (Figure 11). Fertilisation rate was significantly higher in Actifish compared to both treatments using water (one-sided paired t-test $T=2.52$ $p<0.05$ and $T=2.6$ $p<0.05$ for normal and delayed resp.).

The proportion of fertilised eggs with symmetric cleavage patterns ranged from 62% to 89% overall. No significant differences were found between treatments. However, visually it seems that symmetry was more consequently high when using pre-activated sperm (Figure 11).

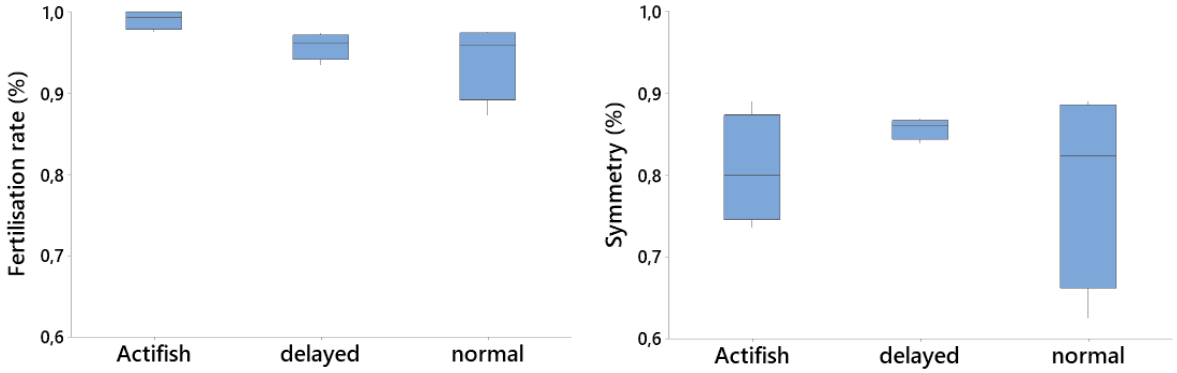

Figure 13. Fertilisation rate (left) and appearance of symmetric cleavage patterns (right) in rainbow trout eggs from one female fertilised using four different males. Fertilisation was achieved using three different activation methods.

Yet again, we saw that fertilisation rates are improved by using Actifish instead of water as the activation medium during fertilisation. We did also see some tendencies towards increased blastomere symmetry when using pre-activated sperm for fertilisation, i.e. selection for long-lived spermatozoa. These results carry some uncertainty due to the low number of observations, but are interesting enough to encourage further studies.

3. Effects of sperm selection on fertilisation rates and embryo survival.

This study was a validation and expansion of the previous fertilisation experiment on rainbow trout, which was performed in the spring of 2019 (above). We wanted to, again, see how fertilisation rates are affected by use of Actifish vs. water as activation medium, and also the use of pre-activated (long-lived) sperm. The study consisted of two parts:

1. A small scale fertilisation trial was performed using eggs from one female and semen from six males to measure the fertilisation rates achieved during "normal", "aided", and "delayed" fertilisation (see previous study).
2. A larger scale experiment evaluating the overall egg production outcome in response to standard fertilisation procedure in water ("normal") vs. the use of pre-activated sperm ("delayed"). Eggs batches from 10 females were each split in two halves and fertilised with one of five males. The eggs were then incubated in 20 separate hatching trays under standard conditions and remaining number of offspring were determined at eying and hatching.

Our previous tests indicated a slight advantage of using Actifish instead of water as the activation medium for both Arctic charr and rainbow trout. The current results, however, show a very clear improvement in fertilisation rates when using Actifish compared to water (one-way paired t-tests $T=2.5$ $p<0.05$ and $T=3.8$ $p<0.05$ for normal and delayed fertilisation resp.). Median fertilisation rates were 98% for Actifish, 45% for water, and 18% for pre-activated sperm in water. The differences in median values are mainly due to the results of two males. Fertilisation with sperm from these three males resulted in 91% to 99% in Actifish, compared to maximum 5% in water. Our interpretation of these result is that the use of a chemically optimised activation medium has the ability to enhance the performance of low quality sperm samples.

No significant difference in either eyeing or hatching success was found between the normal and delayed fertilisation method (paired t-test $p>0.05$). Median values of eyeing success were 93% vs. 76% for normal and delayed fertilisation, and 91% vs. 75% for hatching. Unfortunately, no estimation of fertilisation rate was made for these egg batches, and therefore it is not possible to discern any potential difference in survival between treatments. I.e., if there were a positive effect of sperm selection on offspring viability, it could be masked by lower fertilisation rates in this treatment.

In conclusion, no positive effects of sperm selection could be detected. Therefore, it seems to be more important to the overall success to maximise fertilisation rates. This is efficiently achieved by using chemically optimised activation media (only Actifish tested here).

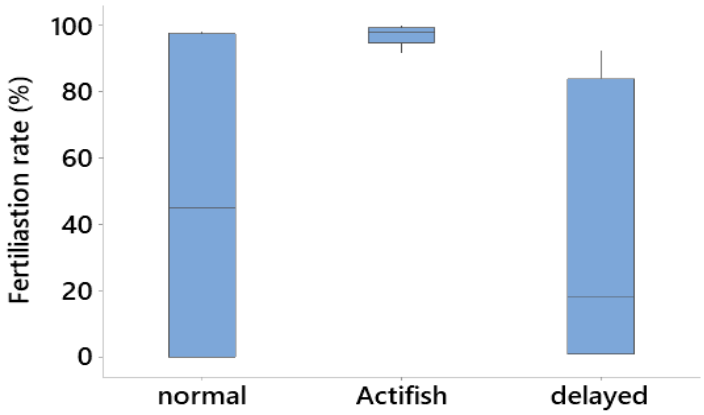

Figure 14. Fertilisation rates in rainbow trout eggs from one female using semen from six different males following three different fertilisation methods – activation in water (normal) and Actifish after mixing eggs and semen, and adding pre-activated sperm (delayed). Boxplots present median (horizontal line), 50% of values (box) and max/min values (whiskers).

Conclusions

Based on the results from the studies presented in this report and the available literature on the topic, we recommended the following treatments and procedures to be followed during artificial reproduction of Arctic charr, rainbow trout and other closely related salmonid species. For a detailed description of the entire procedure, please see appendix 1.

- Rinsing of newly stripped eggs in $NaHCO_3$ before fertilisation can be advantageous by preventing potential negative individual effect of ovarian fluid on sperm performance, especially when single males are used for fertilisation. Rinsing of the eggs is particularly advantageous for egg batches that are not of the highest quality. The presence of leaked cell content from ruptured eggs may otherwise have severe negative effects on fertilisation rates.
- Freshwater should not be used as the activation medium. All other solutions tested in our study resulted in higher fertilisation rates, with no distinct internal differences. However, there are previous studies that report on advantages of using buffered (pH 8.5-9) solutions like D532 compared to pure saline (NaCl) solution, with addition advantages of adding $CaCl_2$ and caffeine.
- We cannot, at this point, recommend application of sperm selection (longevity) as a method to increase offspring viability. Although we did see higher degree of blastomere symmetry in eggs fertilised with pre-activated sperm, we have no data to support any overall positive effects on offspring viability under routine hatchery production. We deem it more likely that fertilisation rates will suffer negative influence from this method, based on our set-up (activation time, egg:sperm ratio, etc.).

Acknowledgements

The compilation of this report and performance of the studies presented herein were achieved through funding from the Swedish government, via the national breeding programs for Arctic charr and rainbow trout, and from the Kolarctic CBC, via the research project Arctaqua.

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Appendix 1: Protocol for artificial fertilisation of Arctic charr

Materials

- Sedation tanks
- Anaesthesia
- Strainers
- Cups
- Fertilisation vessels (bowl/bucket)
- Rinsing solution
- Activation medium
- (Disinfectant e.g., Buffodine + 0.9% NaCl solution)
- Paper (or textile) towels
- Gloves
- Styrofoam boxes w. lids
- Markers and notepad

Preparations

- Rinsing solution: Mix a 1.0% $NaHCO_3$ solution, using clean fresh water.
- Activation medium: mix a ready-to-use solution from stock solution and deionised water (recipes of stock solutions in appendix 2).
- Mix anaesthesia into sedation tank (e.g., Tricaine Methanesulfonate; 2-4 mL stock solution (conc. 25g/L) per litre)
- **NOTE: make sure all solutions are of the same temperature as the rearing tank water!**

Procedure

- Move the fish (a few at a time) straight from the rearing tank to the sedation tank.
- Once sedated, briefly immerse the fish in clean water.
- Pat the genital and surrounding area dry with a towel.
- Strip the eggs from a female into a strainer.
- Rinse the eggs in $NaHCO_3$ solution and move to a clean dry fertilisation vessel.
    - If needed, store cool and covered awaiting fertilisation (e.g. pooling of batches)
- Following the same handling as the female, strip semen from a male into a dry and clean cup
    - If needed, store cool and covered awaiting fertilisation (e.g. pooling of batches)
- To fertilise, add semen from one male to eggs of one female (family production) or semen from min. two males to pooled egg batches (mass production) and give a gentle stir.
- Add activation medium, appr. 1:1 medium to egg volume and let sit for a few minutes.
- Rinse away excess semen and fill vessel with clean water.
- (Alternatively, disinfect eggs in saline disinfectant solution before adding clean water for hardening)
- Let the fertilised eggs harden in vessel for 2-4 hours.
- Gently break apart clusters that may have formed during hardening and transfer eggs to hatchery trays.
- Incubate Arctic charr eggs at 4-7°C during the first week, and then maximum 7°C.

Appendix 2: Recipes for activation media

Molar composition of alternative activation media (ready-to-use solutions):

- Saline: 125 mM NaCl
- D532: 125 mM NaCl, 20 mM Tris, 30 mM glycine; pH 9.0
- D532 + 1mM $CaCl_2$
- D532 + 10 mM Caffeine
- D532 + 1mM $CaCl_2$, 10 mM Caffeine

Molar masses

Tris 121,14 g/mol

Glycine 75,07 g/mol

Caffeine 194,19 g/mol

$CaCl_2$ 110,98 g/mol

NaCl 58,44 g/mol

10X stock solution 1 L (for 10 L ready-to-use solution)

- Saline: 73 g NaCl
- D532: 73 NaCl, 24,23 g Tris, 22,52 g glycine; pH 9.0
    - + 1.11 $CaCl_2$
    - + 19.42 g caffeine[1]

Dissolve dry chemicals of one alternative above in 1 L deionised water.

[1] Solubility of caffeine in water is ~20 g/L at room temperature. Hence, for practical reasons we recommend either lower caffeine dose in stock solution or addition of caffeine in ready-to-use solution.