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Improvement of common carp artificial reproduction using enzyme for elimination of egg stickiness

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Abstract

This study summarizes optimization of techniques for common carp artificial propagation including improvements of activation solution (AS), the process of insemination, and elimination of egg stickiness. The optimum gamete ratio for good fertilization and hatching rate ranged from 8490 to 23 672 spermatozoa per egg, when dechlorinated tap water was used. Optimal ratio between eggs (weight in g) and AS (in ml) was defined as 1:1 to 1:2. Different concentrations of AS such as NaCl from 0 to 34 mM (0–68 mOsmol kg^{-1}) did not change fertilization and hatching rates. An AS adopted for carp spermatozoa (45 mM NaCl, 5 mM KCl, 30 mM Tris–HCl, pH 8) was compared with other saline AS; only the 51 mM (102 mOsmol kg^{-1}) NaCl solution decreased fertilization and hatching rate. The AS containing 20 mM Tris–HCl at pH 9 increased fertilization and hatching rates compared to dechlorinated tap water of pH 7 or to AS of pH 6 and 7. Adhesiveness from the eggs was successfully removed by incubation in Alcalase DX (PLN 04715) using two successive steps with different enzyme concentrations. The first step with an enzyme concentration of 2 ml l^{-1} was applied from 8 to 20 min after fertilization. Later in a second step, the best time for application of alcalase enzyme at a concentration of 20 ml l^{-1} was for 45 and 60 s at 20 min after fertilization leading to fertilization and hatching rates of 80–87%. The α-Chymotrypsin (EC 3.4.21.1. Merck) was also found effective for elimination of stickiness. Results with α-Chymotrypsin enzyme indicate that the response to success in elimination of stickiness is highly variable mainly due to differences in the environment, quality of water and carp strains.

Résumé

Amélioration de la reproduction artificielle chez la carpe en utilisant des enzymes pour l'élimination de la viscosité des œufs. Cette étude résume l'optimisation des techniques pour la fécondation artificielle chez la carpe, y compris les améliorations des solutions d'activation, du processus d'insémination, et d'élimination de la viscosité des œufs. Pour obtenir de bons taux de fertilisation et d'éclosion, le rapport optimal se situe entre 8490 et 23 672 spermatozoïdes par œuf, avec de l'eau du robinet déchlorée. Le rapport optimal entre les œufs (poids en grammes) et la solution d'activation (en millilitres) est définie comme étant de 1:1 à 1:2. Différentes concentrations de solutions d'activation, telles que NaCl de 0 à 34 mM (0 à 68 mOsmol kg^{-1}) ne modifient pas les taux de fertilisation et d'éclosion. Une solution d'activation adoptée pour les spermatozoïdes de carpe (45 mM NaCl, 5 mM KCl, 30 mM Tris–HCl, pH 8) est comparée à d'autres solutions salines d'activation ; seule, la solution NaCl de 51 mM (102 mOsmol kg^{-1}) diminue le taux de fertilisation et d'éclosion. La solution d'activation contenant 20 mM Tris–HCl à pH 9 augmente les taux de fertilisation et d'éclosion, comparée à l'eau du robinet déchlorée de pH 7 ou à une solution d'activation de pH 6 et 7. La viscosité des œufs est éliminée avec succès, par incubation dans l'alcalase DX (PLN 04715) avec deux étapes successives et différentes concentrations d'enzyme. La première étape, avec une concentration d'enzyme de 2 ml l^{-1} a été appliquée de 8 à 20 min après fertilisation. Dans une seconde étape, le meilleur temps d'application de l'alcalase, 20 ml l^{-1}, a été de 45 et 60 s à 20 min après fertilisation, conduisant à des taux de fertilisation et d'éclosion de 80–87 %. La α-chymotrypsine (EC 3.4.21.1. Merck) a été aussi efficace pour l'élimination de la viscosité des œufs mais avec des chances de succès beaucoup plus variables, dues aux différences environnementales, à la qualité de l'eau et aux différentes souches de carpe.

Keywords: Reproduction; Insemination; Aquaculture; Enzyme; Common carp; *Cyprinus carpio*

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1. Introduction

Controlled reproduction of common carp started in the 1950s, after establishing practical application of technologies for artificial propagation (Woynarovich, 1962; Woynarovich and Woynarovich, 1980; Rothbard, 1981; Horvath et al., 1984). Various studies have reviewed the physiology of carp sperm (Billard et al., 1986, 1995), eggs and fertilization (Linhart et al., 1995), endocrine control of gametogenesis and induction of spawning (Yaron, 1995) and artificial insemination Saad and Billard (1987), Billard (1988, 1990) and Billard et al. (1995).

Procedures of artificial insemination and incubation were proposed by Woynarovich (1962). The method was based upon treating gametes in various solutions. Eggs and sperm were mixed in an activating solution of 4 g NaCl and 3 g urea per l of water. Adhesiveness was removed by continuous stirring for 1.5 h in the same solution. A tannic acid treatment lasted for 20 s. This technique was later improved (Woynarovich and Woynarovich, 1980) by the use of a second solution of 4 g NaCl + 20 g urea per l, which reduced the treatment time. Soin (1976) published other modifications including the use of Milk for desticking. Khan et al. (1986) reported that full cream milk powder (20–25 g l^{-1}) was the most efficient in removing the adhesiveness of eggs and it gave the best results in terms of fertilization and hatching rates compared to the methods of Woynarovich. The main hatcheries in central Europe have applied these modifications currently to produce 100–200 millions of sac fry. Two to three minutes after fertilization, the eggs are placed to a Zuger jar with milk, and the content is kept mixing by air bubbling for 60 min. Then running water is introduced to rinse the milk during 10 min (Billard et al., 1995).

The present work was undertaken to re-examine various procedures for artificial insemination in common carp, *Cyprinus carpio*. The objectives of this study were to enhance fertilization and hatching rates through optimization of activation solution (AS), process of insemination, activation of gametes and elimination of eggs stickiness.

2. Materials and methods

2.1. Broodstock handling and gametes collection

The reproduction and culture of common carp was carried out at the Department of Fish Genetics and Breeding, RIFCH, University of South Bohemia at Vodnany in the Czech Republic in 2001–2002. Five- to 7-year-old male and female broodstock were maintained in separate ponds until used in the experiments. Broodfish suitable for stripping were selected in May and stocked separately in the hatchery in 4 m^3 tanks with water flow rate of 0.2 l s^{-1}, temperature of 18–22 °C and 6–7 mg l^{-1} O_2.

For each experiment, 12 males were injected with carp pituitary (CP) at 1 mg kg^{-1}, 24 h before stripping at 21 °C. Sperm was collected individually from each male, kept in thin layer in cell culture vessels under aerobic condition at 0 °C and proportionally pooled from five males prior to fertilization.

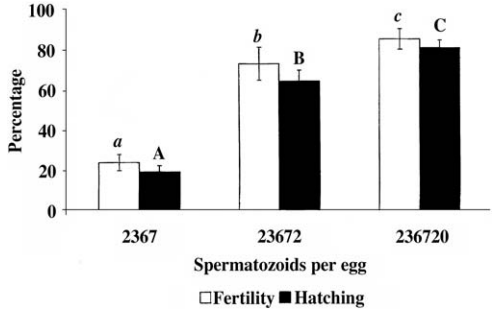

Fig. 1. The effect of increasing number of spermatozoa (2367–236 720 spermatozoa) per egg on fertilization and hatching rates with dechlorinated tap water. Groups with a common superscript do not differ significantly ($P < 0.05$).

Eight females were used for each experiment and injected with CP at the dose of 0.4 mg kg^{-1} and then at 2.1 mg kg^{-1}, 24 and 12 h before stripping, respectively. The females were checked every 3 h after the second injection for ovulation, and stripped into separate dishes; the best egg batches were selected. Ova were stored under aerobic conditions at 17–19 °C for 2–4 h prior to artificial insemination (Rothbard et al., 1996). The eggs of at least four females were proportionally pooled just prior to conducting experiments.

Prior to the fertilization experiments with sperm pooled sperm, sperm concentration was counted with Thoma cell hemocytometer under Olympus microscope BX 41 (400×) and mean number was expressed per 20 squares of Thoma cell. Three batches of approximately 0.3 g (around 200 eggs) of unfertilized eggs were weighed to the nearest 0.0001 g, and then fixed in 4% formaldehyde for later counting and determination of mean egg weight. Number of eggs was then calculated in each experiment from the weight of eggs and

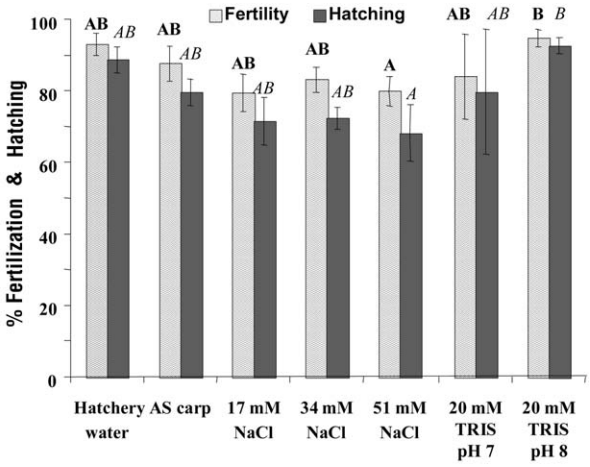

Fig. 2. Fertilization and hatching rates after artificial insemination in dechlorinated tap water; in solutions with increasing NaCl concentration; in buffered water pH 7 and 8 of 20 mM Tris–HCl; and in AS for carp (45 mM NaCl, 5 mM KCl and 30 mM Tris–HCl, pH 8; Saad and Billard, 1987). Sperm was used on the level of 13 000 spermatozoa per egg. Groups with a common superscript do not differ significantly ($P < 0.05$).

expressed as number of eggs per gram of eggs. Before injection and gamete collection, the males and females were anesthetized in a solution of 2-phenoxyethanol (1:1000).

2.2. Determination of optimal sperm/egg ratio (Experiment 1)

The quantity of sperm per egg for fertilization was 2367, 23 672 and 236 720 spermatozoa per egg, what represented 10, 100 and 1000 µl of sperm, respectively. Five grams of eggs with 586 eggs per 1 g were placed into a dish of 50 ml and accurate volume of sperm (10, 100 and 1000 µl of sperm) with estimated number of spermatozoa was dropped by micropipette. Before activation of eggs with water, seminal plasma was added at volumes of 990, 900 and 0 µl into experiments with 10, 100 and 1000 µl of sperm, respectively. Seminal plasma was made free of spermatozoa by means of centrifugation at 2500 × *g* for 5 min at room temperature. Then the dish was placed on shaking table with constant rotation 200 min^{-1}, 10 mm deflection and 5 ml of dechlorinated tap water at 22 °C was added. Two minutes later, approximately 200–300 fertilized eggs were placed with replication (four times) into a special incubator cage of 200 ml supplied with UV-sterilized recirculated dechlorinate tap water at 22 °C, 9 mg l^{-1} O_2.

2.3. Effects of different activation solutions (AS) (Experiment 2)

Five grams of eggs with 770 eggs per 1 g were placed into a dish of 50 ml and accurate volume of sperm with 13 000 spermatozoa per egg was dropped by micropipette. Then the dish was placed on shaking table with constant rotation 200 min^{-1}, 10 mm deflection and 5 ml of different AS at 22 °C were tested. Solutions differing with NaCl concentration (0, 17, 34 and 51 mM), solutions with 20 mM Tris–HCl of pH 7 and/or 8, then dechlorinated tap water as control and carp AS (45 mM NaCl, 5 mM KCl and 30 mM Tris–HCl, pH 8, Saad and Billard, 1987) were also used. Two minutes later, approximately 200–300 fertilized eggs were placed with replication (four times) into a special incubator cage of 200 ml supplied with UV-sterilized recirculated dechlorinate tap water at 22 °C, 9 mg l^{-1} O_2.

2.4. Determination of pH in activation solution (Experiment 3)

Five grams of eggs with 885 eggs per 1 g were placed into a dish of 50 ml and two levels of sperm quantity per egg were dropped by micropipette. The importance of AS pH on fertilization was more clearly defined under conditions of low numbers of sperm (4500) per egg together with the first control. On the other hand, the capacity of eggs to be fertilized was maximized with high number of spermatozoa (2 250 000 per egg) in repeated controls 2 and 3 (Fig. 3). Then the dish was placed on a shaking table; the rotation rate was 200 min^{-1} with a 10 mm deflection. Solutions of 5 ml of

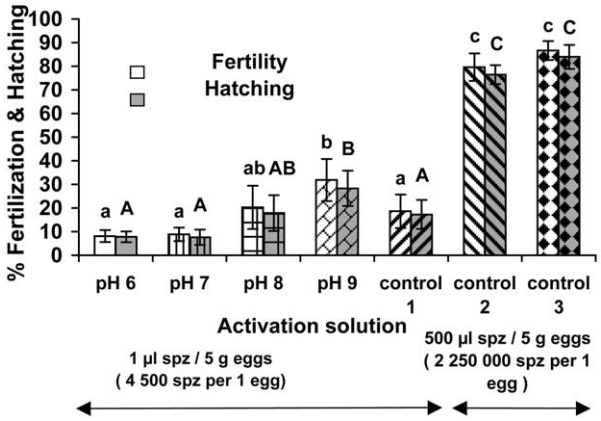

Fig. 3. Fertilization and hatching rates after artificial insemination with different pH of AS containing 20 mM Tris–HCl, dechlorinated tap water with concentration 4500 spermatozoa per egg. Sperm was used on two concentration levels, 4500 and 2 250 000 spermatozoa per egg. Groups with a common superscript do not differ significantly ($P < 0.05$).

20 mM Tris–HCl at 22 °C, were tested at different pH of 6, 7, 8 and 9 and with three controls. Controls were activated with dechlorinated tap water. Two minutes later, approximately 200–300 fertilized eggs were placed with replication (four times) into a special incubator cage of 200 ml supplied with UV-sterilized recirculated dechlorinate tap water at 22 °C, 9 mg l^{-1} O_2.

2.5. Effect of different ratio of AS volume and egg quantity (Experiment 4)

Five grams of eggs with 885 eggs per 1 g were placed into a dish of 50 ml and accurate volume of sperm with 8490 spermatozoa per egg was dropped by micropipette. Then the dish was placed on shaking table with constant rotation 200 min^{-1}, 10 mm deflection and accurate volume of 1.66 ml (1:0.33), 2.5 ml (1:0.5), 5 ml (1:1), 10 ml (1:2) and 15 ml (1:3) of dechlorinated tap water 22 °C were added. Two minutes later, approximately 200–300 fertilized eggs were placed with replication (four times) into a special incubator cage of 200 ml supplied with UV-sterilized recirculated dechlorinate tap water at 22 °C, 9 mg l^{-1} O_2.

2.6. Application of enzymes α-Chymotrypsin (EC 3.4.21.1. MERCK) and Alcalase DX (PLN 04715) for elimination of egg stickiness (Experiment 5)

Initially, seven proteolytic and polysaccharide enzymes were tested for ability to effectively eliminate egg stickiness. The α-Chymotrypsin (EC 3.4.21.1. MERCK) and Alcalase DX (PLN 04715) were selected as effective ones and also as less expensive enzymes, which could be used in practical condition.

Twenty grams of eggs with 567 eggs g^{-1} were placed into a dish of 250 ml and accurate volume of sperm with 140 130 spermatozoa per egg was dropped by micropipette. Then the dish was placed on shaking table with constant rotation 200 min^{-1}, 10 mm deflection and 20 ml of dechlorinated tap water at 22 °C was added.

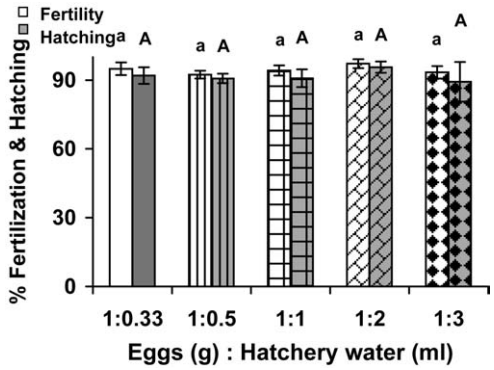

Fig. 4. Fertilization and hatching rates using different ratio of volume of hatchery water to weight of eggs with the concentration 8490 spermatozoa per egg. Groups with a common superscript do not differ significantly ($P < 0.05$).

At 2, 4 and 6 min after activation, redundant water was discarded and additional 20 ml hatchery water was added. Fifty milliliters of α-Chymotrypsin (EC 3.4.21.1. MERCK) in concentration of 0.024 ml l^{-1} was added 8 min after activation. Fifteen minutes after activation, redundant solution was discarded and additional 50 ml of the above α-Chymotrypsin in concentration of 0.024 ml l^{-1} was added again. Finally, 16 (CH1), 18 (CH2), 20 (CH3) and 22 min (CH4) after activation, 50 ml of α-Chymotrypsin in concentration of 0.24 ml l^{-1} was added with similar exposure for 60 s.

When using Alcalase DX (PLN 04715) enzyme, the procedure was similar to the previous treatment until 8 min after activation. Fifty milliliters of Alcalase DX (PLN 04715) in concentration of 2 ml l^{-1} was added 8 min after activation. Fifteen minutes after activation, the diluted enzyme was discarded and additional 50 ml of Alcalase DX in concentration of 2 ml l^{-1} was added again. Finally, 20 min after activation, 50 ml of Alcalase DX in concentration of 20 ml l^{-1} was added for exposure of 45 (A1), 60 (A2), 75 (A3) and 90 s (A4), respectively (Fig. 5).

The first control (C 1) was fertilized and at 2, 4 and 6 min after gamete activation, redundant water was discarded and additional 20 ml dechlorinated tap water was added. Water from eggs was discarded and additional 20 ml hatchery water was added. Eggs were incubated in cages since 8 min after gamete activation. The second control (C 2) was similar until 8 min after gamete activation. Eight minutes after activation, milk solution with concentration of 40 g l^{-1} dry powder was added for 60 min and since 70 min after gamete activation; the eggs were incubated in cages and jars.

The eggs after treatments were properly rinsed four times with 500 ml of hatchery water. After elimination of enzyme, 200–300 eggs were placed with replication (four times) into an incubator cage of 200 ml supplied with UV-sterilized recirculated dechlorinate tap water at 22 °C, 9 mg l^{-1} O_2. The rest of eggs were filled in the Zuger incubation jar of 2 l volume supplied with UV-sterilized recirculated dechlorinate tap water at 22 °C, 9 mg l^{-1} O_2 for observation of the success of desticking.

2.7. Calculation of fertilization and hatching

The eggs were counted in each cage and during incubation, dead eggs were counted and removed, then hatched fry were counted, usually up to 4.5 days of incubation at 22 °C. The percentage of fertilization rate (F_r) was then calculated for each cage from the total number of eggs placed in the cage (E_t) minus dead eggs (E_d) collected up to 24 h after fertilization as follows:

$$F_r = 100\ [(E_t - E_d)/E_t]$$

The hatching rate (H_r) percentage was calculated for each cage from the total number of eggs placed in the cage (E_t) and divided with number of hatched larvae (H_l) as follows:

$$H_r = 100\ (H_l/E_t)$$

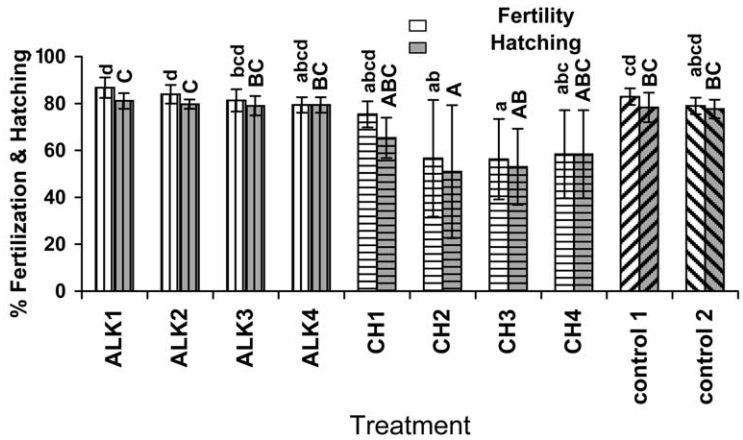

Fig. 5. The different time exposition of 0.024 ml l^{-1} α-Chymotrypsin enzyme (EC 3.4.21.1. MERCK) and 20 ml l^{-1} Alcalase DX enzyme (PLN 04715) for elimination of egg stickiness with the concentration of 140 130 spermatozoa per egg. During the second step, α-Chymotrypsin was used for 1 (CH1), 3 (CH2), 5 (CH3) and 7 min (CH4) exposures. The Alcalase DX enzyme was added at 20 min after activation with exposure for 45 (ALK 1), 60 (ALK 2), 75 (ALK 3) and 90 s (ALK 4), respectively. Groups with a common superscript do not differ significantly ($P < 0.05$).

2.8. Data analysis

Means of the data acquired were evaluated from four replicates. Statistical significance was assessed using multiple analysis of variance (ANOVA, Statgraphics version 5), followed by multiple comparison Tukey HSD range test. Probability values <0.05 were considered significant.

3. Results

3.1. Determination of optimal sperm/egg ratio (Experiment 1)

The fertilization and hatching rates (82% and 80%, respectively) were significantly higher for 236 720 spermatozoa per egg, than for other ratios (Fig. 1). The fertilization and hatching rates were 70% and 64%, respectively, with the level of 23 672 spermatozoa per egg and to 23% and 20%, respectively, with the level of 2367 spermatozoa per egg. ANOVA showed significant effects of the number of spermatozoa per egg ($P < 0.0001$) on the fertilization and hatching rate.

3.2. Effect of different activation solutions (Experiment 2)

The highest fertilization and hatching rate (96% and 93%, respectively) was found for AS of 20 mM Tris–HCl, pH 8 with concentration 13 000 spermatozoa per egg but was only significantly different from one solution. Fertilization and hatching rates were significantly lower in 51 mM NaCl AS, where fertilization and hatching rates were 67% and 63% (Fig. 2).

3.3. Determination of pH in activation solution (Experiment 3)

The highest fertilization and hatching rates (30% and 28%, respectively) with concentration 4500 spermatozoa per egg, was for AS pH of 9. Lower levels of 15–16% fertilization and hatching rates were found with control 1, when low numbers of spermatozoa were used (Fig. 3). Fertilization and hatching rates were only on the level 9–7% with AS of pH 6 and 7 and also using low number of spermatozoa. The quality and fertilizability of eggs were very good that was demonstrated by control 2 and 3 with 80–90% of fertilization and hatching rate, when 2 250 000 spermatozoa per egg were used (Fig. 3).

3.4. Effect of different ratio of volume of activation solution and egg quantity (Experiment 4)

No relationship was observed between the ratios of dechlorinated tap water volume: egg quantity represented by their weight. Results varied between 90% and 96% in the range of ratios 1:0.33 to 1:3 with concentration of 8490 spermatozoa per egg (Fig. 4). ANOVA showed insignificant effect of the ratio of AS:egg quantity ($P < 0.1$–0.4) on fertilization and hatching rates.

3.5. Application of two enzymes α-Chymotrypsin (EC 3.4.21.1. MERCK) and Alcalase DX (PLN 04715) for elimination of egg stickiness (Experiment 5)

The duration of 45–60 s treatment with alcalase enzyme was applied 20 min after gamete activation yielding fertilization and hatching rates of 80–87% with concentration of 140 131 spermatozoa per egg. However, the use of α-Chymotrypsin (EC 3.4.21.1. MERC) was not effective for elimination of stickiness and there was a significant decrease in fertilization and hatching rates compared to controls 1 and 2 (Fig. 5).

4. Discussion

4.1. Conditions of sperm storage

Sperm of common carp can be stored in vitro at 2–5 °C for 2 days (Belova, 1981; Jähnichen, 1981; Hulata and Rothbard, 1979) without decrease in fertilization. However, environmental conditions of sperm storage must be correctly adjusted, because ATP is consumed during sperm storage leading to a slight decrease after 24 h (Saad et al., 1988). If availability of O_2 and substrates for sperm is limited, it can be provided artificially in vitro, by exposition to an O_2 atmosphere in ratio 1:10 and storage at 0 °C as sperm was stored prior fertilization.

4.2. Artificial insemination

Composition of diluents for sperm activation and fertilization was developed by Woynarovich and Woynarovich (1980) and later modified by Saad and Billard (1987). Woynarovich and Woynarovich (1980) used AS containing 4 g (68 mM) of NaCl and 3 g of urea per l. Later, Saad and Billard (1987) in a more detailed study developed AS containing 45 mM NaCl, 5 mM KCl, and 30 mM Tris–HCl pH 8. Saad and Billard (1987) included KCl as important component favorable for motility of sperm (Morisawa et al., 1983; Redondo-Muller et al., 1991), but carp eggs were found not to tolerate more than 5 mM KCl (Saad and Billard, 1987). Saad and Billard (1987) formulated a media with optimal osmotic pressure where all ova were fertilized in a range of 100–150 mOsmol kg^{-1} when the number of spermatozoa per egg was high (20 000–25 000). Also, fertilization was not affected by a pH of 7–9. According to our results, it can be concluded that the minimum number of spermatozoa for good fertilization and hatching rate ranges 8500–25 000 spermatozoa per egg, according to quality of eggs (Figs. 1 and 4). Various AS with NaCl 0–34 mM (0–68 mOsmol kg^{-1}) did not change fertilization or hatching rates. Also our results did not verify those of Saad and Billard (1987) using an AS for carp (45 mM NaCl, 5 mM KCl, and 30 mM

Tris–HCl pH 8) did not change the results. Only 51 mM (102 mOsmol kg^{-1}) NaCl significantly decreased fertilization and hatching rates. The two experiments showed increasing fertilization and hatching rates when using higher pH, firstly pH 8 (Fig. 2) and secondly pH 9 (Fig. 3) instead of dechlorinated tap water with pH 7. An overview of our results showed that dechlorinated tap water or low concentration of buffer solutions at pH 8 or 9 and 10 000–20 000 spermatozoa per egg are the best conditions for AS. Then the question is raised about the differences between our results and those of Saad and Billard (1987). They can be explained by the strategy of methodology during experiments. Saad and Billard (1987) used Petri dishes for basic experimental conditions where the ratio between weight of eggs and volume of AS was 1:30–40 and later they adapted these results to the practical approach. However, under practical conditions the ratio between weight of eggs and volume of AS ranges in 1:0.5–2. Results obtained in these conditions could be influenced by the high mass of eggs and by the content of seminal fluid. Also, the practical approach to carp culture in Europe generally employs milk for elimination of stickiness and it is well known that during this procedure, a low ratio of milk volume to volume of eggs is needed. If the quantity of milk is high, results of desticking are usually not successful. The strategy of our study was opposite to that of Saad and Billard (1987) and we tried firstly to adapt our experimental conditions to the practical ones. The ratio between the weight of eggs and volume of AS was 1:1 in all cases with constant time and rotation level during the procedure of mixing gametes and solutions. Ratio 1:1 used under practical conditions as well as in our experiment was confirmed to be feasible with success for artificial insemination procedure.

4.3. Artificial insemination and elimination of stickiness under practical conditions

Based on our results, our recommended procedure for artificial insemination is as follows.

4.3.1. Fertilization

The minimum volume of short-term stored sperm under aerobic conditions used for insemination was 1 ml of sperm per 1 kg of short-term stored ova (3–4 h in stable temperature 19–21 °C; Rothbard et al., 1996). That volume of sperm represented 236 720 spermatozoa per egg. Also, 0.1 ml of sperm per 1 kg of eggs was found sufficient for good fertilization and hatching rates (Figs. 1, 2 and 4) when 8490–23 672 spermatozoa guaranteed successful fertilization and hatching rates. The mixture of eggs and sperm was directly activated with 1 or 2 l of activating solution made of 20 mM Tris–HCl, pH 9 or of dechlorinated tap water or clean hatchery water at an optimum temperature 22 °C. It was mixed during 2 min and later 1 l of dechlorinated tap water was added. After 2 min, the redundant solution was poured out and another 1 l of dechlorinated tap water (or clean hatchery water) was added. This procedure was repeated again at 4 and 6 min, and desticking process with milk or enzyme was started at 8 min. During that time the eggs were hydrated and swollen rapidly. Egg sticking was prevented during that period by constant mixing.

4.3.2. Desticking

The procedure for egg desticking was developed many years ago by Soin (1976) and later improved by Khan et al. (1986). In Czech Republic, the fish farming practice employs powder milk containing in 100 g of powder 27.2% of fat, 26.6 g of albumin, 37.2 g milk sugars, 5.8 g of ions, 0.2 g of lecithin and 3 g of water for preparation solution with concentration 40 g of powder milk per l of dechlorinated tap water or clean hatchery water. The milk solution is added slowly with intensive mixing in volume of 1 l kg^{-1} of freshly stripped eggs. Later usually at 15 min after activation when the eggs have swelled, additional milk is slowly added. If too much milk is added stickiness of eggs is not eliminated, therefore, it is necessary to add only small quantities of milk solution. The swelling process lasts in general for 60 min. After that time, hatchery water is slowly added within 10 min to replace milk solution and finally, the eggs are transferred to Zuger jars. Carp eggs will swell three to four times in milk than their original volume but using the method of Woynarovich and Woynarovich (1980) with urea, the swelling factor was increased six to nine times (Horvath et al., 1984).

4.3.3. Enzyme treatment

The Alcalase DX (PLN 04715) was successful in removing adhesiveness. Optimum ratio between eggs and alcalase (2 ml of enzyme per 1 l of dechlorinated tap water) was 1:2.5 (g eggs:ml enzymes) with mixing from 8 to 15 min after activation and after 15 min additional 2.5 part of enzyme solution (2 ml of enzyme per 1 l of dechlorinated tap water) was added. Twenty minutes after activation, the enzyme solution was poured out from the eggs and more concentrated alcalase solution (25 ml l^{-1} of dechlorinated tap water) was added in the rate 1:2.5 for 60 s exposure only. At 21 min after activation, the eggs were rapidly rinsed with water and transferred to Zuger jars for incubation. Fertilization and hatching rates were similar between enzyme treatment and milk treatment. The results of the study were repeated in the Czech Republic commercial condition with similar results.

Traditional technique for destickiness of eggs in common carp can be successfully shortened from 70 to 21 min when using enzyme treatment instead of milk or urea. A proteolytic enzyme (alcalase, Merck EC 3.4.21.14) has also been successfully used for elimination of stickiness in European catfish, *Silurus glanis* eggs (Linhart et al., 2002) and tench, *Tinca tinca*(Linhart et al., 2000, 2003), and it is now used routinely in hatcheries of the Czech Republic and France. Enzyme treatment in both species increased hatching success and decreased the time of egg handling from 1 h using milk/clay in tench or only clay in European catfish treatment, to about 2 min.

5. Conclusion

The present work optimized procedures for artificial insemination of common carp, *C. carpio* with increase in fertilization and hatching rates, optimization of AS, process of insemination, activation of gametes and elimination of eggs stickiness with a practical proposal for artificial reproduction in fish hatcheries.

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References

Belova, N.V., 1981. Ecological-physiological peculiarities of semen of pond, carps. II. Change in the physiological parameters of spermatozoids of some carps under the influence of environmental factors. J. Ichthyol. 21, 70–81.

Billard, R., 1988. Artificial insemination and gamete management in fish. Mar. Behav. Physiol. 14, 3–21.

Billard, R., 1990. Artificial insemination in fish. In: Lamming, G.E. (Ed.), Marshall' Physiology and Reproduction, CRC press, Toronto, pp. 870–888.

Billard, R., Gatty, J.L., Hollebecq, M.G., Marcel, J., Saad, A., 1986. Biology of gametes, eggs and embryos. In: Billard, R., Marcel, J. (Eds.), Aquaculture of Cyprinids. INRA, Paris, pp. 151–164.

Billard, R., Cosson, J., Perchec, G., Linhart, O., 1995. Biology of sperm and artificial reproduction in carp. Aquaculture 129, 95–112.

Horvath, L., Tamas, G., Tolg, I., 1984. Special methods in pond fish husbandry. Akademia Kiado, Halver Corporation, Budapest, Seattle.

Hulata, G., Rothbard, S., 1979. Cold storage of carp semen for short periods. Aquaculture 16, 267–269.

Jähnichen, H., 1981. Kurzeitkonservierung von Karpfensperma als weitere Möglichkeit der Rationalisierung der künstlichen Vermehrung von Karpfen (*Cyprinus carpio* L.). Z. Binnenfisch. DDR 28, 244–245.

Khan, H.A., Gupta, S.D., Reddy, P.V.G., Sahoo, S.K., 1986. Use of milk, urea, sodium sulphite and human urine for degumming fertilized eggs of common carp, *Cyprinus carpio* L. Aquac. Hung. (Szarvas) 5, 47–54.

Linhart, O., Kudo, S., Billard, R., Šlechta, V., Mikodina, Y.V., 1995. Morphology composition and fertilization of carp eggs. Aquaculture 129, 75–93.

Linhart, O., Gela, D., Flajšhans, M., Duda, P., Rodina, M., Novák, V., 2000. Alcalase enzyme treatment for elimination of egg stickiness in tench, *Tinca tinca* L. Aquaculture 191, 303–308.

Linhart, O., Štěch, L., Švarc, J., Rodina, M., Audebert, J.P., Grecu, J., Billard, R., 2002. The culture of the European catfish, *Silurus glanis* L. in Czech Republic and in France. Aquat. Living Resour. 15, 139–144.

Linhart, O., Gela, D., Flajšhans, M., Rodina, M., 2003. Proteolytic enzyme treatment: an improved method for elimination of egg stickiness in tench, *Tinca tinca* L. in aquaculture. J. Appl. Ichthyol. 19, 17–18.

Morisawa, M., Suzuki, K., Shimizu, H., Morisava, S., Yasuda, K., 1983. Effects of osmolality and potassium on motility of spermatozoa from freshwater cyprinid fishes. J. Exp. Biol. 107, 95–103.

Redondo-Muller, C., Cosson, M.P., Cosson, J., Billard, R., 1991. In vitro maturation of the potential for movement of carp spermatozoa. Mol. Reprod. Dev. 29, 259–270.

Rothbard, S., 1981. Induced reproduction in cultivated cyprinids. The common and the group of Chinese carps. I. The technique of induction, spawning and hatching. Bamidgeh 33, 103–121.

Rothbard, S., Rubinsthein, I., Gelman, E., 1996. Storage of common carp, (*Cyprinus carpio* L.) eggs for short durations. Aquac. Res. 27, 175–181.

Saad, A., Billard, R., 1987. Composition et emploi d'un dilueur d'insémination chez la carpe, *Cyprinus carpio*. Aquaculture 65, 67–77.

Saad, A., Billard, R., Theron, M.C., Hollebecq, M.G., 1988. Short term preservation of carp (*Cyprinus carpio*) semen. Aquaculture 71, 133–150.

Soin, S.G., 1976. Two new methods for elimination of egg stickiness. Rybnoe Chozjajstvo 10, 18–21 (in Russian).

Woynarovich, E., 1962. Hatching of carp eggs in Zuger-glasses and breeding of carp larva until and age of 10 days. Badmidgeh (Israel) 14, 38–46.

Woynarovich, E., Woynarovich, A., 1980. Modified technology for elimination of stickiness of common carp *Cyprinus carpio* eggs. Aquac. Hung. 2, 19–21.

Yaron, Z., 1995. Endocrine control of gametogenesis and spawning induction in the carp. Aquaculture 129, 49–73.