Alcalase enzyme treatment affects egg incubation and larval quality in pikeperch (Sander lucioperca)

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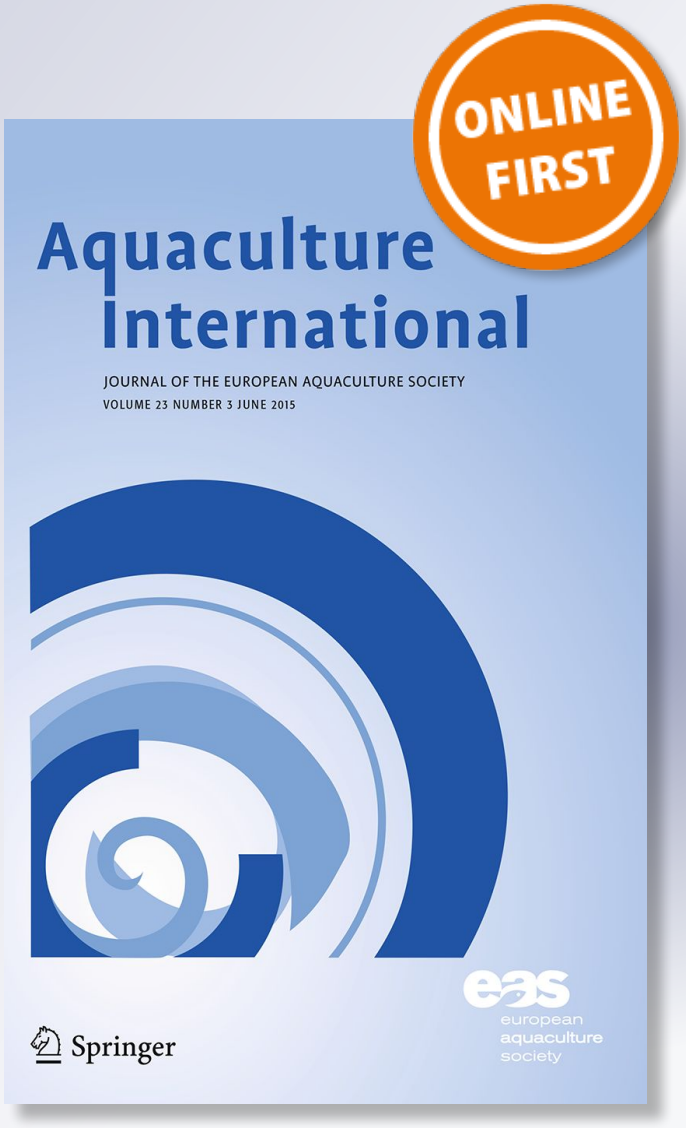

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Alcalase enzyme treatment affects egg incubation and larval quality in pikeperch (*Sander lucioperca*)

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Abstract

Although egg de-adhesion has been the subject of research in pikeperch of late, the possible effect of this technological procedure on larval viability under intensive rearing conditions has not yet been evaluated. The aim of the present study was to evaluate the effect of the Alcalase enzyme on egg incubation and larviculture success compared to a commonly used procedure with milk and kaolin clay suspension. Preliminary research was conducted in order to find the minimal exposure time of eggs in Alcalase enzyme solution for the total elimination of adhesiveness. Further on, stripped eggs from three females were divided into two equal portions, and each portion was treated according to the abovementioned procedures. Efficiency of the procedures was evaluated and compared through egg incubation and larviculture. Alcalase-treated eggs exhibited significantly shorter incubation time (121 ± 12 h vs. 157 ± 10 h), hatching period (16 ± 7 h vs. 48 ± 21 h) and lower embryo survival (82.5 ± 2.4% vs. 87.7 ± 1.4%) with a significantly higher hatching rate (98.5 ± 1.0% vs. 72.0 ± 35.3 %). The larviculture yielded significantly lower production efficacy in eggs treated with Alcalase manifested as the share of larvae with an inflated swim bladder in the total number of stocked eggs (5.8 ± 2.4%) compared to larvae hatched in eggs treated with milk and kaolin (20.1 ± 11.9%). The Alcalase enzyme treatment reduced the incubation time and diminished the larval performance; therefore, its application in eggs of pikeperch should be reconsidered.

Keywords Alcalase · Milk · Kaolin · Egg de-adhesion · Hatching · Larviculture

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Abbreviations:

A	Alcalase enzyme egg de-adhesion treatment
DPF	day post-fertilisation
FOM	final oocyte maturation
hCG	human chorionic gonadotropin
M+K	milk plus kaolin egg de-adhesion treatment
RAS	recirculation aquaculture system

Introduction

For decades now, pikeperch (*Sander lucioperca*) has been described as a promising candidate for diversification of European freshwater aquaculture and is especially suitable for intensive rearing in recirculation aquaculture systems (RAS). Therefore, development of the methods of pikeperch husbandry has been increasing substantially, including artificial reproduction (Zakęś and Demska-Zakęś 2005; Rónyai 2007; Müller-Belecke and Zienert 2008), larviculture (Kestemont et al. 2007; Szkudlarek and Zakęś 2007; Policar et al. 2013) juvenile on-growing (Zakęś et al. 2006a; Rónyai and Csengeri 2008; Wang and Kestemont (2009) and since recently, the induction of gonadal maturation in totally controlled conditions (Hermelink et al. 2011, 2013, 2016). Although the abovementioned methods need further improvement and refinement, the available technologies have already enabled the start of pikeperch farming commercialisation (Steenfeldt et al. 2015).

During the pikeperch artificial reproduction, after gamete collection and prior to egg stocking in the incubation jar, there is an unavoidable procedure—egg adhesiveness removal. In order to properly maintain the eggs in the incubating jars, the adhesiveness of pikeperch eggs must be totally eliminated (Demska-Zakęś et al. 2005; Kucharczyk et al. 2007). This procedure has been the subject of numerous studies in both pikeperch (Zakęś et al. 2006b; Ronyai and Gal 2008; Żarski et al. 2015; Ljubobratović et al. 2017a; Křišťan et al. 2017) and its North American relative, walleye (*Sander vitreus*) (Krise et al. 1986; Krise 1988; Summerfelt et al. 2011). However, some aspects of described procedures, such as low embryo survival, low hatching or extensive labour and time requirements, can be perceived as ineffective. Out of all described procedures and substances involved, protease enzymes have been described as a most promising solution to both issues of excessive time consumption and poor hatching success (Krise et al. 1986; Krise 1988; Zakęś et al. 2006b; Křišťan et al. 2017; Ljubobratovic et al. 2017a). Amongst the paucity of protease enzymes on the market, Křišťan et al. (2017) described the Alcalase enzyme (Merck EC 3.4.21.14) as a suitable product with the most promising outcome in pikeperch eggs. The aptness of Alcalase for this purpose is most likely due to its high activity at relatively low temperatures and appropriate pH values in the circumstances of fish breeding (Sweeney and Walker 1993). Along with the reports for pikeperch, studies on other species confirmed that this enzyme offered a very effective solution for adhesiveness elimination in the time range of 5–10 min post-fertilisation (Linhart et al. 2000; Linhart et al. 2003).

Alongside with parameters of egg incubation success, it could be very important to determine if and to what extent the technological features of the process of artificial reproduction could affect either gamete, embryo or larval quality. To our knowledge unique, the study by Krise et al. (1986) found that the method of stickiness removal can significantly disturb the outcome of larval rearing. Although very effective in terms of time consumption

and hatching, the feature of the enzymes most described in a large proportion of the conducted studies is the reduction of incubation time, which resulted in earlier hatching compared to other methods (Krise et al. 1986; Křišťan et al. 2017; Ljubobratović et al. 2017a). Early hatch in pikeperch larvae is characterised by significantly smaller size (Steenfeldt et al. 2011). Additionally, a study by Zakęś et al. (2006b) noticed that protease enzymes can penetrate the chorion structure. Lastly, possible premature hatching was explained to have negative consequences on early survival in fish (Becker et al. 1983; Small and Wolters 2003). Therefore, it is worthwhile to consider and evaluate if this egg de-adhesion procedure could affect the newly hatched larvae.

The aim of this study was to evaluate the effect of adhesiveness removal in pikeperch eggs with the protease enzyme Alcalase in commercial scale conditions on embryo survival, hatching dynamics and larval performance.

Materials and methods

Broodstock husbandry and procedure of artificial reproduction

Wild pikeperch breeders from the oxbow of river Körös were harvested in December 2016 and transported to an earthen pond at the Research Institute for Fisheries and Aquaculture NAIK HAKI, Szarvas, Hungary. In total, 170 breeders with a total biomass of 300 kg were stocked in 600 m^2 pond supplied with prey fish in the amount of 50% pikeperch biomass. In March 2017, with pond water temperature at 12 °C, breeders were transported to the RAS facility of the institute in two batches; first batch consisted of six females (individual body weight, 0.8–1.4 kg) and four males (individual body weight, 1.1–1.8 kg) and the second batch was brought in a week later and consisted of five females (individual body weight, 2.8–4.0 kg) and three males (individual body weight, 1.5–2.5 kg).

The following day after transportation, fish were hormonally treated with a single injection of human chorionic gonadotropin (hCG) (Choragon, Ferring International Center S.A., Switzerland). Prior to hormonal injection and during all further handling procedures, fish were anaesthetised with a 0.3-mL L^{-1} solution of 2-phenoxyethanol. The procedure of artificial reproduction was performed according to the protocol detailed by Ljubobratović et al. (2018). Once the eggs were collected, a sample of 200–300 dry eggs was activated with hatchery water and their quality evaluated as explained by Żarski et al. (2012). Only the eggs of females with 100% deformations during cortical reaction were used for the study. Therefore, eggs of four and three females from the first and second batch, respectively, were used for the study. Dry eggs were mixed with fresh, plain sperm in a ratio of 1 mL sperm per 100 g of dry eggs in a dry plastic bowl. After a minute of fine mixing with a plastic spoon, activation was done with clean hatchery water at 15 °C.

Preliminary testing of minimal Alcalase exposure time

The first batch of breeders was used for this part of the study. Given that enzyme activity might depend on water quality properties (Eed 2013), preliminary testing of the minimal enzyme exposure time for total elimination of egg adhesiveness was carried out. Enzyme concentration (1.5 mL L^{-1} of Alcalase enzyme) and temperature of the bath (20 °C) were set in accordance with the study by Křišťan et al. (2017). Due to previous results with water hardness and egg

de-adhesion in our hatchery (Ljubobratović et al. 2017a), the addition of NaCl to the enzyme solution was reduced to 0.1%. Two minutes after fertilisation, water was carefully drained, and fertilised eggs of each female were separated into three batches of similar amounts (40–50 g). Half a litre of enzyme solution was used per 100 g of eggs. Eggs of two females were treated with the enzyme for 1, 2 and 3 min, whilst the eggs of other two females were treated with the enzyme 5, 10 and 15 min, thus duplicating each of the exposure times. After the enzyme treatment, eggs were washed with clean hatchery water at 15 °C and stocked into separate 7 L Zug jars. Following stocking in the jar, egg adhesiveness was visually monitored in jars every 6 h and at the first observation of egg clumping, the number of hours elapsed after fertilisation was recorded.

Effect of Alcalase treatment on embryo survival and hatching

In order to evaluate the effect of enzyme treatment (A) on embryo survival and hatching, the procedure was compared to a milk + kaolin procedure (M+K). M+K treatment has been commonly used at the site of NAIK HAKI for last 3 years (Ljubobratović et al. 2014; Ljubobratović et al. 2017b) and has been successfully applied at several hatcheries in Hungary, Romania and Belgium. In order to follow industrial conditions, relatively big females (second batch) were used in this study, thus making the amount of dry eggs per replication in the range 140–300 g. Two minutes after fertilisation, fertilised eggs of each female were separated into two equal batches, and each batch was treated with one of the experimental treatments:

- A: Eggs were stirred in 1.5 mL L^{-1} Alcalase enzyme solution at 20 °C for 5 min. After the enzyme treatment, eggs were washed with plain hatchery water at 15 °C. Total procedure time from fertilisation to stocking the eggs in the incubation jar lasted 10 min.
- M+K: Eggs were stirred in milk solution (1 L of milk with 3.5% fat content mixed with 7 L hatchery water) for 30 min. Then, milk was drained from the eggs and 25 mL L^{-1} kaolin clay solution (Szipöcz Kft, Budapest, Hungary) was added to the eggs for an additional 3-min bath. Afterwards, eggs were washed with plain hatchery water until most of the remaining clay was washed off. The water temperature remained at 15 °C throughout the procedure. The total procedure from fertilisation to stocking the eggs into the incubation jar lasted 40 min.

After the de-adhesion procedure, eggs from each replication (three per treatment) were stocked into separate 7 L Zug jars (six jars in total), and the filling level of eggs was marked on the jar to record the volume after sedimentation finished. At 72 h post-fertilisation, the eggs' level in the jar was marked and the proportion of live eggs was evaluated by microscopic observation of three samples of 100–150 eggs from each female. The 72-h embryo survival rate was estimated as follows:

$$\text{72h embryo survival rate} = \frac{\text{volume of eggs 72h after fertilisation}}{\text{volume of eggs at the time of stocking into Zug jar}} \times \text{mean percentage of live eggs}$$

The volume of eggs in each jar was also measured after hatching and was completed with an accuracy of ± 10 mL. Egg incubation in jars was carried out in a flow-through system supplied

with well water. The water temperature during egg incubation was 15.3 ± 0.6 °C and oxygen saturation in the outflow of the incubators was kept at 100–120%. High oxygen saturation in the jars was maintained in order to prevent premature hatching caused by oxygen depletion.

When hatching was noted by several thousand swimming larvae in the jar, a sample of 100 live eggs from each jar was stocked into separate Petri dishes (size 60 × 10 mm) placed in the 0.5 L plastic bowl filled with plain hatchery water. Bowls were placed in a room with a constant air temperature at 15 °C. After placement in the hatching bowls, newly hatched larvae were harvested out of the bowls every 12 h and counted. The egg incubation time was recorded at the time when the cumulative number of hatched larvae reached over 50% of the total hatch. Hatching duration was calculated as the time from stocking the eggs into the hatching bowls until the time when the last swimming larva was harvested out. When most of the hatched larvae had been counted, 30 larvae per replication were photographed with a stereoscopic microscope. The total length of the larvae was calculated using ImageJ 1.34 software (Rasband 1997–2011) as described by Ben Khemis et al. (2014). Finally, the hatching rate was calculated as the share of harvested larvae in the initially stocked number of eggs in the hatching bowls, whilst the hatching index was defined as the product of multiplying the 72-h embryo survival and hatching rates (Żarski et al. 2015).

Effect of Alcalase treatment on larviculture in RAS

In order to evaluate the effect of enzyme treatment (A) on larviculture success, newly hatched larvae were stocked into the larval rearing RAS for further 1-month rearing. Therefore, 84 h post-fertilisation, a volumetric evaluation of eggs from each jar was performed. Two millilitres of eggs from each jar were gently placed in the Petri dish, and the total number of live eggs in the sample was recorded. This procedure was repeated three times for each jar. When most of the hatched larvae had been counted in the 0.5 L hatching bowl, 12,500 eggs were syphoned from the jar and stocked into a 4-L bowl filled with hatchery water. The bowl with the eggs was immediately transferred to the larval rearing RAS. In the following hour, larvae hatched and they were released to the rearing tank. Eggs from each replication were stocked into a separated tank, making six tanks for the evaluation of the larval rearing success. In that sense, as during the incubation, larvae from each female represented one replication for both treatments.

From hatching until 37 days post-fertilisation (DPF), larvae were reared in the larviculture RAS. The larval rearing system consisted of 12 rearing tanks, a PolyGeyser bead filter, a moving bead bioreactor and a trickling filter. Each larval rearing tank had a cylindrical-conical shape with black-coloured walls, a white conical bottom and a rearing volume of 250 L, making stocking density 50 larvae L^{-1}. From hatching until the 20 DPF, the photoperiod was kept on a 14:10 light–dark cycle, where the light intensity on the surface of the water during the light period ranged between 5 and 15 lux. From 21 DPF, constant light was maintained with light intensity in the range of 20–40 lux. Light intensity was measured with a Voltcraft MS-1300 digital luxmeter (Rapid Electronics Limited, Severalls Lane, UK). Water flow was gradually increased, from 1.2 L min^{-1} at the start of rearing to 6 L min^{-1} by the end of the trial. In order to promote swim bladder inflation, the water surface at each tank was cleaned by a surface sprayer with a flat fan nozzle. Tank hygiene was maintained by extracting the uneaten food, faeces and dead fish through the 0.2 L cleaning cup positioned at the end of the cylinder bottom. This process was enabled by an independent tap used to drain the sediment on the cup's bottom twice per day. Tank walls were cleaned twice per week with a sponge and salt.

From 10 to 20 DPF, larvae were exclusively fed with newly hatched *Artemia* nauplii. During the first 3 days of exogenous feeding, larvae were fed with *Artemia fransciscana* nauplii (AF origin, INVE, Dendermonde, Belgium). Further on, *Artemia salina* (GSL origin, INVE, Dendermonde, Belgium) were used. The initial amount of given *Artemia* was 100 nauplii per larvae per day. This amount was gradually increased to 300 nauplii per larvae per day until 16 DPF and was kept at this level until 21 DPF. From 22 DPF, the amount of given nauplii was gradually reduced by 20% per day, thus being totally excluded from the diet from 26 DPF. Live food was given by hand in six equal daily portions every 4 h. Weaning of larvae to dry feed started at 21 DPF. As a dry diet, Otohime (Marubeni Nisshin Feed Co. Japan) starters were used. During the first 4 days, starter B1 was used (pellet size, 250–360 μm), whilst starter B2 (pellet size, 360–640 μm) was used during the following 7 days and C1 feed (pellet size, 580–840 μm) was given further on until the end of the trial. Based on the producer's specification, crude composition of B feed was the following: crude protein, 56.3%; crude fat, 15.9%; crude fibre, 2.6%; crude ash, 13.5%; calcium, 2.5%; and phosphorus, 2.3%; whilst for the C feed, crude composition was the following: crude protein, 58.3%; crude fat, 12.9%; crude fibre, 1.6%; crude ash, 15.0%; calcium, 2.7%; and phosphorus, 2.5%. The initial amount of dry feed was 20 g $tank^{-1}$ day^{-1} and was gradually increased to 70 g $tank^{-1}$ day^{-1} until the end of the trial.

At 37 DPF, all the larvae were harvested out of the tank and selected based on the presence or absence of an inflated swim bladder. Separation of the larvae was performed in a 0.01-mL L^{-1} 2-phenoxyetanol solution as described by Steenfeldt (2015). All the larvae from each group were counted. In a random sample of 30 larvae with inflated swim bladders, individual total length and body weight were measured, and at the same time, the occurrence of mouth deformities on larvae was visually evaluated. On a random sample of 200 fish with inflated swim bladders, the occurrence of spinal deformities was assessed as well. Larviculture productivity was evaluated by three parameters: survival rate, production efficacy and effective yield. Survival rate was counted as the ratio of total number of harvested larvae in the total number of stocked eggs. Production efficacy represents the number of harvested juveniles with an inflated swim bladder as a percentage of the total number of stocked eggs, whilst the effective yield presents the number of harvested juveniles with inflated swim bladder in the total water volume of the rearing tank.

During the larval rearing, water oxygen saturation and temperature were monitored daily on the outflow of each rearing tank, whilst ammonium-nitrogen, nitrite-nitrogen, nitrate-nitrogen content and water pH were assessed twice per week. Thus, mean water oxygen saturation was 103.3 ± 6.6% and 101.8 ± 6.2% in A and M+K tanks, respectively, whilst the mean temperature was 17.6 ± 1.1 °C in tanks of both treatments. Ammonium-nitrogen, nitrite-nitrogen and nitrate-nitrogen content in the outflow water were 0.15 ± 0.07 (max, 0.28), 0.04 ± 0.02 (max, 0.07) and 13.9 ± 3.6 (max, 18.7) mg L^{-1}, respectively, whilst the water pH was 8.4 ± 0.2.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Prior to analysis, all the percentage variables were arcsine transformed. Not all of the analysed variables fulfilled the normality assumption based on the Shapiro–Wilk test, nor were their variances homogenous based on Levene's test. As well, there was an outlier replication (female) in M+K treatment from hatching onwards. Therefore, differences between the groups were analysed using non-parametric Mann–Whitney *U* tests (Zimmerman 1994; Nahm

2016). The significance level was set to $p \leq 0.05$. Analyses were performed using SPSS 22.0 software (IBM, New York, NY, USA).

Results

Preliminary testing of minimal Alcalase exposure time

At the time of stocking of the eggs into the Zug jars, adhesiveness was not noticed in any of the treatments. However, egg clumping was noticed 12, 18 and 24 h later in the 1-, 2- and 3-min exposure time treatments, respectively, in agreement in both replicates. There was no observed clumping amongst 5-, 10- and 15-min exposure time treatments in both replicates from the time of stocking until the hatching.

Effect of Alcalase treatment on embryo survival and hatching

Although both treatments yielded rather a high embryo survival 72 h post-fertilisation (82.5 ± 2.4% and 87.7 ± 1.4% in A and M+K treatments, respectively), significant differences were noticed between the treatments ($p = 0.046$). A significantly higher hatching rate was found in A treatment (98.5 ± 1.0%) compared to M+K (72.0 ± 35.3%), where in one female, a low hatch was noticed (31.4%) compared to the other two (95.4% and 89.3%). Incubation time in A treatment was significantly shorter compared to the M+K treatment. Likewise, hatching duration was significantly shorter in the A treatment. Mean total length in newly hatched larvae from the M+K treatment was 20% higher compared to the A treatment (Table 1). Faster and more sudden hatching was noticed in the A treatment, which is illustrated in Fig. 1.

Effect of Alcalase treatment on larviculture in RAS

After 1 month of nursing, significantly higher ($p = 0.050$) production efficacy was found in M+K treatment with an average of more than three times higher compared to the A treatment. Likewise, significantly higher ($p = 0.050$) productive yield was found in M+K treatment, yielding nearly four times more juveniles with inflated swim bladder per litre of rearing

Table 1 Egg incubation success with respect to egg de-adhesion treatment in pikeperch. Data are presented as mean ± standard deviation (SD). Three replicates of each treatment

Parameter	A[a]	M+K[b]	*p* value
Embryo survival (%)	82.5 ± 2.4	87.7 ± 1.4	0.046
Hatching (%)	98.5 ± 1.0	72.0 ± 35.3	0.050
Hatching index (%)[c]	81.3 ± 2.6	63.3 ± 31.2	0.513
Incubation time (h)	121 ± 12	157 ± 10	0.050
Hatching duration (h)	16 ± 7	48 ± 21	0.043
Larvae size at hatching (mm)	4.0 ± 0.1	4.8 ± 0.2	0.050

[a] A – 4 min after fertilisation eggs were washed in 1.5 mL L^{-1} Alcalase enzyme for 5 min

[b] M+K – 4 min after fertilisation eggs were stirred in 1:7 milk:water solution for 30 min followed by 3-min bath in 25 mL L^{-1} kaolin bath

[c] Hatching index – product of embryo survival and hatching

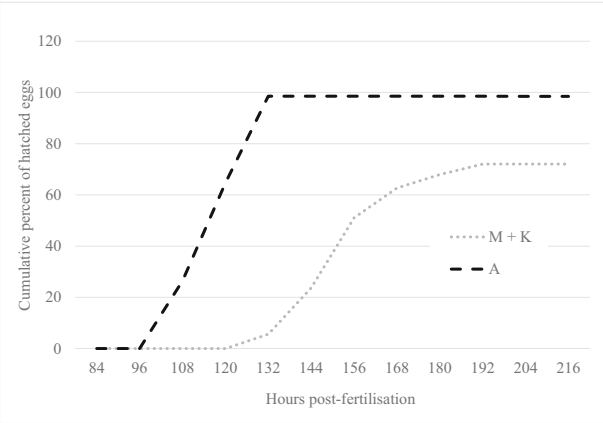

Fig. 1 Mean cumulative hatch after adhesiveness removal with Alcalase enzyme and milk plus kaolin treatments in pikeperch eggs. A – 4 min after fertilisation, eggs were washed in 1.5 mL L^{-1} Alcalase enzyme solution for 5 min; M+K – eggs were stirred in 1:7 milk:water solution for 30 min followed with 3-min bath in 25 mL L^{-1} kaolin. Dashed line presents the mean cumulative percent of hatched eggs at the given time (number of hours post-fertilisation) in eggs treated with 1.5 mL L^{-1} Alcalase enzyme solution for 5 min; dotted line presents the mean cumulative percent of hatched eggs at the given time (number of hours post-fertilisation) in eggs treated with 1:7 milk:water solution for 30 min followed with 3-min bath in 25 mL L^{-1} kaolin

volume compared to A treatment. There were no significant differences in the deformity rate between the treatments, although the replication with low hatching in the M+K treatment yielded a rather high share of juveniles with spinal deformities compared to the other two in both M+K (38.2% compared to 12.0% and 6.8%) and A treatments (37.2% compared to 11.2% and 12.6%). There were no significant differences in other assessed parameters (Table 2).

Table 2 Production parameters of month-old pikeperch juveniles with larvae obtained after Alcalase enzyme (A) and milk plus kaolin (M+K) treatments for egg adhesiveness removal. Data are presented as mean ± standard deviation (SD). Three replicates per each treatment

Parameter	A[a]	M+K[b]	*p* value
Survival (%)	32.1 ± 16.9	55.7 ± 17.0	0.127
Production efficacy (%)[c]	5.8 ± 2.4	20.1 ± 11.9	0.050
Effective yield (pc L^{-1})[d]	2.9 ± 1.2	11.5 ± 5.9	0.050
Final weight (mg)	115.8 ± 8.6	121.2 ± 16.8	0.827
CV_{weight} (%)	38.9 ± 9.5	33.3 ± 6.0	0.513
Final length (mm)	23.7 ± 1.1	23.9 ± 0.9	0.827
CV_{length} (mm)	11.8 ± 2.3	10.1 ± 1.8	0.275
Spine deformities (%)	20.3 ± 14.6	19.0 ± 17.0	0.827
Mouth deformities (%)	12.2 ± 1.9	13.3 ± 3.3	0.637

[a] A – 4 min after fertilisation eggs were washed in 1.5 mL L^{-1} Alcalase enzyme solution for 5 min

[b] M+K – 4 min after fertilisation eggs were stirred in 1:7 milk:water solution for 30 min followed by 3-min bath in 25 mL L^{-1} kaolin

[c] Production efficacy – number of harvested juveniles with inflated swim bladder as a percentage of the total number of stocked eggs

[d] Effective yield – number of harvested juveniles with inflated swim bladder in the total water volume of the rearing tank

Discussion

In many cultured species, it is of crucial importance to totally remove the adhesiveness in eggs prior to stocking in the incubation jar (Krise et al. 1986; Al Hazzaa and Hussein 2003; Linhart et al. 2003; Demska-Zakęś et al. 2005; Siddique et al. 2016). Remaining stickiness results in egg clumping, which can lead to improper oxygen distribution amongst the incubated embryos. This could further weaken the eggs and finally cause mortality. Compared to a previously published study describing the efficacy of Alcalase enzyme treatment for egg de-adhesion in pikeperch (Křišťan et al. 2017), longer enzyme exposure time was needed for total elimination of adhesiveness in our study. On the one hand, the reason for this disagreement might lie in different water quality used in these two studies. However, on the other hand, the reason might be of rather technological nature. Both present and study by Krise (1988) agreed that 5 min is the shortest effective exposure time for enzymes, and both described adhesiveness recurring up to 1-day post-fertilisation in case of insufficiently treated eggs. Whilst the study by Křišťan et al. (2017) was performed under experimental conditions, present and the study by Krise (1988) formed commercially comparable conditions, what might explain the reason for later noticed complications, not visible in case of small sample size.

The success of egg incubation in this study was in agreement with previous studies on egg de-adhesion in pikeperch (Demska-Zakęś et al. 2005; Żarski et al. 2015; Křišťan et al. 2017); thus, the quality of the gametes and technological procedures used in this study were of acceptable quality. Although both treatments led to rather an acceptable embryo survival, it was still significantly lower in Alcalase-treated eggs. The mode of action of external proteolytic enzymes on the structure of a freshly fertilised egg has not yet been closely studied, although preliminary investigations found that it can penetrate the chorion structure (Zakęś et al. 2006b). With this regard and described catalytic activity of proteases leading to hydrolysis of peptide bonds (Li et al. 2013), alteration of the egg envelope's structure and functions cannot be excluded. Thus, perhaps the enzyme action could disturb early egg development to some extent and thus lower its viability. From the other side, in the Alcalase enzyme–treated eggs, almost all surviving embryos hatched, resulting in significantly higher hatching in this treatment. Low hatching in one female finally led to a significant decrease of this parameter in the M+K treatment. The reason for low hatching success in this female might lay in a maternal effect as in the other two replicates of M+K treatment high hatching were observed. Further on, in this replication of the M+K treatment, the lowest larval survival and production efficacy as well as a higher deformity rate were observed compared to the other two females. Although the lower survival may be the consequence of lower hatching (as the eggs were hatched in the larval rearing tank), what is especially intriguing is the phenomenon observed in the case of spinal deformities. In both treatments, a roughly three times higher share of larvae with spinal deformities was observed in the mentioned female that noted low hatching in M+K treatment. Therefore, although M+K treatment decreased the hatching success, it somehow indicated a diminished quality of stocking material as the same pattern of increased spinal deformities was seen in both treatments. Therefore, such an outcome of reduced hatching could not be necessarily described as adverse as it indicates which material to exclude from further culture.

The issue of premature hatching is rarely investigated in fish. It was reported to lead to total mortality in the case of improper dewatering in *Oncorhynchus tshawytscha* eggs (Becker et al. 1983) as well as in the case of overdosed hydrogen peroxide treatment in the eggs of *Ictalurus punctatus* (Small and Wolters 2003). Both studies indicate this phenomenon occurs as a result

of some disturbing factor during the incubation. However, there is a rather important question to ask in the case of pikeperch: When are the embryos ready to hatch? Several studies demonstrated that in this species, hatching might take days (Kucharczyk et al. 2007; Żarski et al. 2015; Ljubobratović et al. 2017a). Additionally, such a phenomenon of prolonged hatching duration was investigated from the aspects of larval viability and cannibalism (Steenfeldt et al. 2011), where the authors did not find an effect of incubation time on larviculture success. Two studies investigating the usage of enzymes for egg de-adhesion reported a shorter incubation time in such treated eggs (Křišťan et al. 2017; Ljubobratović et al. 2017a) compared to other treatments. Steenfeldt et al. (2011) found earlier hatched larvae to be shorter compared to those hatched later. Similarly, in the present study, larvae in the M+K treatment were hatched later and were significantly longer at the time of hatching compared to larvae in eggs treated with Alcalase enzyme. Coming back to the previously mentioned premature hatching due to disturbing factors, the query is whether the enzyme treatment could be defined as disturbing to embryonic development. With respect to lower embryo survival and further significantly reduced production efficacy during larviculture (e.g., larval viability), such a conclusion could be supposed. However, there is another option to consider as well. It might be that the egg envelope is pre-digested and narrowed due to the Alcalase treatment, which leads to an initial amount of the secreted hatching enzyme chorionase to finish digestion of the envelope before the embryos are mature enough to swim properly. Nevertheless, additional studies are needed in order to further conclude which of the possible effects led to a significant decrease of larval viability in the present study: Was it only the early hatching due to Alcalase that weakened the egg envelope, or did the enzyme treatment in general disturbed the entire embryogenesis process, thus leading to larvae of lower quality? Regardless of the exact mechanism, the egg de-adhesion method clearly had an effect on larval rearing success in pikeperch. This outcome is in agreement with a similar study in walleye (Krise et al. 1986). Although the evaluation of the basic parameters of incubation success indicated a negative effect of Alcalase enzyme on embryo survival, it was not obvious. Moreover, based on the egg incubation parameters only, this treatment could have been suggested as an advanced method with respect to shortened procedure duration, higher hatching rate, increased simultaneous hatching (shorter hatching duration) and higher hatching index. Thus, once again, the importance of evaluation of larval rearing success in connection to modifications of artificial reproduction technology was pointed out in the case of percids (Krise et al. 1986; Dabrowski et al. 2000; Żarski et al. 2011; Ljubobratović et al. 2017b). Low survival rate during the larval phase is the most critical issue in pikeperch culture (Mani-Ponset et al. 1994; Ruuhijärvi et al. 1991; Hilge and Steffens 1996). Therefore, with respect to a significantly higher number of larvae with inflated swim bladder found in M+K treatment, the Alcalase enzyme treatment should be considered less effective.

To conclude, the Alcalase enzyme treatment reduced the incubation time, which led to a significantly smaller size of newly hatched larvae. Although the main parameters of incubation success did not suggest a lower efficacy of Alcalase treatment, the significantly lower production efficacy found during larviculture clearly indicated that newly hatched larvae from this treatment were of lower quality compared to eggs treated with milk and kaolin. Thus, although offering the labour reduction, usage of enzymes as an egg de-adhesion substance in pikeperch should be reconsidered as a standard hatchery procedure.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed by the authors.

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