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Effects of different stripping methods of female and activation medium on fertilization success in northern pike (*Esox lucius*)

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ABSTRACT: In this study, the quality of northern pike eggs collected by the traditional method (hand stripping) and the pneumatic method (air stripping) was compared. Additionally, different activation solutions (Billard solution, hatchery water, and Woynarovich solution) were tested for their effects on egg fertilization under artificial conditions. After the eggs were collected, the Pseudo-Gonado-Somatic Index (PGSI) was measured. Although the values of the PGSI in the samples obtained with use of air stripping were lower (13.8 ± 3.9%), they did not differ statistically from those obtained by hand stripping (16.5 ± 5.4%). Hatchery water and Woynarovich solution were found to be the most suitable solutions for sperm activation using the Computer-Assisted Sperm Analysis (CASA) system as compared to the Billard solution. Hand stripping was found to negatively affect the percentage of fertilized eggs and the percentage of hatched larvae in samples fertilized in hatchery water and Woynarovich solution. When the traditional method of egg collection was used, there were no differences in the percentage of fertilization and the percentage of hatched larvae between Billard solution (54.0 ± 21.5% and 44.1 ± 21.9%, respectively), hatchery water (60.0 ± 22.5% and 55.9 ± 22.8%, respectively), and Woynarovich solution (72.0 ± 25.8% and 69.0 ± 23.9%, respectively) treatments. Air stripped eggs showed a higher fertilization rate when hatchery water or Woynarovich solution was applied (86.2 ± 9.3% and 92.4 ± 3.9%, respectively). Also hatching rate was the highest in these samples (83.0 ± 8.4% and 88.3 ± 6.2%). The application of the pneumatic method and Woynarovich solution for northern pike artificial fertilization resulted in higher fertilization and hatching rates as compared to other techniques. Because this was successful in northern pike, the use of air stripping is a promising option for artificial reproduction in other fish species.

Keywords: air stripping; fish; eggs; sperm; Woynarovich solution

INTRODUCTION

Northern pike (*Esox lucius*) broodstocks are usually caught in lakes or rivers and then transported to a hatchery, where the fish are kept in captivity until they reach sexual maturity. The traditional method of collecting northern pike eggs is called hand stripping and is based on manually massaging the abdomen of the female to strip off the ova. In salmonid fish, this procedure can result in

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broken eggs, which decreases the pH of ovarian fluid and releases egg yolk, both of which negatively affect egg fertilization (Dietrich et al. 2007). One successful approach to reduce the negative effects derived from broken eggs is to use saline solution for fertilization (Wojtczak et al. 2007). These solutions not only prevent from clothing caused by egg yolk released from broken oocytes which might block the micropyle of eggs, but also prolong the time during which eggs can be fertilized due to reduced osmotic shock.

Pneumatic methods of egg collection were first applied in Australia in 1957 (Zurbuch 1965). The method is based on the injection of gases (air, nitrogen or oxygen) into the body cavity to expel eggs using gas pressure. This technique has been successfully applied without any negative side effects in salmonid species and is quicker and easier for both the females and the stripper (Zurbuch 1965). To date, this method has only been used for species that ovulate eggs into the body cavity (e.g. salmonids).

The principal aim of this study was to test the usefulness of the air stripping method in pike reproduction, and to determine whether the hand stripping method can increase egg quality. Moreover, we tested the effects of saline-based solutions on the fertility of eggs obtained after hand and air stripping compared to fresh hatchery water.

MATERIAL AND METHODS

Wild spawners (age 3–5 years, body weight 0.5–2 kg) were caught in Dgał Wielki Lake using gillnets immediately before spawning (3–9 days prior to the experiment). After catching, the males and females were separated and placed in separate lake cages with a volume of about 1 m^3 in the Department of the Sturgeon Research hatchery in Pieczarki (northeastern Poland). Males were recognized by releasing sperm by gentle abdominal pressure and females by the shape of the abdomen and urogenital region. The water temperature in the holding cages was maintained in the range of 7–10°C. The final maturity of the females was checked every 3 days. Mature females (in which eggs flowed under gentle abdominal pressure) were taken for artificial reproduction. Fish were not subjected to hormonal treatment; only fish (male and female) which matured naturally were used in these experiments. Before manipulations, fish were anaesthetized using 2-phenoxyethanol (Sigma-Aldrich, St. Louis, USA) at 0.5 ml/l.

Application of traditional (hand stripping) and pneumatic (air stripping) methods for northern pike egg collection. For this experiment, only fish in which eggs were freely released from the ovary and had a regular shape (same size) and colour (no transparent eggs in the batch) were selected. Eggs from sixteen females were stripped using the traditional method (hand stripping, $n = 8$) or the pneumatic method (air stripping, $n = 8$) (Figure 1) and then separated into dry plastic bowls. Immediately after collection, each egg sample was subjected to fertilization. Sperm for fertilization was collected about 10 min before spawning started and stored on crushed ice. To hand strip the eggs, the fish was held slightly on her side, tail down; gentle hand pressure was applied to the abdomen, moving toward the vent. The stream of eggs was directed into a clean dry bowl positioned so that water from the fish did not drip onto the eggs. For the pneumatic method, a 0.8 mm diameter needle

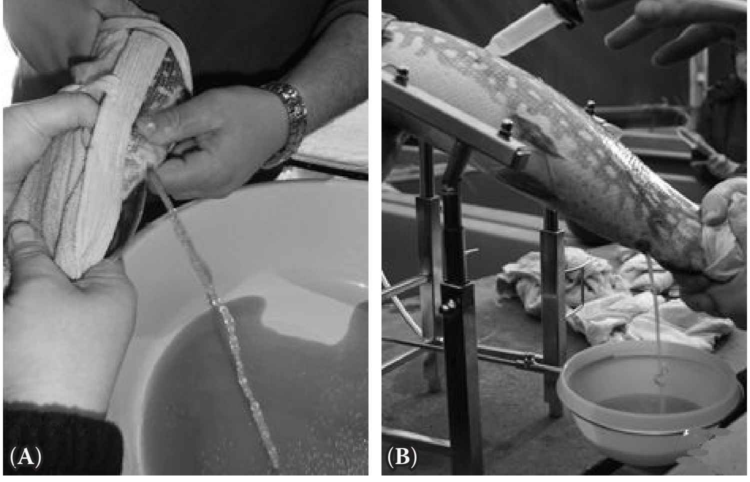

Figure 1. Different methods of northern pike *Esox lucius* L. egg collection: traditional (hand stripping) (**A**), pneumatic (air stripping) (**B**)

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was inserted between the pectoral and pelvic fins, and air was pumped in under a pressure of 0.5 Pa. The air flow (at 0.5 l/min) gently pushed the eggs from the ovary to be collected. Both air pressure and air flow were previously adjusted and tested to achieve best efficiency and avoid fish mortality (data not shown). After the eggs were collected using either method, the Pseudo-Gonado-Somatic Index (PGSI = weight of eggs/weight of fish prior to spawning × 100%) was measured and calculated. The pH of the eggs was measured using an Orion Star 5 m equipped with an Orion Ross Ultra electrode (Thermo Scientific, Waltham, USA). Since northern pike eggs contain only a minute level of ovarian fluid, separating the eggs from the ovarian fluid was impossible. Therefore, the pH was measured in the total volume of collected eggs, including ovarian fluid. Spawning all individuals took about 30 min.

All experimental procedures were approved by the Local Animal Ethics Committee in Olsztyn, Poland (No. 24/2011/N of 30/03/2011 from 2011 to 2016).

Effects of Billard solution, hatchery water, and Woynarovich solution on northern pike sperm motility. Sperm were collected by gentle massage of the abdominal region of male pike (n = 4). During sperm collection, special care was taken to avoid contamination with urine, faeces or blood. Sperm samples were activated using one of three solutions: Billard solution containing 125 mM NaCl, 20 mM Tris buffer, 30 mM glycine, and 1 mM $CaCl_2$ at a pH of 9.0 and 350 mOsm/kg; hatchery water (HW) at a pH of 7.4 and 10 mOsm/kg; or Woynarovich solution containing 68 mM NaCl and 50 mM urea at a pH of 7.7 and 180 mOsm/kg. To prevent sperm samples from sticking to the microscope slide, each solution contained 0.5% bovine serum albumin (BSA).

A Computer-assisted Sperm Analysis (CASA) system was used to determine the percentage of motile sperm (MOT, %), sperm with progressive motility (PRG, %), curvilinear velocity (VCL, μm/s), average path velocity (VAP, μm/s), straight linear velocity (VSL, μm/s), amplitude of lateral head displacement (ALH, μm), movement linearity (LIN, %; LIN = VSL/VCL × 100 %), and beat cross frequency (BCF, Hz). To measure CASA parameters, 1 μl of each sperm sample was diluted in 9 μl of simple immobilizing solution at pH 9.5 containing 130 mm NaCl, 40 mM KCl, 3.3 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 2.5 mM $NaHCO_3$ (Kobayashi et al. 2004). Next, 1 μl of sperm suspension was activated with 25 μl of the selected solution and placed on a Teflon-coated slide with 12 wells 30 μm in depth and 5 mm in diameter (Tekdon Inc., Myakka City, USA). Recordings were made approximately 6 s after sperm activation with the use of a Basler 202K digital camera (Basler, Ahrensburg, Germany) integrated with an Olympus BX51 microscope (Plan FL N 20X/0.5 NH ph1 lens; Olympus, Tokyo, Japan). The recording speed was 46.6 frames per s and 200 frames from each recording were analyzed using CRISMAS software (Image House Ltd., Copenhagen, Denmark) to measure sperm motility parameters. Sperm motility was measured twice and the averages of each set of sperm samples and each CASA parameter were calculated based on two measurements. During CASA analysis, the semen samples were kept on ice at 4°C.

Effect of Billard solution, hatchery water, and Woynarovich solution on the fertilization of northern pike eggs collected using the traditional (hand stripping) and pneumatic (air stripping) methods. For fertilization, samples of eggs (approximately 100 eggs in each sample) were collected after using the traditional (n = 8) and pneumatic (n = 8) collection methods. Next, samples of sperm taken from males (n = 4) were pooled and added to the egg samples in a suboptimal ratio of 200 000 sperm to one egg, assuring high fertility (Kowalski, unpublished data). Sperm concentrations (range 13.9–20.2 × 10^9/ml) had previously been measured using a Bürker chamber after sperm solutions were diluted 1 : 2000 with 0.7% NaCl (Sigma Aldrich). Five ml of Billard solution, HW, and Woynarovich solution were used as fertilizing solutions for eggs collected using the traditional and pneumatic methods. Ten minutes after fertilization, the egg samples were carefully washed twice in HW. The samples were then submerged in HW and incubated in an experimental incubation unit separately for each treatment and replication at a temperature of 12°C. After incubation, the percentage of fertilized eggs and, later, the percentage of hatched eggs were calculated for each treatment.

Statistical analysis. The PGSI, pH of eggs, and CASA parameters were calculated using arithmetic means and standard deviations. For the CASA parameters, the differences between values observed with the different activation solutions used for northern pike sperm activation and fertilization were determined by one-way analysis of variance

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(ANOVA) and *post-hoc* Tukey's tests. The data showed a normal distribution, as required for these procedures, and percentages were transformed using the arcsin function. Differences between the PGSI and the pH of eggs after using the different solutions were determined by the non-parametric Mann-Whitney test. Two-way analysis of variance (ANOVA) was used to test the significance of differences between the percentage of fertilized eggs and the percentage of hatched larvae obtained from hand-stripped and air-stripped eggs in the three different sperm activation media. Statistical analysis was carried out using GraphPad Prism software (Version 6.02).

RESULTS

Efficiency of the traditional (hand stripping) and pneumatic (air stripping) methods of northern pike egg collection. The PGSI values observed after hand stripping (16.5 ± 5.4%) and air stripping (13.8 ± 3.9 %) of eggs did not differ significantly ($P > 0.05$). Female survival rate after egg collection by hand stripping and air stripping was 100% using both methods.

Effects of Billard solution, hatchery water, and Woynarovich solution on northern pike sperm motility. The percentage of motile sperm in HW and Woynarovich solution used for northern pike sperm activation was greater than 60%, which was significantly higher than the percentage of motile sperm using Billard solution (35%; $P < 0.05$; Table 1). Motility duration was the longest ($P < 0.001$) when Woynarovich solution (Woynarovich and Woynarovich 1980) was applied (124 ± 15 s), and the lowest in HW (41 ± 19 s). There were no differences in PRG values between the solutions used for sperm activation. The VCL value was lower in Billard solution than in HW. The highest VAP was observed when sperm were activated with HW (132.5 ± 19.1 μm/s). There were no differences in VSL values between solutions used for sperm activation. The ALH value was the lowest ($P < 0.05$) in samples activated with Billard solution. The linearity of movement was lower ($P < 0.05$) in HW if compared to Billard solution. There were no differences recorded between the values of BCF in all samples.

Effects of Billard solution, hatchery water, and Woynarovich solution on northern pike fertilized eggs collected using the traditional (hand stripping) and pneumatic (air stripping) methods. Two-way analysis of variance (ANOVA) indicated that the effect of diluents was significant ($F = 22.87$; $P < 0.05$) as well as the effect of stripping method ($F = 18.99$; $P < 0.05$) when eyed stage percentage was used as dependent variable. There were no interactions between both factors ($P = 0.32$). The effect of diluent was not significant ($F = 21.21$; $P = 0.22$) when hatching rate was used as dependent variable. However, the stripping method showed high influence ($F = 25.21$; $P < 0.001$) on hatching results. No interaction was found when hatching rate was used as dependent variable ($F = 1.22$; $P = 0.67$). The percentage of fertilized eggs and hatched larvae after using Woynarovich solution as a fertilization medium was higher than that observed

Table 1. Computer-assisted Sperm Analysis (CASA) parameters of the northern pike *Esox lucius* L. recorded in Billard solution, hatchery water, and Woynarovich solution. Data are means ± SD

CASA parameter	Activation solution		
	Billard	hatchery water	Woynarovich
MOT (%)	34.7 ± 23.1^a	68.3 ± 32.1^b	62.7 ± 35.2^b
PRG (%)	4.6 ± 4.3^a	3.0 ± 3.0^a	5.2 ± 4.9^a
VCL (μm/s)	102.4 ± 12.1^a	159.3 ± 27.9^b	140.0 ± 42.9^{ab}
VSL (μm/s)	53.6 ± 18.5^a	59.9 ± 12.3^a	56.8 ± 17.7^a
VAP (μm/s)	83.3 ± 19.6^a	132.5 ± 19.1^b	105.5 ± 32.2^c
ALH (μm)	0.8 ± 0.1^a	1.7 ± 0.3^b	1.3 ± 0.4^b
LIN (%)	54.6 ± 11.0^a	37.1 ± 1.7^b	41.3 ± 7.8^{ab}
BCF (Hz)	10.6 ± 2.5^a	11.2 ± 1.1^a	11.3 ± 1.5^a

MOT = sperm motility, PRG = sperm progressive motility, VCL = curvilinear velocity, VSL = straight linear velocity, VAP = average path velocity, ALH = amplitude of lateral head displacement, LIN = movement linearity, BCF = beat cross frequency

$^{a–c}$values with different letters indicate significant differences between groups ($P < 0.05$)

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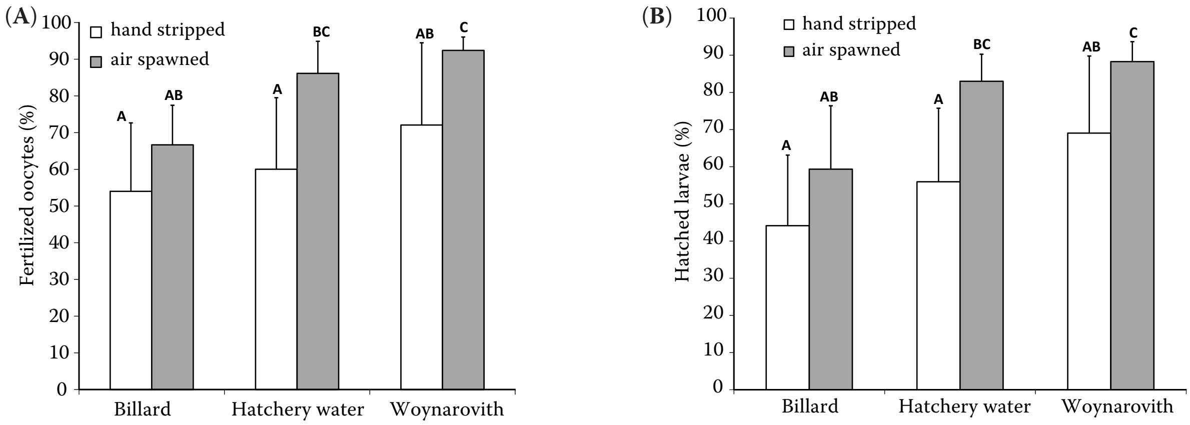

Figure 2. Percentage of fertilized eggs (**A**) and percentage of hatched larvae (**B**) of northern pike *Esox lucius* L. collected with traditional (hand stripping) and pneumatic (air stripping) methods after fertilization in Billard solution, hatchery water (HW), and Woynarovich solution

$^{A–C}$different letters indicate significant differences between groups ($P < 0.05$)

after fertilization using Billard solution (Figure 2). The percentage of eggs fertilized and hatched after the application of HW showed intermediate values and was comparable to the results obtained with both Billard and Woynarovich solutions. When the traditional method of egg collection was used, there were no differences in the percentage of fertilization and percentage of hatched larvae using Billard solution (54.0 ± 21.5% and 44.1 ± 21.9%, respectively), HW (60.0 ± 22.5% and 55.9 ± 22.8%, respectively), and Woynarovich solution (72.0 ± 25.8% and 69.0 ± 23.9%, respectively) (Figure 2).

DISCUSSION

In the present study, we have demonstrated that the pneumatic method of egg collection (air stripping) results in higher percentages of fertilized eggs and hatched larvae compared to the traditional method (hand stripping). The combination of collecting eggs by air stripping and fertilizing eggs in Woynarovich solution is the most promising option for northern pike artificial reproduction.

The current results indicated no differences between the quantities of eggs collected using the traditional and pneumatic methods. Despite the lack of differences between the pH of eggs among the groups, we found that eggs collected with the pneumatic method had a higher fertilization rate. In northern pike, gentle abdominal massage is commonly used for egg collection, and a small volume of ovarian fluid is observed (Billard and Marcel 1980). Therefore, in this species, eggs may be particularly susceptible to damage as a result of manual massaging of females during egg collection. In salmonid species, in which ovarian fluid constitutes 10–30% of egg mass, broken eggs decrease the pH of ovarian fluid and reduce egg quality (Dietrich et al. 2007). It is plausible that northern pike eggs might also be sensitive and hand stripping can decrease their quality, but the pH of eggs is not an efficient diagnostic tool in this species.

In northern pike, the maximum percentage of motile sperm was noted in the solution with osmolality values ranging from 125 to 235 mOsm/kg (Alavi et al. 2009). Our results indicate that northern pike sperm motility parameters such as MOT, VAP, and ALH were significantly higher when Woynarovich solution (180 mOsm) was used to activate sperm, compared to Billard solution (350 mOsm). Using HW (10 mOsm) to activate sperm gave the highest VAP value, but the other parameters were similar to those measured when Woynarovich solution was used. Previous studies on salmonid fish have indicated that higher values of sperm velocity are correlated with a higher fertilization success rate (Gage et al. 2004). As HW quality and composition vary between locations, it is reasonable to advise the use of Woynarovich solution when artificial fertilization of pike eggs is concerned.

In our study, eggs that were air stripped had the highest fertilization rate and percentage of hatched larvae. Moreover, Woynarovich solution was found to be the most effective defined fertilization solution for northern pike reproduction. Currently, HW is the most commonly used fertilization solution (Szabo

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2003); however, buffered solutions, such as a simple saline solution (100 mM NaCl, 10 mM Tris, pH 9.0), are also used in hatcheries for northern pike sperm activation and to increase the fertilization rate of eggs (Lahnsteiner et al. 1998; Zhang et al. 2010). It is worth noting that Woynarovich solution was successfully used as a fertilization solution not only for the fertilization of cyprinid fish eggs, but also for those of percid fish. In Eurasian perch (*Perca fluviatilis*), Woynarovich solution was found to be the most suitable medium for the controlled insemination of eggs, compared to Billard solution and HW (Zarski et al. 2012). Our results corroborate these data as northern pike eggs had a higher fertilization rate when Woynarovich solution was used as compared to Billard solution.

The efficiency of egg collection using the traditional (hand stripping) and pneumatic (air stripping) methods was similar, but the egg quality, as measured by the fertilization rate, was higher in samples obtained with the pneumatic method. Moreover, the Woynarovich solution was found to be effective as a sperm and egg activator, providing a promising option for artificial pike reproduction. Although HW led to similar fertilization results as those obtained with Woynarovich solution, using a defined solution is more predictable in terms of the final outcome than the use of HW which might vary depending on the source. Further studies should focus on egg quality and damage to eggs collected using the traditional and pneumatic methods and establish quality indicators to allow for an early assessment of egg condition.

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