Effects of stocking density, feeding technique and vitamin C supplementation on the habituation on dry feed of pikeperch (*Sander lucioperca*) pond reared juveniles

Ljubobratović U.[1*]; Kucska B.[2]; Sándor Z.[1]; Peteri A.[1]; Rónyai A.[1]

Received: September 2015 Accepted: July 2016

Abstract

Influence of three different stocking densities, vitamin C supplementation of *Daphnia* spp. and feeding practice (i.e. mechanical, hand) on the success of dry feed habituation of pond reared pikeperch juveniles was investigated through one month trial. Pond reared pikeperch juveniles were harvested 42 days post-fertilisation (mean individual weight 1.1 ± 0.3g) and stocked into the experimental recirculation system. For the stocking density trial, fish were stocked in three different initial densities of 0.5, 1 and 1.5 fish L^{-1}, while the trial on live food supplementation and feeding practice was performed with two two-group comparisons with one common control treatment. All treatments were performed in 3 replicates. Analysis of variance did not reveal any significant differences in the assessed parameters between the tested stocking densities. However, Pearson correlation for the habituation success was strong in the course of increased density ($r^2 = 0.829$, $p = 0.006$). Vitamin C supplementation led to increased survival, habituation success and growth, although the differences were not significant ($p > 0.05$). Similarly, there were no significant differences between the hand and mechanical feeding in the habituation success ($p = 0.860$). Based on the results of present study and previous results reported for habituation of pikeperch and walleye pond nursed juveniles, stocking densities of 4 to 8 fish L^{-1}, feeding with dry feed with worms supplied manually and possible enrichment with vitamin C of the given food could be suggested for the successful commercial pikeperch dry feed habituation.

Keywords: Pikeperch, Habituation, Dry feed, Stocking density, Vitamin C.

1-Research Institute for Fisheries and Aquaculture NAIK HAKI, Anna liget 8, H-5540, Szarvas, Hungary.

2-Kaposvár University, Faculty of Agricultural and Environmental Sciences, H-7400 Kaposvár, Guba Sándor u. 40, Hungary.

*Corresponding author's email: u.ljubobratovic@gmail.com

Introduction

Since the pikeperch (*Sander lucioperca*) has been described as the promising candidate for diversification of European aquaculture, many studies have been pointed to the production of juveniles habituated on dry feed. In order to produce such a stocking material, two methods have been described – intensive larviculture (Kestemont *et al*., 2007; Szkudlarek and Zakęś, 2007) and habituation of pond reared juveniles to the formulated feeds (Szkudlarek and Zakęś, 2002, Bódis *et al*., 2007; Horváth *et al*., 2012; Policar *et al*., 2013). Since the larviculture is connected to the several critical points which may lead to low survival (Ruuhijärvi *et al*., 1991; Hilge and Steffens, 1996), explained alternative of dry feed habituation has been found as an effective and technologically less demanding method which is suitable for seasonal production. Three studies have been investigating the effect of stocking density on the effectiveness of the habituation of pikeperch juveniles on the dry feed. While Szkudlarek and Zakęś (2002) (mean individual weight 0.65 g, tested densities 0.99 to 2.31 g L^{-1}) and Molnar *et al*. (2004) (mean individual weight 0.91 g, tested densities 0.91 to 2.08 gL^{-1}) did not find any effect of the stocking density on the effectiveness of rearing, Policar *et al*. (2013) (mean individual weight 0.42 g, tested densities 0.42 to 3.36 g L^{-1}) found that there is a significant relationship between initial stocking density and survival. Previously mentioned studies used different feeding techniques during habituation period (i.e. hand feeding and mechanical feeder) and they reported differing outcomes in sense of loses during the habituation period. Vitamin C supplementation in food has been proved to have a significant influence on growth and health of fish larvae and juveniles (Merchie *et al*., 1997; Kolkovski *et al*., 2000; Kestemont *et al*., 2007). In such a harsh period as can be the transition from live to inert diets in predatory fish, it is worth concerning that this positive effect could be important for final success.

In this study, we aimed to examine the effects of stocking density, vitamin C supplementation of the live food and feeding practice on the final success of dry feed habituation of pikeperch fry which were previously reared in pond conditions.

Materials and methods

Fish origin, pond rearing and experimental design

Reproduction of wild caught pikeperch breeders was performed through tank spawning on the nests as described by Rónyai (2007). On the second day post-fertilisation, nests with fertilized eggs were stocked into 0.17 ha pond fertilized with cow manure. Forty two days post-fertilisation, 5000 juveniles (mean individual weight 1.1±0.3g) were harvested from the pond and transported to the experimental indoor facility of Research Institute for Fisheries and Aquaculture NARIC HAKI. Fish were stocked into two 300 L tanks supplied with pond water and were disinfected with 30 minute formalin bath (concentration 100 mg L^{-1}). Afterwards fish were distributed in twenty 275 L tanks in an experimental recirculation system. Tanks were covered with black plastic sheet with an opening part of 25 × 10 cm used for feeding. This was done according to recommendations by Howey *et al*. (1980).

Habituation procedure

Experiment lasted for 30 days. For the habituation on formulated food, Aller Futura EX (Aller Aqua A/S, Allervej 130, 6070 Christiansfeld, Denmark, 0.8-1.3 mm pellet size, 64% protein, 14% fat, 10.2% ash, 1.3% fibre, 1.5% phosphorus) was used. During first 15 days together with dry feed, fish were offered with live food, *Daphnia* spp. and *Tubifex* spp. (*Daphnia* and *Tubifex* further on) and the feeding regime was different for each consecutive 5 days period as follows:

- Day 1 to day 5 - 2.5% of initial biomass dry feed was offered and supplemented with 20% of initial tank biomass *Daphnia;*
- Day 6 to day 10 - 5% of initial biomass dry feed was offered and supplemented with 5% of initial tank biomass *Tubifex* and 5% of initial tank biomass *Daphnia*;
- Day 11 to day 15 - 5% of initial biomass dry feed was offered and supplemented with 2.5% of initial tank biomass *Tubifex.*

Further on, dry feed was exclusively given in the amount 5% of initial biomass from day 16 to day 30. Dry feed was given by the mechanical feeder (FIAP belt feeder Aquacultur Fishtechnik, Germany) from 9:00 to 19:00, constantly. Live food was given by hand in four equal meals given at 09:00, 12:00, 15:00 and 18:00.

Experimental design

Two parallel trials were performed simultaneously in the same system (altogether 18 tank units), set in completely randomized design.

Trial 1 – Effect of stocking density on the habituation success of pikeperch fry.

In order to examine the effect of stocking density on the habituation of pond reared juveniles three treatments were compared in triplication (altogether nine tanks):

- Low – initial stocking density of 150 fish per tank (≈0.5 fish L^{-1});
- Medium – initial stocking density of 300 fish per tank (≈1 fish L^{-1});
- High – initial stocking density of 450 fish per tank (≈ 1.5 fish L^{-1}).
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Trial 2 – Effect of feeding technique and vitamin C enrichment

Stocking density in the second trial was 140 fish per tank. In order to examine the effect of feeding technique and live food enrichment with vitamin C, trial was composed of two two-group comparisons. One treatment “Feeder” was used as the common control for both comparisons, as follows:

- Control (“Feeder”) – fish were fed with standard procedure which is previously described;
- Vitamin C (“VitC”) – fish were under the same feeding regime while the given *Daphnia* was enriched with vitamin C prior to application;
- Hand feeding (“Hand”) – instead of constant mechanical feeding fish were fed

the same amount given by hand, each three hours starting from 08:00 until 20:00.

Sampling, data collection and analyses

Prior to stocking in the experimental system, a sample of 30 fish was measured for individual body weight (±0.05g) and individual total length (±1 mm). After each 5 days of training (day 6, 11, 16, 21 and 26), obvious cannibals (visually determined by the deformed stomach shape) were extracted from the tanks and further counted in the cannibalism rate. Tanks were cleaned twice per day, at 07:00 and 19:00 and the number of dead individuals was recorded. At the end of the experiment on day 31, all fish were measured for individual body weight (±0.05g) and the sample of 10 habituated fish per tank was measured for individual total length (± 1 mm). It was obvious that some fish were trained and the others were starved by the phenotypic appearance, as explained by Horváth *et al*. (2012). Therefore, fish were divided in two groups, trained and starved.

In order to enrich *Daphnia* with vitamin C, a portion of live food was treated with 1g L^{-1} solution of L-ascorbic acid (Sigma-Aldrich, St. Louis, MO) for one hour prior to administration. On day 1, 2 and 3, a sample of 1g of enriched and non-enriched *Daphnia* was taken for the analysis of vitamin C content. On day 13 one fish per tank was taken from VitC and Feeder treatments and sacrificed by overdosed clove oil anaesthetisation for analysis of vitamin C content in the fish. Laboratory analysis of live food and fish tissue samples were carried out as described by Papp *et al*. (1998).

Oxygen saturation and temperature were measured daily in the rearing tanks. Nitrogen compounds and pH were checked twice per week on the sample taken from the sedimentation tank prior to daily cleaning. Oxygen was kept above 60% saturation, while the temperature was kept on 23.6 ± 0.6 ˚C (range 22.2-26.7 ˚C). Average ammonium-nitrogen, nitrite-nitrogen, nitrate-nitrogen and pH were 0.14 ± 0.05 mg L^{-1}; 0.02 ± 0.00 mg L^{-1}; 6.58 ± 2.10 mg L^{-1}, and 8.71 ± 0.26, respectively and all were in acceptable range for pikeperch during the trials (range 0.10-0.24 mg L^{-1}; 0.02-0.03 mg L^{-1}; 3.39-9.39 mg L^{-1}; 8.33-9.00, respectively).

Statistical procedures

Data are presented as mean ± standard deviation. In the first trial, statistical analysis was carried out based on one-way analysis of variance (ANOVA, $p \leq 0.05$). Prior to analysis, all the probability data were arcsin transformed. In order to test the distribution, one-sample Kolmogorov-Smirnov test was used and further homogeneity of variances was analysed with Levene’s test. All the analysed data fulfilled these requirements for ANOVA. In order to test the relationship between the habituation success and stocking density, bivariate (Pearson) correlation was performed. In the second trial, statistical analysis was performed by Student’s t-test (unpaired samples) for two two-group comparisons. For both comparisons, “Feeder” treatment was used as control. Statistical analyses were done using SPSS 22.0 software (IBM, New York, NY, USA).

Results

In the trial on initial stocking density mean habituation success was between 12.7 and 17.8%, while the SGR of habituated fish was rather constant on the 3.6 % day^{-1} without significant differences among the treatments in any of the analysed parameters (Table 1). Bivariate correlation between the weaning success and stocking density showed strong positive dependence (r^2 = 0.829, p = 0.006). Significant differences among the analysed culture parameters were not noticed in the trial on vitamin C supplementation, yet the higher mean final weight in the VitC treatment was close to the significance (p=0.060). Significant higher ascorbic acid content was recorded in the treated groups of *Daphnia* compared to control, but the differences in the fish tissue content of the substance were not significant (Table 2). There were no significant differences between the mechanical and hand feeding treatments in any of the analysed parameters, however rather low *P*-value was recorded in growth rate (p=0.056) being higher in the mechanical feeding treatment (Table 3).

Table 1: Effect of different initial stocking density on the success of dry feed habitation based on the assessed parameters (values are given as mean ± SD (n=3)).

Parameters	**Low**	**Medium**	**High**
Survival (%)	26.9±4.3	31.0±4.3	29.9 ± 2.2
Habituation success (%)	12.7±2.4	15.2±3.9	17.8 ± 4.8
Mortality (%)	42.4 ± 0.1	44.8 ± 0.1	40.2 ± 0.1
Final weight of habituated fish (g)	3.2 ± 0.4	3.1 ± 0.2	3.1 ± 0.2
Final length of habituated fish (mm)	76.3 ± 4.7	74.5 ± 2.8	73.2 ± 0.9
CV_{weight} (%)	31.3 ± 8.3	31.3 ± 1.7	31.1 ± 3.1
SGR (% day^{-1})	3.6 ± 0.4	3.6 ± 0.2	3.6 ± 0.2
Weight starved fish (g)	0.96 ± 0.0	0.89 ± 0.1	0.92 ± 0.1
Cannibalism (%)	30.7 ± 2.4	24.2 ± 2.9	29.9 ± 9.2

Initial body weight: 1.1±0.3g. Initial length: 51.6±10.7mm.
Low – initial stocking density of 0.5 fish/l; **Medium** - initial stocking density of 1 fish/l; **High** - initial stocking density of 1.5 fish/l. SGR: specific growth rate; CV_{weight}: in-tank coefficient of variation for mean weight for the habituated fish.

Table 2: Effect of vitamin C enrichment of *Daphnia* spp. during dry feed habituation on the assessed rearing parameters (values are given as mean ± SD (n=3)).

Parameters	VitC	Feeder	P-value
Survival (%)	35.7±2.5	32.6±1.5	0.135
Mortality (%)	22.6 ± 7.2	27.4 ± 3.5	0.930
Habituation success (%)	16.2±3.3	11.4±0.7	0.107
Final weight (g)	3.5±0.3	3.2±0.1	0.060
Final length (mm)	76.4±0.6	75.2±9.1	0.240
SGR (% day^{-1})	3.9 ± 0.3	3.6 ± 0.1	0.118
Cannibalism (%)	40.0±3.6	31.7±7.2	0.148
CV $_{weight}$ (%)	31.5±1.5	32.5±8.5	0.890
Daphnia ascorbic acid content (μg g^{-1})	7.2 ± 1.0*	0.6±0.2*	0.000*
Fish ascorbic acid content (μg g^{-1})	8.0±2.1	5.4 ±2.0	0.191

Initial body weight: 1.1±0.3g. Initial length: 51.6±10.7mm. Asterisk denotes parameter differed significantly between the two groups (independent t-test, p<0.05) Feeder: fish were fed with non-enriched *Daphnia* spp; VitC: fish were fed with enriched *Daphnia* spp. SGR: specific growth rate; CV $_{weight}$: in-tank coefficient of variation for mean weight for the habituated fish.

Table 3: Effect of feeding technique during dry feed habituation on the assessed rearing parameters (values are given as mean ± SD (n=3)).

Parameters	Hand	Feeder	P-value
Survival (%)	39.0±13.5	32.6±1.5	0.483
Mortality (%)	29.3 ± 18.5	27.4 ± 3.5	0.933
Training success (%)	11.2±3.9	11.4±0.7	0.860
Final weight habituated fish (g)	3.0±0.0	3.2±0.1	0.060
Final length habituated fish (mm)	73.4±2.1	75.2±9.1	0.240
SGR (% day^{-1})	3.4 ± 0.1	3.6 ± 0.1	0.056
Cannibalism (%)	41.7±4.8	31.7±7.2	0.148
CV $_{weight}$ (%)	29.0±14.2	32.5±8.5	0.701

Initial body weight: 1.1±0.3g. Initial length: 51.6±10.7mm. Feeder - formulated feed was administrated by the automatic feeders; Hand - formulated feed was administrated by hand. SGR: specific growth rate; CV $_{weight}$: in-tank coefficient of variation for mean weight for the habituated fish.

Discussion

Based on the one-way analysis of variance there were no significant differences in the weaning success among the stocking density treatments in the first trial. These results are in agreement with previous studies on pikeperch (Szkudlarek and Zakęś, 2002; Molnar *et al*., 2004) and walleye (Kuipers and Summerfelt, 1994). Nevertheless, based on the correlation analysis, there is a positive significant dependence between the habituation success and initial stocking density. Similar outcome was found by Policar *et al*. (2013). Common for present and other studies which did not find significant effect of the stocking density is usage of lower stocking densities (0.5 – 4 fish per L^{-1}) compared to previously mentioned study. It might be that on densities of 4 and more fish L^{-1} this factor exhibits more pronounced effect. Nevertheless, even though the effect was not revealed through one-way ANOVA, strong relationship was found through bivariate correlation that might be more reliable indicator for studies on stocking density. Similar statistical method was previously used to explain the effect of stocking density on pikeperch larviculture (Szkudlarek and Zakęś, 2007). Taking into account results achieved by Policar *et al*. (2013) and the protocol explained for walleye by Summerfelt *et al*. (2011), we may imply that stocking densities of 4-8 fish per litter are acceptable for commercial scale fry habituation.

Live food enrichment with vitamin C in pikeperch and walleye larviculture lead to lower deformity rate and increased growth and stress resistance (Kolkovski *et al*., 2000; Kestemont *et al*., 2007). All assessed parameters in our study showed differences which are not in the range of statistical significance ($p > 0.05$, independent t-test). However, low *P*-values were achieved for some parameters such as survival, habituation success, SGR and final weight (Table 2). These differences in the direction of vitamin C enrichment are suggesting that it could have some positive effect. Perhaps rather low habituation success is the reason for not revealing more pronounced influence of this important nutritional supplement. Therefore, we may suggest need for further studies. Analysis of vitamin C content demonstrated that 1 g L^{-1} of ascorbic acid solution in the water with exposure of one hour may lead to significant enrichment of *Daphnia* nauplii. Even though not statistically significant, this enrichment and 10 days of supplementation with vitamin C led to obvious accumulation of ascorbic acid in fish tissue as it was 148% higher in the supplemented fish compared to control (8.0±2.1 µg g^{-1} and 5.4 ±2.0 µg g^{-1} for VitC and Feeder, respectively).

With respect to above indicated influence of specific nutritional component such as vitamin C on the juvenile pikeperch, using high value natural food rich in ω-3 fatty acids such as *Chironomus* spp. (Bogut *et al*., 2007; Kamler *et al*., 2008) could lead to more advantageous results of dry feed habituation. The advantage of *Chironomus* spp. over *Daphnia* spp. was previously found by Bódis *et al*. (2007) and may be one of the reasons for lower habituation success in the present study. In our trials, both *Daphnia* spp. and *Tubifex* spp. were used during the habituation period. Based on our observations worms could have an additional advantage over the planktonic crustaceans connected to the swimming ability of the given live food. While the *Daphnia* spp. is immediately dispersed over the rearing volume, worms stay rather concentrated close to the feeding site which might lead to better concentration of the fish at the feeding spot.

Even though mechanical feeding has been described as a rather preferable method of feed application in walleye habituation (Summerfelt, 1996; Summerfelt *et al*., 2011), there is an advantage of hand feeding in pikeperch habituation methodology. Explained usage of frozen chironomids in the feed which led to best published results in this technology (Policar *et al*., 2013) is highly dependable on hand feeding as the bloodworms could be mixed with formulated diets prior to administration (Horváth *et al*., 2012). Therefore, similar obtained results on the habituation success of 11.2±3.9% and 11.4±0.7% for hand and mechanical feeding, respectively, with high P - value (p=0.860) are witnessing that hand feeding would not lead to lower final success (Table 3). However, lower growth and higher cannibalism achieved in the hand feeding group could be perhaps explained by higher feed ingestion once the fish were habituated due to longer feed availability in mechanically fed groups and therefore this feeding technique could be more preferable in the post-habituation period.

Habituation success of the present study is lower than that of previously published studies (Szkudlarek and Zakęś, 2002; Policar *et al*., 2013). There are several possible explanations for this outcome. In our study, we used bigger juveniles compared to other studies which might lead to lower habituation according to the study of Policar *et al*. (2013). Moreover, in the first 5 days of habituation *Daphnia* spp. was exclusively used as the supplementation feed and according to the results of Bódis *et al*. (2007) using both *Tubifex* spp. and *Chironomous* spp. leads to higher acceptance of dry feed. Finally, with this observation on the effect of stocking density, we may imply that higher density would further increase the success. Nevertheless, there is a difference between the earlier mentioned reports and present study. Besides the survival, which is the main parameter of success, we have included the parameter of habituation success, similar to previous report on young-of-the-year pikeperch (Horváth *et al*., 2012). This feature was rather visible on the fish as they got dry feed exclusively for 15 days and obvious cannibals were regularly taken out from the tanks. Finally, similar results are still occasionally present in the commercial size pikeperch habituation (Zoltán Horváth, H & H Carpio Ltd., personal communication).

In conclusion, based on the correlation analysis outcome it seems that increased stocking density leads to increased habituation success. Vitamin C enrichment led to increased success and growth of the fish, although differences were not statistically significant. Hand feeding did not lead to lower habituation success. Hence, stocking densities with 4 to 8 fish per litter and hand feeding with mixed formulated feed and worms by possible enrichment with vitamin C could be suggested for commercial scale habituation of pond nursed pikeperch juveniles. However, it is strongly required that further studies clarify the details of technology for a reliable and safe habituation methodology of pond reared pikeperch.

Acknowledgments

This study was financed by the Aquaexcel TNA project as a part of the research project "*Improved pikeperch propagation*", Application Ref: 0045/03/06/18. Special acknowledgment is addressed to EU FP-7 project AQUAREDPOT (reference number: 316266) for supporting the researchers involved. Gratitude goes to Zoltán Horváth (H & H Carpio Ltd.) for constructive communication which helped presentations of the collected data.

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