# A comparison of activating solutions with hatchery water in artificial insemination of rainbow trout (*Oncorhynchus mykiss*)

*Research Article*

**Abstract**

In the propagation of rainbow trout, the available water supply used for incubation of fertilized egg is generally used for also spermatozoa being activated to reach eggs. The aim of the present study was to assess comparisons of the effects of two lab-made activating solutions and hatchery water on progressive sperm motility percentage (%), duration of progressive sperm motility (s), and fertilization success in artificial insemination of rainbow trout. For this purpose, an activating solution (A1) containing 60 mM $NaHCO_3$, 50 mM Tris (pH=9.0) and another activating solution (A2) containing 20 mM Tris, 30 mM glycine, 125 mM NaCl, (pH=9.0), and also hatchery water (HW) were used for activation of spermatozoa and fertilization.The average motility percentages of samples activated by HW, A1 and A2 were observed >90% with no significant differences, while the durations of progressive motility were found to be significantly different as 22.5±0.7 s, 30.0±1.4 s and 30.5±0.7 s respectively. The lowest average fertilization rate (64.6±1.4 %) was obtained using HW, while those values were 89.4±5.1 and 91.3±0.6 % using A1 and A2 respectively. Consequently, both motility durations and fertilization rates obtained by using A1 and A2 were significantly higher than those values of obtained by HW.

**Key Words:** Activating solutions, hatchery water, artificial insemination, rainbow trout

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## Introduction

The reproductive function in teleosts fish is unique in several ways such as wide variation in the amount of spermatozoa produced, the periods during which spermatogenesis occurs differ widely (Billard, 1986). Moreover, in most of them, the sperm cells which are immotile in the testis are activated only after release into their living environment for a short period (from 30 s to several minutes) of forward motility (Scott and Baynes, 1980).

Motility of spermatozoa is the main reason affecting the fertilization success. This motility is generally initiated by osmolality in marine fish and many freshwater fish while spermatozoa of Salmonids and Acipenserids is activated by the low $K^+$ concentration (5 mM) in combination with osmolality. When spermatozoa released and meet with the natural medium (fresh water, sea or brackish water) their motility are limited by some factor such as temperature and pH, ions (Cosson, 2004; Alavi and Cosson, 2005; Alavi and Cosson, 2006). Spermatozoa directly released in the natural medium are subjected to also other factors such as thermic shock, micropolluants, and toxic residues. This stage of reproduction is very susceptible to being damaged (Billard, 1988).

Spermatozoa and eggs are simply put together and available water supply (fresh or salt water) as an external medium is generally added in artificial insemination. Especially in the traditional propagation of freshwater species as a rainbow trout (*Oncorhynchus mykiss*), hatchery water is used for spermatozoa being activated to reach eggs (Billard and Jensen, 1996).

The present investigation was conducted with the purpose of comparing the effects of two lab-made activating solutions and hatchery water on progressive sperm motility percentage (%), duration of progressive sperm motility (s), and fertilization success in artificial insemination of rainbow trout.

## Materials and Methods

### *Fish and collection of the gametes*

Female and male rainbow trout (*Oncorhynchus mykiss*) individuals were obtained from a commercial fish hatchery located in Muğla, Turkey. All fish were fed with the same diet. Gametes were collected during the spawning in December since it is accepted as middle of the spawning season for the region Six males (two years of age) and three females' (three years of age) gametes were obtained by gentle abdominal massage, avoiding any contamination Gamete collection does not harm the fish as a routine application in the fish farm. December is accepted as middle of the spawning season for the region. Both sperm and eggs samples were mixed and pooled samples obtained for each before the experiments.

***Activating solutions, evaluation of the post-thaw motility of the spermatozoa and fertilization***

An activating solution (A1, Billard, 1992) containing 60 mM sodium bicarbonate ($NaHCO_3$), 50 mM Tris pH=9.0 and another activating solution (A2, Lahnsteiner, 2000) containing 20 mM Tris, 30 mM glycine, 125 mM NaCl, pH=9.0, and also hatchery water (HW) were used for activation of spermatozoa and fertilization. These two solutions are commonly used in previous studies. Temperature (11.0±0.1 °C) of activating solutions was adjusted to the same of HW. Percentages of progressive motility (%) and durations of progressive motility (s) were determine under a phase-contrast microscope at 200× magnification immediately while fertilization rates were calculated using a stereomicroscope at 20× magnification eighth days after the insemination. The sperm motility percentages were estimated as the percentage of cells that exhibited progressive forward movement (Horváth, et al., 2003). The durations of motility were determined as the times until forward movement stopped and circular movement began. The percentages of sperm motility were assessed using an arbitrary scale with 10% interval increments in which non-motility was recorded as 0% (modified from Borges, et al., 2005). The evaluation of motility characteristics was performed subjectively by 3 different researchers examining 5 different microscopic fields. Three aliquots of each sample were determined by each researcher, and the average motility characteristics were then calculated and sperm samples were diluted 1:400 with A1, A2 and HW during motility measurements. Fertilization was performed at approximately 300.000:1 sperm-to-egg ratios. To adjust sperm volume, an immobilizing solution (110 mM NaCl, 28.18 mM KCl, 1.22 mM $MgSO_4.7H_2O$, 1.77 mM $CaCl_2.2H2O$, 10.05 mM Bicine, and 9.99 mM Hepes, pH=8.2) was used (Robles,et al.,2003).A1, A2 and HW were added to the eggs in plastic cups (350 ± 20 eggs). Next, the sperm sample was immediately added and the gametes were gently mixed for 60 s. After 5 min to allow fertilization to occur, the eggs were rinsed with HW and incubated for 15 min to water-harden the eggs, and then transferred to hatchery trays supplied with constant water flowing continuously through the system. Eight days after fertilization, at least 100 eggs were taken from each replicate placed in petri dishes containing a clearing solution of acetic acid:methanol:distelled water (1:1:1 v/v/v). After a 10 min, fertilized eggs were distinguished from unfertilized eggs by the presence of a clearly back bone. The fertilization success of a sample obtained from an activating solution was estimated by calculating the percentage of fertilized eggs in relevant replicates (Geffen and Evans, 2000). Fertilization tests were carried out in triplicate for each for each activating solutions.

***Statistical analysis***

All values are represented mean±standard deviation. Statistical differences in the durations of sperm progressive and fertilization rates were tested using one-way ANOVA, following Tukey's HSD post-hoc, at the 0.05 probability level. Statistics were performed using SPSS software version 20.0

**Results and Discussion**

Many teleost fish spermatozoa vary from the spermatozoa of mammalian species in terms of some specific properties such as they are immotile in testis, their motility is activated by releasing into the water, and their progressive motility occurs within minutes (Kime, at al., 2001). The improvement and maintenance of this short duration of motility are critical for determining fertilization success. In general, the percentages of progressive motility (%) and the durations of progressive motility (s) which determined also in this study are the two clearest parameters for assessing sperm quality.

The average sperm density of the pooled samples was calculated as $10.46 \pm 0.50 \times 10^9$ spermatozoa/ml. The effects of different activating solutions (HW, A1 and A2) on the percentage and duration of progressive sperm motility in rainbow trout are shown in Fig. 1. No significant differences ($P > 0.05$) were found in the average motility percentages of samples activated by HW, A1 and A2 ($P > 0.05$) even though a slight increase was noted in motility percentages of A1 and A2 was noted. In contrast, the average motility durations have shown significant differences ($P < 0.05$). The highest motility durations were achieved using A1 and A2 (30.0±1.4 s and 30.5±0.7 s, respectively). Those values were significantly higher than the average motility duration obtained by HW (22.5±0.7 s). Cejko, et al., (2013) compared sperm motility parameters determined by 4 different activating solutions with those values achieved by distilled water in *Cyprinus carpio* sperm. They found some differences among motility parameters, particularly the percentage of motile sperm (82.7%) and percentage of progressively motile sperm (approximately 40%). The highest values were found sperm samples activated by a activating solution containing 100 mM NaCl and 10mM Tris, (pH=9) while other activating solutions were lower pH values and had different contents while they did not report any fertilization rates. In our study, similar to motility durations, significant difference ($P < 0.05$) in the fertilization rates was found between HW and the others (Fig. 2). The lowest average fertilization rate (64.6±1.4 %) was obtained using HW, while those values were 89.4±5.1 and 91.3±0.6 % using A1 and A2 respectively.

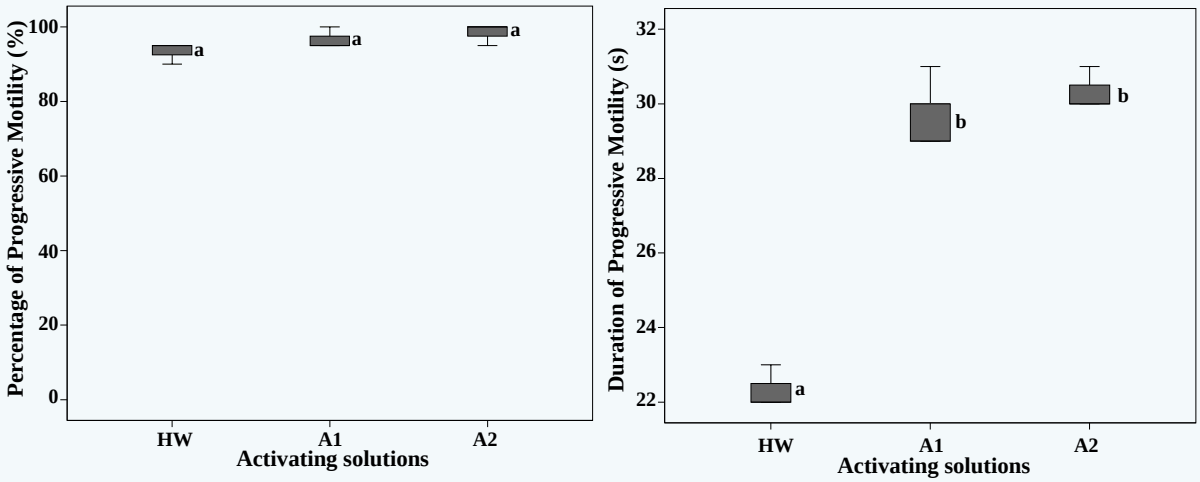


**Figure 1.** The percentage (A) and duration (B) of progressive motility levels in rainbow trout (*Oncorhynchus mykiss*) as a function of different activating solutions. In the box plots, different superscripts indicate the differences ($P < 0.05$).

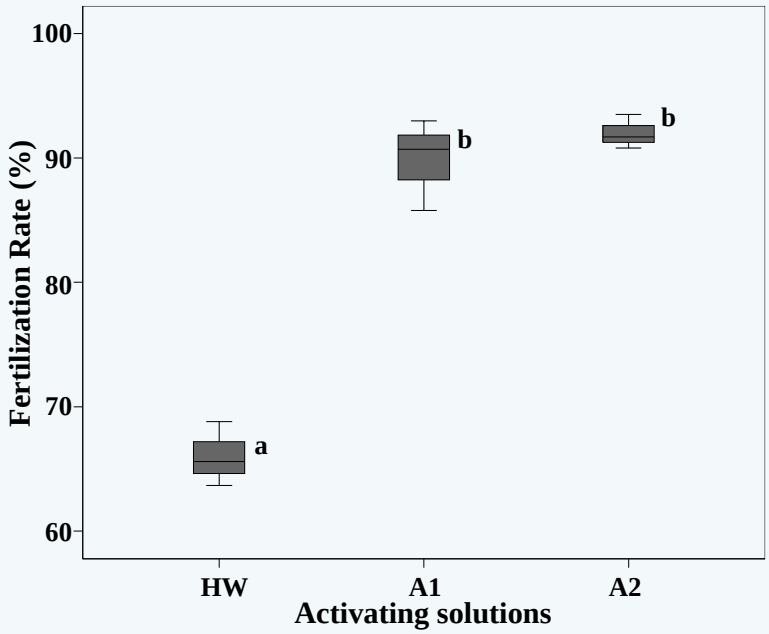


**Figure 2.** Fertilization rates obtained from sperm samples of rainbow trout (*Oncorhynchus mykiss*) activated with different activating solutions. In the box plots, different superscripts indicate the differences ($P < 0.05$).

Sperm activating solutions are beneficial not just for aquaculturist, also are useful also for researchers. Obviously, motility parameters are more precisely determined using with them. The activating solutions used for spermatozoa of fish species living in marine and fresh water have different contents. Even though fish are

living the same environment, the activation solutions could be species depending. Compositions of activating solutions using commonly in activation of spermatozoa of some freshwater fish species were listed in Table 1. As seen in Table 1, osmolality of activating solutions used for freshwater fish are provided by mainly NaCl. Having regard to these studies listed Table 1, the advantages found in our study seem to arise from two eventual reasons; pH and osmolality. Optimum pH and onmolality values are key elements to trigger and improve motility (Cosson, 2004; Alavi and Cosson, 2005; Alavi and Cosson, 2006). Activating solutions has broader buffering capacity than water. Thus, these solutions are very likely to prevent pH alterations emerge from during spermatozoa activation.

## Conclusions

Choosing a proper activating solution can play an extremely useful role in artificial insemination of fish species. Fertilization rates could be increased when the proper activating solutions are used instead of the hatchery water. Besides, preparation of these solutions is usually simple, rapid, and cheap as opposed to common belief. In this study, the effects of two lab-made activating solutions on sperm motility characteristics and fertilization rates were compare with those values obtained by the hatchery water during the artificial insemination of rainbow trout. As with all activating solutions designed for use with relevant fish species, they are in need of much more improvement and study. Moreover, a proper activating solution should be designated for less-studied or new species

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**Table 2.** Compositions and pH levels of activating solutions using commonly in activation of spermatozoa of potential aquaculture freshwater fish species.

| Species | Compositions of Activating Solutions | References |
|---|---|---|
| ***Freshwater fish*** | | |
| *Alburnus alburnus* | 100mM NaCl, 10mM Tris<br>pH 9.0 | Lahnsteiner, et al., 1996 |
| *Chalcalburnus chalcoides* | 50 mM NaCl, 20 mM Tris<br>pH 9.0 | Lahnsteiner, et al., 1999 |
| *Clarias macrocephalus* | ~70 mM NaCl | Vuthiphandchai, et al., 2009 |
| *Cyprinus carpio* | 68 mM NaCl, 50 mM urea<br>pH 7.7<br><br>86 mM NaCl<br><br>5mM KCl, 45mM NaCl, 30mM Tris<br>pH 8.0<br><br>45 mM NaCl,5 mM KCl,30 mM Tris-HCl<br>pH 8.2 | Billard, et al., 1995<br><br>Kucharczyk, et al., 2008<br><br>Perchec, et al., 1996<br><br>Li, 2013 |
| *Danio rerio* | 40 mM NaCl, 20 mM HEPES<br>pH 8.5 | Ingermann, et al., 2011 |
| *Oncorhynchus mykiss* | 125 mM NaCl, 0.1 $CaCl_2$, 30 Tris<br>pH 8.5<br><br>125 mM NaCl, 1 mM $CaCl_2$, 20 mM Tris, 30 mM glycine<br>pH 9.0<br><br>140 mM NaCl<br>pH 9.0 | Cosson, et al., 1995<br><br>Billard, 1977<br><br>Ubilla and Valdebenito, 2011 |
| *Sander lucioperca* | 120 mM NaCl<br><br>119 mM $NaHCO_3$ , 0.5% BSA | Jarmolowicz, et al., 2010<br><br>Cejko, et al., 2008 |
| *Silurus glanis* | 17 mM NaCl, 5 mM Tris–HCl<br>pH 8.0 | Linhart, et al., 2005 |

*BSA; bovine serum albuminn