Deliverable Report

Deliverable No:	D16.2		**Delivery Month:**	36
Deliverable Title	Determination of the effect of nutritional factors on pikeperch larval rearing			
WP No:	16		**WP Lead beneficiary:**	P9. UL
WP Title:	Larval husbandry - pikeperch			
Task No:	16.1		**Task Lead beneficiary:**	P9. UL
Task Title:	Optimal combinations of factors to improve larval rearing			
Other beneficiaries:	P3.IRTA	P21.DTU	P29.ASIALOR	
Status:	**Delivered**		**Expected month:**	24
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Objective: The overall aims of WP16. Larval husbandry-pikeperch were to identify the bottlenecks for larval survival and growth, and to establish an optimal combination of factors, which could give the best performance of pikeperch larval populations. The objective of ***D16.2 Determination of the effect of nutritional factors on pikeperch larval rearing*** was to determine the effects of four nutritional factors and their interactions on pikeperch larval rearing using a multifactorial approach. The present deliverable aims to investigate the effects of four nutritional factors on the performance of pikeperch larvae. These include (a) the timing of the onset and (b) duration of the weaning period, (c) the method of food distribution and (d) implementing or not a co-feeding approach. This will standardize and improve the feeding strategy, as the current literature indicates a diverse range in feeding protocols now employed in pikeperch larval rearing (Steenfeldt, 2015).

Introduction

There exists a strong incentive to increase the production of intensive aquaculture while limiting the impact on dwindling natural resources. To this end, European aquaculture aims to promote efficient and sustainable production of safe seafood of the highest quality and nutritional value. The European Project DIVERSIFY identified six new fish species, which could be eligible as potential future species for European fish farming. Among these candidates, the fresh water species pikeperch (*Sander lucioperca*) was selected.

Pikeperch has gained attention as a promising new species in intensive fish farming (Nyina-wamwiza et al. 2005) with great economic potential. Until now several bottlenecks have prevented the success of larval rearing. Three major bottlenecks have been identified to be: (1) high mortality mainly due to cannibalism, (2) high occurence of deformities and (3) large size heterogeneity between larval cohorts at various ontogenic development stages. Our research group at P9. University of Lorraine, UL, France has lead the work studying the optimal combination of factors that can improve the production of juvenile pikeperch.

Materials and Methods

Multifactorial experiment

A multifactorial experimental design was developed to study simultaneously the effect of selected factors and their interactions on pikeperch larval rearing using a factorial design. As this study aims to produce an

optimal set of factors for fish farming, practical constraints were taken into account (P29.ASIALOR) and the protocols used for each of the four factors are in accordance with previous data (**Table 1**).

1. The beginning of weaning: We wanted to compare early weaning (10 days post-hatching, dph) to the commonly used later weaning at 16 dph (Hamza et al., 2007). In commercial intensive rearing of pikeperch, weaning as early as possible is targeted, in order to reduce the costs of feeding *Artemia*. However, precocious weaning seems to induce lower larval growth (Steenfeldt, 2015).

2. The method of food distribution: After a survey of the literature, it appears that the frequency and type of daily feedings are highly variable among the different studies reported. The feeding frequency mainly depends on the photoperiod to which the fish are exposed. Moreover, the food distribution can include either discontinuous, discrete meals given, for example every 30 min for one or two hours (Ostasrewska et al., 2005; Hamza et al. 2008, 2010, 2012; Kestemont et al. 2007; Ljubobratović et al., 2015; Król and Zakęś, 2016), or continuous feeding using automatic feeders (Szkudlarek et Zakęś, 2007; Lund et al., 2012). We compared the effect of discontinuous feeding of 7 meals day^{-1} (one meal every 90 min) to continuous feeding (using peristaltic pumps for the live preys and band feeders distributing artificial diet) over a 12 h period.

3. Implementing or not a co-feeding approach: There are studies showing co-feeding of live and inert diets to larval pikeperch (Hamza et al., 2007; Szkudlarek et Zakęś, 2007; Ljubobratovic et al., 2015; Król and Zakęś, 2016) or only offering live food (Lund et al., 2012, 2014). On the other hand, there are also reports (Szkudlarek et Zakęś, 2007; Król and Zakęś, 2016) of implementing both a co-feeding approach and early weaning (4 dph). We tested the effect of co-feeding the larvae with live food and a 100-µm inert diet (Larviva Pro-wean, Biomar, Denmark) at 3.5 g day $^{-1}$ 6 days before the weaning period.

4. The weaning duration: We tested two weaning durations corresponding to a rapid (3 days) or slow (9 days) feeding transition from *Artemia* nauplii to artificial diets. A slow transition was applied by Kestemont et al. (2007) and Lund et al. (2014), whereas other protocols are based on a longer duration (Hamza et al., 2007; Lund et al., 2012).

Table 1. Applied modalities for each factor.

Factor	Modality 1	Modality 2
Beginning of weaning	10 dph	16 dph
Method of distribution	Continuous	Discontinuous
Co-feeding	Yes	No
Weaning duration	3 days	9 days

The study was carried out in 8 tanks of a ten (two rows of 5 tanks) 700-l indoor recirculating aquaculture system (RAS) using mechanical and biological filters, as well as a UV sterilization unit (**Fig. 1**). The tanks were stocked with 30,000 larvae each (ca. 43 larvae l^{-1}).

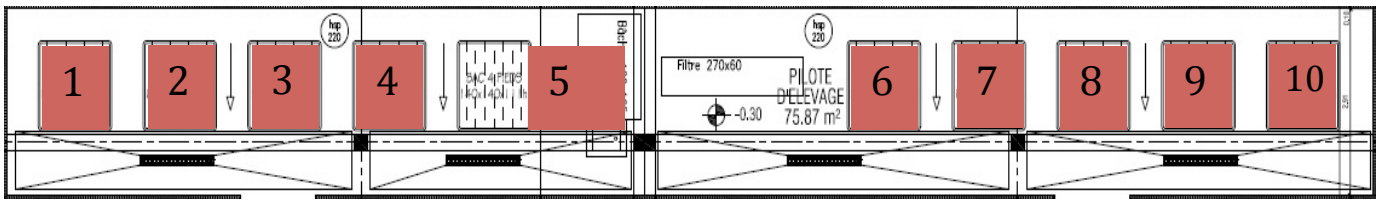

Figure 1. Diagram of the experimental facilities. The closed loop consists of 10 tanks and a central filter. Tanks 2 to 9 are used during the experiment. Tanks 1 and 10 are used as additional moving-bed biofilter.

Biological materials: pikeperch larvae

Larvae (240,000) were obtained from P29. ASIALOR (Pierrevillers, France) and transferred to the P9. UL experimental platform (UR AFPA, Vandœuvre-lès-Nancy, France). The larvae were then distributed to 8 tanks, where water temperature was initially kept at 15-16°C.

Standardized rearing conditions

Photoperiod was fixed at 12h of light and 12h of darkness (Hamza et al. 2007) with a progressive increase of light intensity (from 0 to 50 lx) during the first 30 min, stabilizing at 50 lx (see Deliverable D16.1) until the last 30 minutes of the photoperiod where the light intensity decreased from 50 to 0 lx. Temperature was the same for all tanks ranging between 16 and 20°C (Hamza et al. 2007; Kestemont et al. 2007; Szkudlarek and Zakęś 2007). Temperature was initially increased incrementally by 1°C day^{-1} until 20°C. Dissolved oxygen was maintained above 7 mg l^{-1} and salinity was fixed at 0.7-0.9 ‰.

Distribution of tested factors in the tanks

The experimental combinations of factors were applied according to an experimental matrix (**Table 2**).

Table 2. Experimental matrix applied.

Tank	Combination	Beginning of weaning (dph)	Distribution method	Co-feeding	Weaning (days)
2	2	10	continuous	Yes	3
3	1	16	continuous	Yes	9
4	3	10	discontinuous	Yes	9
5	7	16	discontinuous	Yes	3
6	0	10	continuous	No	9
7	4	16	continuous	No	3
8	5	10	discontinuous	No	3
9	6	16	discontinuous	No	9

Measures, sampling and observations

The physical-chemical properties of the water were monitored regularly to ensure optimal conditions: Oxygen level was checked every morning (> 7 mg); ammonia and nitrite concentrations were titrated twice a week and pH was also measured twice a week. Water pH was corrected by regular inputs of $NaHCO_3$.

The experiment lasted 49 days (from 5th February until 24th March 2016). Larvae were sampled every 7 days and designated as days after first feeding (daff), which begun 4 dph: T0, T7, T14, T21, T28, T35, T42 and T49. During sampling, larvae were siphoned into a basin, then pipetted, counted and distributed in the different sampling tubes. Larvae siphoned in excess were returned to their original tank. This method allowed limiting losses due to sampling with a net. Sampled larvae in the tubes were then sacrificed by a lethal dose of MS222, excess water was removed with a fine mesh dip net and larvae were placed in 4% buffered formalin.

The parameters measured included: (a) Morphometric measures (total length (TL), body weight (W), coefficient of variation of total length (CV TL) and coefficient of variation of weight (CV W) done on 30 larvae tank^{-1} sampling date^{-1}; (b) Observations during 5 min day^{-1} of each tank in order to detect cannibals. Cannibals were identified and counted as individuals responsible for a direct attack or with a big abdomen (for the last weeks). (c) At the end of experiment, the total fish biomass was weighed for each tank and the % swim bladder inflation measured by separating fish with or without a swim bladder by lightly anesthetizing them with MS222 (70 mg l^{-1}) followed by transfer to salted water (20 g of salt l^{-1}) according to Jacquemond (2004a, b).

Statistical analyses

For this multifactorial experiment, statistical analyses were carried out using the statistical software Analys (Kobilinsky, 2000; Gardeur et al., 2007). The detection of the potentially active effects of tested factors on the output variables was given by Daniel's graphics (Half Normal probability plot of basal estimation function, Daniel, 1959) using an oversaturated model of variance analysis. The interactions between 3 or more factors were considered insignificant. When an interaction between two factors was found significant ($P < 0.05$), the potential single effects of these factors were also considered insubstantial. Data are presented as means ± confidence interval (95%) calculated with the standard errors of the means based on mean square errors.

Results

Multifactorial experiment

Effects on total length, body weight and size heterogeneity

At T7 daff (11 dph), no significant effect was identified.

At T14 daff (18 dph), three significant interactions were identified by multifactorial analysis. A similar effect of the interaction between the method of distribution and co-feeding was observed on total length ($F = 121$; $p = 0.008$) and on weight ($F = 20.8$; $p = 0.045$) (**Figure 2a, b**). The method of food distribution had no effect on mean weight of larvae when no co-feeding was applied. Conversely, a higher mean weight was observed when co-feeding was applied with continuous feeding distribution (compared to a discontinuous one). Moreover, the multifactorial analysis showed also a significant interaction between the beginning of weaning and the co-feeding on the coefficient of variation of weight ($F = 36$; $p = 0.027$) (**Figure 2c**). When no co-feeding was applied, no effect of the beginning of the weaning was observed. In contrast, when co-feeding was applied, higher coefficients of variation for the weight were observed when the weaning started at 16 dph.

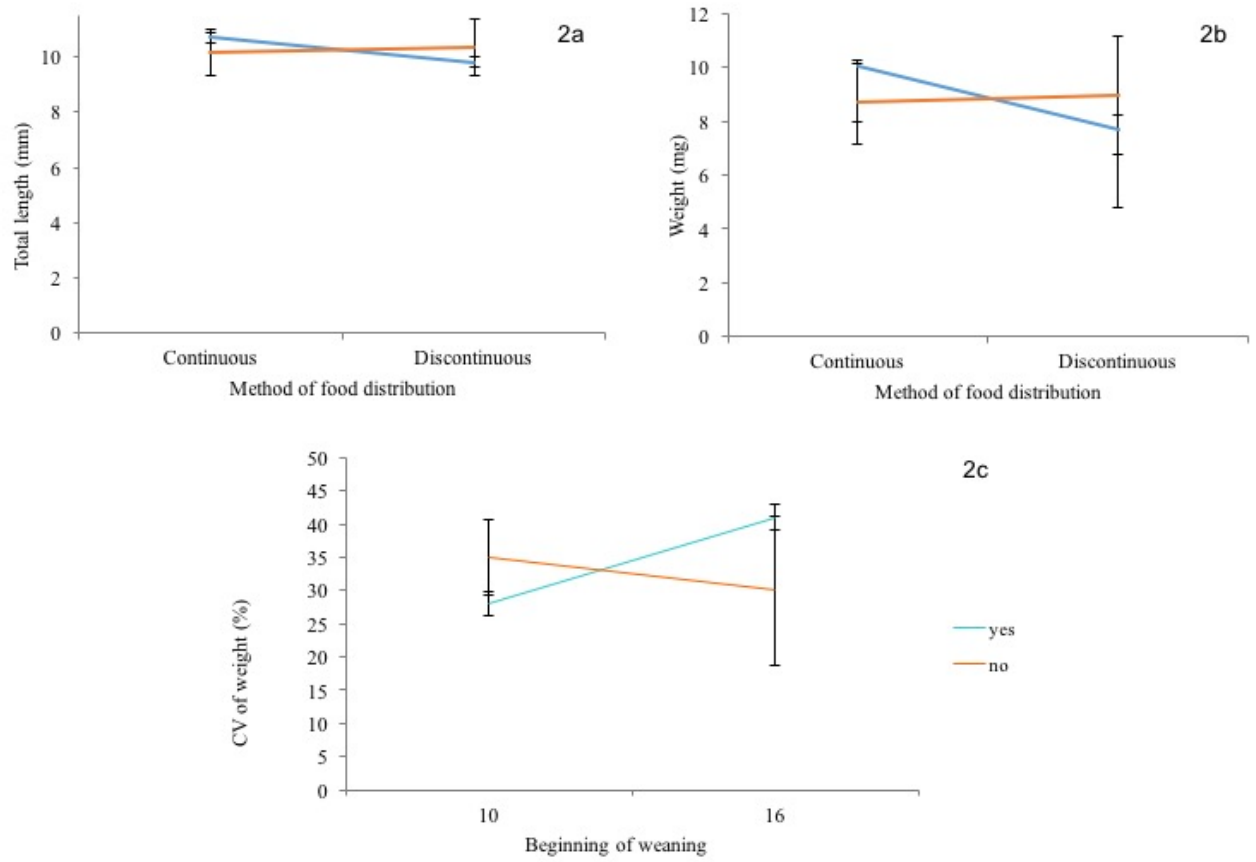

Figure 2. Significant interactions observed at T14 daff (18 dph)

At T21 daff (25 dph), the multifactorial analysis provided a significant interaction between the beginning of weaning and the weaning duration on total length of larvae (F = 15; p = 0.018). Larvae were significantly larger with weaning applied at 16 dph and lasting 9 days compared to the three other combinations (**Figure 3a**).

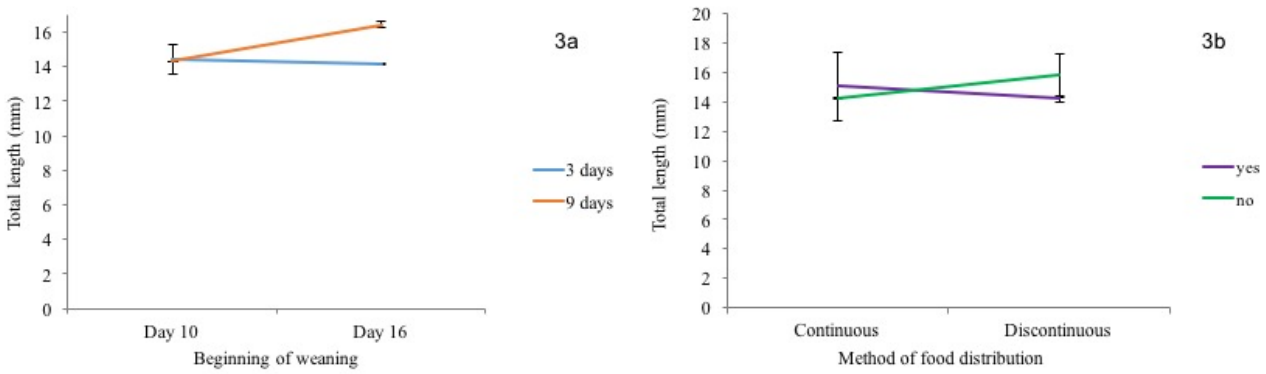

Figure 3. Significant interactions at T21 daff (25 dph)

Furthermore, as observed at T4, there was a significant interaction between the method of distribution and co-feeding on total length of larvae (F = 28.1; p = 0.034) (**Figure 3b**). A significant simple effect of the beginning of weaning was recorded on the coefficient of variation for the weight (F =14.8; p = 0.031). The larvae were more homogenous when weaning began at 10 dph (30 ± 2.6 %) compared to 16 dph (37 ± 3.9%) (not shown).

At T28 daff (32 dph), there was a significant effect of the time of weaning on weight (F = 10.6; p = 0.031). Indeed, larvae were heavier when weaning begun at day 16 (20 dph) than day 10 (14 dph) (93.4 ± 32.6 mg and 57.05 ± 16.8 mg, respectively) (not shown). Furthermore, there was a significant interaction between the method of food distribution and weaning duration on the coefficient of variation of weight (F = 47.5; p = 0.037). Larvae weights were more homogeneous with a continuous distribution and nine days of weaning (**Figure 4**). There was a non-significant (F= 6.4; p = 0.065) interaction between the beginning of weaning and the weaning duration.

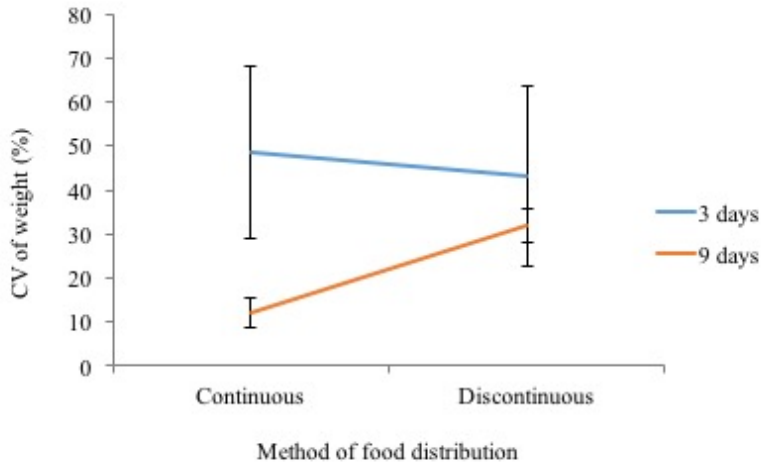

Figure 4. Interaction between the type of food distribution and weaning duration at T28 (32 dph)

There was no effect found by a multifactorial analysis on T35 (39 dph) and T42 (46 dph) fish. However, at T35, there was a non-significant (P>0.05) interaction between the beginning of weaning and the weaning duration on weight (F = 5.2; p = 0.08) and on total length (F = 5.3; p = 0.08). Furthermore, a simple effect of the method of distribution on the coefficient of variation of weight (F = 4.6; p = 0.09) was also on the limit to be significant. Larvae were more homogeneous in weight with discontinuous feeding (mean CV = 29%) than with continuous feeding (CV = 47%).

For T49 daff (53 dph), the multifactorial analyses provided two significant effects of the interaction between the beginning of weaning and weaning duration on weight (F = 11.8; p = 0.041) and length (F= 214; p = 0.005). The results showed that weaning, which begun at 16 dph and lasted 9 days, produced heavier and larger larvae (**Figure 5a, 5b**). Concerning larval size, there was a significant interaction between the weaning duration and the method of distribution (F = 63.8; p = 0.015) (**Figure 5c**). There was also a significant effect of the interaction between the beginning of weaning and co-feeding on the coefficient of variation of weight (F = 24.8; p = 0.016). The group of larvae with more homogenous weight began weaning at 16 days with or

without co-feeding (**Figure 5d**). With early weaning at day 10, larvae were more homogeneous in weight when no co-feeding was applied.

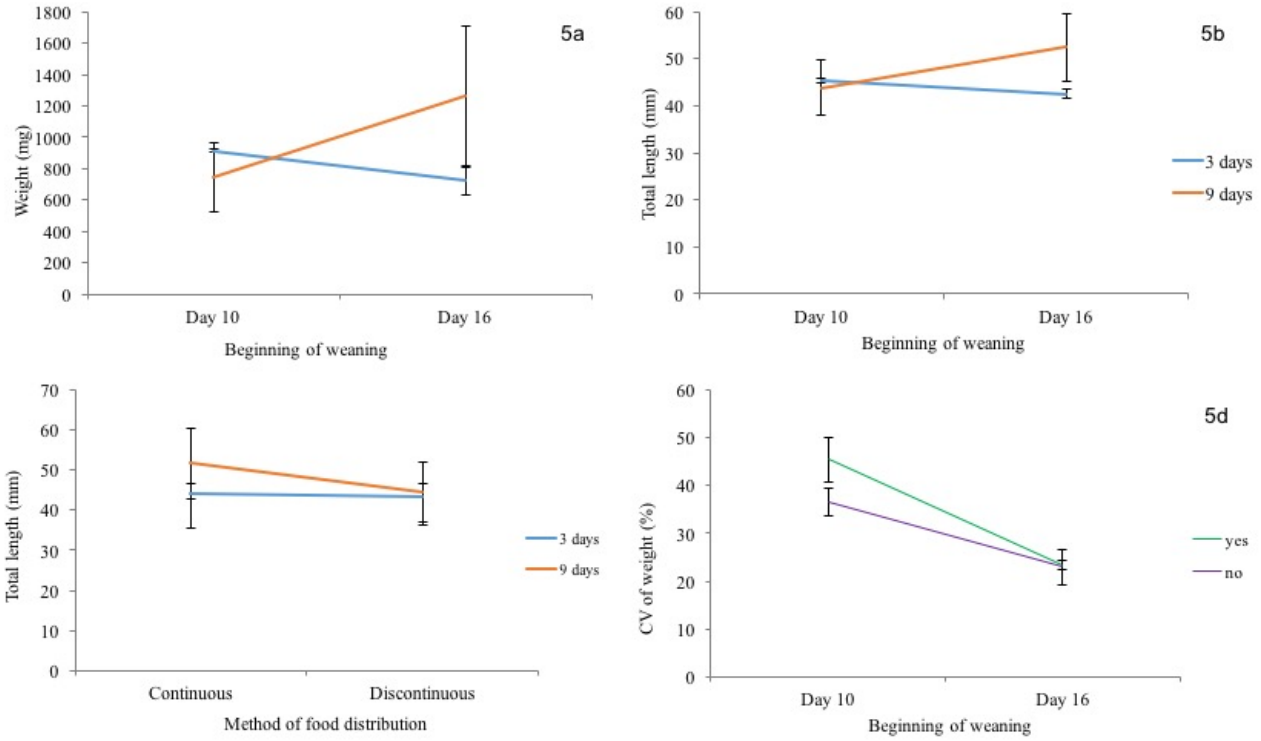

Figure 5. Significant interactions at T49 daff (53 dph)

Furthermore, there was a significant simple effect of the method of distribution of food on the coefficient of variation for weight ($F = 24.8$; $p = 0.02$). Indeed, with discontinuous feeding, the groups of larvae were more homogenous (30.5 %) than with continuous feeding (33.8 %) (not shown).

Effects on growth rate

The mean specific growth rate (SGR) for all tanks was 15.6 % day^{-1} between the first sampling date (T0, 4 dph) and the last one (T49, 53 dph). Tank 3 had the best SGR with 16.7 % day^{-1} and the worst SGR was observed in tank 4 with 14.9% day^{-1}. At the end of the experiment, larvae of tanks 3 and 9 had a mean weight higher than the other tanks (1502.0 ± 359.3 mg and 1022.2 ± 211.6 mg, respectively) (**Figure 6**). The multifactorial analyses provided a significant effect of the interaction between the beginning of weaning and weaning duration ($F = 18.8$; $p = 0.023$) (**Figure 7**). In **Figure 6** the highest growth rate was observed when weaning began at 16 days for a duration of 9 days, which corresponds to tanks 3 and 9.

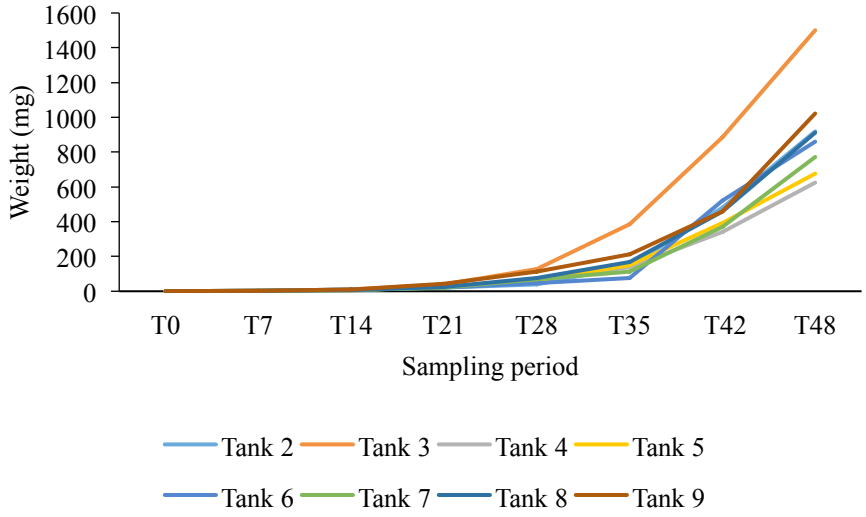

Figure 6. Growth curve of pikeperch larvae submitted to eight combinations of factors.

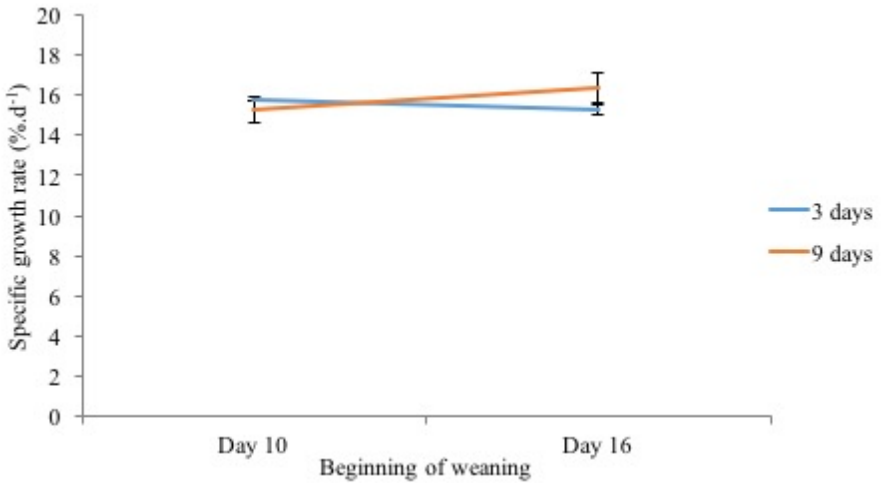

Figure 7. Significant interaction on the specific growth rate (SGR).

Effects on cannibalism, swim bladder inflation rate and final biomass

There was no effect provided by the multifactorial analysis on cannibalism (number of cannibals observed). However, two periods of cannibalism (28-34 dph and 49-53 dph) were determined (**Figure 8**). Furthermore, we observed for the first time that there were two types of cannibalism distinguished by whether the cannibal attacked the tail or head of the cannibalized larvae. Attacking the flank of a fish was also considered as an attack (**Figure 8**). There was no cannibalism observed before 14 dph.

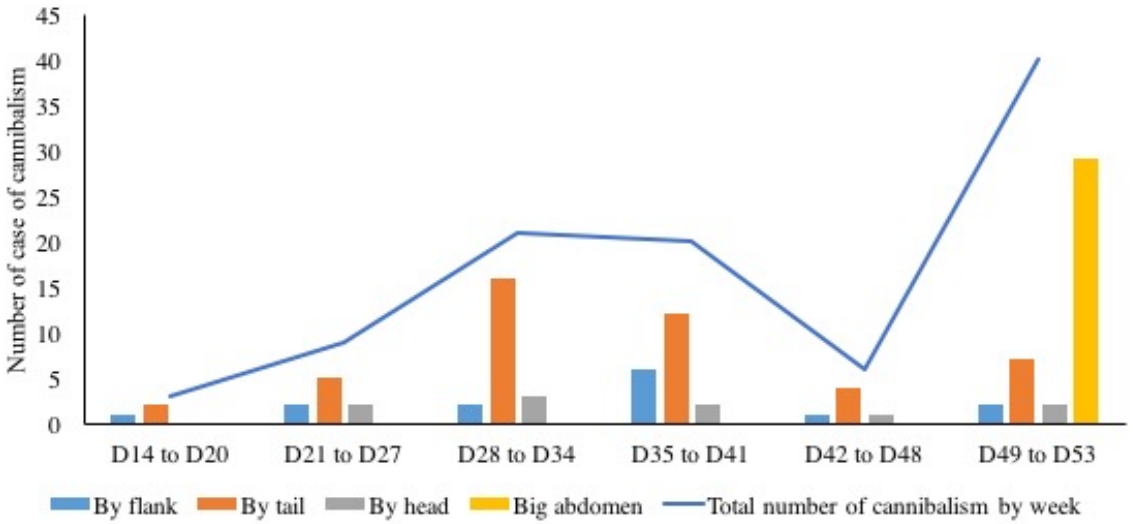

Figure 8. Chronology of cannibalism from 14 dph to 53 dph.

On swim bladder inflation, the weaning duration had a significant effect (F = 12.4; p = 0.024). Indeed, within 3 days of weaning (tanks 2, 5 7 and 8), the % inflation of swim bladder reached 18.2% whereas after 9 days of weaning (tanks 3,4, 6 and 9), 67.8% of the fish exhibited a swim bladder (**Table 3**).

The multifactorial analyses showed simple effects of the method of food distribution (F = 12.9; p = 0.037) and weaning duration (F = 10.9; p = 0.046) on final biomass. Indeed, with a continuous food distribution (tanks 2,3,6 and 7) mean final biomass was 1108 ± 588.2 g whereas with a discontinuous distribution (tanks 4, 5, 8 and 9) it was 1850.7 ± 705.9 g. Furthermore, after 3 days of weaning (tanks 2, 5 7 and 8) mean final biomass was 1138.5 ± 363.8 g whereas after a 9 day weaning period (tanks 3,4, 6 and 9), it was 1820.3 ± 731.1 g.

The best survival rates were recorded in tanks 9 (13.1%), 4 (11.3%) and 3 (10.5%).

Table 3: Summary of swim bladder inflation, final body weight, tank biomass and survival recorded at 53 dph.

Tanks	Swim bladder inflation (%)	Final biomass (g)	Mean weight (mg)	Survival (%)
2	22.51	1026	919.27	5.5
3	98.11	1962	1502.31	10.5
4	15.97	2110	623.57	11.3
5	10.43	1361	677.11	7.3
6	86.29	766	861.50	4.1
7	24.63	678	770.95	3.6
8	15.17	1489	913.10	8.0
9	70.81	2443	1022.20	13.1

Discussion

In this experiment, compared to results obtained in the first trial (see ***D16.1 Determination of the effect of environmental factors on pikeperch larval rearing***), it was demonstrated that weaned juveniles of 1.0-1.5 g mean body weight can be produced in 53 days, with good survival (0.3-2.6% of first trial *vs* 3.6-13.1% in second trial). By implementing several combinations of factors (tanks 3, 4 and 9), the overall percent survival was above 10%. To the best of our knowledge, this is the first time larval pikeperch have been reared successfully in large tanks (700 l) and over an extended period (53 dph), including the initial phase of feeding on live prey and the weaning period. Studies on pikeperch reported in the literature were carried out for relatively short durations (2-3 weeks) and in small water volumes (20-60 l) examining a specific rearing phase (e.g. weaning) (Ostaszewska et al., 2005; Kestemont et al., 2007; Szkudlarek and Zakęś, 2007; Hamza et al., 2008; Lund et al., 2014; Ljubobratović et al., 2015; Król and Zakęś, 2016). It must be also noted that according to the protocol developed for Eurasian perch (*Perca fluviatilis*) (Jacquemond et al., 2004a et b), variable levels of swim bladder inflation were recorded at 53 dph, ranging from ca. 10% to 100%. However, the higher survival and swim bladder levels obtained in tanks 3 and 9 are very promising. The multifactorial analysis showed that a longer weaning duration (3 *vs* 9 days) increased mean swim bladder inflation (18% *vs* 67%) and final biomass increase (+ 62%). In addition, discontinuous feeding increased best the final biomass produced in tanks (+ 66%). The practice of co-feeding (6 days) and the onset of the weaning period (at 10 or 16 days dph) had no effect on the final biomass and the percent of inflated swim bladders, while the method of food distribution only affected the level of swim bladder inflation.

During the course of the experiment, mean SGR was 15.6% day^{-1}, ranging from 14.9 to 16.7% day^{-1}. Although the experimental design of the present study differs from other studies, the current results of the SGR compare well with Kestemont et al. (2007), who reported a range from 16.4 to 18.5% day^{-1}, which was measured in 11 to 37 dph fish reared at 20-21°C and Hamza et al. (2012) who found a SGR from 12.1 to 16.1% day^{-1} in 10 to 34 dph fish reared at 21-23°C. On the other hand, these findings were higher than those by Hamza et al. (2007) who calculated 5.9 – 12.8% day^{-1} in 4 to 36 dph larvae reared at 19-20°C and Król and Zakęś (2016) that found 12.2 to 12.8% day^{-1} in 15 to 43 dph larvae reared at 20.0°C. However, Lund et al. (2012, 2014) reported higher SGR values (19.3-21.6% day^{-1}) in 1-27 dph pikeperch reared in cooler water (16-18^{o}C). There was a strong interaction between the onset of weaning (10 *vs* 16 dph) and its duration (3 *vs* 9 days) on mean larval size and weight at 25 and 53 dph which were higher when fish were weaned later with longer weaning duration. In 32 dph fish the timing of the onset of weaning was also significant (simple effect). Kestemont et al. (2007) argued that in order to achieve good growth and survival with low deformities, the onset of weaning should take place at 19 dph and not at 12 or 26 dph. This emphasizes the importance of the timing of weaning on the normal ontogeny of the digestive tract and the appearance of a functional digestive activity (Dabrowski, 1992; Cuvier-Péres and Kestemont, 2002). Pikeperch larvae growth was also influenced by the interaction between the method of food distribution and whether or not co-feeding was implemented. In fact, when co-feeding was applied, no effect of the method of food distribution was observed, whereas in the absence of co-feeding, the larvae were heavier and larger with continuous feeding. On the other hand, this effect was not conserved after 25 dph suggesting that this interaction is effective only during the weaning period.

The regulation of the size or weight heterogeneity is of major importance to limit the impact of cannibal individuals in predatory fish (Kestemont et al., 2003). Numerous biotic and abiotic factors influence the heterogeneity of a population. The present study suggested that a later start of weaning increased weight heterogeneity (simple effect observed at 25 dph). However, the influence of all the feeding factors on size and weight heterogeneity is complex and varies according to fish growth and/or age. At 18 dph, a significant interaction between the timing of the onset of weaning and the presence of co-feeding was found. On the other hand, in the absence of co-feeding, there is no effect of the timing of weaning on weight heterogeneity. Pikeperch larvae are more homogeneously sized (25-30%) when larvae are weaned later, if compared to larvae weaned early (40-45%). However at 18 dph, the weaning period is not totally finished in some tanks (weaning at 16 dph) so one must be cautious interpreting the significant effects. However, as significant lower coefficients for weight were noted at 53 dph when the larvae were weaned later, the effect of the

timing of the weaning on fish heterogeneity is confirmed. In contrast, the effects of the method of food distribution remain unclear as a higher homogeneity in weight is recorded with continuous feeding (interaction with the weaning duration) at 32 dph, whereas pikeperch batches were more homogenous with a discontinuous feeding at 39 and 53 dph.

Two main types of cannibalistic behavior appear sequentially during larval ontogeny and have been described as type I and type II. In type I cannibalism, the prey is very large relative to the cannibal's size and is incompletely ingested and digested. Type II cannibalism refers to the complete ingestion of a prey by a larger cannibal (Baras 2012). From a pure biomechanical perspective, type I cannibalism can start as soon as the structures enabling suction feeding are developed (i.e. a suspensorium, hyoid bar and operculum), which means as early as the start of exogenous feeding in some fish species. However, food processing becomes progressively efficient as the buccal apparatus becomes more sophisticated and ossified. The caudal peduncle of a larva is shallow and always narrower than its gape height. Consequently, type I cannibalism requires no size differential and in some cases, the cannibal is slightly smaller than its victim (Baras 2012). In predatory fish larviculture, the feeding strategy can modulate the impact of cannibals on mortality. Recently, Ljubobratović et al. (2015) carried out an experiment focusing only on the weaning strategy using 11 dph larvae (20 l circular tanks, from 11 dph to 27 dph) and calculated survival rates between 21 and 43% with cannibalism rates of 25-45% according to various weaning strategies. In our study, we observed no cannibalism before 14 dph and then two periods of intense cannibalism (first between 28 and 34 dph and second between 49 and 53 dph). The elimination of cannibals or the practice of size grading over these periods could be an approach to increase survival (Szczepkowski et al., 2011). An alternative to reduce cannibalism is a more favourable diet composition, such as when Król and Zakęś (2016) supplemented L-tryptophan to the diet, which reduced mortality.

In conclusion, the results suggest that a later onset and longer duration of weaning followed by discontinuous feeding will improve larval survival, growth and reduce deformities in pikeperch populations.

Deviations: Initially, the deliverable D16.2 was expected on month 24, it means that it is submitted with a delay of 12 months. This delay is explained by the fact that we have repeated this experiment, because of a very high level of mortality observed is the first experiment done in May 2015 (probably due the high increase of the water salinity).

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