frontiers | Frontiers in Marine Science

ORIGINAL RESEARCH
published: 01 April 2022
doi: 10.3389/fmars.2022.864536

Effects of First Feeding Regime on Gene Expression and Enzyme Activity in Pikeperch (*Sander lucioperca*) Larvae

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Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: 28 January 2022
Accepted: 11 March 2022
Published: 01 April 2022

Citation:
Imentai A, Gilannejad N, Martínez-Rodríguez G, López FJM, Martínez FP, Pěnka T, Dzyuba V, Dadras H and Policar T (2022) Effects of First Feeding Regime on Gene Expression and Enzyme Activity in Pikeperch (Sander lucioperca) Larvae. Front. Mar. Sci. 9:864536. doi: 10.3389/fmars.2022.864536

The present study investigates the effects of different feeding regimes with rotifers (*Brachionus plicatilis*) and *Artemia salina* on the gene expression and digestive enzymes in pikeperch (*Sander lucioperca*) larvae at 17 days post-hatch (DPH) over a period of 13 days. Five experimental feeding protocols were performed in four replicates. At 4 DPH, the larvae (total length= 5.62 ± 0.03 mm, body weight = 0.66 ± 0.16 mg) were divided into five experimental groups (2-L tanks) at initial density of 100 larvae per liter. Light intensity on the water surface was 90-100 lux and photoperiod was set at 13L: 11D (07:00 to 20:00 h). Water temperature, pH, and dissolved oxygen (DO) were measured before each feeding and the values were 17.8 ± 0.17°C, 7.3 ± 0.04 and 88.5 ± 2.53%. The fish larvae at 5 days post-hatch (DPH), were initially fed with rotifers (*Brachionus plicatilis*) for 3 days and from 8 to 17 DPH were fed with rotifers/*Artemia* for different time periods as follows: (A) only rotifers; (B) 8–13 DPH rotifers/14–17 DPH *Artemia*; (C) 8–10 DPH rotifers/11–17 DPH *Artemia*; (D) only *Artemia*; (E) a combination of rotifers and Artemia. Frozen paste of algae was added to the larval tanks twice a day (2 x 300,000 cells/mL). Rotifers and *Artemia* were provided as live feed to larvae three times a day with residual counts prior to each feeding. Feeding densities were steadily increased based on residual counts, performed prior to each feeding. The expression of genes related to intestinal development and maturation (*aminopeptidase N, anpep; leucine aminopeptidase 3, lap3; intestinal-type alkaline phosphatase, alpi*), together with key pancreatic digestive proenzymes (*trypsinogen 1, try1; chymotrypsinogen b, ctrb; carboxyl ester lipase precursor, cel; phospholipase a2, pla2g1b; pancreatic alpha amylase, amy2a*), were assessed. Additionally, the activity of six enzymes (trypsin, lipase, alkaline phosphatase, amino peptidase, amylase, and chymotrypsin) were determined. The highest expression of two genes related to intestine (*lap3; anpep*) were observed in the fish fed a combination of rotifers and *Artemia* from 8 DPH

(Group E). The expression of *amy2a, ctrb, pla2g1b, try1* was significantly lower in larvae fed rotifers until 14 DPH and replaced by *Artemia* afterwards (Group B). The specific activity of brush border membrane enzymes (alkaline phosphatase and aminopeptidase N) increased with combination of rotifers and *Artemia* in larval diet (Group E), indicating a more efficient functionality of digestive structures. The groups fed only with rotifers till 17 DPH (Group A) (38 ± 4.07%) and larvae fed with rotifers till 14 DPH followed by feeding with *Artemia* till 17 DPH (Group B) (36 ± 5.25%) showed significantly ($P<0.05$) lower survival rates than the other groups (54-67%). The group fed only with rotifers (Group A) showed significantly lower specific growth rate (SGR) than the other groups, and the highest SGR was found in the group fed with combination of rotifers and *Artemia* after 3 day rotifer feeding (Group E). The highest standard length (8.32 ± 0.48 mm) was obtained by combined feeding of rotifers and *Artemia* after 3 day of initial rotifer feeding. Combination of rotifers and *Artemia* from 8 DPH (Group E) could be considered a more appropriate diet for first feeding pikeperch larvae compared with later introduction of *Artemia*, as indicated by the higher expression of genes and activities of digestive enzymes. Our findings provide new insight into the effect of temporal sequence of rotifers and *Artemia* on the expression of genes and activities of digestive enzymes in pikeperch larvae.

Keywords: *Artemia*, digestion, feeding protocol, euryhaline rotifer, pikeperch larvae

INTRODUCTION

Among the species belonging to the percid family, the pikeperch (*Sander lucioperca* L.) has been recognized as a potential candidate of European inland aquaculture mainly because of the delicious flesh and high growth rate (Schulz et al., 2007; Kristan et al., 2016). Although a vast research data has been reported on pikeperch culture under controlled conditions (Policar et al., 2013; Policar et al., 2016; Khendek et al., 2018; Baekelandt et al., 2019; Penka et al., 2021), successful rearing of larvae is a major problem in intensive culture (Policar et al., 2019). The main challenges of the larval culture are high mortality and quality of cultured larvae (Policar et al., 2019). The survival rates of pikeperch during the larval stage are below 20%. In order to reduce the mortality rates of pikeperch larvae reared under controlled conditions, feeding protocols must be improved. Live prey is required during two first weeks after mouth opening of pikeperch. Afterwards, pikeperch can we successfully weaned. Earlier weaning of pikeperch, beginning from 12th DPH results in higher mortality and deformity of fish (Kestemont et al., 2007). Although *Artemia* is a common diet in fish larviculture, the nutritional profile, size and inconsistent in biochemical composition, makes it less suitable candidate for first feeding. In this respect, recent studies showed the importance of larval first feeding with rotifers (*Brachionus plicatilis*) with a supplementation of microalgae to provide a better growth and higher survival (Yanes-Roca et al., 2018; Imentai et al., 2020; Yanes-Roca et al., 2020a; Yanes-Roca et al., 2020b). It was reported that higher growth and survival was reached when larvae fed with *B. plicatilis* at the beginning of exogenous feeding and afterwards replaced with *Artemia* or a combination of two during first feeding (Imentai et al., 2020). Although successful protocol for first feeding is described based on the survival and growth parameters, the molecular basis of pikeperch larval nutrition during this crucial stage is largely unknown.

Expression of genes related to the digestive function is regulated at multiple levels and is species (Yúfera et al., 2018) and age-specific (Wang et al., 2006). The synthesis of digestive enzymes can be modulated by genes, hormones and nutrition (Peres et al., 1998). It has been reported that pancreatic trypsin, chymotrypsin, elastase, carboxypeptidase A, B and lipase of Japanese flounder (*Paralichthys olivaceus*) are expressed at first feeding (Srivastava et al., 2002). As well for the larvae of eel (*Anguilla japonica*) pancreas start to synthesize all digestive enzymes by the onset of exogenous feeding (Kurokawa et al., 2002).

Digestion is a critical for the growth and survival of fish larvae (Govoni et al., 1986). During this complex process, the ingested food is hydrolyzed by secretion of specific digestive enzymes into the gastrointestinal tract (Gilannejad et al., 2021). At mouth opening, pikeperch larval stomach is not yet developed, for which extra-cellular degradation of ingested prey in the intestine is mainly done by pancreas-derived enzymes (Hamza et al., 2015). In many fish species, larvae pancreatic (trypsin, amylase) and intestinal (leucine-alanine peptidase, Alkaline phosphatase, aminopeptidase N) enzyme activities usually increase at mouth opening and might vary in response to the

Abbreviations: Actb, actin beta; alpi, intestinal-type alkaline phosphatase; amy2a, pancreatic alpha amylase; anpep, aminopeptidase N; cel, carboxyl ester lipase precursor; bile-salt activated lipase; ctrb, chymotrypsinogen b; chymotrypsin b precursor; eef1a, eukaryotic elongation factor 1-alpha; lap3, leucine aminopeptidase 3; pla2g1b, phospholipase a2; try1, trypsinogen 1; trypsin 1 precursor; AP, Alkaline phosphatase; AN, Aminopeptidase N.

nature and composition of the diet (Infante and Cahu, 1994; Hamza et al., 2007; Hamza et al., 2015).

Trypsin, chymotrypsin and amylase are among the key pancreatic enzymes that have been found in the larvae of Teleostei (Hoehne-Reitan and Kjorsvik, 2004). Trypsin is an important indicator of nutritional condition that is directly connected to protein metabolism. Its activity has been detected during the larval development of most studied marine fish (Cahu and Infante, 1995; Nolting et al., 1999; Infante and Cahu, 2001; Garcia-Gasca et al., 2006) and in some freshwater species (Chakrabarti et al., 2006; Silveira et al., 2013; Lahnsteiner, 2017; Palinska-Zarska et al., 2020). Activity of trypsin along with other pancreatic enzymes such as lipase and amylase are generally used as indexes of digestive function and maturation (Infante and Cahu, 1994; Ribeiro et al., 1999; Zambonino Infante and Cahu, 1999; Infante and Cahu, 2001).

Imentai et al. (2020) evaluated the growth, survival rate, and development of digestive organs using histological methods in *S. lucioperca* larvae under different feeding regimes with different times of shift in live preys rotifer to *Artemia*, at 17 DPH, age at which stomach has not been developed yet. The objectives of the present study were to determine the gene expression and activity levels of major digestive enzymes in *S. lucioperca* larvae in the same experimental design. Comparison between gene expression and actual activity of the different enzymes could provide a better understanding of the regulation mechanisms. To achieve this aim, expression of key pancreatic digestive proenzymes (*trypsinogen 1, trypsin 1 precursor, try1; chymotrypsinogen b, chymotrypsin b precursor, ctrb; carboxyl ester lipase precursor; bile-salt activated lipase, cel; phospholipase a2, pla2g1b; pancreatic alpha amylase, amy2a*), together with genes related to intestinal development and maturation (*intestinal-type alkaline phosphatase, alpi; leucine aminopeptidase 3, lap3; aminopeptidase N, anpep*) were assessed. Likewise, the activity of key enzymes: trypsin, lipase, alkaline phosphatase, amino peptidase, amylase and chymotrypsin were determined at 17 DPH.

MATERIALS AND METHODS

Live Feed Culture

The rotifers *Brachionus plicatilis* used in this study were cultured in 50-L flat-bottomed tanks (n=3) at ambient temperature and salinity 30 ± 5 g L^{-1} using a batch culture protocol and fed *Nannochloropsis* sp. paste (Nanno 3600, ReedMariculture Inc., USA) at a rate of 1 mL L^{-1} of culture twice a day. The *Artemia* nauplii (Micro artemia cysts, Ocean Nutrition™, Belgium) were hatched (20-24 h) onsite following provided manual from the producer and were harvested every morning. After harvesting, the densities of both rotifers and *Artemia* (individuals mL^{-1}) were estimated using a Sedgewick-Rafter counting chamber under a light microscope.

Fish and Experimental Design

Pikeperch larvae were reared at the Experimental Fish Facility of the Faculty of Fisheries and Protection of Waters, University of South Bohemia, Czech Republic. Newly hatched pikeperch larvae originating from nest spawning of pond-cultured broodstock (Malinovskyi et al., 2018) were acclimated to experimental RAS with water temperature of 15 ± 0.5°C at 3 DPH. At 4 DPH, the larvae (total length= 5.62 ± 0.03 mm, body weight = 0.66 ± 0.16 mg) were divided into five experimental groups with four replicates (2-L tanks) at initial density of 100 larvae per liter. All larvae at 5 DPH were initially fed with rotifers for 3 days and thereafter from 8 to 17 DPH were divided to 5 (A-E) different feeding regimes (**Figure 1**) and fed with rotifers and *Artemia* as follows: (A) larvae fed only with rotifers till 17 DPH; (B) larvae fed with rotifers till 14 DPH followed by feeding with *Artemia* till 17 DPH; (C) larvae fed with rotifers till 11 DPH followed by

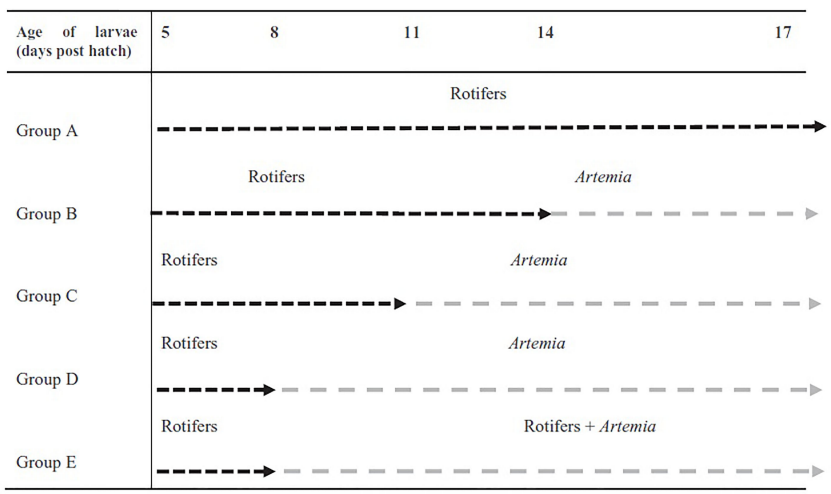

FIGURE 1 | The applied feeding regimes for pikeperch larvae throughout the experiment (Imentai et al., 2020).

feeding with *Artemia* till 17 DPH; (D) larvae fed only with *Artemia* till 17 DPH; (E) larvae fed a combination of rotifers and Artemia till 17 DPH.

Frozen paste of algae was added to the larval tanks twice a day (2 x 300,000 cells/mL), beginning at 5 DPH and continuing throughout the experiment according to the mentioned protocol. Rotifers and *Artemia* were provided as live feed to larvae three times a day with residual counts prior to each feeding. Feeding densities were steadily increased based on residual counts, performed prior to each feeding (**Supplementary Table 1**). In order to improve larval feeding efficiency, prior to each feeding water flow was stopped and restarted in each tank after 2 h. Flow rates started at 100 mL/min and increased with time (**Supplementary Table 1**).

Water temperature, pH, and dissolved oxygen (DO) were measured before each feeding with a pH/temperature tester (HI98129, Hanna Combo) and an oxymeter (OxyGuard International A/S, Farum, Denmark) and the values were 17.8 ± 0.17°C, 7.3 ± 0.04 and 88.5 ± 2.53%, respectively. Total ammonia and nitrite concentrations were determined twice a week and maintained below 0.5 and 0.1 mg L^{-1}, respectively. Light intensity on the water surface was 90-100 lux and photoperiod was set at 13L: 11D (07:00 to 20:00 h). Salinity was 2 ± 0.2 g L^{-1} which was kept constant by adding salt (Instant Ocean® Sea Salt) to the water of the whole culture system. Tanks were cleaned twice per day by siphoning out the faeces and dead larvae.

Sampling Procedure

At the end of the trial (17 DPH) all larvae were accounted for, and samples were collected to assess the larvae growth. Growth and survival rates were calculated using the following formula:

Specific growth rate (SGR, % day^{-1}) = 100 ($\ln TL_f - \ln TL_i$)/t where TL_i and TL_f are initial and final total lengths, respectively, and t is the period in days.

Survival rate (%) = Final fish number/Initial fish number × 100

Sixty individuals per treatment were randomly collected and immediately frozen in liquid nitrogen and then stored at -80°C for enzyme activity and gene expression assays. For gene expression analyses, frozen larvae were transferred into an appropriate volume (1/10 w/v) of RNA*later*®-ICE (Ambion Life Technologies), according to the manufacturer's instructions, and were kept at -80 °C until RNA extraction.

Gene Expression of Digestive Proenzymes

The ZFIN Zebrafish Nomenclature Guidelines for gene and protein names and symbols (available here) were followed in this manuscript. Primers were designed using the Primer3 software v.0.4.0 (available here) and were synthesized by IDT® (Belgium).

Total RNA was extracted from individual whole larvae, homogenized by a Polytron® PT 1200 E with a dispersing tool PT-DA 03/2EC- E050 (Kinematica AG), and the NucleoSpin® XS kit (Macherey-Nagel). An on-column RNase-free DNase step was included to eliminate or reduce the genomic DNA. RNA quality and concentration were measured using the Bioanalyzer 2100 with the RNA 6000 Nano kit (Agilent Technologies, LifeSciences), and the Qubit® 2.0 Fluorometer with a Qubit® RNA BR Assay Kit (Invitrogen, Life Technologies), respectively. Samples with RNA integrity number higher than 8.0, were reverse transcribed using the qScript™ cDNA synthesis kit (Quanta BioSciences), where 250 ng of each total RNA sample was used as input in a total reaction volume of 20 µL. Afterwards, each sample was diluted with 10 mM Tris-HCl, 0.1 mM EDTA (pH 8) to a final concentration of 1.25 ng μL^{-1}. QPCR reactions were run in Hard-Shell® Low-Profile Thin-Wall 96-Well Skirted PCR plates, with Microseal® B Adhesive Seals (BioRad), with 5 ng of cDNA (assumed from RNA input), 200 nM of each one of the forward and reverse primers (**Supplementary Table 2**), and IQ™ SYBR® Green Supermix (BioRad), in a final volume of 10 µL. Cycling program of 95°C, 10 min; [95°C, 15 s; 60°C, 30 s] × 40 cycles; melting curve 60-95°C, 0.5°C/5 s, was performed in a CFX Connect™ and a CFX 96 Real-Time Detection System with BioRad CFX Maestro Software v2.0 (BIORAD Laboratories).

Relative gene expression was calculated by the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) corrected for efficiencies (Pfaffl, 2001), where *actin beta* (*actb*) and *eukaryotic elongation factor 1-alpha* (*eef1a*) were used as internal reference genes to normalize the results, and a pool of cDNA samples belonging to the control group (C) were used as the calibrator to correct for inter-assay variations on all the plates. Adequacy of the *actb* and *eef1a* as internal control genes was confirmed by the target stability value (M = 0.61) and the coefficient of variance (CV = 0.21), established by the BioRad CFX Maestro Software. To verify the amplification efficiency, before analysis of the samples, 1:10 serial dilution of the calibrator (5 ng to 50 fg) was used to construct the calibration curves, with several pairs of primers. No template control (NTC) and no reverse transcriptase control (NTR) were included in the curves to discard the presence of artifacts and genomic DNA contaminations. Primers with a curve interpolating at least three points over six, efficiency (E) within the range of 90-110%, and a coefficient of determination (R^2) higher than 0.98 were selected for the QPCR analyses. Details regarding the sequences of the primers, amplicon size, amplification range, and accession number of their sources are detailed in **Supplementary Table 2**.

Enzyme Activity Analyses

The activity of different digestive enzymes was determined using both fluorometric and colorimetric methods on samples formed by 3 pools of 15 larvae for each treatment. The fluorometric methods were applied to the determination of trypsin, chymotrypsin and lipase activities while colorimetric methods were used to determine amylase, alkaline phosphatase and aminopeptidase. Trypsin was assayed using Boc-Gln-Ala-Arg-7-amido-4-methylcoumarin hydrochloride and chymotrypsin was determined using ala-ala-phe-7-amido-4-methylcoumarin according to Rotllant et al. (2008). In both cases, a 10 µL volume of the diluted homogenate was mixed with 5 µL of the substrate and 195 µL of 50 mM Tris–HCl, 10 mM CaCl2 buffer (pH 8.0) in microplate wells. Fluorescence was measured at Λ_{ex}/Λ_{em} of 380/440 for 5 min. Lipase activity was quantified using 4-methylumbelliferyl butyrate following the method described by Vaneechoutte et al. (1988). A stock solution was made by

dissolving 100 mg of MUB in 10 mL DMSO, to which 100 µL Triton X-100 was added; the mixture was stored at −80°C. This stock solution was diluted in phosphate buffer pH 7.0 reaching a final concentration of 0.4 mM MUB. A 10 µL volume of the diluted homogenate (1:10) was added to the microplate well and mixed with 250 µL of MUB 0.4 mM. Fluorescence was measured at $\Lambda_{ex}/\Lambda_{em}$ of 355/460 nm. In these cases one unit of activity corresponded to 1 µmol of either methylcoumarin or methylumbelliferone released per min. Amylase activity was measured using soluble starch as substrate and 3,5-dinitrosalicylic acid (DNS) as specific reagent to determine reducing sugars, according to Miller (1959). 250 µL of starch solution and 250 µL of diluted sample with citrate buffer 50 mM at pH 6.5 were added to the test tubes which were incubated at 25°C for 15 min. Thereafter, 500 µL of DNS was added to the mixture and the tubes were placed in a bath at 100°C for 5 minutes; after cooling to room temperature, 5 ml of distilled water was added and the absorbance was read at 540 nm. The calibration curves were made with concentrations ranging from 0 to 2 gL^{-1} of glucose in citrate buffer. One unit of enzyme activity was defined as the amount of enzyme needed to release 1 µmol of glucose per minute under standard assay conditions. Leucine aminopeptidase was determined according to Maraux et al., (1973) using sodium phosphate buffer 50 mM at pH 7.2, and the substrate leucine p-nitroanilide (0.1 mM dissolved in DMSO), being readings carried out at 410 nm. Enzymatic activity was defined as 1 µmol of nitroanilide released per minute. Alkaline phosphatase activity was determined following the method described by Bergmeyer (1974) using a solution of 2% 4-nitrophenyl phosphate in NaOH-glycine 100 mmol l-1 buffer at pH 10.1. The reaction was stopped by adding a solution of NaOH (0.05 N) and the absorbance was measured at 405 nm. One unit of activity was defined as the liberation of 1 µmol of nitrophenyl per minute.

Statistical Analyses

All data are presented as mean ± the standard deviation (SD). Data were analyzed with the program RStudio (R Core Team, 2014). Data were analyzed for possible outliers using the Grubbs' test (ESD method). Normal distribution of data and homogeneity of variance were confirmed by Shapiro-Wilk and Levene's tests, respectively. Comparisons for gene expression were made using one-way ANOVA followed by Tukey *post-hoc* test when significant differences were found at $p<0.05$. In addition, PCA were carried out in order to summarize data structure and to integrate the information obtained through the measurement of multiple gene expression and enzyme activities. These analyses were carried out using the software Statgraphics Centurion (Statgraphics Technologies, The Plains, VI.EE. UU.).

RESULTS

The survival rate was significantly higher in larvae from experimental groups C (54%), D (55%) and E (67%) ($p<0.05$) (**Table 1**). The group fed only with rotifers (Group A) showed significantly lower specific growth rate (SGR) than the other groups, and the highest SGR was found in the group fed with combination of rotifers and *Artemia* after 3 day rotifer feeding (Group E). The highest value of standard length (8.32 ± 0.48 mm) was found in the group fed with combination of rotifers and *Artemia* after 8 DPH (group E) (**Table 1**).

Gene Expression of Digestive Proenzymes

Gene expressions data are presented in **Figure 2**. Among the eight studied genes, seven showed significant differences in expression between dietary treatments and one (*alpi*) presented no significant interaction in expression among the experimental groups ($p<0.05$). The expression of *anpep, lap3* and *try1* was significantly upregulated in larvae fed rotifers for initial 3 days followed by feeding a combination of rotifers and *Artemia* (Group E) compared with other treatments ($p<0.05$). Although no significant, a similar trend was observed for *alpi* expression where larvae with feeding regime E showed also a higher value. The expression of *amy2a, ctrb, pla2g1b*, and *try1* was significantly lower in larvae fed rotifers until 14 DPH and replaced by *Artemia* afterwards (Group B).

Results of the PCA carried out on gene expression data are summarized in **Figure 3**. The combination of several variables into two principal components explained 80.8% of total variability observed among data. The PC1 in vertical axis was defined by expression of genes encoding digestive enzymes (*amy, cel, ctr, try* and to a lesser extent *pla*) which showed a clear correlation among them. The PC2 in the horizontal axis was defined by the expression of indicators of intestinal maturation; *anpep* and to a lesser extent *alpi* and *lap3*. The pattern of distribution of the samples belonging to each experimental group along the two axes evidenced some clear trends. A large proportion of A-labeled individuals were grouped in the area corresponding to negative values defined by the vectors in PC1, this meaning they present low values of expression of the

TABLE 1 | Survival, SGR and standard length of *S. lucioperca* larvae at 17 (n= 60) days post hatch (DPH).

Parameter	Groups				
	A	B	C	D	E
Survival (%)	38 ± 4.07^{b}	36 ± 5.25^{b}	54 ± 4.58^{a}	55 ± 5.39^{a}	67 ± 5.96^{a}
SGR_{TL} (% day^{-1})	19.58 ± 4.52^{b}	19.62 ± 4.83^{ab}	19.67 ± 3.91^{ab}	19.68 ± 4.02^{ab}	22.36 ± 4.97^{a}
Standard length (mm)	7.78 ± 0.47^{b}	7.69 ± 0.50^{b}	7.85 ± 0.50^{b}	7.76 ± 0.49^{b}	8.32 ± 0.48^{a}

Values are presented as mean ± SD. Values in the same row with different superscript letters are significantly different (P<0.05).

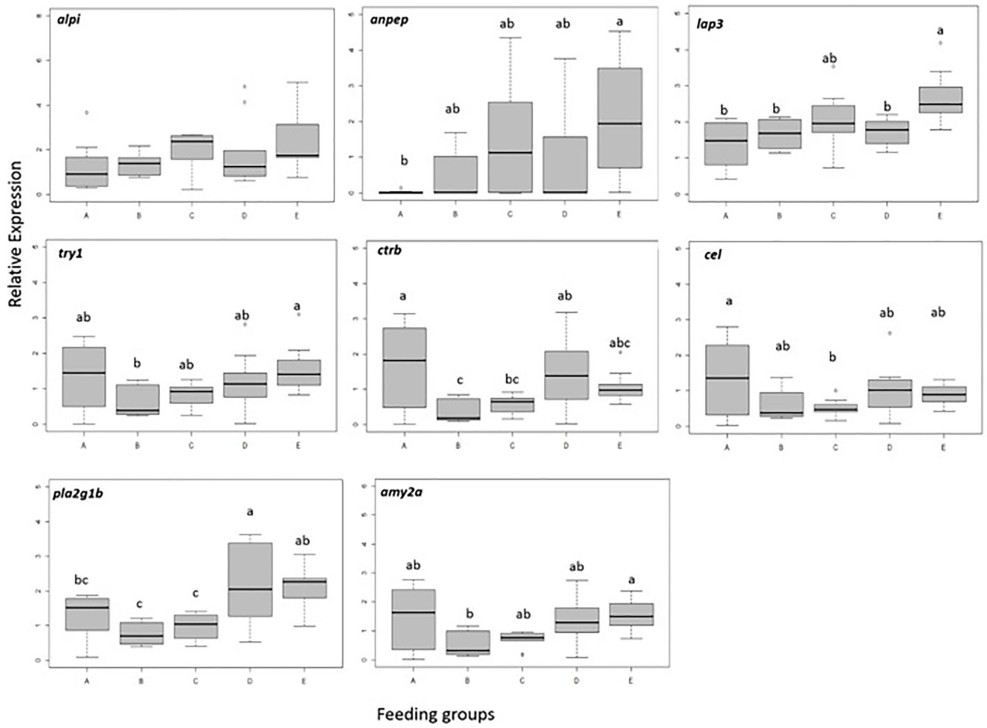

FIGURE 2 | Relative expression of genes in pikeperch larvae at the end of experimental period (17 DPH) at different feeding regimes (n=10 larvae per group). Different letters denote significant (p<0.05) differences among feeding groups. Box limits correspond to upper and lower quartiles, whiskers to minimum and maximum values, horizontal bar to the median, and points show outliers outside the 1.5 times interquartile range.

digestive proenzymes. In contrast, B-labeled individuals were clustered in the opposite area, indicating higher values of expression for most digestive proenzymes. The remaining groups of individuals labelled C, D and E showed intermediate values of expression of such genes. In addition, the expression of genes related to maturation of the brush border membranes appeared to be low in groups A and B, high in the E-labeled group, and those in groups C and D showed a great variability and not a clear pattern.

Enzyme Activity

The effect of different feeding regimes on the activities of digestive enzymes in pikeperch larvae is detailed in **Figure 4**. At 17 DPH, from the six tested digestive enzymes, two (amylase and chymotrypsin) did not show any activity while the rest displayed a significant difference among groups. The highest trypsin activity was observed in larvae from experimental groups B and E ($p<0.05$). There was no significant difference in trypsin activity among the other groups ($p<0.05$). Lipase activity was significantly lower in larvae from treatment A compared with the other groups ($p<0.05$). Data also revealed a significantly lower activity of alkaline phosphatase (AP) in larvae from group A (p<0.05). Whereas AP activity was highest in larvae from groups D and E ($p<0.05$). Similar to AP, Aminopeptidase N (AN) highest activity levels were found in larvae from treatments D and E ($p<0.05$).

The PCA analysis performed with data from enzyme activities is detailed in **Figure 5**. In this case, the combination of different values of enzyme activity into two principal components accounted for 90.2% of total variability observed among data. The PC1 in the horizontal axis discriminated between individuals with generally high or low enzyme activities. The pattern of distribution of the data clearly segregated A-labeled samples, which presented the lower levels of activities and also the B-labeled samples, which presented high activities of trypsin and lipase but very low of AP and AN. Samples in groups C, D and E presented equilibrated profiles of both luminal and intestinal enzymes.

DISCUSSION

At present, molecular studies in pikeperch larvae are limited. In this study, the analysis of expression of key genes of digestive proenzymes and activities of selected digestive enzymes was conducted to evaluate the mechanisms involved in digestion under different feeding regime from 4 till 17 DPH of pikeperch larvae.

Several studies have reported the ontogenetic development of the digestive system of pikeperch (Kowalska et al., 2006; Hamza et al., 2007). In pikeperch larvae both pancreatic and intestinal

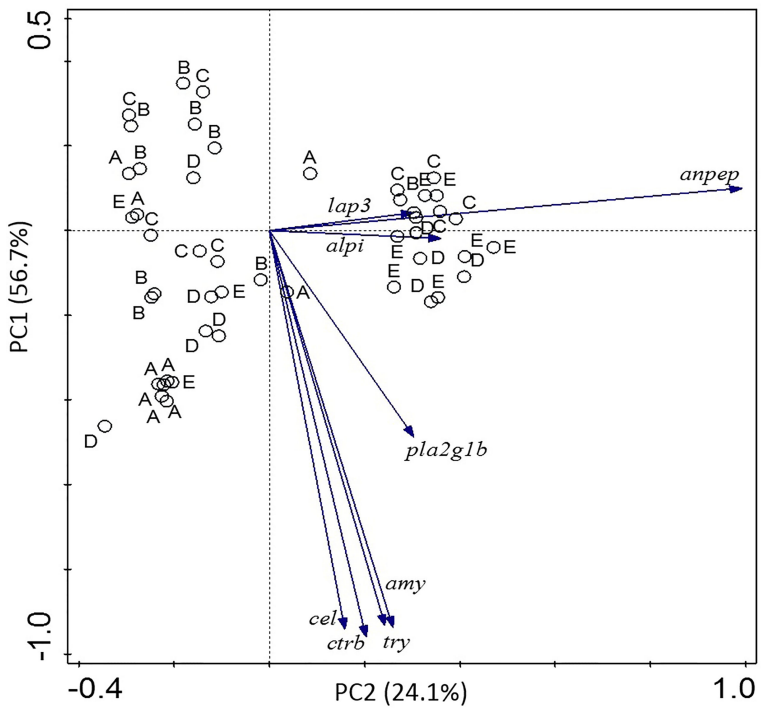

FIGURE 3 | Principal component analysis (PCA) correlation biplot based on the first two principal components (PC1 and PC2) generated from gene expression of different digestive enzymes and indicators of intestinal maturation in the different experimental groups.

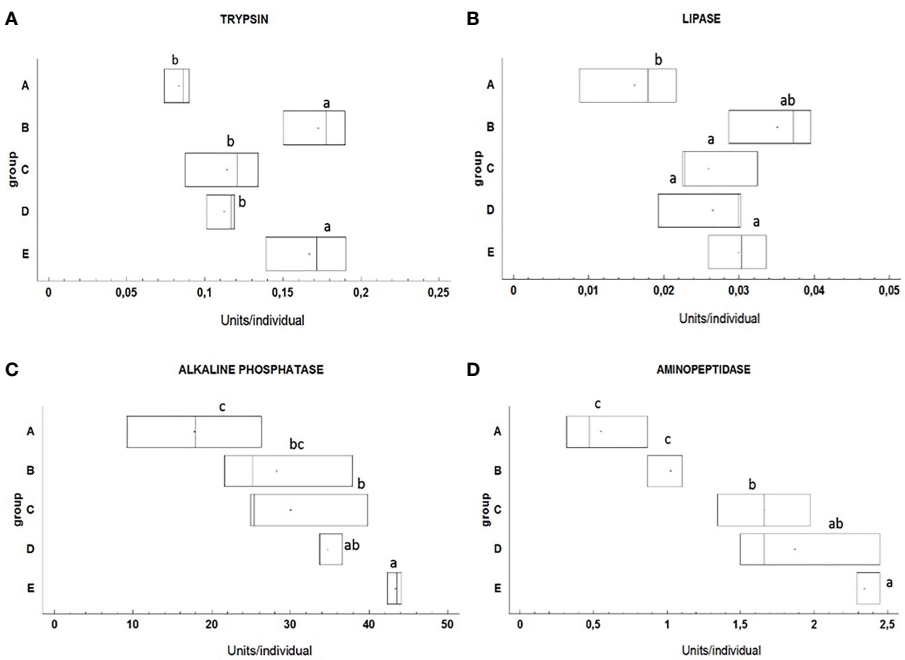

FIGURE 4 | The activities of trypsin **(A)**, lipase **(B)**, alkaline phosphatase **(C)** and aminopeptidase **(D)** in pikeperch larvae at the end of experimental period (17 DPH) at different feeding regimes (n=3 pools of 15 larvae per group). Different letters indicate significant differences among groups (p<0.05). Box limits correspond to upper and lower quartiles, horizontal bar to the median, and points show mean values.

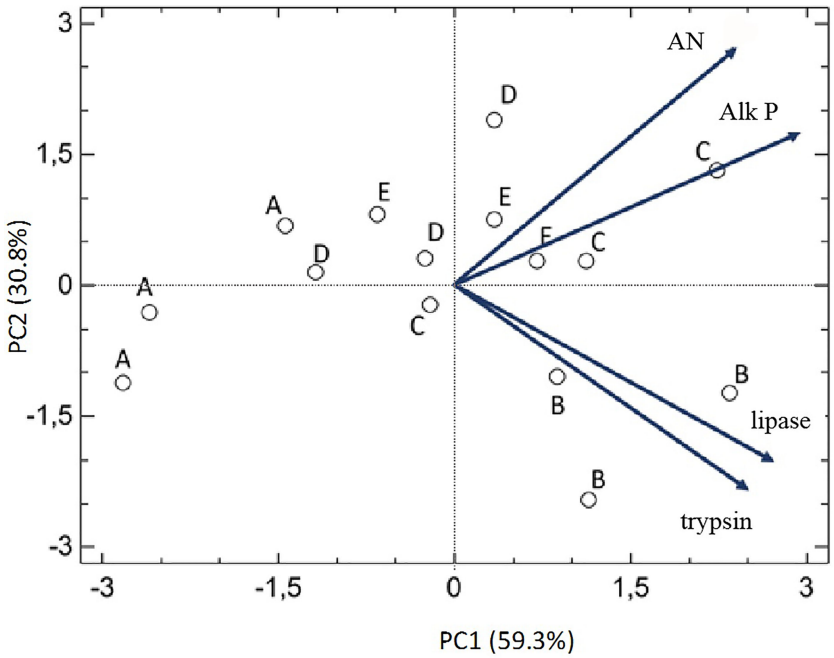

FIGURE 5 | Principal component analysis (PCA) correlation biplot based on the first two principal components (PC1 and PC2) generated from the activity of different digestive enzymes in the different experimental groups.

enzyme activities already appear at hatching (Hamza et al., 2007). Therefore, it is most likely that these activities are genetically programed (Hamza et al., 2015). Leucine aminopeptidase (Lap) is located on the brush border membrane and its activity indicates the intestine develop into the adult mode of digestion, improving luminal digestive capacity (Infante and Cahu, 2001). In the present study, *lap3* showed the highest expression in larvae fed rotifers and *Artemia* from 8 DPH until 17 DPH (Group E). Similarly, expression levels of *anpep* were high in Group E. The intestine *lap3* and *anpep* genes expression results obtained for pikeperch larvae in this study was agreement with the survival rate and histological analysis of intestine in study published by Imentai et al. (2020). In contrast, an upregulation of *anpep* expression was observed in larvae of Atlantic bluefin tuna fed rotifers compared with copepods, which could be due to the enrichment of rotifers (Betancor et al., 2017). In our study, rotifers that were fed on *Nannochloropsis* sp. and *Artemia* were immediately used after hatching. Moreover, in this study a diluted paste of *Nannochloropsis* sp. was added to all larval tanks. Previous studies have reported that addition of algae during rearing larvae increases growth, survival and has a beneficial effect on the development of digestive tract (Naas and Harboe, 1992; Navarro and Sarasquete, 1998; Skjermo and Vadstein, 2020). Similarly, in this study the highest aminopeptidase (AN) activity was also detected in groups D and E. This together with the significant upregulation of *anpep* in Group E indicates a better efficiency of the digestive processes and the intestinal absorptive capacity in these treatments (Infante and Cahu, 2001). AN is an important intestinal brush border enzyme (Eichholz, 1968) involved in the fermentation process, and its main role is to facilitate the absorption and transport of nutrients through the enterocytes (Cahu and Infante, 2001). Conversely, *alpi* expression was insignificant to different prey shift time in *S. lucioperca* larvae. Alkaline phosphatase (AP) is an esterase, and its activity is produced by phosphorylated substrates such as phospholipids and phosphoproteins. AP has often been considered as a sensitive indicator of the nutritional status of larvae (Cahu and Infante, 1994; Ribeiro et al., 2002; Kamaszewski et al., 2014). In the present study, its specific activity was significantly higher in larvae fed *Artemia* alone or in combination with rotifer from 8 DPH (Groups D and E). This together with the trend of higher *alpi* expression level in Group E may suggest a better development and intestinal maturation process in these feeding regimes (Infante and Cahu, 2001). Ribeiro et al. (2002) reported higher AP activity in *Solea senegalensis* larvae fed *Artemia* compared with artificial compound diet.

Previous studies have shown that pancreatic enzymes such as trypsin, lipase, phospholipase, or amylase are regulated at a transcriptional level during larval development (Cahu and Infante, 2001; Kurokawa et al., 2002; Cahu et al., 2003). In this study the lipase activity was significantly higher in fish fed *Artemia* compared with larvae fed exclusively on rotifers. While amylase specific activity are usually reported in pikeperch larvae from hatching (Hamza et al., 2015) and

chymotrypsin is detected during first feeding (Ljubobratovic et al., 2020), our attempts to detect those enzymes by using a highly sensitive fluorometric substrate and colorimetric method has failed. Similar results were found in *Chelon labrosus* larvae in which, in spite of detection of *ctr* transcripts, activity levels were inexistent (Gilannejad et al., 2020). According to the authors, higher levels of chymotrypsin activity might be necessary in larger animals.

The expression of *pla2g1b*, one of the genes involved in the digestion of lipids, was shown in this study to be influenced by prey shift time of pikeperch larvae. In our study, *pla2g1b* was upregulated in larvae fed rotifers and *Artemia* from 8 DPH until 17 DPH continuously (Group E). On the contrary, down regulation of *pla2g1b* expression in our study, showing that introducing *Artemia* later than 11 DPH (Groups A, B and C) might inhibit the hydrolysis of the sn-2 position of glycerophospholipids (Okamura et al., 2021). In our study, *try1* expression increased with earlier introduction of *Artemia* into diet and was down regulated in larvae fed *Artemia* from 14 DPH (Group B). This decrease in the trypsinogen expression could be attributed to the increased contribution of the acid digestion (Srichanun et al., 2013).

Trypsin is an important indicator of nutritional condition that is directly connected to protein metabolism. It is mainly influenced by protein content and amino acid profile of the diet (Grendell and Rothman, 1981; Tseng et al., 1982). In the present study, *try1* showed the highest expression in larvae fed rotifers and *Artemia* from 8 DPH until 17 DPH (Group E) and corresponds to higher specific activity in this group. While for Group B, larvae fed *Artemia* from 14 DPH *try1* expression and specific activity were opposite. This could be attributed to differences in post transcriptional/translational regulatory mechanism leading to higher activity in this group. Hamza et al. (2007) reported the trypsin activity in pikeperch from hatching and observed its significantly higher activity in larvae fed *Artemia* compared with the weaned larvae.

Principal component analysis (PCA) is a technique for reducing the dimensionality of large datasets that are often difficult to interpret. It has been applied in a wide number of studies to synthetize information obtained from the analysis of multiple digestive enzymes. As an example, PCA was used to evaluate information obtained from the whole spectrum of activity of digestive enzymes in different species of Annelidae in order to identify their food preferences (Cichocka et al., 2021). Also, the digestive enzyme profile was integrated with other parameters (growth related genes, immune and stress indicators) to identify biological responses to the inclusion of camelina meal in diets of red seabream *Pagrus major* (Mzengereza et al., 2021). In addition, PCA served to discriminate functionality of different regions in the digestive system of different species like the limpet *Megathura crenulata* (Martin et al., 2011) or the common octopus (Bastos et al., 2020). In the present study, PCA was used to integrate the above-described information obtained from the expression of selected genes and activities of digestive enzyme in groups of pikeperch larvae receiving different feeding patterns. Results of PCA performed both on gene expression and enzyme activities were greatly coincident and clearly identified a less favorable status in larvae fed only with rotifers (A) which showed low enzyme activity and poor intestinal maturation. Feeding larvae with rotifers till 14 DPH followed by *Artemia* till 17 DPH (group B) seems to have a positive impact on the expression and activity of digestive enzymes, but not on the intestinal maturation, while in contrast, the combination of rotifers and *Artemia* till 17 DPH positively influenced markers of intestinal maturation and activity of brush border enzymes, although not so clearly the gene expression and activity of luminal digestive enzymes.

It is well documented that activities of digestive enzymes can vary depending on species, age, and diet, among others (Buddington and Doroshov, 1986; Infante and Cahu, 2001). At the early stages when the stomach has not been developed yet, the intestine and pancreas play a key role in food storage, protein hydrolyses, emulsification and feed transit modulation, all these processes being regulated by complex neuroendocrine mechanisms (Holmgren and Olsson, 2009). Pikeperch larvae, from the beginning of exogenous feeding, have a poor or moderately developed digestive system and mostly during the feeding period its enzymes activities are increasing (Lahnsteiner, 2017). The results based on activities of digestive enzymes showed that earlier shift time to *Artemia* and combination of rotifers and *Artemia* during first feeding larvae leaded to a higher activity of analyzed enzymes. This could be mainly attributed to a different nutrient composition of diets. Several studies have previously reported the difference at the protein level and amino acid profile between live preys, e.g. rotifers and *Artemia* (Lubzens et al., 1989; Chakraborty et al., 2007; Yanes-Roca et al., 2018). *Artemia* is a protein source with high digestibility in fish larvae (Rønnestad and Conceição, 2005), however without enrichment it does not fully cover the nutritional needs of larvae at the beginning of first feeding. While enriched *Artemia* is difficult to ingest particularly for a small mouth size larvae (Majoris et al., 2018). Although, the same prey can become suitable diet once gape size increases (Yufera and Darias, 2007). Yanes-Roca et al. (2018) suggested that rotifers are suitable for pikeperch larvae from the beginning of exogenous feeding (4-5 DPH) until 12 DPH due to their size and nutritional value. However, exclusive rotifer feeding from 12 DPH is a limit for pikeperch larvae which should be fed by combination of rotifers and *Artemia* from this age and later by *Artemia* forward to weaning.

In conclusion, the findings in this study showed that the activity of digestive enzymes and the gene expression was affected by feeding regime. The activities were increased in larvae that were co-fed or replaced on *Artemia* from 8 DPH until 17 DPH. This might indicate that combination of two diets promotes appetite or that the larvae were at the sub-optimal feeding status. The present findings corroborate previous study (Imentai et al., 2020), that feeding pikeperch larvae with *B. plicatilis* from 5 to 8 DPH and afterwards exclusively with *Artemia* or combination of rotifers and *Artemia* till 17 DPH can ensure high survival, growth rates, better development of digestive organs, and increased activity of digestive enzymes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by EU-harmonized Animal Welfare Act of the Czech Republic.

AUTHOR CONTRIBUTIONS

AI designed the study, performed the experiment, analyzed data, and wrote the manuscript. NG and GM-R analyzed the gene expression data and contributed to the revision of the manuscript. FML and FM analyzed the digestive enzymes data and FML contributed to the revision of the manuscript. TPě contributed to the statistical analysis. VD and HD gave valuable advice. TPo supervision and funding acquisition. All authors have read and approved the final version of this article.

FUNDING

The study was financially supported by the Ministry of Agriculture of the Czech Republic, project NAZV QK22020144.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.864536/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.