RESEARCH PAPER

Comparison of the Procedures for Adhesiveness Removal in Pikeperch (*Sander lucioperca*) Eggs with Special Emphasis on the Effect of Tannic Acid

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Abstract

The methods for removal of adhesiveness in pikeperch eggs were compared in this study. Following the hormonal induction with carp pituitary, eggs of three females were used in each of two trials. Firstly, three common methods were tested: tannic acid, enzyme and milk. Tannic acid treatment led to poor hatching (2.5±0.5%), significantly lower than the other treatments (75.5±19.3% and 72.3±28.8%, enzyme and milk, respectively). In order to find the source of negative outcome, in the second trial the influence of water hardness on the tannic acid bath was tested through three treatments with varying concentrations of disodium ethylenediaminetetraacetic acid salt (NA$_2$EDTA). Softening of the tannic acid bath did not lead to improved hatching rates (5.5±1.3%, 3.5±1.5% and 2.7±1.3% for 0, 400 and 800 ppm of EDTA, respectively). Evaluation of the effects of tannin and EDTA on water chemistry revealed that the use of tannin can alter the embryonic development and hatching process. While de-adhesion procedure with milk appeared to be impractical, the application of tannic acid bath leads to variable and lower hatching outcomes. Thus, usage of enzymes was found to be the most promising method which should be further described for straightforward implementation in practice.

Keywords: Hatching, tannin, enzymes, milk, water hardness.

Introduction

An important element of commercial pikeperch artificial propagation is the removal of egg adhesiveness. This specific procedure aiming at deactivation of adhesive properties of the eggs takes place straight after the artificial fertilisation and is crucial for effective mass incubation in commercial scale incubators (Demska-Zakęś, Zakęś, & Roszuk, 2005; Kucharczyk, Kestemont, & Mamcarz, 2007). In artificial conditions, non-effective adhesiveness removal results in egg clumping and sticking for the walls of the incubation jars which leads to low availability of oxygen for some of the eggs and further embryo mortality and fungal infections. There are both family- and species-specific mechanisms of egg attachment (Riehl & Patzner, 1998) which implies that specific methods need to be developed for the elimination of this feature of eggs. Methods for removal of the adhesiveness are best described for common carp eggs due to its long history of artificial propagation (Woynarovich, 1962, 1964; Schoonbee & Brandt, 1982; Khan, Gupta, Reddy, & Sahoo, 1986). The widely-used method in carp culture is the salt and urea treatment (90 min time duration) with a subsequent short tannic acid bathing (3-4 times 10 sec) for hardening the remaining mucus on the surface of egg envelope (Woynarovich, 1964; Meske, Woynárovich, Kausch, Lühr, & Szablewski, 1968; Woynarovich & Horváth, 1980). Recently, there have been reports on the rather unexplored substances for egg de-adhesion, such as pineapple juice (Thai & Ngo, 2004) which are promising candidates for testing in other species as well. Finally, some investigations have addressed the differing fertilisation solutions in several species (Schoonbee & Prinsloo, 1984; Billard, Cosson, Perchec, & Linhart,, 1995; Legendre, Linhart, & Billard, 1996).

The salt and urea treatment with a short tannic acid bath has also been used in pikeperch culture (Bokor, Horvath, Horvath, & Urbany, 2008). However, due to its long lasting, efforts have been made to find a shorter method. Several studies have tested usage tannic acid on its own as a de-adhesion procedure (Demska-Zakęś *et al.*, 2005; Rónyai & Gál, 2008; Żarski *et al.*, 2015). In a pioneer study, Demska-Zakęś *et al.* (2005) revealed that this commonly-used substance in culture of many species can lead to undesirable results such as low hatching rates or irregular hatching of pikeperch larvae. Similar

problems were reported by Rónyai & Gál (2008). Żarski *et al.* (2015) were the first to report high hatching rates after using of solely tannic acid to remove adhesiveness in pikeperch eggs. They found that application of tannic acid after the completion of egg swelling leads to significantly improved hatching rates. Accordingly, reported mean hatching rates of last three mentioned studies in the most successful treatments ranged from 52.3 to 94.9 %, while two of them reported irregular hatching with egg chorion remaining on the head of larvae.

The use of enzymes has been proposed as a way to reduce the time required to remove egg adhesiveness in many species (Krise, Bulkowski-Cummings, Shellman, Kraus & Gould, 1986; Linhart, Rodina, Gela, Flajšhans, & Kocour 2003a). It has been demonstrated that for pikeperch, washing their eggs with enzymes offers an effective new approach (Zakęś, Demska-Zakęś, Roszuk, & Kowalska, 2006; Křišt'an, Blecha, & Policar, in press), resulting in hatching rates between 70 and 90 %. Still, reported effective enzyme solutions in pikeperch and walleye varied from 0.01 to 0.5 % with application time ranging from 2 to 30 minutes (Krise *et al.*, 1986; Zakęś *et al.*, 2006.; Křišt'an *et al.*, in press).

The usage of milk or milk powder in common carp (Linhart, Rodina, Gela, Kocour, & Rodriguez, 2003) and Siberian sturgeon (Feledi, Kucska, & Rónyai, 2011)), led to hatching rates above 80%. To our knowledge, usage of milk exclusively as the egg de-adhesion substance in pikeperch was not reported.

Mentioned de-adhesion procedures with tannic acid, enzymes and milk were not previously compared in a common study in pikeperch eggs. Thus, these widely used methods for adhesiveness removal with tannic acid (Żarski *et al.*, 2015), enzyme (Křišt'an *et al.*, in press) and milk (Feledi *et al.*, 2011) were tested in the present study. With purpose to further explain the failure of hatching using tannic acid, the influence of modification of water hardness by adding additional chelating agent (disodium ethylenediaminetetraacetic acid, Na_2EDTA) into tannic acid solution was examined.

Materials and Methods

Broodstock Origin and Management

The study was composed of two trials which were carried out over two consecutive years. Wild pikeperch breeders originating from the oxbow of the Körös River were harvested and transferred to wintering ponds. At the beginning of the natural spawning season, breeders were transported to the indoor facility of the Research Institute for Fisheries, Aquaculture and Irrigation, Szarvas, Hungary. Following transportation, fish were exposed to thermal induction by gradually heating water from 10°C to 16°C over four days. Fish were kept at this temperature for a further three days prior to hormonal

induction. Females were injected with two consecutive injections; each of 3 mg kg^{-1} CPE (Carp pituitary extract), and time frame between two injections was 36 hours Males were injected with single injection of CPE in the concentration of 2 mg kg^{-1} at the time of second female injection. Prior to each injection as well as prior to stripping procedure, all the fish were anaesthetised with clove oil solution, which was consisted of mixed 96% ethanol (Sigma-Aldrich) and clove oil (Aromax, Hungary) in ratio 1:1, further diluted in hatchery water in concentration 0.1 ml L^{-1}.

Eggs from three females (mean individual mass 2.1±0.4 kg and 2.3±0.1 kg in first and second trial, respectively) and milt from fours males (mean individual mass 1.4±0.5 kg and 1.7±0.7 kg in first and second trial, respectively) were used in each trial. Eggs of each female were used as one replication, altogether three females making three replications of three treatments in each trial.

Experiment I

Eggs of each female were divided into three batches, fertilised with the milt of two males and treated with different de-adhesion methods. Egg adhesiveness was removed for each batch according to three treatments:

1. Tannin (T): fertilised eggs were mixed in clean hatchery water for 30 minutes, changing water every five minutes. After 30 minutes, a 750 mg L^{-1} solution of tannic acid (Molar Chemicals Kft, Budapest, Hungary) was applied twice for 30 seconds. Eggs were washed between and after treatments.
2. Enzyme (E): two minutes after fertilisation a 0.5 ml L^{-1} solution of protease enzyme (product number P4860, Sigma-Aldrich, St. Louis, Missouri, USA) was added to previously washed eggs with an exposure time of two minutes.
3. Milk (M): two minutes after fertilisation eggs were treated with milk solution (1 L 3.5% fat milk diluted in 7 L hatchery water) for 60 minutes, changing the solution every 20 minutes. Eggs were gently stirred continuously.

After the de-adhesion procedure was finished, each batch of eggs (replication) was placed in a separate 7 L Zug jar (altogether 9 jars) supplied with flow-through aerated well water. Around 48 hours post fertilization a sample of one hundred eggs was taken from each jar and embryo survival was determined by the microscopic observation. Upon hatching, 200 eggs were taken from each jar and placed in separate 15 L washbowls which were floating in a flow-through tank supplied with the same water as the hatching jar to maintain temperature. Half the water from the washbowls was refreshed with tank water every eight hours. Every three hours, newly hatched larvae were counted and harvested from washbowls. Hatching dynamics were evaluated in order to define the period in which most of the

hatching took place what would additionally describe the effect of different methods. During egg incubation, water temperature was maintained at 16.3±0.3°C and the oxygen saturation level was kept above 80%.

Experiment II

Eggs of each female were fertilised with the milt of two males and stirred in clean hatchery water for 30 minutes, changing the water every five minutes. After 30 minutes of stirring, eggs from each female were divided into three batches (one replication of each treatment) and bathed according to treatments:
1. T-Control: eggs were bathed in tannic acid solution in clean hatchery water;
2. T-EDTA-400: eggs were bathed in tannic acid solution with 400 mg L^{-1} EDTA solution in hatchery water;
3. T-EDTA-800: eggs were bathed in tannic acid solution with 800 mg L^{-1} EDTA solution in hatchery water.

Tannic acid concentration in all treatments was 700 mg L^{-1} and exposure time was twice for 40 seconds. For the T-EDTA treatments, the appropriate concentration of EDTA disodium salt (Sigma-Aldrich, St. Louis, Missouri, USA) was diluted in hatchery water prior to dilution of the tannic acid (Hungaropharma Zrt, Budapest, Hungary). In order to view the effect of additional chelating agent, Na_2EDTA as a practical water softener was used. In both trials same water source was used for the fertilisation and eggs incubation which was carried out in the flow-through system. Water quality parameters were evaluated on the inflow well water. Additionally, impact of tannic acid addition on the clean and softened water was analysed through water hardness, pH and alkalinity.

Immediately after the placement of the eggs in separate 7 L Zug jars (altogether 9 jars), a sample of 200 eggs was taken from each replication and placed in a separate net chamber hanging above the respective incubation jar (altogether 9 chambers, one on each jar), which prevented flushing of the unfertilised eggs. At 48 and 84 hours after fertilisation, eggs were taken out from the chambers for the evaluation of embryo survival. Evaluation of the embryo survival in both trial was evaluated as the number of eggs reaching the neurula stage in relation to the total number of analysed eggs using a

stereomicroscope at 10-fold magnification.

After the evaluation of the 84 hours embryo survival, the chambers were cleaned, disinfected and dried. Afterwards, 150 eggs from all treatments were taken from the incubation jars (altogether nine) and placed into the separate net chambers hanging on the top of the respective jars to estimate the hatching. Every four hours, chambers of all treatments were checked for hatched larvae which were harvested. During egg incubation, water temperature was stable at 16.5±0.3°C and the oxygen saturation level was above 80%.

Statistical Analyses

All the percentage data were arcsine transformed prior to statistical analyses. One-sample Kolmogorov-Smirnov test was used to test data distribution and homogeneity of variances was assessed with Levene's test. The differences between the treatments in the variables with unknown distribution (fertilisation rate in experiment I and hatching period in both trials) and in the variables with non-homogeneous variances (hatching rate in experiment I) were examined with Mann-Whitney U nonparametric test (P≤0.05, asymptotic significance). The variables with normal distribution and homogenous variances (first day hatch, 48 and 84 hours fertilisation rate and hatching rate in experiment II) were analysed with one-way analysis of variance (ANOVA). In experiment II, the relationship between water hardness in the tannic bath and embryo survival 48 and 84 hours post-fertilisation, hatching rate, and hatching period was analysed with Pearson's bivariate correlation (P≤0.05). Statistical analyses were done using SPSS22.0 software (IBM, New York, NY, USA).

Results

Experiment I

There were no significant differences in the mean 48hours embryo survival among treatments. Mean hatching rate in T treatment was significantly lower compared to E and M treatments (Table 1). Significantly higher first day hatch (number of larvae hatched during the first 24 hours of hatching; Figure 1) was found in E treatment (116.3±31.3) compared to milk and tannic acid treatments (34±21.6 and 0.7±1.2, respectively). Hatching duration was

Table 1. The effect of different de-adhesion procedures on hatching (mean ± standard deviation)

Parameter/Treatments	Tannic acid	Enzyme	Milk
48 h embryo survival (%)	84.1±24.1	88.0±9.6	94.1±2.6
Hatching rate (%)	2.5±0.5[a]	75.5±19.3[b]	72.3±28.8[b]
First day hatch (N)	34±21.6[b]	116.3±31.3[a]	0.7±1.2[b]
Hatching period (hours) ((hours)	36.7±26.4[a]	89.0±2.1[b]	93.0±1.7[b]

[*] Values with different superscript are different with a significance level of P≤0.05

significantly shorter in T treatment, while there were no differences in this parameter between E and M treatments. First hatch was observed between 96 and 100 hours post-fertilisation in all treatments.

Experiment II

There were no significant differences in the mean embryo survival and hatching rate among treatments. Hatching rate in all replications was below 7 %. The hatching period was significantly lower in T-EDTA-400 treatment compared to other treatments which were not statistically different between each other (Table 2). The relationship between water hardness in the tannic acid bath and studied parameters was weak for all parameters except for hatching rate which was strongly positively correlated (r=0.697, P<0.05).

The chemical composition data of the source water and mixtures of tannin and Na_2EDTA were determined by accredited methods. Data obtained for mineral ion composition and calculated hardness data are given in Table 3. As the main observations we have to emphasize, that the addition of tannins – due to their complexation effects – decreased the availability of Ca^{2+} and Mg^{2+} for complexometric titration and – due to the acidic character of tannins – the pH of water decreased.

Discussion

De-adhesion method with eggs bath in tannic acid solution in both clean and softened water led to low hatching. In terms of used concentration, exposure time and time of application after fertilisation, method applied in this study was similar to previously successfully explained by Żarski *et al.* (2015) and it was in the line with optimal variant found by Demska-Zakęś *et al.* (2005). Based on several reports on the chemistry of water soluble tannic acid (a mixture of esters of glucose with gallic acid and 3-galloylgallic acid or longer galloyl gallic acid side chains), two explanations for the failure of hatching and irregular hatching could be speculated. First, the tannin products used may have different properties depending on the origin and supplier since these compounds are known to have a variety of molecular weights (Hagerman *et al.*, 1998) and usual characterization of tannin provided by suppliers refers only to the final ratio of glucose to gallic acid molecules (Makkar & Becker, 1993). The reports on hatchery used tannins usually have no characterization of tannins except providing the name of supplier (Demska-Zakes *et al.*, 2005; Hosseini & Khara, 2015; Zarski *et al.*, 2015). Second, the reason may lie in different water quality parameters. Special caution has been already urged with usage of tannic
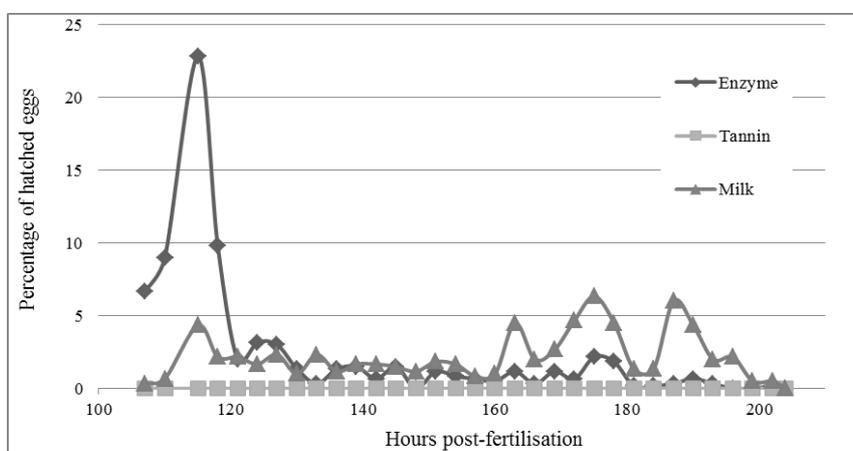

Figure 1. Percentage of hatched larvae over time in Experiment 1.

Table 2. The effect of different de-adhesion methods on fertilisation, hatching and deformity rate (mean ± standard deviation)[*]

Parameter/Treatments	T-Control	T-EDTA-400	T-EDTA-800
48 h embryo survival (%)	66.8 ± 7.2	66.5 ± 10.0	63.7 ± 11.9
84 h embryo survival (%)	65.3 ± 8.4	65.2 ± 8.8	60.0 ± 12.3
Hatching rate (%)	5.5 ± 1.3	3.5 ± 1.5	2.7 ± 1.3
Hatching period (hours)	49 ± 22[a]	12 ± 4[b]	60 ± 4[a]

[*] Values with different superscript are different with a significance level of P ≤ 0.05
T-Control—control tannic acid treatment without water softening
T-EDTA-400—tannic acid treatment with water softened with 400 mg L^{-1} of EDTA
T-EDTA-800—tannic acid treatment with water softened with 800 mg L^{-1} of EDT

Table 3. Water quality parameters of the water source used in trials and impacts of tannin and Na₂EDTA water hardness, pH and alkalinity *

Water samples	KisKut (source water)	T-700	T-1400	T-4200	T-700 + EDTA-400	T-700 + EDTA-800
pH	7.15	7.09	6.94	6.46	6.65	6.26
Conductivity ($\mu S\ cm^{-1}$)	1010	-	-	-	-	-
Ammonium-Nitrogen ($mg\ L^{-1}$)	<0.100	-	-	-	-	-
Nitrite-Nitrogen ($mg\ L^{-1}$)	<0.020	-	-	-	-	-
Nitrate-Nitrogen ($mg\ L^{-1}$)	0.380	-	-	-	-	-
Orthophosphate Phosphorus ($mg\ L^{-1}$)	<0.050	-	-	-	-	-
Total Phosphorus ($mg\ L^{-1}$)	<0.050	-	-	-	-	-
Chloride ($mg\ L^{-1}$)	42.0	-	-	-	-	-
Sulphate ($mg\ L^{-1}$)	185	-	-	-	-	-
Hydrogen carbonate ($mg\ L^{-1}$)	462	443	434	384	389	384
Carbonate ($mg\ L^{-1}$)	0.0	0.0	0.0	0.0	0.0	0.0
Calcium ($mg\ L^{-1}$)	64.5	-	-	-	-	-
Iron ($mg\ L^{-1}$)	<0.05	-	-	-	-	-
Potassium ($mg\ L^{-1}$)	0.62	-	-	-	-	-
Magnesium ($mg\ L^{-1}$)	49.1	-	-	-	-	-
Sodium ($mg\ L^{-1}$)	109	-	-	-	-	-
p – alkalinity ($mmole\ L^{-1}$)	0.0	-	-	-	-	-
m – alkalinity ($mmole\ L^{-1}$)	7.57	7.27	7.11	6.30	6.38	6.30
Total Hardness (°dH)	20.5	11.0	2.2	<1	3.5	<1

* Data from NAIK ÖVKI Environmental Analysis Center (http://www.haki.hu/en/ovki_kak_en) accredited by Hungarian NAH agency (http://www.nah.gov.hu/)
T – 700: KisKut (well water source) with added tannin at 700 mg L⁻¹
T – 1400: KisKut (well water source) with added tannin at 1400 mg L⁻¹
T – 4200: KisKut (well water source) with added tannin at 4200 mg L⁻¹
EDTA – 400 + T-700: KisKut (well water source) softened with EDTA at 400 mg L⁻¹ and added tannin at 700 mg L⁻¹
EDTA – 800 + T-700: KisKut (well water source) softened with EDTA at 800 mg L⁻¹ and added tannin at 700 mg L⁻¹

acid to remove egg adhesiveness in hatcheries which use water of high alkalinity (Hodson & Hayes, 1989; Rottmann, Shireman, & Chapman, 1991). Tannins are known to react with and precipitate proteins in complex process (Kawamoto, Nakatsubo, & Murakami, 1996; Hagerman, Rice, & Ritchard, 1998). Moreover, tannin is known as a chelating agent and it can react with metallic ions *in vitro* (South & Miller, 1997; Andjelković *et al.*, 2006; Quideau, Deffieux, Douat-Casassus, & Pouységu, 2011) and in isolated human plasma (Gryszczyńska *et al.*, 2015). Therefore, variability in hatching may be attributable to differing water hardness among hatcheries with different water sources. Hypothetically, during the tannic acid bath, water with high concentrations of Ca^{2+} and Mg^{2+} could lead to formation of such metal–tannin–protein complexes which could be difficult to break down by hatching protease, chorionase. Based on the concentrations of mentioned metals revealed in the inflow water (Table 3.), such an assumption could be feasible. Thus, in our second trial water was softened using a common water softener (EDTA) acting as additional chelator in the tannic acid bath. Nevertheless, it did not lead to any improvement in hatching success. Oppositely, adding of extra chelating agent (EDTA) to tannic acid decreased the hatching rate. These unexpected observations have led to additional water analyses (Table 3). The addition of tannins – due to their complexation effects – decreased the total hardness referring to the lowered

availability of Ca^{2+} and Mg^{2+} during the complexometric determination. These observations suggest the lowered availability of metallic elements participating in ionic homeostasis during the embryonic development. Though the tannic acid bath was performed in softened water, the source of water for the egg incubation did not differ among the treatments. Chelation complex of tannins and calcium is present after tannic acid bath during the incubation and can be, at least partially, deliberated at the hatching phase. Partial deliberation of tannin components by breakage of weak chelate- and hydrogen-bridge bonds from the chelated complexation of the outer layer of chorion can enter into the perivitellin space in the last stage of hatching. Consequently, the formation of chelates possible might cause the impaired activity of chorionase on egg envelope proteins as chorionase is a metalloenzyme sensitive to chelating agents (Hagenmaier, 1974b). Several studies on the physiology of egg activation and fertilisation have described the role of Ca^{2+} as a crucial one (Yamamoto, 1954; Webb & Miller, 2013). Fertilisation and egg swelling took place in the same water in all our treatments. Our results suggest that there may be an influence of Ca^{2+} ions even after egg activation and egg swelling. Accordingly, described difficulties with hatching may be the consequence of an interactive effect between hardened egg shell and improper embryo development due to unfavourable ion content of the

eggs. Both of the suggested hypotheses are indicating that the influence of ion composition of the perivitellin fluid on embryonic development could be an important issue for further studies on percid species

Previously described phenomenon of irregular hatching where the larvae free themselves from the chorion with the tail while the egg shell remains on the head (Demska-Zakęś et al., 2005; Rónyai & Gál, 2008) was observed in our study as well in all tannin treatments (Figure 2).   Although tannin exerts hormetic effects at low concentration (0.1-0.3 mg L^{-1}) on sea urchin embryo, at higher concentration it has embryotoxic effects (De Nicola et al., 2004, 2007) and lowers the mitotic activity of the embryonic cells (Pagano, Castello, Gallo, Borriello, & Guida, 2008). Moreover, the egg envelope (chorion or *zona radiata*) proteins are members of a unique group of structural proteins (Hyllner, Westerlund, Olsson, & Schopen, 2001; Arukwe & Goksøyr, 2003), which can be decomposed during the last stage of hatching by the hatching protease (chorionase) (Yamagami, 1973; Hagenmaier, 1974a,b; Luczynski, Strzczek, & Brzuzan, 1987) or choriolysins (Kawaguchi et al., 2013). Therefore, the phenomenon that egg chorion remains on the head of larvae can probably be related to the impaired activity of chorionase on egg envelope proteins and the above mentioned embryotoxicity.

While tannic acid removes adhesiveness through precipitating complexation process of phenol groups with the glycoproteins and mucopolysaccharides responsible for sticky feature (Mansour, Lahnsteiner, & Patzner, 2009) of egg, enzymes are most likely acting by breaking down peptide groups of the glycoproteins and mucopolysaccharides by hydrolysis(Castro et al., 2011; Li, Yi, Marek, & Iverson, 2013; Tavano, 2013). The use of enzymes in eliminating egg adhesiveness is mainly focused on shortening the time required for the procedure.

Křišt'an et al. (in press) reported high hatching rates with concentrations of 0.5to 2 mlL^{-1} for two minutes exposure time, two minutes after fertilisation. In our study similar method led to similar hatching success, with, however, higher variation, which was present in milk treatment as well. It can be assumed that the reason for higher variation is either in remnant stickiness which was noticed in these two treatments (E and M) or in genetic effect as each replication originated from different environments. A concern raised in this treatment is that most of larvae hatched during the first day of hatching (Table 1, Figure 1) which could be characterised as undeveloped as their eyes were not pigmented at the time of hatching. Similar observation regarding shortened incubation time in enzyme treated eggs was given by Křišt'an et al. (in press). These results indicate a specific effect of enzymes: it can lead to weakening of the egg envelope, probably due to enzyme digestion of the envelope surface. This treatment seems to be most promising from practical aspect as it reduces the total procedure time significantly. Therefore, it would be of interest for pikeperch culturists to examine whether the earlier hatching in this treatment affects the larviculture success. Finally, similar to tannic acid, enzyme activity is dependent on many environmental factors such as temperature and pH (Moore, 1974; Tipton and Dixon, 1979; Eed, 2013; Kumar, Sahu, Pal, Saravanan, & Priyadarshi, 2013). Therefore, prior to application, this treatment as well should be refined on hatchery level.

Tannic acid and enzymes act through chemical reactions between the substances and egg envelope structure, but milk acts mostly by physical binding of milk fat to the sticky surface of egg shell, neutralising adhesiveness. Achieved hatching rate was similar to enzyme treatment (Table 1.).However, as mentioned earlier, remnant stickiness was observed in this treatment even though the stickiness elimination

Figure 2. Remained egg shell cover on the head of newly hatched larvae in tannic acid treatment.

procedure was the longest at in total 60 minutes. Further, bathing and washing the eggs in milk appeared to be the most difficult due to low transparency of the solution. With these regards, this method seems inconvenient from practical aspect.

It was reported that hatching in pikeperch often lasts for days (Kucharczyk *et al.*, 2007). In our study it was the case in all treatments except in the T-EDTA-400 treatment where the hatching period took 8 to 16 hours. Considering the low hatching rate in this treatment we may not imply that it was due to more efficient hatching. Based on our observation, shorter hatching period in tannin treatments in both trials might be connected to the low hatching which started in the later stages of embryo development while many embryos unable to break the egg shell eventually died.

While the usage of milk as de-adhesion substance appears to be impractical, method with tannic acid is rather dependable on water and product quality and therefore should be rather specified on the hatchery level and modified substantially. Adhesiveness removal with enzymes is the most promising method of de-adhesion for commercial scale pikeperch propagation. Therefore, this method should be further refined and explained for straightforward implementation in practice. Finally, its effect of shortened egg incubation time on larval viability should be of interest for further studies.

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