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Insemination, fertilization and gamete management in tench, *Tinca tinca* (L.)

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Abstract. Various procedures for artificial insemination in tench, *Tinca tinca* (L.) were re-examined with evaluation of fecundity of males and females among different tench strains. The objectives of this study were to enhance fertilization and hatching rates through optimization of the activation solution, the insemination process, the activation of gametes, and the elimination of eggs stickiness. Sperm for all experiments was collected directly into immobilization solution of modified Kurokura solution containing 180 mM of NaCl and stored at 2 °C for 2.5–5 h prior to the experiment. When dechlorinated tap water was used for activation a gamete ratio of 1150 spermatozoa per egg showed the best significant fertilisation and hatching rates. Optimal ratio between eggs (weight in g) and activation solution (in cm^3) was 1:1. Different concentrations of activation solutions such as NaCl from 0 to 68 mM (0–136 mOsmol kg^{-1}) without buffer statistically decreased fertilization and hatching rates. The activation solution containing 17 mM of NaCl, 10 mM Tris–HCl, pH 8 and 9 significantly increased fertilization and hatching rates compared to dechlorinated tap water of pH 7 or activation solution containing 17 mM of NaCl, 10 mM Tris–HCl, pH 6 and 7. Adhesiveness of the eggs was successfully removed by incubation in Alcalase and activity: 3.16 Anson units per cm^3.

Introduction

Controlled reproduction of tench started in the 1970s, after establishing technologies for artificial reproduction (Brylinski and Pyka 1976; Kouril and Chabera 1976; Horvath 1977; Horvath et al. 1984; Kouril et al. 1986). Approaches either through basic or applied research were highlighted in a review of tench gamete biology and artificial reproduction by Linhart and Billard (1995).

In tench, as in other cyprinids, temperature is the major environmental factor for the development of gametogenesis and spawning (Pimpicka 1989). A water temperature of 6–9 °C during the winter period, up to mid-February, ensures favorable conditions for the stages of ovogonial proliferation and beginning of vacuolization. Vitellogenesis is slowly stimulated from February to the end of April by increasing the temperature up to 17 °C. The spawning season begins in May when temperature increases from 17 to 22 °C (Horo-

szewicz 1981). Generally spawning starts at temperatures of 19–22 °C (Breton et al. 1980; Pimpicka 1990) with the duration of the spawning season approximately 9 weeks at temperatures up to 25 °C. During summer when temperatures rise up to 25–27 °C, the trophoplasmatic growth of ovocytes increases in the follicles (Horoszewicz 1981). In both males and females, the plasma concentrations of androgens as estradiol-17beta (E-2), testosterone, androstendione, 11-ketotestosterone, 17,20beta, 21-trilhydroxv-4-pregnen-3-one, 17,20beta-dihydroxy-4-pregnen-3-one, 17,20alpha-dihydroxy-4-pregnen-3-one and C-21 steroids significantly increases in the spring and summer with the peak at spawning in early July (Pinillos et al. 2003). The level of steroids and prostaglandins increases not only in the blood plasma but also in water and consequently induces pheromonal communication between female and male (Pinillos et al. 2002). Generally females produce pheromones (complex of E2 hormones) in ovarian follicles, and these are released from body into surrounding water. The pheromones then cause an increasing of maturation-inducing steroid concentration (MISs; Baynes and Scott 1985; Schulz and Miura 2002).

In the controlled spawning process, the sperm (0.4 cm^3) is stripped directly on 100 g of eggs or it is collected into an immobilization solution (IS) (ratio <0.9 sperm:1 IS) (Rodina et al. 2004) and 1 cm^3 of immobilized sperm is mixed with 100 g of eggs. The function of IS is to prevent sperm contamination with urine in order to preserve the sperm fertilization capacity (Linhart et al. 2003b; Rodina et al. 2004). The control sperm stored for 10 h without any IS showed fertilization and hatching rates only at the 6–7% level (Rodina et al. 2004). The first step of insemination and egg activation is that eggs and sperm are stirred together for few seconds and 25 cm^3 of 34 mM NaCl solution or hatchery water are added (Gela et al. 2003). The second step is to eliminate the egg stickiness, upon adding 100 cm^3 of diluted proteolytic alcalase enzyme (Merck EC 3.4.21.14) at concentrations 10.0 cm^3 of enzyme per dm^3 of hatchery water, applied 3 min after fertilization for 2 min with stirring of eggs (Linhart et al. 2003a). The application of enzyme decreases the time necessary for egg handling from almost 1 h, using conventional desticking methods, to just 2 min (Linhart et al. 2000). Furthermore, the application of enzyme results in a significantly higher hatching rate and a shortening of hatching time. In addition, when the newly-hatched larvae are transferred from incubation jars to hatching trays, there is a lesser amount of mud sediments (caused by fine particles in hatchery water) attached to the egg surface (Linhart et al. 2003a). The eggs are then rinsed with hatchery water and transferred to Weiss jars (7–10 cm^3) at 18–23 °C for 60–70 day-degrees of incubation (Linhart and Billard 1995).

The present work was undertaken to establish optimized procedures for artificial insemination in common tench, *Tinca tinca* (L.). The objective of the study was to enhance fertilisation and hatching rate through the optimisation of the activation solution, the activation of gametes, the insemination process and the elimination of egg stickiness.

Material and methods

Broodstock handling and egg collection

Artificial propagation followed methods described by Linhart and Billard (1995) and Linhart et al. (2003a). Five- to seven-year-old broodstock (400–700 g body weight) taken from breeding ponds in April was kept sex-separated in two ponds of 0.01 ha each. Broodstock was assessed for maturity by means of abdominal compression and spermiation which was detected by sperm production. Spawners suitable for stripping were selected in June and kept at the hatchery in 4 m^3 tanks with water flow rate of 0.2 dm^{-3} s^{-1}, temperature of 18–22 °C and 6–7 mg dm^{-3} O_2. Males were treated with single intramuscular injection of carp pituitary extract at a dose of 1 mg kg^{-1} body weight. Females were stimulated for ovulation 30 h prior to egg stripping by injection of GnRH analogue [D-Ala^6, GnRH ProNHEt, Kobarelin] at a dose of 5 μg kg^{-1} b.w. Eggs from 10 females were then pooled, stored at a temperature of 17–18 °C and then used for the experiments. Prior to the fertilization experiments, three batches of approximately 0.2 g (around 300 eggs) of pooled unfertilized eggs were weighed to the nearest 0.0001 g and fixed in 4% formaldehyde for later counting and determination of mean egg weight. On the basis of this, the number of eggs in a sample was expressed as the weight of the sample (g) multiplied by 1474. Before each injection and gamete collection, the males and females were anaesthetized in a solution of 2-phenoxyethanol (1:1000).

Sperm collection and immobilization

Sperm was collected individually (20 males, Figure 1) from the genital papilla into 5 cm^3 syringe with 2 cm^3 of IS (IS; Kurokura 180 = 180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$ and 2.38 mM $NaHCO_3$; Rodina et al. 2004). No more than 1.0 cm^3 of sperm was taken into one syringe to keep dilution rate 2:1 (IS: sperm) in order to prevent spontaneous initiation of motility (Linhart et al. 2003b; Rodina et al. 2004). Sperm was pooled in containers and stored under aerobic condition on ice for 2.5–5 h. Pooled sperm concentration was diluted and counted in a Burker cell haemocytometer under an Olympus microscope BX 41 (400×) and the mean number of spermatozoa counted in 16 squares of the Burker cell was finally expressed as number of spermatozoa per volume unit.

Artificial insemination with test of fertilization and hatching rates

Different approaches to artificial insemination were assessed by fertilization and hatching rates as follows. One gram of eggs (1474 eggs per 1 g) were placed into a 20 cm^3 dish; an accurate volume of sperm with estimated number of

Figure 1. Demonstration of sperm collection from the genital papilla of tench into 5 cm^3 syringe with 2 cm^3 of immobilization solution and 1 cm^3 of sperm.

spermatozoa per cm^3 was dropped on them from a micropipette. The dish was then placed on a shaking table (Figure 2) with constant agitation at 200 rpm and with 10 mm deflection. One cm^3 of dechlorinated tap water or experimental activation solution at 22 °C was added. Two minutes later, approximatley 250–360 eggs were placed into a incubator cage of 200 cm^3 supplied with UV sterilized recirculated tap water at 22 °C, 9 mg dm^{-3} O_2. For each experiment the procedure was replicated four times. The eggs were exactly counted in each cage, and later, during incubation of eggs the dead eggs and hatched larvae were counted, usually up to 4.5 days. 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:

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

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

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

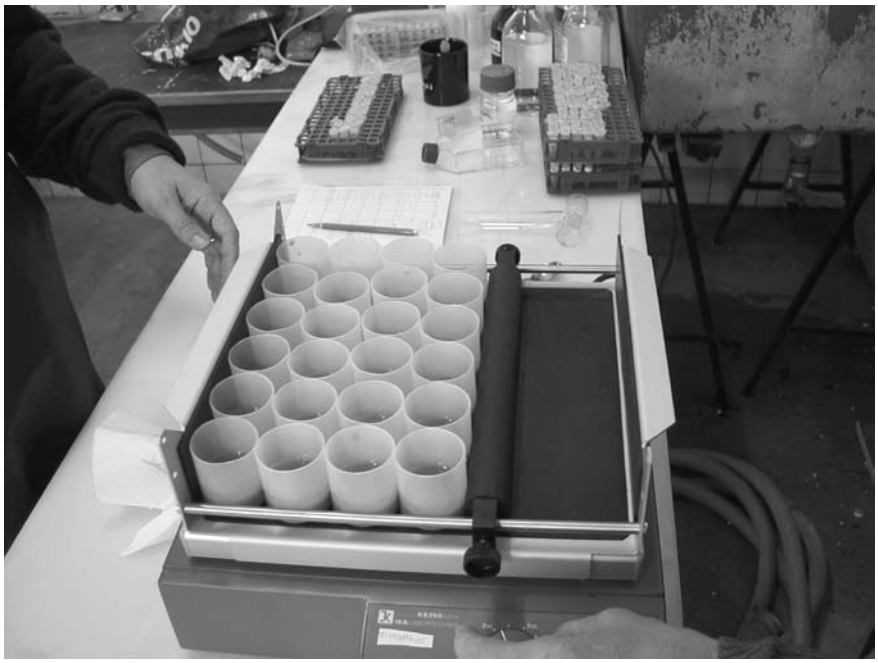

Figure 2. Dishes for fertilization experiments on a shaking table.

Determination of optimal sperm/egg ratio (Experiment 1)

The quantity of sperm per egg for fertilization was 115, 1150, 11,500 and 46,100 spermatozoa per egg, which represents 0.1, 1, 10 and 40 mm^{-3} of sperm, respectively. One gram of eggs was placed into a dish and accurate volume of sperm (0.1, 1, 10 or 40 mm^{-3} of sperm) with estimated number of spermatozoa (115, 1150, 11,500 or 46,100 spermatozoa per egg) was dropped using a micropipette. Before activation of eggs with water, additional IS was added at volumes of 39.9, 39, 30 or 0 mm^{-3} into experiments with 0.1, 1, 10 or 40 mm^{-3} of sperm, respectively. Then the dish was placed on a shaking table and 1 cm^3 of dechlorinated tap water (22 °C) was added.

Effects of different NaCl concentrations in activation solutions (Experiment 2)

One gram of eggs was placed into a dish and an accurate volume of sperm with a low number (500) of spermatozoa per egg was dropped with a micropipette. Then the dish was placed on a shaking table and different activation solutions at volume of 1 cm^3 were tested. The solutions used had differing NaCl concentrations: 0, 17, 34 and 51 mM (in distilled water). Dechlorinated tap water was used as a control. After 2 min, the fertilized eggs were placed in incubator cages with four replicates.

Determination of optimal pH in activation solution (Experiment 3)

One gram of eggs was placed into a dish and 500 spermatozoa per egg were added by micropipette. The importance of the pH, of the activation solution, on fertilization and hatching rate was greater with very low number of sperm (500) per egg used for experiment. Then the dish was placed on shaking table and 1 cm^3 of 17 mM NaCl with 10 mM Tris–HCl at pH 6, 7, 8 and 9 of 22 °C was added. The control was activated with dechlorinated tap water. Two min later, the fertilized eggs were placed in special incubator cages with four replications.

Data analysis

Means of the data acquired were evaluated from 4 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.

Results

Determination of optimal sperm/egg ratio (Experiment 1)

The fertilization and hatching rates (80.6% and 80%, respectively) were significantly the highest for 11,500 spermatozoa per egg, than for other ratios (Figure 3). The fertilization and hatching rates were 42.7% and 42.2%, respectively, with the ratio of 1150 spermatozoa per egg and 17.8% and 17.4%, respectively, with the ration of 115 spermatozoa per egg. The fertilization and hatching also insignificantly decreased to 75.5% and 74.5% at the ration of 46,100 spermatozoa per egg. ANOVA showed significant effect of number of spermatozoa per egg ($p < 0.0001$) on the fertilization and hatching success.

Effects of different NaCl concentrations in activation solutions (Experiment 2)

Dechlorinated tap water (pH 7) and a concentration of 500 spermatozoa per egg gave a significantly highest fertilization rate (expressed as percentage of hatching) of 54.6%. Fertilization and hatching rates were significantly lower in all NaCl solutions from 17 to 51 mM NaCl activation solution, where fertilization and hatching rates were 0.8–9.2% and 0.8–7.2% (Figure 4). ANOVA showed the significant effect of the NaCl concentration in the activation solution on fertilization and hatching success ($p < 0.0001$).

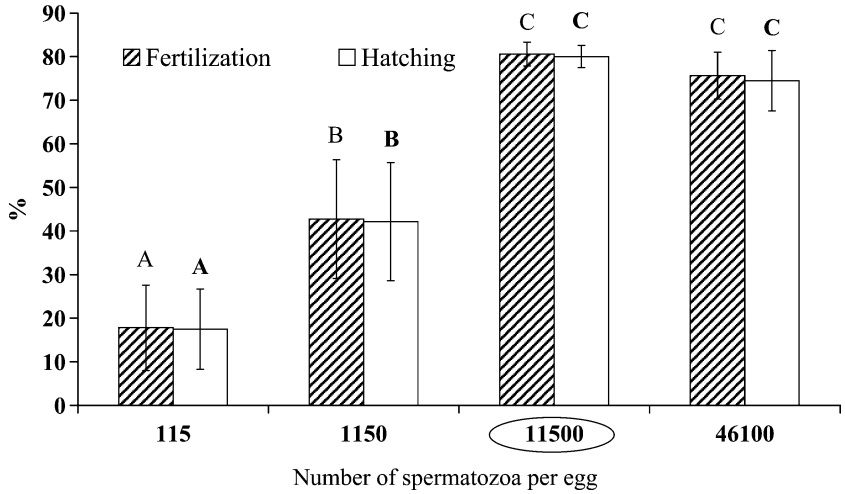

Figure 3. The effect of increasing number of spermatozoa from 115 to 46,100 per egg on the fertilization and hatching success. Mean values of 4 replicates are shown, vertical lines are SD. Groups with a common superscript do not differ significantly ($p < 0.05$).

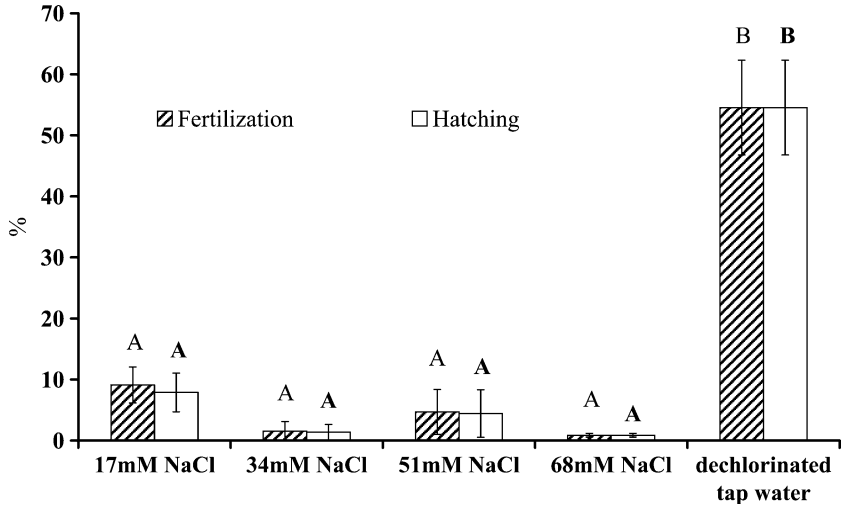

Figure 4. Fertilization and hatching success after artificial insemination with different concentrations of NaCl in distilled water of activation solution with the same level of 500 spermatozoa egg^{-1}. Mean values of four replicates are shown, vertical lines are SD. Groups with a common superscript do not differ significantly ($p < 0.05$).

Determination of the optimal pH of the activation solution (Experiment 3)

The highest fertilization and hatching rates (71.2 and 71.7%, and 71.2 and 71.3%, respectively) with concentration of 500 spermatozoa per egg, was found for activation solutions with pH 8 and 9 (Figure 5). Lower but insignificant levels, of 54.6 and 58.7% hatching rates, were found with dechlorinated tap water (control) and the activation solution at pH 7. Fertilization and hatching rates were only on the level 43.2 and 42.1% with activation solution of pH 6 and also using lower ratio of spermatozoa to egg (Figure 5). The experiment also demonstrated good quality and fertilization capacity of eggs. ANOVA showed significant effect of the pH of activation solution ($p < 0.0001$) on fertilization and hatching success.

Discussion

Conditions of sperm storage

In tench, the low osmolality of urine is the main key factor for spontaneous activation of spermatozoa (Linhart et al. 2003b), as urine exhibits osmolality down to half of that of seminal fluid. Osmolality of contaminated tench sperm was re-equilibrated successfully by Rodina et al. (2004) with storage potential for 10 h owing to the "KUROKURA 180" IS containing 180 mM NaCl. The

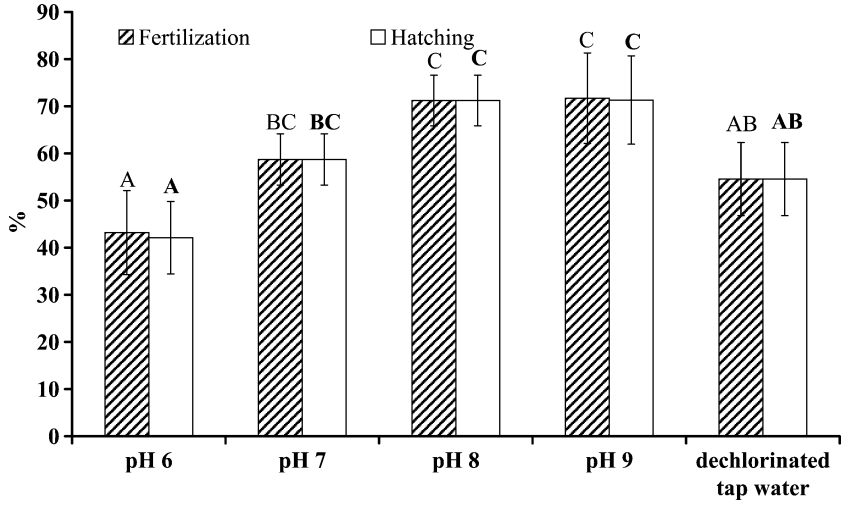

Figure 5. Fertilization and hatching success after artificial insemination with different pH of activation solution containing 17 mM NaCl +10 mM Tris–HCl with the same level of 500 spermatozoa egg^{-1}. Mean values of four replicates are shown, vertical lines are SD. Groups with a common superscript do not differ significantly ($p < 0.05$).

total concentration of soluble compounds in the IS solution was around 190 mM leading to osmolality 343 mOsmol kg^{-1}. The role of the IS in tench is not only to stop movement of spermatozoa spontaneously activated by contaminating urine, but also to allow the recovery of the energetic stores. This prediction would explain why tench spermatozoa do not really need energetic organic components in IS, because the motility period of tench spermatozoa is very short (Rodina et al. 2004). However, environmental conditions of sperm storage must be correctly adjusted, because an energetic component as ATP is consumed during sperm storage (Saad et al. 1988). If availability of O_2 and substrates for sperm is limited, it can be provided artificially *in vitro*, by exposure to aerobic atmosphere (Billard et al. 1995) in ratio 1:10 and storage at 0 °C (Linhart et al. 2003c).

Artificial insemination

Composition of diluents for sperm activation and fertilization was developed by Kouril et al. (1976), Horvath et al. (1984) and Linhart and Kvasnicka (1992). Kouril et al. (1976) and Linhart and Kvasnicka (1992) used activation solution containing 2 g (34 mM) of NaCl with or without milk solution. Horvath et al. (1984) recommended traditional Woynarovich and Woynarovich (1980) method for common carp, using 4 g (68 mM) of NaCl and 3 g of urea per dm^3. Later, Geldhauser (1992) in a detailed study observed that activation solution from Woynarovich and Woynarovich (1980) decreased fertilization success in tench. According to our results, it can be concluded that the minimum number of spermatozoa for optimal fertilization and hatching rate ranges 500 to 10,000 spermatozoa per egg, according to quality of eggs (Figures 3 and 5). Various activation solutions with NaCl 0–68 mM (0–136 mOsmol kg^{-1}) in distilled water dramatically decrease fertilization or hatching rates (Figure 4). NaCl with buffer solution significantly decreased fertilization and hatching rates. The experiment showed increasing fertilization and hatching rates when using higher pH such as 8 and 9 (Figure 5) instead of pH 6. An overview of our results showed that dechlorinated tap water or low concentration of buffer solutions at pH 8 or 9 and 11,000 spermatozoa per egg are the best conditions for activation solution. Then the question is raised about the differences between our results and those of Kouril et al. (1976) and Linhart and Kvasnicka (1992). They can be explained by the strategy of methodology during experiments. Linhart and Kvasnicka (1992) used Petri dishes for basic experimental conditions where the ratio between weight of eggs and volume of activation solution was 1:30–40 and later they adapted these results to the practical field conditions. However, under practical conditions the rate between weight of eggs and volume of activation solution is 1:1. Results obtained in these conditions could be influenced by the high mass of eggs and by the content of seminal fluid. The strategy of our study was opposite to that of Linhart and Kvasnicka (1992). We tried to adapt our experimental

conditions to the practical ones. The ratio between the weight of eggs and volume of activation solution was 1:1 in all cases with constant time and rotation level during the procedure of mixing gametes and solutions. The ratio 1:1 used under practical conditions as well as in our experiment was confirmed to be feasible with success for artificial insemination procedure. Also in the experiment, optimal low sperm : egg ratio was used for discrimination of fertilization effect (Billard and Cosson 1992), when the high fertilization/hatching variability was used as potential predictor of gametes or environmental quality, etc.

Artificial insemination and elimination of stickiness under practical conditions

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

Fertilization (Figure 6). The minimum volume of short-term stored sperm under aerobic conditions used for insemination was 1 cm^3 of sperm collected in IS 1:2 (volume ratio) per 100 g of short-term stored ova. Ova contaminated by urine during stripping should be discarded (Linhart and Billard 1995). The *in vitro* storage of ova should not exceed a few hours at a stable temperature of 17–18 °C and it was advised to carry out the insemination and activation steps as soon as possible after ovulation. Also, 0.1 cm^3 of diluted sperm with IS per 100 g of eggs was found sufficient for good fertilization and hatching rates as documented in Figures 3 and 5, when 500–10,000 spermatozoa guaranteed successful fertilization and hatching rates. The mixture of eggs and sperm was directly activated with 100 cm^3 of activating solution made of 17 mM NaCl + 10 mM Tris–HCl, pH 9 or of dechlorinated tap water or clean hatchery water at an optimum temperature 22 °C. After 2.5 min, the excess solution was poured out and desticking process with enzyme was started at 3 min. During that time the eggs were hydrated and swelled rapidly. Egg sticking was prevented during that period by constant mixing.

Desticking (Figure 5). The procedure for egg desticking with milk and clay suspension was developed by Kouril et al. (1976), later Linhart et al. (2000) used enzyme. In the Czech Republic, fish farming practice employs enzyme diluted in dechlorinated tap water or clean hatchery water. For the elimination of egg stickiness alcalase enzyme is used 3 min. after fertilization. Optimum rates between eggs and diluted enzymes (2 cm^3 of Alcalase enzyme, *Bacillus licheniformis*; CALBIOCHEM cat. no. 12674120, diluted in 998 cm^3 of hatchery water or AS; unpublished results) is 1:1 (g eggs: cm^3 of diluted enzymes) with stirring for 2 min. After 2 min of exposure in enzyme solution, the eggs are rapidly rinsed with hatchery water and transferred to Weis jars. The duration of egg incubation after enzyme treatment was about 4–5 h shorter, than the classical method using milk solution and talc suspension. This prolongation for the classical method may be explained by hardening of the egg envelopes. Hatching is expected to start 3 days (57 D°) after

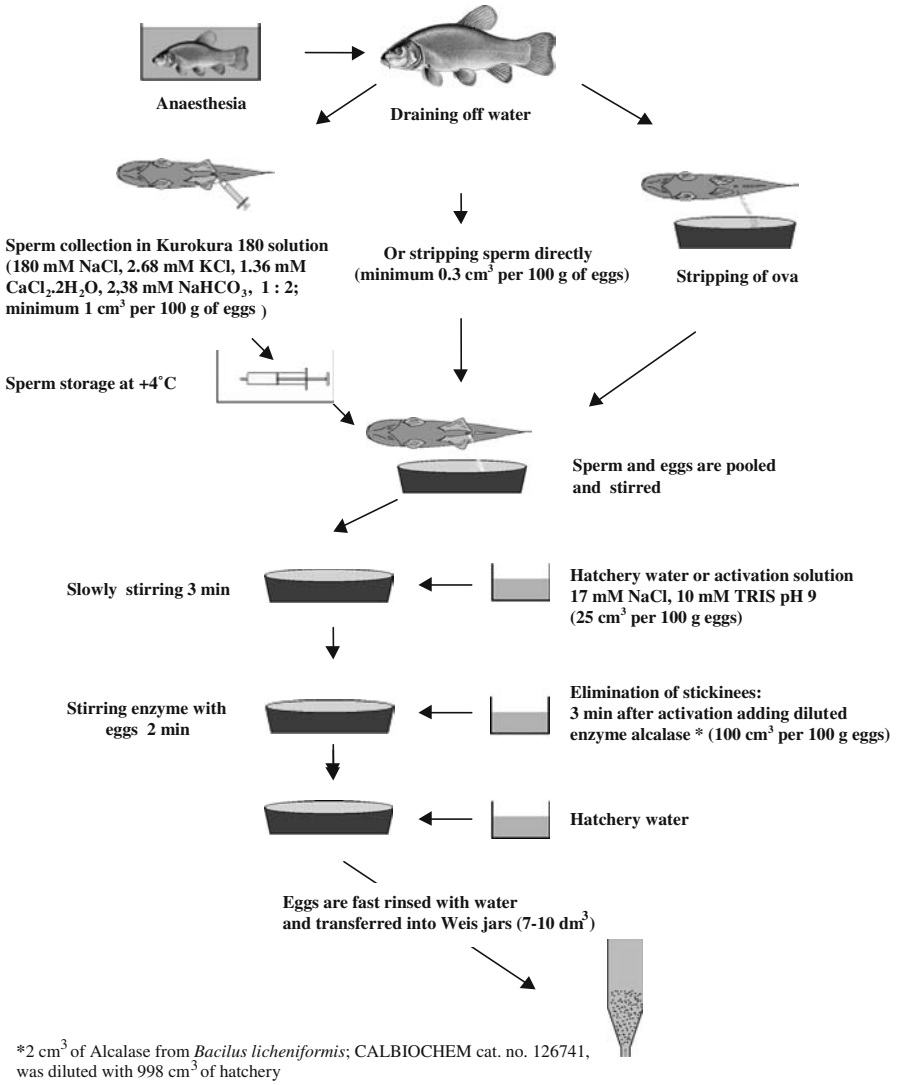

Figure 6. Procedure of the artificial insemination in tench after stripping of hormonally treated broodstock.

incubation at 22 °C. Application of alcalase enzyme was found the easiest approach and the procedure of stickiness elimination itself was 30 times shorter than the time required for stickiness elimination by other procedures (Gela et al. 2003).

The present work describes procedures for artificial insemination of tench, *Tinca tinca* (L.). An increased hatching rate was achieved through optimization of activation solution composition, the process of insemination, activation of

gametes and elimination of egg stickines. A practical scheme for artificial propagation at fish farm hatcheries was proposed.

Acknowledgements

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