ELSEVIER

Aquaculture 191 (2000) 303–308

Aquaculture

www.elsevier.nl/locate/aqua-online

Alcalase enzyme treatment for elimination of egg stickiness in tench, *Tinca tinca* L.

O. Linhart [a,*], D. Gela [a], M. Flajšhans [a], P. Duda [a], M. Rodina [a], V. Novák [b]

[a] *Department of Fish Genetics and Breeding, Research Institute of Fish Culture and Hydrobiology, University of South Bohemia, 389 25 Vodňany, Czech Republic*

[b] *Pond Fish Farm Hluboká Inc., Tyršova 681, 373 41 Hluboká nad Vltavou, Czech Republic*

Received 2 September 1999; received in revised form 17 December 1999; accepted 18 May 2000

Abstract

Enzyme treatment to eliminate egg stickiness in tench was compared with standard methodology in an attempt to increase egg hatching rate under hatchery conditions. Three minutes after activation, eggs were exposed to an alcalase enzyme solution for 2 min. The highest hatching rate of 87.1% was found with 10.0 ml l^{-1} enzyme treatment. Hatching rates of ca. 85% were recorded at 15.0 and 5.0 ml l^{-1}, but hatching rate decreased to 80% at 20.0 ml l^{-1} enzyme. The traditional desticking procedure involving milk/clay treatment gave a hatching rate of 74.1% and required 1 h. Under fish farm conditions, the highest hatching rate of 88.1% was also recorded following treatment of eggs with 10 ml l^{-1} enzyme, while enzyme concentrations of 7.5 and 5.0 ml l^{-1} gave hatching rates of ca. 83%. Treatment with milk/clay solution gave a hatching rate of 30%. ANOVA showed significant differences between enzyme and milk/clay treatments on the hatching rate ($P < 0.0001$).

Keywords: Tench; *Tinca tinca*; Reproduction; Egg stickiness; Alcalase

1. Introduction

The development of tench culture will be dependent on management of the reproductive cycle (Linhart and Billard, 1995; Kouřil, 1998). One problem relates to the stickiness of tench eggs, so that egg stickiness must be reduced before eggs can be

* Corresponding author. Tel.: +42-342-382402; fax: +42-342-382396.
E-mail address: linhart@vurh.jcu.cz (O. Linhart).

0044-8486/00/$ - see front matter
PII: S0044-8486(00)00433-6

incubated successfully in hatchery jars. The traditional method for eliminating egg stickiness involves stirring for 30–40 min in milk solution, then adding a fine clay suspension and stirring for a further 10 min. The eggs are then rinsed with hatchery water and transferred to Weiss jars at 18–23°C for 60–70 degree–days (°C × days) of incubation (Linhart and Billard, 1995).

A proteolytic enzyme (alcalase, Novo Industry) has been used for elimination of stickiness of eggs of the European catfish, *Silurus glanis* L. (Horvath, 1980; Proteau et al., 1994; Linhart et al., 1997), and is now used routinely in hatcheries of the Czech Republic and France (Linhart et al., 1997).

The aim of this study was to examine whether this alcalase technique could be used for eliminating the egg stickiness in tench, *Tinca tinca*, and whether this would increase egg hatching rate and require less time than the traditional milk/clay treatment.

2. Material and methods

An experiment was performed at the University of South Bohemia, RIFCH, Vodňany, and the results verified under hatchery production conditions at Pond Fish Farm Hluboká.

After transfer from ponds to the hatchery, mature males and females were initially separated in tanks at 20–23°C. Artificial propagation followed the methods of Linhart and Billard (1995).

2.1. Laboratory experiment

Nine females and 10 males were used for the experiment. The experiment involved testing the effects of five enzyme concentrations (alcalase enzyme, Merck EC 3.4.21.14) compared to the standard milky/clay treatment. Three replicates were made with eggs from three females. In all experimental replicates, eggs were divided into five batches of 20 g, each containing 15,000–16,000 eggs (3 replicates × 5 treatments = 15 groups).

Sperm of 10 males was collected into plastic (PE) syringes with immobilizing solution (1 part sperm to 2 parts medium: 171.2 mOsmol NaCl, 53.7 mOsmol KCl and 75.1 mOsmol glycine; Linhart and Kvasnička, 1992). Egg batches in small dishes were inseminated with 500 μl sperm in immobilization solution.

For the enzyme test, each batch of inseminated eggs was activated with 10 ml of fresh hatchery water (pH 7.8). Three minutes later, 40 ml of hatchery water containing various concentrations of enzyme were added for 2 min. Concentrations of enzyme were 20.0, 15.0, 10.0 and 5.0 ml/l of hatchery water. The eggs were then rinsed in hatchery water.

The milk/clay treatment follows the description of Linhart and Billard (1995). Each batch of inseminated eggs was activated with 5 ml of solution containing 34 mM NaCl and dissolved milk powder (200 g l^{-1}, 27.2% fat), stirred for 10 s and then left standing for 3 min. Another 5 ml of milk solution was added to the eggs during the 40-min stirring of the eggs. Then, 2 ml of a fine clay suspension (20 g l^{-1}) was added and eggs stirred for another 10 min. The eggs were then rinsed in hatchery water.

Each treated batch was then divided into three samples (each < 300 eggs) and placed into special incubator cages of 200 cm^2, each supplied with recirculated water at 20°C and 9 mg l^{-1} O_2. The remaining eggs (14,000–15,000 eggs) from each batch were transferred to 2-l Weiss jars, supplied with water at 20°C and 8 mg l^{-1} O_2. Dead eggs and larvae hatched in cages were counted and counts used for calculation of hatching rate. The duration of egg incubation to hatching was registered in degree–days (°C × days) to when 50% of larvae hatched. Malformations of hatched larvae were also checked. After 2 h of incubation, eggs were sampled from the Weiss jars and photographed under an Olympus SZ 40 binocular microscope to document changes of the egg envelope.

2.2. Application under fish farm conditions

Twenty-five million eggs were pooled from 500 females during one stripping time. The eggs were divided into 25 samples of 400 g (800,000 eggs) in 10-l dishes. These were inseminated with 7 ml of sperm per dish (sperm pooled from 400 males). Seven samples were used for the milk/clay treatment, and six, nine and three samples for alcalase enzyme concentrations of 10.0, 7.5, and 5.0 ml l^{-1}, respectively. The milk/clay treatment was applied as described before, but on a larger scale.

For the enzyme test, the eggs were activated with 100 ml fresh hatchery water (pH 7.2), then 400 ml of hatchery water containing various concentrations of enzyme was applied as before. The eggs were then rinsed in hatchery water and transferred to 10-l glass Weiss jars supplied with water at 21–22°C, 7–8 mg l^{-1} O_2.

The hatched larvae from each jar were kept separately in hatchery trays until the beginning of exogenous feeding. Numbers of larvae in each tray were then estimated volumetrically and hatching rate was calculated. Malformations of hatched larvae and time of hatching was also checked.

Means of 3–9 replicates were compared and statistical significance was assessed using analysis of variance (ANOVA, Statgraphics version 5) after arcsin transformation, followed by multiple comparison LSD tests. Probability values < 0.05 were considered significant.

3. Results

3.1. Laboratory experiment

Stickiness was eliminated with enzyme concentrations of 15.0, 10.0 and 5.0 ml l^{-1} without destruction of egg envelopes (Fig. 1A), whereas 20-ml l^{-1} enzyme damaged some egg envelopes (Fig. 1B). The clay particles used in the milk/clay treatment covered the egg envelope (Fig. 1C). Time to hatching at 20°C was within the range of 58–71 degree–days and was not significantly different among enzyme concentrations of 15.0, 10.0 and 5.0 ml l^{-1} (Table 1). No larval malformations were observed. The highest hatching rate was 87.1% from the 10.0 ml l^{-1} enzyme treatment, but hatching rates were similar in the 15.0 and 5.0 ml l^{-1} treatments. Hatching rates were significantly lower in the 20.0 ml l^{-1} enzyme (80.0%) and milk/clay (74.1%) treatments

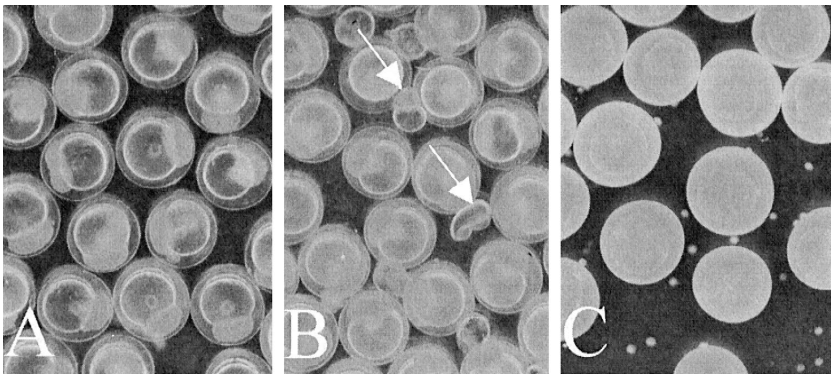

Fig. 1. Micrographs of tench eggs 2 h after fertilization with various treatments: (A) 5–15 ml l^{-1} alcalase; (B) 20 ml l^{-1} alcalase (arrows show damaged envelopes); (C) milk/clay treatment.

(Table 1). ANOVA showed significant effects of enzyme concentration and milk/clay treatments on the hatching rate ($P < 0.0015$) and duration of egg incubation ($P < 0.0003$) until hatching.

3.2. Application under fish farm conditions

The 10.0 and 7.5 ml enzyme/l treatments eliminated egg stickiness, but 5.0 ml l^{-1} was not always completely effective. Time to hatch was delayed 6–12 h at 22°C in the milk/clay treatment compared to the enzyme treatments, because embryos could not break the egg shell. No malformations were observed. The highest hatching rate (88.1%) was found in the 10 ml l^{-1} enzyme, but was about 83% in concentrations of 7.5 and 5.0 ml l^{-1} (Fig. 2). Hatching rate from the milk/clay suspension treatment was only about 30%. ANOVA showed a significant effect of enzyme concentrations and of milk/clay treatment on the hatching rate ($P < 0.0001$). Enzyme treatment decreased the time of

Table 1
Hatching, dead eggs, hatching rate and duration of tench egg incubation in laboratory conditions with alcalase enzyme and milk/clay treatments. Values with a common superscript within a column do not differ significantly ($P < 0.05$)

Treatment	Enzyme per litre of hatchery water (ml)	Number hatching, $\bar{x} \pm$ S.D.	Number of dead eggs, $\bar{x} \pm$ S.D.	%Hatching, $\bar{x} \pm$ S.D.	Duration of eggs incubation until hatching (°C × days), $\bar{x} \pm$ S.D.
Alcalase	20	116.0 ± 41.2	31.3 ± 16.8	80.0 ± 3.7^{b}	58.3 ± 1.5^{a}
enzyme	15	111.0 ± 23.5	20.3 ± 8.2	85.2 ± 3.1^{bc}	60.0 ± 2.0^{ab}
	10	89.3 ± 20.0	13.0 ± 2.2	87.1 ± 1.1^{c}	61.7 ± 2.9^{ab}
	5	135.3 ± 26.5	24.3 ± 7.9	85.1 ± 1.7^{bc}	62.3 ± 2.5^{b}
Milk/clay	0	163.3 ± 38.6	56.7 ± 12.5	74.1 ± 0.7^{a}	71.0 ± 1.7^{c}

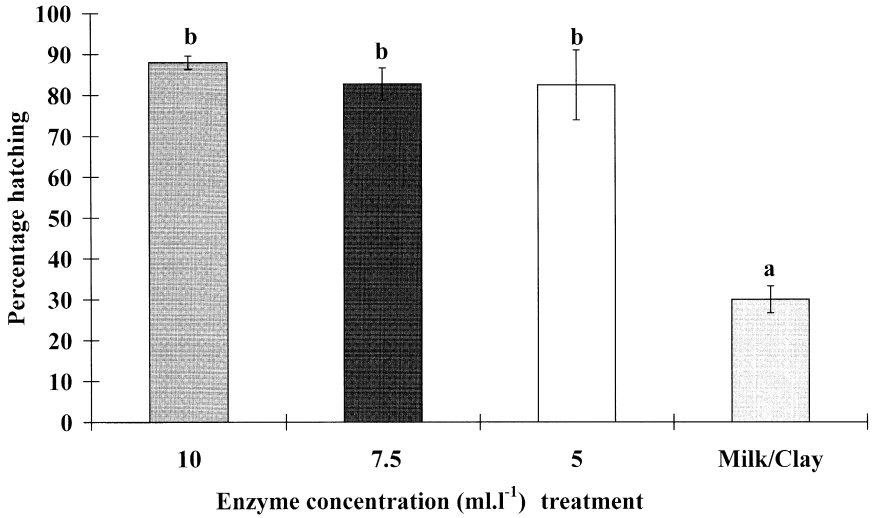

Fig. 2. Hatching rate of tench eggs after elimination of eggs stickiness in experiment under farm conditions using alcalase concentrations of 10.0, 7.5 and 5.0 ml l^{-1} and a milk/clay treatment. Values with a common superscript do not differ significantly ($P < 0.05$).

egg handling, from almost 1 h using milk/clay treatment, to ca. 2 min and also resulted in higher hatching rates, than obtained with the traditional milk/clay method.

4. Discussion

During the experiment under fish farm conditions, 2,520,000 larvae hatched from 8,400,000 eggs (30% of hatching) after desticking of eggs with the traditional milk/clay treatment and 13,780,000 larvae hatched from 16,600,000 eggs (83–88% hatching) from the enzyme-treated eggs. No difference in survival in nursery ponds between the fry from enzyme treatments and milk/clay treatment has been indicated in the subsequent 2 years.

Elimination of egg stickiness is critical for controlled artificial reproduction of phytophilic fish in freshwater aquaculture. The traditional two-step approach, based upon treating eggs with milk solution and clay suspension, may result in inconsistent incubation times and hatching rates (Linhart and Billard, 1995), probably because of varying conditions during propagation and use of the clay suspensions. The clay solution is usually prepared by stirring pieces of clay soil in hatchery water followed by straining and settling. Hence, both the size of clay particles and their concentrations will differ between localities and trials. If the clay suspension is too concentrated, particles stick on the egg envelope, which results in the low hatching rate. Application of enzyme solution shortly after insemination has been used for elimination of eggs stickiness in European catfish, and the results of the present study showed that this method can also be used with tench.

Acknowledgements

We thank Mrs. Marie Pečená and Ivana Samková for technical assistance and Prof. W.L. Shelton for critical comments of the manuscript. This work has been supported by National Agency for Agriculture Research no. 6051 and by the Ministry of Education, CEZ: J06/98:126100001 from the Czech Republic.

References

Horvath, L., 1980. Use of a proteolytic enzyme to improve incubation of eggs of the European catfish. Prog. Fish-Cult. 42, 110–111.

Kouřil, J., 1998. Hormonally induced spawning of tench *Tinca tinca* (L.) females (a review). Pol. Arch. Hydrobiol. 45, 415–433.

Linhart, O., Kvasnička, P., 1992. Artificial insemination in tench, *Tinca tinca* L. Aquacult. Fish. Manage. 23, 183–188.

Linhart, O., Billard, R., 1995. Biology of gametes and artificial reproduction in common tench (*Tinca tinca* L.). A review. Pol. Arch. Hydrobiol. 42, 37–56.

Linhart, O., Billard, R., Kouřil, J., Hamáčková, J., 1997. Artificial insemination and gamete management in European catfish (*Silurus glanis* L.). Pol. Arch. Hydrobiol. 44, 9–23.

Proteau, J.P., Schlumberger, O., Albiges, C., 1994. Nouvelle technique de decollage des oeufs du silure glane (*Silurus glanis*). In: Legender, M., Proteau, J.P. (Eds.), Int. Workshop on the Biological Bases for Aquaculture of Siluriformes, Montpellier, France. 48.