New species for EU aquaculture

Deliverable Report

Deliverable No:	D10.1	**Delivery Month:**	58
Deliverable Title	Recommended Ca/P, vitamins and phospholipids to improve larval development and reduce skeleton alterations in pikeperch		
WP No:	10	**WP Lead beneficiary:**	P21. DTU Aqua
WP Title:	Nutrition – pikeperch		
Task No:	10.1	**Task Lead beneficiary:**	P21. DTU Aqua
Task Title:	Effect of selected dietary nutrients on pikeperch larval development and performance		
Other beneficiaries:			
P16. FUNDP			
P2. FCPCT			
P29. ASIALOR			
Status:	Delivered	**Expected month:**	36
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Objective: (from DOW)

Recommended Ca/P, vitamins and phospholipids to improve larval development and reduce skeleton alterations in pikeperch: This deliverable will recommend the optimum levels of vitamin A, D, E and C and phospholipids that should be included in enrichment products for pikeperch larvae. The deliverable will present the methodology employed and the main results that leaded to the recommended improvements including the consequences in the larval production. The deliverable will describe the effects of phospholipids, LC-PUFAs, Ca/P and vitamins, and will include: a) husbandry variables and fish response to stress tests, organ development and digestive enzymes activities, b) liver proteomics, in situ hybridization and selected genes expression, c) lipid and fatty acid analyses and d) skeleton morphogenesis to recommend dietary Ca/P, vitamins and phospholipid levels to improve larval development and reduce skeleton alterations in pikeperch.

Description: Based on the above three experiments were conducted in D10.1.to determine optimal dietary micro-diet levels of FA, Ca/P and vitamins for larval development and to reduce skeleton alterations in pike perch larvae: A multifactorial screening experiment (1) and two confirmatory experiments based on 1).

An additional fourth and fifth experiment was conducted to determine requirements of phospholipids and LC PUFA in weaning diets for pike perch larvae and influence on stress.

The work has so far generated 1 published paper (1) and 1 manuscript to be submitted to a peer reviewed journal (2):

(1) Lund, I, El Kertaoui, N., Izquierdo, M.S., Dominguez, D., Hansen, B.W., Kestemont, P. (2018). The importance of phospholipids combined with long-chain PUFA in formulated diets for pikeperch (*Sander lucioperca*) Larvae. British Journal of Nutrition, doi:10.1017/S0007114518001794

(2) El Kertaoui, N., Lund I., et al. Key nutritional factors in pikeperch (*Sander lucioperca*) larval development.

As D10.2 also involved performance studies and physiological stress experiments on larval dietary requirement for LC-PUFAs; deliverables D10.1 and D10.2 should be considered as a whole.

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1. Introduction

Pikeperch (*Sander lucioperca*) is recognized as one of a few freshwater species with a great potential for the expansion of the EU aquaculture industry, mainly because of flesh quality and thus a high market value. The major bottlenecks for further expansion of pikeperch culture today include relatively low larval survival (typical 7-20%) (Lund & Steenfeldt, 2011; Lund et al 2014), and high incidence of deformities. The optimal levels for major essential nutrients are still unknown for pikeperch. However, recent studies suggest requirements similar to those of marine carnivorous fish larvae for both phospholipids and long-chain polyunsaturated fatty acids (LC-PUFAs) (Hamza et al., 2015). Moreover, at a physiological level, oxidative risk is particularly high in the fast growing larvae due to the high metabolic rate, oxygen consumption and water content in the larval tissues (Betancor et al., 2012). On the other hand, the ratio among dietary fatty acids, such as eicosapentaenoic (EPA), docosahexaenoic (DHA) and arachidonic (ARA) acids constitutes a critical factor for broodstock and larval performance (Bell and Sargent, 2003, Izquierdo et al., 2005) due to competitive interaction among them. Hence, regardless of the need to study the optimum absolute dietary values for LC-PUFAs in this species, optimum dietary ratios must be defined. Dietary phospholipids tend to be a richer source of essential fatty acid (EFA) than neutral lipids such as triacylglycerols (Tocher, 1995). In addition, phospholipids may be superior to neutral lipids as a source of EFA in larval fish due to improved digestibility (Sargent et al., 1997, 1999). It was shown that intact phospholipids were a more efficient mode of supplying dietary EPA and DHA to sea bass larvae (*Dicentrachus labrax*) than triglycerides (Gisbert et al., 2005; Tocher et al., 2008). Previous studies have demonstrated a positive effect of dietary PL on the pikeperch larvae development, especially regarding their growth rate and digestive capacities (Hamza et al., 2008, 2015). Indeed, phosphoglycerides are particularly important in fish larvae production for their essential function as

necessary components for cellular bio-membranes and organelles formation (Izquierdo and Koven, 2011), and some authors suggest that fish larvae may be unable to efficiently synthesize phospholipids in an enough quantity to cover this high demand and therefore need to be included in the diet (Izquierdo and Koven, 2011). PL has a role as a precursor of second messenger regulating calcium entry into the cells, and it is involved in a signaling system controlling biological processes in the early development of vertebrates (Berridge and Irvine, 1989). In fish larvae, several studies dealing with the impact of first feeding on fish development showed that the dietary incorporation of total phospholipid reduce malformation, especially lordosis and scoliosis, and increase larval resistance to osmotic stress (Kestemont et al., 1996; Hamza et al., 2008; Henrotte et al., 2010). Moreover, during first feeding, several dietary nutrients play a central role in bone development and the posterior appearance of skeletal malformations (Izquierdo et al., 2010, 2013). The effect of interactions between LC-PUFAs and vitamins, specially vitamins E and/or C, has been reported in several fish for both freshwater- and marine larvae, but has not been examined in pike perch. In tandem to the dietary requirement of vitamins (E and C) in fish larvae, they also constitute a part of larval defense against lipid oxidation (Hamre et al., 2010) by their action as antioxidants. The importance of dietary vitamins and minerals are well known for many larval fish, vitamin C supplementation has been reported to reduce deformities and to improve the welfare of juvenile pike perch, but the importance of other vitamins and minerals is still unknown for pikeperch physiology and welfare, and there is still a lack of information on nutrition along larval development. Vitamin A whose antioxidant function has been also described (Palacios et al., 1996), is among the nutritional causative factors of skeletal anomalies in reared fish as well as vitamin D (Boglione et al., 2013). Limited knowledge exists on mineral requirements. Among the minerals studied, Selenium (Se) has been investigated with respect to its antioxidant role in fish larvae (Moren et al., 2011; Betancor et al., 2011, 2012; Saleh et al., 2014), likewise, interactions between Se and vitamin E have previously been demonstrated (Poston et al., 1976; Bell et al., 1985). The interaction between calcium (Ca) and phosphorus (P) remains among the most important between minerals. In fact, authors have suggested that dietary Ca/P ratio should be considered as well as individual dietary levels of minerals (Li et al., 1986), since the ratio between Ca and P affects the uptake of calcium (Moren et al., 2011). As Ca and P exist in a constant ratio in fish bone (NRC, 1993), this suggests needs to be maintained in fish feeds (Hossain and Yoshimatsu, 2014). Today pikeperch farming industry relies on use of commercial feeds developed for marine fish species for which the Ca/P ratio or content may be too high or not optimal.

Therefore, the general objective of this research program was to increase the knowledge on the influence of selected essential nutrients (PL, LC PUFAs, vitamins and minerals) for young developing percid fish using pikeperch fry as biological model.

2. Multifactorial screening experiment for investigating the interactions and effects of dietary fatty acids, vitamins and minerals on early development of pike-perch (*Sander lucioperca*).

Objectives
To determine the importance of various levels and interactions of major essential nutrients; LC-PUFAs, vitamins (Vit A, E, C, D) and minerals (Ca/P, Se) during weaning to dry feeds (25 days post hatching (dph)) by studying the impact by anatomo-histological, biochemical and molecular biomarkers. The specific objectives of the studies were:

a) Investigate the dietary effects of LC-PUFAs and their interactions with vitamins and minerals on larval development and performance, digestive capacity, skeleton malformation, bone mineralization, and molecular markers* of oxidative status (* analysed in a DHA/ARA/EPA confirmatory experiment) and bone development.

b) Determine the combined effect of n-3 and n-6 LC-PUFAs (DHA and ARA) and n-3/n-6 ratio and its influence on larval development, digestive enzymes activity, deformity occurrence and lipid metabolism.
c) To assess the effect of the dietary Ca/P taken into account P and Ca effect especially its implication in bone mineralization.

Hypothesis:

- ✓ Pikeperch larvae show a disturbance of the physiologic function during the early life stage due to the imbalanced diet; mainly developed for marine fish larvae with Ca/P and n-3/n6 LC-PUFAs ratios much higher than those observed in common freshwater fish diets. Therefore, causing increased mortalities and incidence of skeletal deformities.
- ✓ The elevated LC-PUFAs and PL requirements in pikeperch larvae - being a carnivorous fish larvae- is reflected in a high risk of suffering from lipid oxidation as well as the alteration lipid metabolism pointing the need of a high dietary levels of anti-oxidant nutrients.
- ✓ Dietary LC-PUFAs play a role in regulating the development of the digestive tract and consequently influenced the growth and survival rates in pikeperch larvae.

Materials and methods

Experimental design

The experiment, investigated the effect of selected dietary nutrients (fatty acids, vitamins and minerals) on pikeperch larval development and performance. Two modalities (low and high levels) of 8 variables: Ca/P, EPA+DHA, ARA, Vit. E, D, C, A and Se were tested (**Table 1**), in order to identify the most influential nutrients as well as their interactions in pikeperch larvae. The experiment was carried out as a fractional factorial screening, one of the most important designs for studying multiple factors with minimal experimental units as possible (Hamre et al., 2004; Gardeur et al., 2007; Teletchea et al., 2009, Trabelsi et al., 2011). Thus, the 256 runs required for a full 2^8 factorial experiment was reduced to 2^{8-4} using fractional factorial design generated by Planor software (Kobilinsky). High levels of vitamins and Se were chosen to be below the anticipated toxic level while low levels were slightly above the requirements reported in previous studies on pikeperch or based on literature for other species (**Table 1**). For fatty acids and Ca/P, the choice of levels tested was determined based on results obtained in pikeperch larvae (Hamza et al., 2008; Lund et al., 2018).

Table 1: Selected factors and modalities

Factors	Levels	References
Ca/P	0.6 1.2	- Good performance in pikeperch larvae at Ca/P: 0.5-0.6 even with a less dietary protein compared with other diets (Kestemont et al., 2007). - Normal Ca/P for rainbow trout: 0.9-1 (Rodehutscord, 1996), but low: 0.5-06 in condition of low Ca availability (Vielma et al., 1999; Antony Jesu Prabhu et al., 2014a).
EPA+DHA (%)	1.25 3.5	- Best growth and development in pikeperch larvae fed 1.25% EPA+DHA (1.25% vs 2.64%) (Hamza et al., 2008, 2015). - Significant improvement in growth (body weight) in pikeperch larvae fed 2.67 and 3.79% EPA+DHA (Lund et al., 2018).
ARA (%)	0.8 1.6	- Best growth and development in pikeperch at 0.8% ARA vs higher level (Hamza et al., 2008). - Good survival and SGR in sea bass: up to 1.2% (Atalah et al., 2011).

Vitamin E (mg/kg)	1000 3000	- Higher VE requirement (3000mg/kg) due to high HUFA needs during larval stages (Atalah et al., 2012; Izquierdo et al., 2013). - In seabass larvae: 3000mg/kg adequate for good larval performance, avoid muscular lesions, and improved stress resistance (Betancor et al., 2011; Atalah et al., 2012). - Requirement measurements depend to interactions of VE with other nutrients (Hamre, 2011).
Vitamin C (mg/kg)	2000 3600	- 3600 mg/kg improved the protection against peroxidation, decreased TBARS contents, spared vitamin E, and reduced the incidence of muscular lesions (Betancor et al., 2012).
Vitamin A (IU/kg)	8000 30000	- Optimum dietary VA content (based on growth): around 8000–9000 IU VA/kg for Atlantic halibut and Japanese flounder juveniles respectively (Moren et al., 2004; Hernandez et al., 2005). - VA toxic: maximum dose around 45000 IU VA/kg dry diet (Cahu et al., 2003).
Vitamin D (IU/kg)	2800 28000	- 27600 IU VD3/kg: Best result of sea bass larval morphogenesis, and earlier maturation of digestive function (Darias et al., 2010). - VD content in commercial diets used in larval rearing: 2500-2800 IU VD3/kg diet, in agreement with the value recommends by NRC 1993 (2400 IU VD3/kg).
Se (mg)	3 12	- Se concentration in fish around 2-3 mg/kg (Antony Jesu Prabhu et al., 2014b). - Up to 11.65 mg Se/kg improved survival rate, stress resistance and promoted the expression of bone formation and mineralization genes in seabream larvae (Saleh et al., 2014). - Se toxicity has been demonstrated at dietary levels of 10-20 mg/kg (NRC, 2011).

The 16 experimental diets (**Table 2**) represented a unique variant nutrient combination, to obtain a high level of information. No replicates were allotted in this experiment (1 tank per treatment). The experiment was repeated in UNamur facilities because of a high cannibalism related mortality observed in a first experiment carried out at DTU Aqua in Denmark, which blurred results.

Table 2: Experimental factors-modalities (Diet = experimental conditions)

Exp. diets (n°)	Ca/ P	EPA+DHA %	ARA %	Vitamin E mg/kg	Vitamin D IU/kg	Vitamin C mg/kg	Vitamin A IU/kg	Se mg/kg
1	0.6	1.25	0.8	1000	2800	2000	8000	3
2	1.2	1.25	0.8	1000	28000	3600	8000	12
3	0.6	3.5	0.8	1000	2800	3600	30000	12
4	1.2	3.5	0.8	1000	28000	2000	30000	3
5	0.6	1.25	1.6	1000	28000	2000	30000	12
6	1.2	1.25	1.6	1000	2800	3600	30000	3
7	0.6	3.5	1.6	1000	28000	3600	8000	3
8	1.2	3.5	1.6	1000	2800	2000	8000	12
9	0.6	1.25	0.8	3000	28000	3600	30000	3
10	1.2	1.25	0.8	3000	2800	2000	30000	12
11	0.6	3.5	0.8	3000	28000	2000	8000	12
12	1.2	3.5	0.8	3000	2800	3600	8000	3
13	0.6	1.25	1.6	3000	2800	3600	8000	12
14	1.2	1.25	1.6	3000	28000	2000	8000	3

15	0.6	3.5	1.6	3000	2800	2000	30000	3
16	1.2	3.5	1.6	3000	28000	3600	30000	12

Diets and feeding

Sixteen isonitrogenous and isolipidic diets containing different levels of Ca/P, EPA+DHA, ARA, Se, vitamin A, C, D and E were formulated and fabricated by SPAROS as cold extruded feed pellets of 200-400 µm and 400-700 µm.

Diet formulation

Experimental diets were formulated (**Table 3**) using a mix of oil as source EPA, DHA and ARA to reach the required fatty acids content and to equalize the lipid content in each diet. Lutavit E, Lutavit C, Rovimix A and Rovimix D3 were used as vitamin source of vitamin E, C, A and D , respectively. Selplex-Se yeast was used as a source of Se, while Ca/P levels were obtained by changing the P levels in diets using NaH_2PO_4 as source of P). The analysed proximate content of the main important nutrients and vitamins/minerals is shown in **Table 4.**

Feeding

Diets were manually supplied sixteen times per day each 45 min from 8:00 am to 07:30 pm for 14 days. To assure feed availability, daily feed supplied was maintained at 15% of larval wet weight using the 200-400 µm pellets during the first week of feeding, and a mixture of both pellet sizes 200-400 and 400-700 µm during second week (in agreement with the size of pikeperch larvae). Larvae were observed under the binocular microscope to determine feed acceptance.

Rearing conditions

A stock of pikeperch larvae (3dph) were obtained from a spawning broodstock from Viskweekcentrum Valkenswaard located in Leende, Nederland. First, an initial larval rearing was carried out in 3 tanks (500l) from 3 dph until weaning period. Larvae were fed *Artemia nauplii* enriched with DHA Protein Selco® (INVE, Dendermonde, Belgium) each hour (from 8:00 am to 6:00 pm) until they reached 17 days old, followed by a co-feeding period from 18 to 24 dph using *Artemia* nauplii and mixture of the 16 diets (200-400 µm pellets). The multifactorial experiment started with completely weaned larvae in order to avoid any additional stress. Then 25 dph larvae (9.44±4.42 mg) were randomly distributed in 16 experimental tanks respecting a density of 770 larvae $tank^{-1}$ and fed one of the experimental diets for 14 days. All tanks (90 L aquarium) were supplied with filtered water at a rate of 8% h^{-1} to ensure water renewal and maintain high water quality during the trial. Water was continuously aerated. Temperature and oxygen were daily measured; average water temperature along the trial was 20 °C and water dissolved O2 reach 7.8±0.32 mg. Photoperiod was kept at 12h light: 12h dark. Tanks were daily manually cleaned between 03:00 pm and 06:00 pm by a siphon system. During the experiment a daily mortality counting and a regular check out by measuring nitrite and nitrate concentrations were respected.

Table 3: Formulation and proximate composition of the experimental diets
*dietary content per g/100g; **vitamin A&D presented per IU/kg †vitamin C&E presented per mg/kg

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16
Ingredients (%)																
MicroNorse	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0
CPSP 90	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Squid meal	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0
Krill meal (Aker Biomarine)	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Fish gelatin	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Wheat Gluten	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Potato starch gelatinised (Pregeflo)	10.45	12.95	9.15	13.37	8.95	13.27	9.49	13.85	8.68	13.04	9.14	13.46	9.04	13.26	9.58	12.08
Fish oil - SAVINOR	1.20	1.20	0.80	0.80	1.15	1.15	0.00	0.00	1.20	1.20	0.80	0.80	1.15	1.15	0.00	0.00
Incromega DHA 500TG	0.00	0.00	3.40	3.40	0.00	0.00	3.58	3.58	0.00	0.00	3.40	3.40	0.00	0.00	3.58	3.58
VEVODAR	2.10	2.10	2.10	2.10	4.25	4.25	4.25	4.25	2.10	2.10	2.10	2.10	4.25	4.25	4.25	4.25
Soybean oil	3.00	3.00	0.00	0.00	1.05	1.05	0.00	0.00	3.00	3.00	0.00	0.00	1.05	1.05	0.00	0.00
Vit & Min Premix PV02	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Lutavit C35	0.58	1.02	1.02	0.58	0.58	1.02	1.02	0.58	1.02	0.58	0.58	1.02	1.02	0.58	0.58	1.02
Lutavit E50	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60
Rovimix A (5000000 IU/kg)	0.00	0.00	0.44	0.44	0.44	0.44	0.00	0.00	0.44	0.44	0.00	0.00	0.00	0.00	0.44	0.44
Rovimix D3 (5000000 IU/kg)	0.015	0.51	0.015	0.51	0.51	0.015	0.51	0.015	0.51	0.015	0.51	0.015	0.015	0.51	0.015	0.51
Brewer's yeast	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Choline chloride	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Soy lecithin - Powder	6.00	6.00	6.00	6.00	6.00	6.00	4.50	4.50	6.00	6.00	6.00	6.00	6.00	6.00	4.50	4.50
Binder (sodium alginate)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
NaH2PO4	4.20	0.35	4.20	0.35	4.20	0.35	4.20	0.35	4.20	0.35	4.20	0.35	4.20	0.35	4.20	0.35
SelPlex - Se yeast	0.05	0.47	0.47	0.05	0.47	0.05	0.05	0.47	0.05	0.47	0.47	0.05	0.47	0.05	0.05	0.47
L-Taurine	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50

Table 4: Proximate composition **(%)** of the experimental diets

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16
Proximate composition																
Moisture*	6.40	6.20	6.20	6.30	6.50	6.30	6.30	6.50	6.40	6.40	6.30	6.50	6.60	6.40	6.30	6.50
Crude protein*	51.1	51.3	51.5	51.1	51.3	51.1	51.1	51.2	51.0	51.3	51.3	51.0	51.2	51.1	51.1	51.2
Crude fat*	17.4	17.5	17.5	17.6	17.6	17.7	17.7	17.5	17.5	17.5	17.6	17.5	17.6	17.6	17.6	17.5
Crude ash*	7.60	6.60	7.60	6.60	7.50	6.70	7.60	6.60	7.50	6.50	7.60	6.60	7.60	6.60	7.60	6.60
Phosphorus*	1.97	1.06	1.99	1.07	1.96	1.07	2.01	1.05	1.98	1.06	2.01	1.07	1.95	1.06	2.03	1.05
Calcium*	1.24	1.26	1.25	1.24	1.25	1.24	1.25	1.24	1.24	1.24	1.26	1.23	1.23	1.24	1.24	1.24
Selenium*	4.00	14	14	4	15	4	4	14	5.0	14	14	4.0	14	4.0	4.0	14
Vitamin A**	7794	7775	28933	28896	28866	28917	7825	7794	28877	28891	7816	7777	7800	7814	2889	28843
Vitamin C†	1944	3428	3415	1952	1941	3409	3418	1948	3417	1933	1947	3416	3401	1946	1956	3417
Vitamin D3†	2893	28544	2835	28707	28006	2794	28430	2829	28397	2881	28208	2884	2831	28777	2848	28805
Vitamin E **	997	1006	992	1004	997	1001	1006	994	2952	2947	2956	2946	2938	2871	2967	2897

*dietary content per g/100g; **vitamin A&D presented per IU/kg †vitamin C&E presented per mg/kg

Samplings and output variables

Several parameters were evaluated including husbandry variables, biochemical assays, digestive enzymatic activities, organ development and tissue morphology, deformities and gene expression.
Final survival was calculated by individually counting all the living larvae at the end of the experiment. Survival was calculated as the percentage between final and initial number of fish. Mortality did not consider missing larvae due to type II cannibalism (i.e. fish completely ingested, usually head first, by a cannibal), while the mortality due to cannibalism was thus estimated by adding missing larvae to dead larvae due to cannibalism, partly consumed by their conspecifics (N_c+N_m).
Growth was monitored by sampling 40 larvae per tank at the beginning and the end of the experiment. At the end of the experiment the weighed larvae were fixed in 10% buffered formalin and kept until analysis to determine the rate of malformations. The number of sampled larvae was taken into account for survival calculation.
Survival, growth and cannibalism rates were assessed as follows:

Counted mortality (%) =100 (N_d+N_c) N_i^{-1}

Survival (%) =100 N_f N_i^{-1}

Cannibalism (%) =100 (N_c+N_m) N_i^{-1}

Specific growth rate (SGR, % day^{-1}) =100 (LnW_f– LnW_i) ΔT^{-1}

where: N_i and N_f=initial and final number of fish per tank, N_d=number of dead fish without signs of cannibalism, N_c=number of dead fish due to cannibalism (generally type I cannibalism, i.e. fish partly consumed by a cannibal), N_m=number of missing fish at the counting (end of experiment, N_m= N_i-N_d-N_f), W_i and W_f=initial and final body weights (mg), ΔT=duration of the experiment (days).

Proximate analysis

To analyze biochemical composition, larvae in each tank were collected, after a starving period of 12 h, then washed with distilled water and kept at -80ºC until analysis.

Fatty acid composition in diets

The fatty acid composition in diets was determined by extraction of the lipids by a chloroform/methanol mixture, (Folch et al., 1957). Samples were homogenised by a tissue- tearor probe diameter 4.5 mm, Biospec Products, Inc. Trans esterification of the lipids were done by acetyl chloride in methanol at 95 °C. The fatty acid methyl esters were analyzed by gas chromatography–mass spectrometry (GC–MS). Peaks on a given chromatogram were identified by comparison with the retention time of a commercial mix of a known FAME standard, SUPELCO 18919 (4:0–24:0), from SIGMA. Peaks were quantified by means of the target response factor of the fatty acids and 23:0 as internal standard. Fatty acid concentrations were calculated (Chem. Station Ver. E.02.02.1431) based on the quantified peaks of the standard series and the samples as well of dry weight of prey and larvae and expressed as ng $sample^{-1}$. A total of 34 fatty acids were analyzed. For the dietary composition only the 10 most abundant and relevant FAs are presented (**Table 5**).

Table 5. Fatty acid composition (% of TFA) of the 16 experimental feed types

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16
FA																
16:0	13.4	13.2	12.2	12.80	13.30	13.10	10.40	10.70	13.50	13.50	12.00	12.20	13.30	13.50	10.60	10.30
18:0	4.10	4.10	3.50	3.60	4.20	4.20	3.90	3.90	4.10	4.10	3.50	3.40	4.20	4.30	3.90	4.00
Total SFA	27.0	27.10	25.80	26.6	30.10	29.9	26.0	26.50	27.30	27.10	26.10	25.90	30.00	30.60	26.20	26.10
16:1 (n-7)	2.60	2.70	2.70	2.70	2.70	2.60	2.10	2.20	2.60	2.60	2.60	2.60	2.60	2.70	2.10	2.10
18:1 (n-9)	15.50	15.50	11.80	11.90	12.90	12.70	10.10	10.30	15.40	15.40	11.50	11.50	12.90	12.90	10.10	9.90
Total MUFAs	24.35	23.65	21.85	21.40	21.40	21.10	18.30	18.60	23.70	23.90	21.00	21.50	21.00	21.20	18.10	18.00
18:2 (n-6)	16.50	15.70	12.80	13.5	14.30	14.10	10.60	10.60	16.50	16.40	12.50	12.10	14.60	14.30	11.00	10.30
20:4 (n-6) ARA	9.00	9.40	10.30	10.0	17.90	18.20	17.80	17.70	9.20	9.10	11.30	10.30	17.70	18.00	17.40	18.10
Total (n-6) PUFA	26.40	27.00	25.20	26.00	35.40	35.50	31.90	31.50	27.50	27.50	26.20	24.60	35.40	35.50	31.90	31.70
18:3 (n-3)	14.00	14.00	1.40	1.40	5.80	5.60	1.10	1.10	13.70	14.0	1.40	1.30	5.70	5.80	1.10	1.00
20:3 (n-3)	0.80	0.10	0.20	0.30	0.10	0.20	0.30	0.30	0.20	0.20	0.30	0.30	0.20	0.20	0.20	0.20
20:5 (n-3) EPA	3.90	3.90	6.50	6.30	3.90	3.90	5.40	5.40	3.90	3.90	6.20	6.60	3.90	3.50	5.30	5.30
22:6 (n-3) DHA	4.80	4.90	19.80	18.80	4.80	5.00	17.70	17.30	4.90	4.70	19.20	19.90	4.80	4.60	17.40	17.90
Total (n-3) HUFA	9.85	9.25	27.55	26.45	9.15	9.45	24.25	23.85	9.35	9.15	26.75	28.05	9.25	8.65	23.75	24.35
DHA/EPA	1.23	1.26	3.05	2.98	1.23	1.28	3.28	3.20	1.26	1.21	3.10	3.02	1.23	1.31	3.28	3.38
DHA/ARA	0.53	0.52	1.92	1.88	0.27	0.27	0.99	0.98	0.53	0.52	1.70	1.93	0.27	0.26	1.00	0.99
EPA/ARA	0.43	0.41	0.63	0.63	0.22	0.21	0.30	0.31	0.42	0.43	0.55	0.64	0.22	0.19	0.30	0.29
Oleic/DHA	3.23	3.16	0.60	0.63	2.69	2.54	0.57	0.60	3.14	3.28	0.60	0.58	2.69	2.80	0.58	0.55
(n-3)/(n-6)	0.93	0.91	1.19	1.11	0.44	0.44	0.82	0.81	0.87	0.87	1.11	1.23	0.44	0.43	0.80	0.82

Histological analysis

At the beginning and end of the experimental trial 10 pikeperch larvae from each tank were collected and fixed in 10% buffered formalin. For its histological process larvae were dehydrated through graded alcohols (70-96º) thanks to Histokinette 2000 tissue processor (Leica, Nussloch, Germany), then xylene and finally embedded in paraffin wax (Jung Histoembedder, Leica, Nussloch, Germany).
Larvae were cut through a series of 5 µm sections and paraffin section was mounted every 50µm. for histological analyses, slides were stained with hematoxylin, eosin, safran (HES) and examined using light microscopy in a range of magnifications (10-40x).

Enzymatic assays

The heads and tails of pikeperch larvae were removed to isolate the digestive segment on a glass maintained on ice (0 °C), and then stomach region was separated with the intestinal segments. Pooled samples from each tank were homogenized in 10 volumes (v/w) cold distilled water. Assay of the cytosolic peptidase, leucine alanine peptidase (leu-ala) was performed following the method of Nicholson and Kim (1975) using leucine-alanine (sigma-Aldrich, St LLuis, MO, USA) as substrate. Alkaline phosphatase (AP) and aminopeptidase N (AN), two enzymes of brush border membrane, were assayed according to Bessey et al. (1946) and Maroux et al. (1973) using p-nitrophenyl phosphate (Sigma-Aldrich) and L-leucine p-nitroanalide (Sigma-Aldrich) as substrates, respectively.
Pepsin was assayed by the method of Worthington (1982) modified by Cuvier-Péres and Kestemont (2002). Trypsin and amylase activities were assayed according to Holm et al. (1988) and Metais and Bieth (1968), respectively such as described by Gisbert et al. (2009). Protein was determined using the Bradford (1976) procedure. Enzyme activities are expressed as specific activities (U or mU mg protiein^{-1}).

Skeleton anomalies and related gene expression

To determine the presence of skeletal anomalies, 50 larvae per tank were fixed and stored in buffered (10% phosphate) formalin at the end of the experiment. Staining procedures with alizarin red (**Table 6**) were conducted to evaluate the skeletal anomalies following a modified method from previous studies (Izquierdo et al., 2013). Classification of skeletal anomalies was conducted according to Boglione et al. (2001). Anomalies were expressed as frequency of total severe anomalies and specific anomalies, such as jaw deformities, scoliosis, lordosis, kyphosis, prehaemal and caudal vertebrae, within each dietary group.
Total RNA from larvae samples (average weight per sample 60mg) was extracted using the Rneasy Mini Kit (Qiagen). Total body tissue was homogenized using the Tissue Lyzer-II (Qiagen, Hilden, Germany) with QIAzol lysis reagent (Qiagen). Samples were centrifuged with chloroform for phase separation (12000g, 15min, 4ºC). The upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into an RNeasy spin column, where total RNA bonded to a membrane, and contaminants were washed away by RW1 and RPE buffers (Qiagen). Purified RNA was eluted with 30µl of RNase-free water. The quality and quantity of RNA were analysed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and by electrophoresis of total RNA in a 1% agarose gel. Synthesis of cDNA was conducted using the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Primer efficiency was tested with serial dilutions of a cDNA pool (1, 1:5, 1:10, 1:15, 1:20 and 1:25). Product size of the real-time q PCR amplification was checked by electrophoresis analyses using PB322 cut with HAEIII as a standard. Real-time quantitative PCR was performed in an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) using RAG1 as the house-keeping gene in a final volume of 20µl per reaction well, and 100ng of total RNA reverse transcribed to complementary cDNA. Each gene sample was analyzed once per gene. The PCR conditions were the following: 95ºC for 3min 30sec followed by 40 cycles of 95ºC for 15sec, 61ºC for 30sec, and 72ºC for 30sec; 95ºC for 1min, and a final denaturing step from 61ºC to 95ºC for 10sec. Data obtained were normalized and the Livak method (2–ΔΔCt) used to determine relative mRNA expression levels. Pikeperch specific gene primers were designed after searching the NCBI nucleotide database and using the Oligo 7 Primer Analysis software (Molecular Biology Insights, Cascade, CO, USA). Within the oligo used,

RAG1, Twist2, Mef2c and Sox9 were designed by sequence analysis of these previous published in other fishes. Sequence alignment by MEGA 7 software was realized and conserved domains obtained were used to designed primers with Primer3 (v. 0.4.0) program and subsequent sequencing of PCR products and BLAST of them. Detailed information on primer sequences and accession numbers is presented in **Table 7**.

Table 6: Staining protocol

Step	Duration	Significance of the step	Solution (100ml)
KOH 0,5% + H_2O_2 3%	7h- overnight	Depigmentation	KOH 0,5% 85-90ml H_2O_2 3% 10-15ml
Alcian Blue	1-1.5h	Cartilage staining	80 ml ethanol 20 ml glacial acetic acid 10 mg Alcian blue
Ethanol 95%	1 h	Hydration	95ml absolute ethanol 5ml distilled water
Ethanol 95%	1 h		95ml absolute ethanol 5ml distilled water
Ethanol 95%	1 h		95ml absolute ethanol 5ml distilled water
Ethanol 75%	1 h		75ml absolute ethanol 25ml distilled water
Ethanol 40%	1 h		40ml absolute ethanol 60ml distilled water
Ethanol 15%	1 h		15ml absolute ethanol 85ml distilled water
Distilled water	1 h– overnight		Distilled water
Trypsin solution	3-5h (Until the first vertebrae are seen)	Tissue digestion	90mg pancreas porcine trypsin 70ml distilled water 30ml saturated solution of Na2B4O7
Alizarine red	24h – Until the bone is stained (have to dye the fins and part of the skull)	Bone staining	saturated solution of Alizarine red in 75ml absolute ethanol and 25ml distilled water
Ethanol75%	Quick wash	Wash	75ml absolute ethanol 25ml distilled water
Trypsin solution	12-24 hours, Alternating with 1% KOH after 4 hours	Tissue digestion and clearing	90mg pancreas porcine trypsin 70ml distilled water 30ml saturated solution of Na2B4O7
KOH 1%	Alternating with trypsin after 4 hours		KOH 1%
Glycerin KOH 1:3	12-24 hours	Fixation and clearing	25ml Glycerin 75ml KOH (0.5%)
Glycerin KOH 3:1	12-24 hours		75ml Glycerin 25ml KOH (0.5%)
Glycerin	12-24 hours	Storage	Pure glycerin and some grains of thymol

Table 7: Sequences of primers used for gene expression analysis

Gene	Nucleotide Sequence	Accesion nº	Amplicon	Tm
RAG1	F: 5'-AGCCAAAGCCAAACTCAGAA-3'	KC819903	150	60
	R: 5'-TCACGCACCATCTTCTCATC-3'			
Twist2	F: 5'-CCCCTGTGGATAGTCTGGTG-3'	--	226	60
	R: 5'-GACTGAGTCCGTTGCCTCTC-3'			
Mef2c	F: 5'-GCGAAAGTTTGGCCTGATGA-3'	--	180	60
	R: 5'-TCAGAGTTGGTCCTGCTCTC-3'			
Sox9	F: 5'-TCCCCACAACATGTCACCTA-3'	--	163	60
	R:5'-AGGTGGAGTACAGGCTGGAG-3'			

Statistics

Statistical analyses were done following appropriate methods to a fractional factorial design as developed by Kobilinski (2000) and Gardeur et al. (2007). Calculations were done using the Planor-Analys software developed by Kobilinski (2000). The interactions between 3 or more factors were considered insubstantial. When an interaction between two factors was found significant ($P < 0.05$), the potential single effects of these factors were also considered insubstantial. All the significant effects were then tested with the Statistica™ software for windows (Stat Soft, USA) with one- or two-ways ANOVA. When significant ($P < 0.05$), means were compared according to the Tukey post hoc test.

Results and discussion

Husbandry and skeletal deformities

At the end of the experiment, the highest survival was recorded in larvae fed high Ca/P (**Fig. 1, A**), but final weight and specific growth rate (SGR) were significantly lower in larvae fed high Ca/P, associated with a higher incidence of kyphosis and pectoral deformities in these larvae (**Fig. 1, B**).

The higher kyphosis and pectoral element deformities were recorded in larvae fed high Ca/P levels (**Fig. 2, A and B**). In these sense, the growth was probably reduced, in these larvae, due to the higher incidence of bone deformities, since skeletal deformation may affect various ecophysiological performances of fish larvae such as swimming behavior and feeding efficiency. Indeed, high incidence of kyphosis is often reported as typical consequences of P deficiency in fish larvae (Fjelldal et al., 2016).

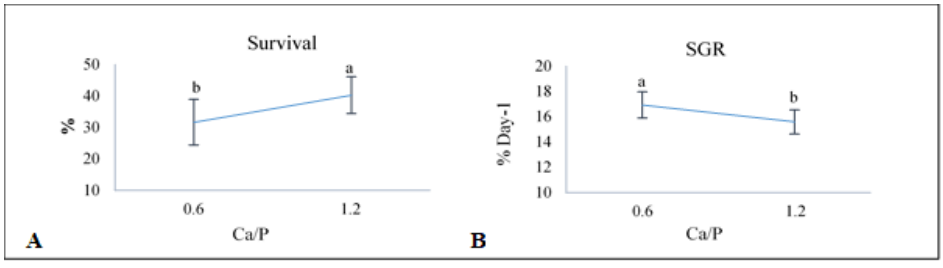

Figure 1: Effect of dietary Ca/P on Husbandry variables: Survival (A); Survival (B). Only graphs with significant effects are shown. Results are expressed as the Mean ± SD (n=8). Different letters denote statistically significant differences between treatments.

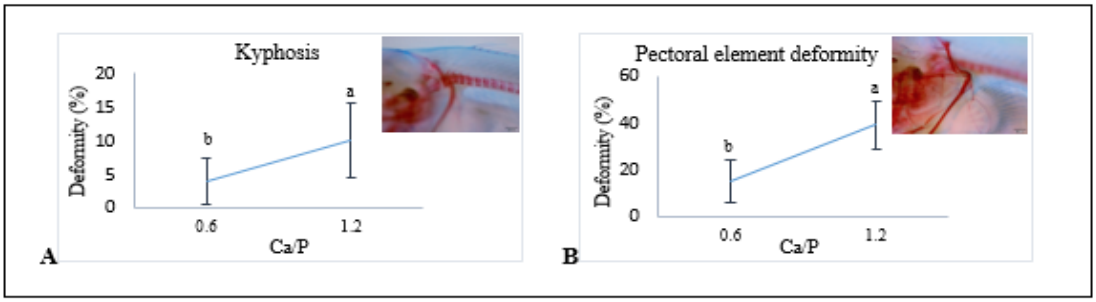

Figure 2: Effect of dietary Ca/P on larval deformities: Kyphosis (A) and Pectoral element (B). Only graphs with significant effects are shown. Results are expressed as the Mean ± SD (n=8). Different letters denote statistically significant differences between treatments.

Results showed that the increase in EPA+DHA reduced jaws deformities in larvae fed 3600 mg Vit. C, (**Fig. 3,B**), a high Vit. C dietary content seemed to be efficient in reducing the incidence of deformities when high levels of n-3 HUFA (especially DHA) were included in the diets pointing the antioxidant function of this vitamin (Betancor et al., 2012). In addition, a possible explanation may be related to dietary effect on the ossification of cartilaginous- origin bone process, since vitamin C affects collagen synthesis in structural organs such as cartilage and bone (Padayatty et al., 2003).

In this study, high vitamin C associated with high EPA+DHA level resulted in higher cannibalism rate, mainly because of a decrease in incidence of jaws deformities in these larvae (**Fig. 3,A**).

The increase of EPA+DHA seemed to reduce the prevalence of scoliosis in larvae fed 0.8% ARA (**Fig. 3,C**), while the high levels of ARA had the opposite effect. Beside scoliosis, high levels of EPA+DHA and ARA increased lordosis, while the decrease in EPA+DHA with the high ARA level reduced skeletal deformities (**Fig. 3, C and D**). In this regard, a possible explanation can be related to EPA+DHA/ARA, suggesting the importance of a balanced n-3 HUFA/n-6 HUFA ratio in this species.

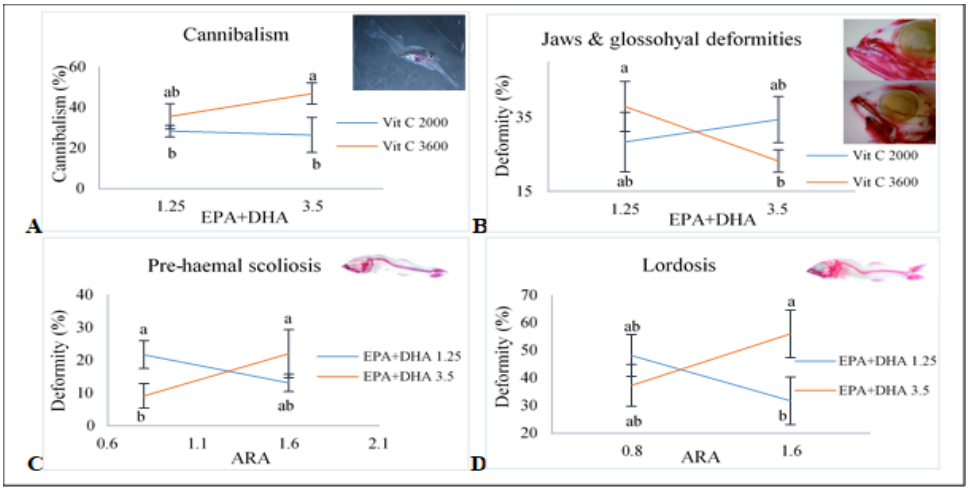

Figure 3: Effect of fatty acids and vitamin C on Larval deformities and cannibalism, A: EPA+DHA and vitamin C interaction effect on cannibalism rate; B: EPA+DHA and vitamin C interaction effect on jaws deformities; C: EPA+DHA and ARA interaction effect on pre-haemal scoliosis; D: EPA+DHA and ARA interaction effect on lordosis. Only graphs with significant effects are shown. Results are expressed as the mean ± SD (n=4). Different letters denote statistically significant differences between treatments.

Activity of digestive enzymes

A differential expression pattern in the ontogenetic development of digestive enzyme system was observed depending on the dietary content. Our results showed that a high dietary n-3 HUFA content enhanced trypsin activity in larvae fed a low Ca/P level (**Fig. 4,B**). Previous studies showed that n-3 HUFA are potent stimulators of cholecystokinin (CCK) secretion (Little et al., 2007); thus EPA+DHA effect may reflect the endocrine modulation of the pancreatic digestive function which is regulated by CCK (Saleh et al., 2013, Kamaszewski et al., 2014). In fact, trypsin is secreted as a trypsinogen activated by an enterokinase requiring Ca ions. Consistently, the better utilization of Ca under specific condition of HUFA and Ca/P should be further investigated. Moreover, the opposite interaction found between EPA+DHA and Ca/P could be linked to the effect of Ca/P on growth. A higher aminopeptidase activity was also observed in larvae fed low Ca/P (**Fig. 4, A**), this result may be related to the growth improvement obtained in these larvae, denoting a higher maturation status of the gut (Zambonino Infante and Cahu, 2001; Zambonino-Infante et al., 2008).

Pepsin and the intestinal enzymes (leucine alanine, aminopeptidase and alkaline phosphatase) activities were negatively correlated with ARA levels (**Fig. 4, C, D, E, F**). High ARA level in the diet may delay enterocyte maturation (Yuan et al., 2015), thus pointing to its potential involvement in regulating the development of the digestive tract.

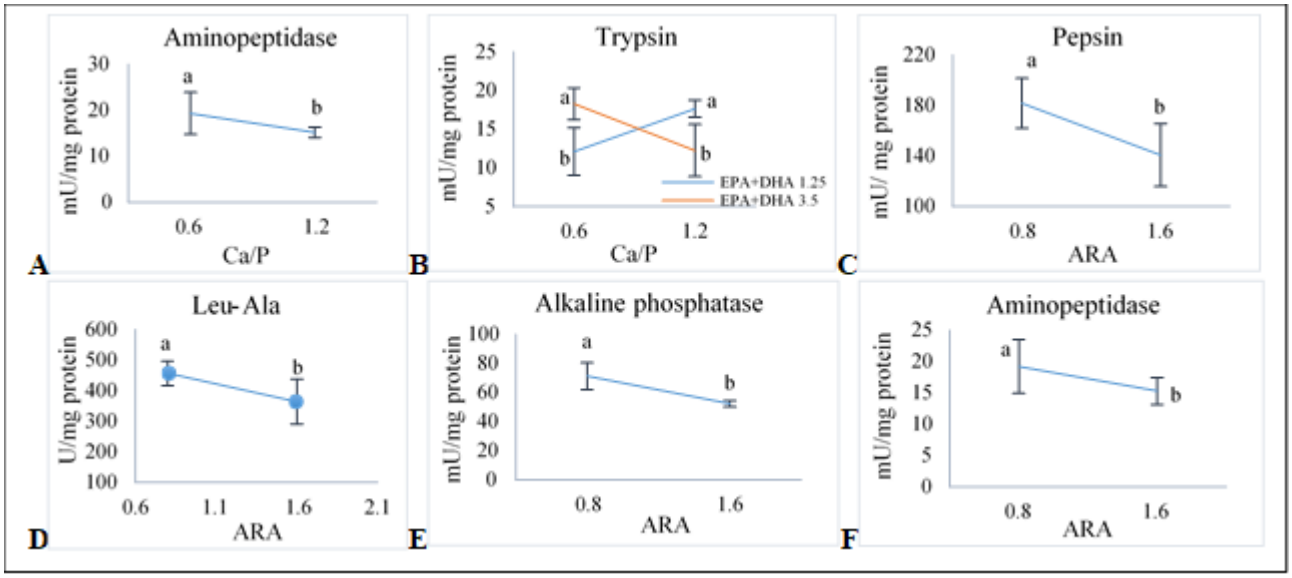

Figure 4: Larval enzymatic activity (aminopeptidase, trypsin, pepsin, leucine alanine and alkaline phosphatase) of 39 dph pikeperch larvae fed the different experimental diets. Only graphs with significant effects are shown. Results are expressed as the Mean ± SD (single effect: n=8; interaction effect: n=4). Different letters denote statistically significant differences between treatments.

Fatty acids larval content

In term of fatty acid body content, DHA, EPA and the total n-3 HUFA were significantly higher in the group of larvae fed the high n-3 HUFA treatment (3.5%) as consequence of the abundance of these fatty acids in diets indicating that the fatty acid levels in the diet affect its content in larval tissues. Previous studies also

showed that the fatty acid composition reflect the composition of fatty acids in the diet (Montero et al., 1996; 2003; 2005, 2008; Izquierdo et al., 2000; 2003, 2005)

On the other hand, the increase in dietary ARA seemed to reduce EPA content in larval tissues as well as EPA/ARA ratio (**Fig. 5, A, B**); this result might indicate a selective deposition and retention of HUFAs in pikeperch larvae, likely due to the inhibition of EPA incorporation by dietary ARA (Bell et al., 1995). In fact, the relation among dietary EPA and ARA has been proposed to be a critical factor for larval performance due to competition interaction among them (Bell and Sargent, 2003, Izquierdo et al., 2005).

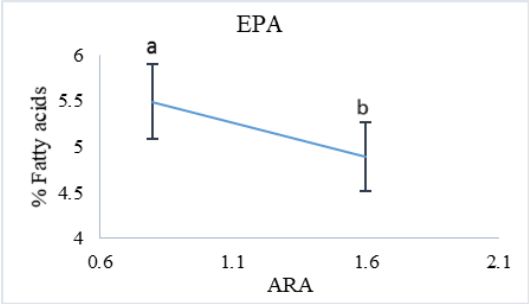

Figure 5: Larval fatty acid content of 39 dph pikeperch larvae fed the different experimental diets. Only graphs with significant effects are shown. Results are expressed as the Mean ± SD (single effect: n=8; interaction effect : n=4). Different letters denote statistically significant differences between treatments.

Molecular study

Gene expression results reflected the decrease of twist expression in larvae fed high EPA+DHA with Se supplement (**Fig. 6, A**). This result reflects the antioxidant role of Se, since the twist gene antagonizes osteoblast formation, and usually induced at post-proliferative stage of osteoblast differentiation.

Mef2c22 was over expressed in larvae fed high vitamin C levels (**Fig.6, B**); this points to the effect of vitamin C on the ossification process, and the collagen synthesis as mentioned above.

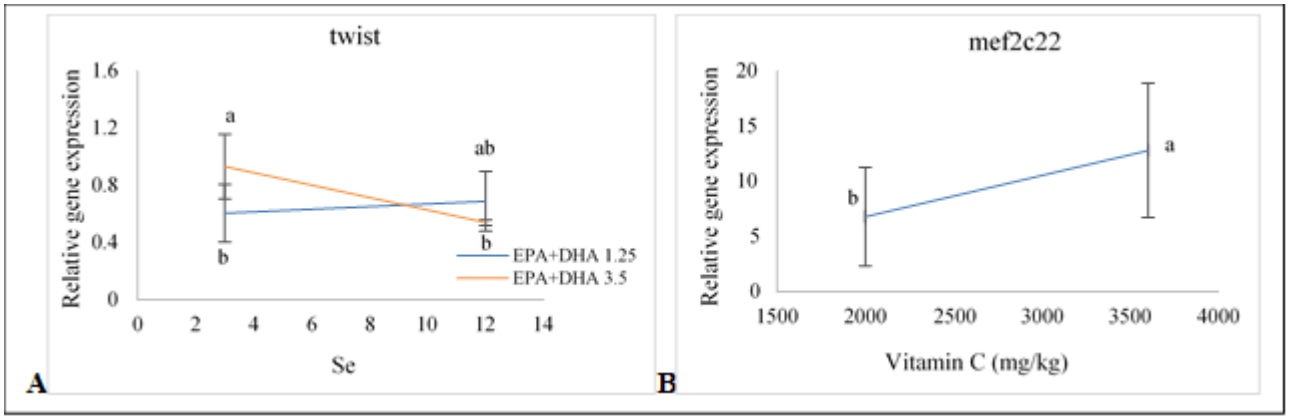

Figure 6: Twist (A) and msf2c2 (B) gene expression measured in larvae fed the 16 experimental diets. Onlygraphs with significant effects are shown. Results are expressed as the Mean ± SD (single effect: n=8; interaction effect: n=4). Different letters denote statistically significant differences between treatments.

Histological study

Histological changes were observed in the anterior intestine samples from 3000 mg kg-1 vitamin E diets group. The changes were characterized by an increased enterocyte vacuolization accompanied by the displacement of nuclei (**Fig. 7,A**). Regarding the liver, high Ca/P resulted in an increase of lipid vacuole accumulation in larvae fed low n-3 LC-PUFA level (**Fig. 7,B**). Larvae fed diets combining low n-3 LC-PUFA and low Ca/P contents displayed very condensed hepatocytes with centered nucleus and marked cytoplasm staining, with a scarce deposition of lipid reserves. On the contrary, the increase in Ca/P did not affect the lipid vacuole deposition in larvae fed higher LC-PUFA levels (**Fig. 7,B**). No other histopathologies were observed in larval tissue.

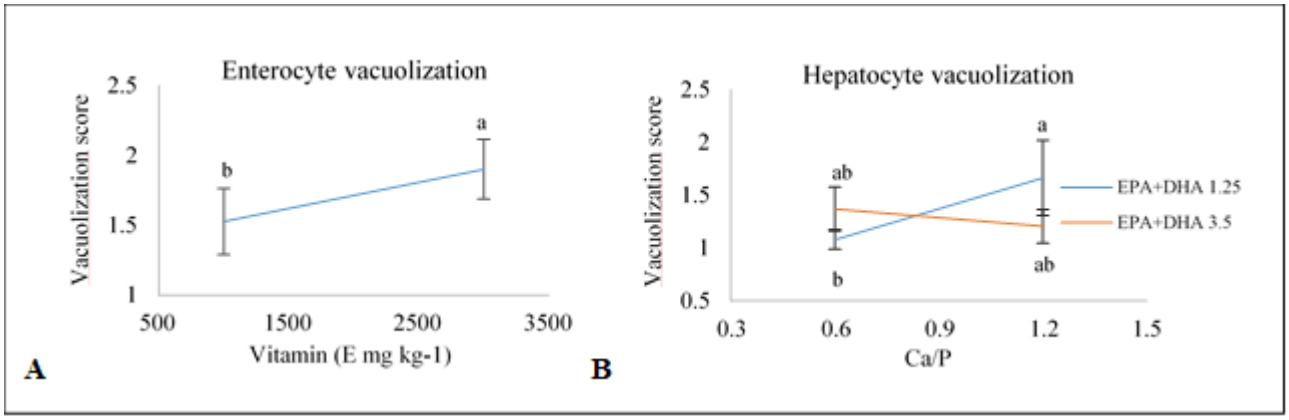

Figure 7: (A) Enterocyte and (B) hepatocyte vacuolization observed in pikeperch larvae fed different diets. Score (1) not observed; (2) mild vacuolization; (3) severe vacuolization. Only graphs with significant effects are shown. Results are expressed as the Mean ± SD (single effect: n=8; interaction effect: n=4). Different letters denote statistically significant differences between treatments.

Conclusion

In light of the results obtained in the multifactorial experiment; Ca/P, fatty acids and their interaction seem to be key nutritional factors influencing pikeperch larval development. However, only two levels of Ca/P and fatty acids were tested in the multifactorial experiment. Therefore two confirmatory experiments testing gradual levels of the dietary EPA+DHA/ARA and Ca/P ratios were performed based on the multifactorial screening results.

3. DHA/EPA/ARA experiment

Based one the multifactorial exp. a confirmatory experiment investigated the combined effect of graded levels ARA with two DHA dietary levels (low and high) in early weaning diets on larval performance, digestive capacity, biochemical composition, oxidative status, skeletal deformities and bone mineralization of pikeperch larvae. The study was conducted at DTU Aqua in Denmark.

Methodology

Larvae and rearing conditions

Larvae were obtained from AQUPRI, Denmark as newly hatched. Larvae were fed on unenriched *Artemia* nauplii (AF and EG strain) (INVE, Dendermond, Belgium) until they reached 14dph, followed by a co-feeding period from 15 to 17 dph using *Artemia* nauplii and mixture of the experimental diets.
At 18 dph larvae were randomly distributed into 18 experimental tanks (50 L) at a density of 1350 larvae tank^{-1} in a flow through system with adjustable light and temperature control
Larvae in each tank were fed with one of six experimental diets tested in triplicates 8 (3 tanks per diet). To assure feed availability, daily feed supplied was maintained at app. 15-20 % of larval wet weight per tank during the first week (200-400 µm/ 400-700 µm) and 10-15 % per tank during the rest of experiment period. Daily feed was administered by automatic feeders from 8 A.M to 6 P.M. and fed approximately every 20-30 min. Feed was fed in surplus and daily bottom of tanks were vacuum cleaned to remove feed waste.
Final survival was calculated at 40 dph by individually counting all the living larvae at the end of the experiment. Growth was determined by measuring body weight of 30 fish tank-1 at the beginning, the middle and at the end of the trial.

Diet composition

Six diets with two levels of DHA (low and high levels) and 3 levels of EPA/ARA (1, 2 and 4) were tested. Diets were formulated by SPAROS, Portugal (**Table 8**).

Table 8. Dietary composition of the experimental diets (calculated values).

As feed	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Crude protein, % feed	54.2	54.2	54.2	54.2	54.2	54.2
Crude fat, % feed	20.2	20.2	20.2	20.2	20.2	20.2
Starch, % feed	9.7	9.7	9.7	9.7	9.7	9.7
Ash, % feed	9.0	9.0	9.0	9.0	9.0	9.0
Total P, % feed	1.67	1.67	1.67	1.62	1.62	1.62
Ca, % feed	1.52	1.52	1.52	1.52	1.52	1.52
Ca/P	0.91	0.91	0.91	0.93	0.93	0.93
LNA (C18:2n-6), % feed	0.53	0.40	0.33	0.50	0.37	0.30
ALA (C18:3n-3), % feed	0.13	0.13	0.13	0.10	0.10	0.10
ARA, % feed	1.20	0.59	0.30	1.19	0.59	0.30
EPA, % feed	1.19	1.19	1.19	1.22	1.22	1.22
DHA, % feed	0.61	0.61	0.61	2.49	2.49	2.49
EPA/ARA	0.99	2.00	3.95	1.02	2.07	4.12
DHA/EPA	0.52	0.52	0.52	2.04	2.05	2.05
Total phospholipids, % feed	7.76	7.76	7.76	6.22	6.22	6.22

Enzymatic assays

The enzymatic analyses were performed as described in M & M for the multifactorial experiment above Enzyme activities are expressed as specific activities (U or mU mg protein^{-1}).

Skeleton anomalies and related gene expression

To determine the presence of skeletal anomalies, 50 larvae per tank were fixed and stored in buffered (10% phosphate) formalin at the end of the experiment. As for the enzymatic analyses the analytical procedures were similar as described in M &M for the multifactorial experiment above.

Results and discussion

Larval FA composition analysed at larval age 40 dph seemed to follow the dietary composition with respect to LC PUFAlevels of DHA, EPA and ARA, **Table 9.**

Table 9. Analysed average main FA composition of larvae at 40 dph, (% TFA± sd) (TFA; mg g^{-1} w.w.± sd.). different letters within a row denote a significant (P<0.05) difference.

Diet	D1	D2	D3	D4	D5	D6
TFA	52.5±3.4	61.1±4.5	53.6±11.9	43.6±5.7	44.2±9.1	53.0±4.5
FA %						
14:0	1.8±0.2	1.7±0.4	1.5±0.1	1.5±0.2	1.7±0.5	1.8±0.4
16:0	23.6±2.1	25.7±2.5	27.2±3.1	26.3±4.3	23.6±3.6	25.1±3.8
18:0	5.6±0.2	5.9±0.5	6.0±0.5	7.1±0.6	6.1±0.8	4.7±0.8
Total SFA	33.4±3.5	35.4±4.2	36.7±4.0	36.3±5.8	34.2±5.7	33.6±5.6
16:1(n-7)	2.4±0.1	2.4±0.3	2.4±0.1	2.0±0.2	2.2±0.2	5.6±0.8
18:1(n-9)	14.0±1.5	20.0±1.5	22.2±1.0	12.8±0.3	20.6±1.2	19.7±2.5
20:1 (n-9)	0.9±0.1	1.0±0.2	1.1±0.1	0.8±0.2	1.0±0.1	0.9±0.3
Total MUFAs	18.8±2.3	25.1±2.6	27.7±1.8	17.4±1.1	25.2±1.7	28.1±3.8
18:2(n-6)	14.4±0.2	14.3±1.0	14.6±1.2	10.7±1.8	12.5±2.3	13.5±2.7
20:4(n-6)	21.5^{a}±0.3	12.4^{b}±0.6	7.4^{c}±0.7	20.8^{a}±1.0	12.8^{b}±1.5	6.9^{c}±0.3
Total(n-6)	37.9^{a}±0.9	28.7^{b}±2.1	24.3^{c}±2.8	33.5^{a}±3.3	27.3^{b}±4.2	22.8^{c}±3.6
18:3(n-3)	0.8±0.0	0.8±0.1	0.8±0.0	0.6±0.1	0.7±0.1	0.7±0.1
20:5(n-3)	7.8^{ab}±0.4	9.3^{bc}±0.7	10.1^{c}±0.7	7.8^{ab}±0.9	8.8^{bc}±0.9	9.9^{c}±0.9
22:6(n-3)	3.2^{a}±1.4	2.9^{a}±1.4	2.8^{a}±0.7	5.3^{b}±0.6	6.7^{b}±1.3	6.9^{b}±1.2
Total(n-3)	12.7±2.1	14.3±2.3	13.7±1.8	15.8±1.9	17.3±2.6	18.5±2.6
DHA/EPA	0.4	0.3	0.3	0.7	0.8	0.7
(n-3)/(n-6)	0.4	0.5	0.6	0.5	0.6	0.8

The growth was similar between the different groups of larvae with no significant differences in growth at 32 dph (**Fig. 8)**, while at the end of the experiment at dph 40 juveniles fed D4, exhibited a lower growth performance compared to the larvae fed D2.
Overall survival at 40 dph was similar with a tendency for a better survival for D4, and there may be some interaction with the growth recorded in these larvae. The actual mortality registered (dead larvae siphoned) showed no differences between groups but was affected by the highly cannibalistic behavior of pikeperch larvae.

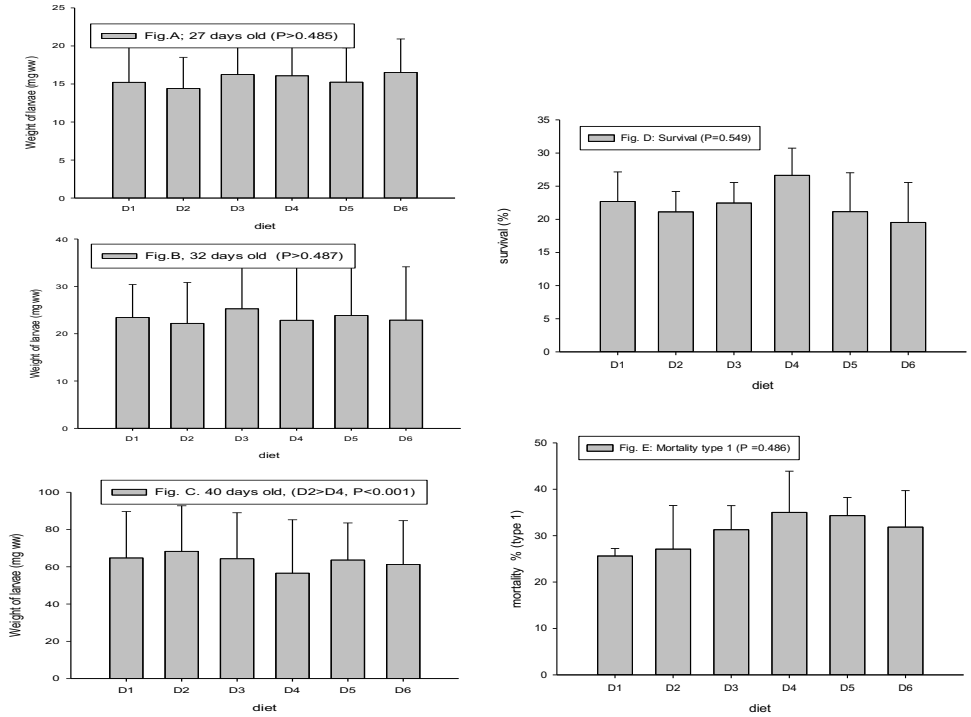

Figure 8: Husbandry response at different time (days of feeding 9, 14, 22) by use of the 6 different experimental diets corresponding to larval age 27, 32, 40 dph. Different letters denote statistically significant differences between treatments.

Pepsin specific activity was influenced by the dietary DHA content, being higher at 22 dph in larvae fed diet 6 than in diets 1, 2 and 3 (**Fig. 9, A**). At 40 dph, the increase in dietary EPA/ARA levels enhanced the trypsin activity in fish fed low DHA level (**Fig. 9, C**). No differences evidenced within alkaline phosphatase and aminopeptidase activities between treatments, however a decrease in AP activity was observed at 32 dph in all groups. In this sense, the higher activity of AP recorded at 22 dph, could be explained by an alteration in the secretion process due to the weaning effect (Cahu and Zambonino, 1994; Hamza et al., 2007).

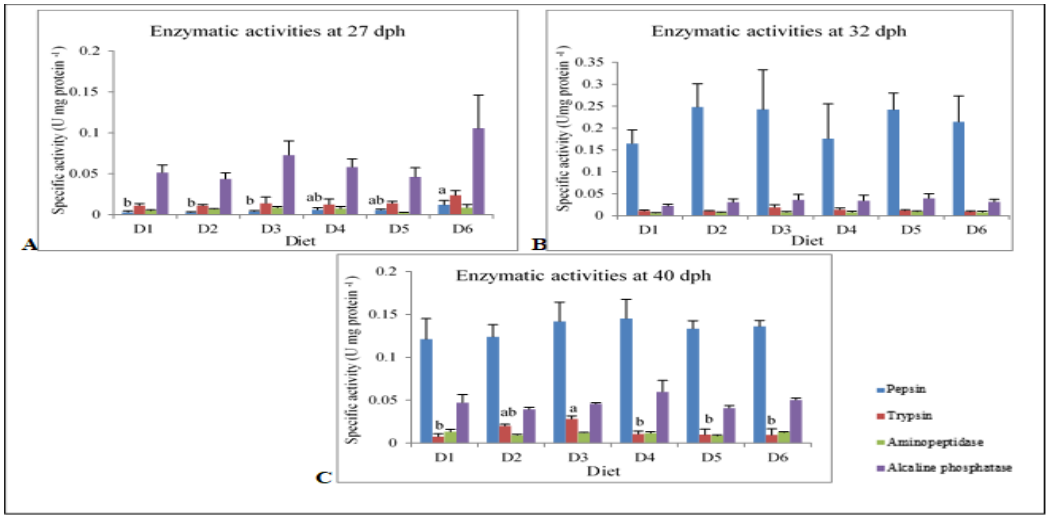

Figure 9: Larval enzymatic activity (aminopeptidase, trypsin, pepsin, and alkaline phosphatase) of pikeperch larvae fed the different experimental diets. Different letters denote statistically significant differences between treatments.

4. Confirmatory Ca/P experiment

Based on the multifactorial results, another feeding experiment was conducted to check the effect of graded levels of Ca/P ratio regardless the Ca and P levels. The experiment investigated the dietary Ca/P effect not only by varying one of the two minerals, but also varying both.

Experimental diet composition

Six diets with three Ca/P levels (0.3, 0.6 and 1.2) were tested (**Table 10**). Triplicate groups of 21-dph pikeperch larvae were fed the different experimental diets during two weeks.

Table 10. Proximate composition of experimental diets (%).

As fed basis	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Crude protein	51.16	51.15	51.14	51.14	51.16	51.17
Crude fat	18.46	18.46	18.46	18.46	18.46	18.46
Fiber	0.16	0.16	0.16	0.16	0.16	0.16
Starch	9.97	8.02	4.20	4.21	11.48	15.17
Ash	9.04	10.96	14.72	12.95	8.46	6.18
Total P	**2.68**	**2.68**	**2.68**	**3.97**	**2.01**	**1.01**
Ca	**0.80**	**1.61**	**3.21**	**1.20**	**1.20**	**1.20**
Ca/P	**0.30**	**0.60**	**1.20**	**0.30**	**0.60**	**1.19**

Methodology

Final survival was calculated by individually counting all the living larvae at the end of the experiment. Survival was calculated as the percentage between final and initial number of fish. Growth was monitored by sampling 30 larvae per tank at the beginning and the end of the experiment. At the end of the experiment the weighed larvae were fixed in 10% buffered formalin and kept until analysis to determine the rate of malformations. The number of sampled larvae was taken into account for survival calculation.
Assays of digestive enzymatic activities and skeletal deformity evaluation were conducted according to the literature as described above.

Results and discussion

Low growth was observed in larvae fed diet 6, containing the highest Ca/P ratio and the lowest P % (**Fig. 10**). Meanwhile, no significant differences were observed in digestive enzymatic activities (**Table 11**). In this regard, the need for a low Ca/P ratio in pikeperch larval diets has been suggested (Kestemont et al., 2007).

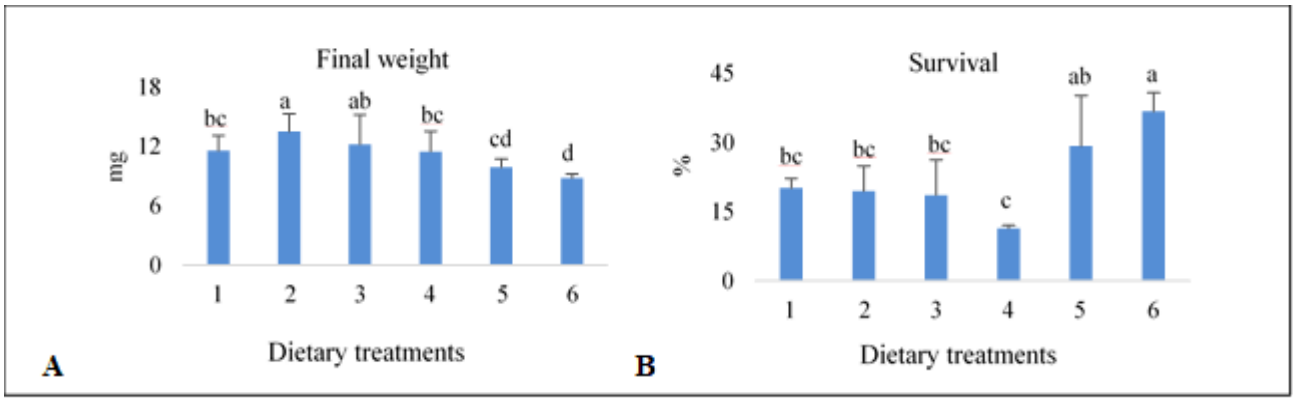

Figure 10: Effect of dietary Ca/P on husbandry variables, (A) final weight, (B) survival. Different lettersdenote significant differences ($p<0.05$)

Table 11: Larval specific enzymatic activities (mU/mg protein). Different letters within a row denote a significant ($P<0.05$) difference

mU/mg protein	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Trypsin	16.8±3.8	16.1±3.8	17.6±2.7	19.5±2.0	15.6±3.8	16.6±0.3
Alkaline phosphatase	48.9±8.1	48.4±10	58.5±27.1	57.1±6.7	48.8±22.4	46.6±18.5
Aminopeptidase	4.7±0.0	5.4±0.7	9.6±2.7	9.6±2.7	11.3±4.6	9.3±4.8

Regardless Ca/P dietary levels, growth and larval survival were significantly affected by the P dietary content (**Fig. 11**), suggesting the importance of a balanced Ca/P and P diet for pikeperch larvae.

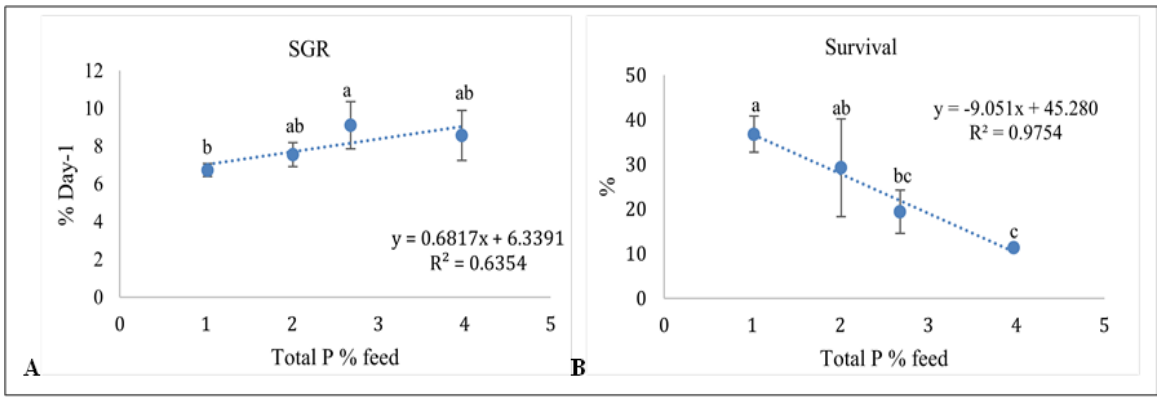

Figure 11: P effect on husbandry variables, (A) larval growth, (B) survival. Different letters denote a statistically significant difference between treatments.

Conclusion

The study highlights the importance of Ca/P ratio as one of the major nutrient factors of interest during the early larval development in pikeperch. In addition, it appears that total P % content should be considered in determining the optimal Ca/P level. Further analyses are still needed to achieve a better understanding of the results obtained, such as the effect of graded levels of Ca/P as well as dietary P content on skeletal deformities.

5. Requirements of phospholipids and n-3 LC PUFAs in pikeperch larvae

The optimal requirements for phospholipids have so far not been determined, thus two experiments were carried out The main objectives of the first study (I) was to examine optimal levels of soy bean lecithin (SBL) derived PL in formulated extruded starter feeds for pikeperch larvae and determine additional effects of inclusion of TAG n-3 LC PUFAs on larval performance and larval development. A second supplementary experiment (II) was additionally carried out to investigate the dietary effect on juvenile physiology and stress response.

Materials and methods

Origin and treatment of pike perch larvae

Fertilized eggs of pikeperch were obtained from Aquapri Innovation, Egtved, Denmark and transferred in oxygenated plastic bags to DTU Aqua at the North Sea Research Centre, Denmark. Eggs were incubated in upflow McDonald type incubators until hatching by a flow through system at 17.5°C with use of heated freshwater from a temperature controlled 10 m^3 reservoir. Larvae were distributed into two 0.5 m^3 fibre glass tanks upon hatching and kept in these tanks at 19.5-20.3 °C until 10 DPH at a constant low light intensity of approximately 30 lux. From 3- 10 DPH larvae were fed newly hatched unenriched *Artemia* nauplii (strain MC460, *Artemia* Systems, kept at 5° C after hatching) for 2 x 8 hours through a peristaltic pump (Longer pumps BT300-2J) to secure that live *Artemia* nauplii were always present ad libitum in the tanks. At 10 DPH all larvae were mixed and moved to another temperature controlled freshwater flow- through larval system consisting of 18 conical tanks of 46 L each. Each tank was initially stocked with 17 larvae/L and larvae were counted into each tank and reared at 20-20.5° C until 30 DPH. The experiment was carried out with 6 dry feed diets in a triplicate set up with 3 tanks per feed type (total of 18 tanks). Oxygen saturation and temperature were monitored daily by a hand-held Oxyguard meter from Oxyguard, Birkerød, Denmark. During the experiment oxygen was (7.8 ±0.4 mg/L^{-1}) and temperature (20.2±0.1 °C). The flow rate was kept at 15 L/h/ tank and checked daily. Constant dimmed light (i.e. 30-40 lux at water surface) was provided by electrical led bulbs installed over each tank.

Preparation of feed and feeding procedure

Six diets were formulated to be isonitrogenous and isoenergetic (**Table 12**). The six diets contained 3 levels of PL (PL1, PL2, PL3), while three of the diets were further supplemented by 3 levels of DHA (PL1H1, PL2H2, PL3H3). Soybean lecithin powder was used as PL source and Algatrium DHA70 as supplemented DHA source (+EPA) (**Table 12**), these two sources gradually replaced olive oil in the diets. Experimental diets were manufactured by SPAROS (Olhão, Portugal). All powder ingredients were initially mixed and grinded in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Subsequently, the oils were added, diets humidified with 25% deionized water and agglomerated by a low-shear and temperature controlled extrusion process (model P55, Italplast, Italy). The resulting pellets (0.8 mm) were dried in a convection oven for 3 h at 40ºC (model OP 750-UF, LTE Scientifics, United Kingdom), crumbled (model 103, Neuero Farm, Germany) and mechanically sieved (model Eco-Separator, Russell-Finex. United Kingdom) to particle size ranges (400-600 µm and 800 µm). From 10 to 17 dph triplicate groups of larvae were co-fed one of 6 experimental dry feed together with newly unenriched hatched EG *Artemia* nauplii (*Artemia* Systems) from 8 a.m-22 p.m. During this period *Artemia* feeding was daily successively postponed by 1 hour. During dph 10-17 larvae were fed 400-600 µm (initially further grinded in a mortar to 200-250 µm) and this was gradually exchanged with 600-800 µm during the second week. A mixture of these two

size ranges was applied for the remaining of the experiment until 30 dph. Dry feed was daily administered by 18 programmable automatic screw-twin feeders (designed for the purpose), which allowed very small quantities to be fed at short specific time intervals (app. 15 minutes) during the entire feeding period. Dry feeds were administered in equal amounts and in surplus daily for all tanks; estimated to approximately 25% of expected larval biomass in the first weeks; decreasing to 10-15 % during the last week. Daily the bottom of each tank was vacuum cleaned for feed remaining; dead *Artemia* and dead larvae and collected in a bucket. The numbers of dead larvae per tank day^{-1} were subsequently registered.

Table 12 Dietary composition and analytical content of the 6 experimental diets.

Diet Ingredients (%)	PL1	PL2	PL3	PL1H1	PL2H2	PL3H3
MicroNorse Fish Meal[a]	45	45	45	45	45	45
CPSP 90[b]	7	7	7	7	7	7
Squid meal[c]	13	13	13	13	13	13
Fish gelatin[d]	1	1	1	1	1	1
Wheat Gluten[e]	4.4	4.4	4.4	4.4	4.4	4.4
Wheat meal[f]	6.1	5.9	5.6	6.1	5.9	5.6
Algatrium DHA70[g]	0.0	0.0	0.0	0.55	2.0	3.4
Olive oil[h]	18.9	12.1	3.4	18.35	10.1	0.0
Vitamin & Mineral Premix PV01[i]	1.0	1.0	1.0	1.0	1.0	1.0
Soy lecithin powder[j]	3.0	10.0	19.0	3.0	10.0	19.0
Binder (guar gum)[k]	0.2	0.2	0.2	0.2	0.2	0.2
Antioxidant powder (Paramega)[l]	0.2	0.2	0.2	0.2	0.2	0.2
Antioxidant liquid (Naturox)[m]	0.2	0.2	0.2	0.2	0.2	0.2
Analysed content (% WW)						
Crude protein	54.1	54.7	55.6	54.1	55.8	55.3
Crude lipid	26.8	25.9	24.6	26.6	25.6	24.8
NFE + fibre (substracted)	3.0	3.0	2.8	2.8	3.1	3.2
Dry matter (DM)	93.0	93.0	93.1	93.6	92.8	93.5
Ash	9.1	9.4	10.0	9.0	9.3	10.2
Phosphorus	1.30	1.27	1.31	1.28	1.29	1.30
Calcium	1.84	1.85	1.85	1.83	1.84	1.85
(% of protein)						
Lysine	4.20	4.22	4.19	4.16	4.21	4.17
Methionine + Cysteine	1.90	1.93	1.90	1.91	1.87	1.89
Taurine	0.52	0.50	0.51	0.52	0.52	0.51
(% ww)						
Phosphatidylcholine (PC)	1.40	2.61	4.31	1.42	2.68	4.29
Phosphatidylethanolamine (PE)	0.43	1.22	2.20	0.40	1.14	1.87
Phosphatidylinositol (PI)	0.44	1.28	2.44	0.43	1.28	2.48
Total phospholipids(TPL)	3.73	8.19	14.38	3.70	8.32	14.51

[a] MicroNorse Fish Meal[a]: 71% crude protein. 9.8% crude fat. Tromsø Fiskeindustri AS. Norway. [b]Soluble fish-protein concentrate (CPSP 90): 84% CP and 12% fat (Sopropêche. Boulogne-Sur-Mer. France). [c] Squid meal: Super prime squid meal: 80% crude protein. 3.5% crude fat. Sopropêche. France. [d] Fish gelatin: 88% crude protein. 0.1% crude fat. LAPI Gelatine SPA. Italy. [e] Wheat gluten: VITAL 83.7% crude protein. 1.4% crude fat. ROQUETTE Frères. France. [f] Wheat meal: 11.7% crude protein. 1.6% crude fat. Casa Lanchinha. Portugal. [g] Algatrium DHA70: Brudy Technologies. Spain. [h] Olive oil: Henry Lamotte Oils GmbH. Germany. [I] PVO40.01 premix for marine fish (Premix Lda. Viana do Castelo, Portugal). Vitamins (per kg diet): 100 mg DL-alpha tocopherol acetate. 25 mg sodium menadione bisulfate. 20.000 IU retinyl acetate. 2.000 IU DL-cholecalciferol. 30 mg thiamin. 30 mg riboflavin. 20 mg pyridoxine. 0.1 mg B_{12}. 200 mg nicotinic acid. 15 mg folic acid. 1.000 mg ascorbic acid. 500 mg inositol. 3 mg biotin. 100 mg calcium

panthotenate. 1.000 mg choline chloride. and mg betaine. 500. Minerals (per kg diet): 2.5 mg cobalt sulfate. 1.1 mg copper sulfate.

Samplings

Growth, phospholipid and FA composition, digestive enzymatic activity, radiographic analysis and gene expression

A representative number of larvae (2 x 100) were sampled at hatching, (0 dph) and at 10 dph for FA analysis and 50 larvae sampled for initial weight measurement. At 30 dph likewise 2 x 10 larvae per tank were sampled for phospholipid and FA analysis and additionally 35 larvae per tank for growth measurements. At 1 and 10 dph a total of 200 larvae and 90 larvae respectively were sampled for analysis of digestive enzymes as well as 10 larvae per replicate at 30 dph. In addition 2 x 10 larvae per replicate were sampled for proteomics at 30 dph. All these larvae were killed by an overdose of benzocaine and treated as above and immediately frozen at – 80 °C prior to examination. At 30 dph additionally 10 larvae per tank were sampled for larval organ and skeleton development for specific staining of bone and cartilage and were posterior to sedation fixed and stored in 10 % phosphate buffered formaldehyde until analysis. In order to quantify the gene expression involved in skeletal development 10 larvae per replicate were sampled, sedated and stored in RNA-later overnight and frozen at -80 °C until analysis.

Analysis and proximate composition of feeds

Representative samples of the six diets were homogenized using a Krups Speedy Pro homogenizer 244 and analyzed for dry matter (DM) and ash (NMKL, 1991), crude protein 245 (ISO, 2005; crude protein; Kjeldahl N×6.25), and crude lipid (Bligh and Dyer, 1959). Nitrogen-free extract (NFE) was calculated as DM less the sum of crude protein, crude lipid, and ash. The proximate composition of the diets was similar in terms of protein, lipid, NFE and ash content and with almost identical values of the essential amino acids; lysine, methionine and cysteine and the sulfur- containing non-protein amino acid taurine (**Table 12**).

Fatty acid composition in diets and pikeperch larvae

The fatty acid composition in diets and larvae was determined by extraction of the lipids by a chloroform/methanol mixture, (Folch et al., 1957). The method has been described above in the previous referred experiments.

A total of 34 fatty acids were analyzed. For the dietary composition only the 22 most abundant and relevant FAs are presented (**Table 13**). The main difference in dietary composition of FA (% TFA) (18:1 n-9) ; (18:2n-6); (18:3n-3); (EPA); (DHA), and consequently total MUFAs, total n-6 PUFAs and total n-3 PUFAs The 18:2n-6 and 18:3n-3 content increased by the increase in supplementation of SBL; for which 18:2n-6 is the dominant FA (i.e. 55 % TFA, data not shown) with the additional content of some 18:3n-3 α- linolenic acid (3% TFA). Diets PL1H1-PL3H3 contained significantly increased levels of EPA and DHA (as compared with diets (PL1-PL3), as due to the supplement (0.6% - 3.4 %) of Algatrium DHA 70.

Table 13. Mean analysed TFA content (mg g^{-1} d.w.± SD) and FA composition (% of TFA) of the 6 experimental diets formulated to have several PL and DHA levels, (n=2)

Diet	PL1	PL2	PL3	PL1H1	PL2H2	PL3H3
TFA	109.4	54.3	34.1	187.8	69.7	46.9
FA						
14:0	0.7±0.0	0.8±0.0	1.0±0.00	0.7±0.0	0.79±0.0	1.0±0.0
16:0	13.0±0.2	14.6±0.1	17.8±0.01	12.9±0.2	13.8±0.0	15.2±0.1
18:0	2.6±0.0	2.7±0.0	2.9±0.0	2.5±0.0	2.4±0.0	2.3±0.0

20:0	0.3±0.0	0.2±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.10±0.0
22:0	0.1±0.0	0.2±0.0	0.3±0.0	0.1±0.0	0.1±0.0	0.2±0.0
24:0	0.1±0.0	0.1±0.0	0.2±0.0	0.1±0.0	0.1±0.0	0.1±0.0
Total SFA	17.0±0.3	18.9±0.1	22.6±0.1	16.8±0.1	17.7±0.0	19.3±0.1
16:1(n-7)	1.5±0.0	1.4±0.0	1.4±0.0	1.5±0.0	1.4±0.0	1.3±0.0
18:1(n-9)	62.1±0.2	50.0±0.1	27.0±0.0	60.1±0.4	42.6±0.1	12.4±0.0
20:1 (n-9)	1.6±0.0	1.7±0.0	2.0±0.0	1.5±0.0	1.7±0.0	1.9±0.0
22:1(n-9)	0.2±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.2±0.0	0.2±0.0
24:1(n-9)	0.9±0.0	1.1±0.0	1.3±0.0	1.3±0.1	2.6±0.0	4.5±0.0
Total MUFAs	66.4±0.2	54.5±0.1	32.0±0.0	65.5±0.4	48.5±0.0	20.4±0.0
18:2(n-6)	10.1±0.0	18.6±0.1	34.5±0.0	9.6±0.2	17.5±0.1	31.9±0.1
18:3(n-6)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
20:2(n-6)	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
20:3(n-6)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
20:4(n-6)	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.3±0.0
Total(n-6)	10.4±0.0	18.9±0.1	34.9±0.0	9.9±0.2	17.9±0.0	32.4±0.1
18:3(n-3)	1.1±0.0	1.7±0.0	3.1±0.0	1.0±0.0	1.6±0.0	2.8±0.0
20:3(n-3)	0.0±0.0	0.0±0.0	0.1±0.0	0.0±0.0	0.0±0.0	0.1±0.0
20:5(n-3)	1.5±0.0	1.8±0.0	2.3±0.0	1.6±0.1	2.5±0.1	3.6±0.0
22:6(n-3)	3.6±0.1	4.2±0.0	5.1±0.0	5.2±0.2	11.7±0.0	21.4±0.2
Total(n-3)	6.2±0.1	7.7±0.0	10.5±0.0	7.9±0.3	15.9±0.0	27.9±0.1
DHA/EPA	2.4±0.1	2.3±0.0	2.2±0.1	3.3±0.0	4.8±0.2	5.9±0.1
(n-3)/(n-6)	0.6±0.0	0.4±0.0	0.3±0.0	0.8±0.0	0.9±0.0	0.9±0.0

Phospholipid NMR analysis of feed and pikeperch larvae

Quantification of phospholipids in diets and larvae were done by Spectral Service AG, Germany by use of ^{31}P-NMR spectroscopy using an internal standard according to method previously described (Menses and Glonek, 1988). Preweighed homogenous subsamples of feed or larvae (30 DPH) were used and prior to analysis larvae for each replicate were freeze dried for 24 h on a Adolf Kühner AG Beta 2-16. NMR analyses were carried out on a Bruker DRX 600 spectrometer (Bruker Biospin GmBH, Germany), resonating at 600.13 MHz for ^{1}H and 150.90 for ^{13}C, using a 5 mm BBO probe at 297 K. For the quantification of phospholipids in the samples a defined amount of the test substance and coresponding internal standard (exactly weighed) were dissolved in CDCL3, methanol and aqueous CS-EDTA solution (0.2 m, pH 7.5). After 30 min shaking the organic layer was separated by centrifugation and measured with 31 P-NMR (ref). The integrated signals of the test substance and of the internal standard TPP (triphenylphosphate) were used for calculation.

Enzyme assays

The heads and tails of pikeperch larvae were removed to isolate the digestive segment on a glass maintained on ice (0 °C), and then stomach region was separated with the intestinal segments. Please refer to the previous description in this Deliverable for the analytical procedure of enzymatic analyses.

Skeleton anomalies and gene expression

Skeleton analyses were conducted on fixed 30 dph pikeperch larvae. These were stained with alizarin red to evaluate the skeletal anomalies and vertebral mineralization following methods modified from previous studies. Classification of skeletal anomalies was conducted according to Boglione et al. (2001). Anomalies were expressed as frequency of total severe anomalies and specific anomalies, such as cranial, dentary, branchiostegal, prehaemal and caudal vertebrae, within each dietary group. The effects of the different weaning diets on the axial skeleton mineralization were evaluated considering the total number of completely mineralized vertebral bodies within a larval size class.

Total RNA from larvae samples (average weight per sample 60mg) was extracted using the Rneasy Mini Kit (Qiagen), the method has been previously described. Pikeperch specific gene primers were designed after searching the NCBI nucleotide database and using the Oligo 7 Primer Analysis software (Molecular Biology Insights, Cascade, CO, USA). Within the oligo used, RAG1, ALP, Twist2, Mef2c and Sox9 oligonucleotide primers were designed by extracting fish mRNA sequences from NCBI, aligning with MEGA 7 software and identifying conserved domains, to which primers were designed using Primer3 (v. 0.4.0) program. Target specificity was tested by sequencing the resulting amplicons and comparing to the NCBI sequence database using BLAST

Proteomics

Ten larvae per tank were collected at the end of the experiment and immediately stored at -80 °C. Later, larvae were dissected on a glass maintained at 0°C and the whole liver was extracted and immediately frozen in liquid nitrogen and kept at -80°C until analysis.

Proteins were extracted in 1:10 w:v DLA buffer (7 M urea, 2M thiourea, 4% CHAPS, 30 mM Tris/HCl, pH8.5), and cell were lysed by 2 x 10 s sonication on ice. The soluble protein fractions were harvested by centrifugation at 12,000 x g for 15 min at 4°C. The pH of the protein extract was adjusted to 8.5 by addition of the appropriate volume of 50mM NaOH and protein concentration was evaluated using the Pierce 660 nm protein assay kit as described by the manufacturer. Minimally labelling of samples (containing 25 µg of solubilised proteins) was performed on ice for 30 min in the dark using three cyanine dyes (Cy2, Cy3, Cy5), and quenched with 1 mM lysine for 10 min. Cy2 was used as the internal standard and was composed of equal amounts of proteins from each sample while Cy3 and Cy5 were used to individually label the samples. The three labelled mixtures were combined and the total proteins were added v:v to reduce buffer for 15 min at room temperature, in the dark. These mixtures were then cup-loaded on immobilized pH gradient strips (24 cm, pH 4–7; GE Healthcare) passively rehydrated overnight with a rehydration buffer. The isoelectric focusing was performed with an Ettan™ IPGphor II isoelectric focusing unit (GE Healthcare) at 20 °C for a total of 68,000 Vh. IPGs strips were then reduced (1% DTT) and alkalized (2.5% iodoacetamide) in equilibration buffer just before being loaded onto a 10%, 24 cm, 1 mm thick acrylamide gel. The strips were run in an Ettan™ DALTsix electrophoresis unit (GE Healthcare) at constant 0.5 W/gel. The gels were then scanned with a Typhoon 9400 scanner (GE Healthcare) at wavelengths specific for the CyDyes (488 nm for Cy2, 532 nm for Cy3, and 633 nm for Cy5). Image analysis, with a resolution of 100 µm, was performed using the DeCyder BVA 5.0 software (GE Healthcare). Data were analysed using one-way analysis of variance (ANOVA) followed by the Tukey test (using the Statistica 5.5 software, StatSoft, Inc. 2000) when significant differences between groups were found (pb 0.05). For peptide sequencing and protein identification, preparative gels including 150 µg of proteins of mixed samples were performed (pH 4–7 IPG strips, 10% acrylamide). Gels were post-stained with 10% krypton overnight after twice 30 min of fixation in 40% ethanol, 10% acetic acid. The proteolytic digestion was performed on excised spots by the addition of 3 µL of modified trypsin (Promega, Leiden, Netherlands) suspended in 100 mM NH4HCO3 cold buffer. Proteolysis was performed overnight at 37 °C. The supernatants were collected and kept at −20 °C prior to analysis. Peptides were analysed by using nano-LC-ESI–MS/MS maXis Impact UHR-TOF (Bruker, Bremen, Germany) coupled with a nanoLC UltiMate 3000 (ThermoFisher). Scaffold (version Scaffold-4.6.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. All MS/MS samples were analysed using Mascot (Matrix Science, London, UK; version 2.4) and X!Tandem (The GPM, thegpm.org). Peptide and protein identifications were accepted if they could be established at greater than 95 and 99% probability respectively, as specified by the Peptide Prophet algorithm (Keller et al. 2002) and the Protein Prophet algorithm (Nesvizhskii et al. 2003), respectively. Proteins were functionally annotated using AgBase-Goanna (McCarthy et al. 2006) as described in Roland et al. (2013) The GO categories for biological processes were then sorted to determine the most common ones represented by the proteins identified. Statistical analyses were performed using the Statistica 5.5 software (StatSoft, Inc. 2000), the differences in the abundance of the spots between groups were analysed using one-way analysis of variance (ANOVA 1) followed by a Tukey test with 5% probability significant level.

Juvenile physiology and welfare indicators (II)

An additionally experiment (II) with use of the same diets as described were performed to investigate dietary effects on pike perch juvenile physiology and welfare indicators. This involved blood cortisol, ratio of brain serotonin. The various dietary nutritional profiles were linked to tolerance of sub lethal physiological stressors of larvae like hypoxia using an approach where oxygen is measured in post larvae following a stressful event, which provide insight into metabolic costs on recovering from different stressors based on dietary treatment. This allows for assessing different severities or durations of repetition of a stress event . Neural development was measured using visual and mechano sensory acuity during avoidance responses.

Methodology

Another batch of larvae was used for this experiment (II). These larvae were obtained by AquaPri at 21 dph and fed with unenriched *Artemia* and start weaned to a commercial dry feed from dph 15 and co-fed with enriched *Artemia.* From dph21 until 80 dph they were fed one of the 6 exp. diets as described above. Tanks and experimental set up was similar as in the previous PL experiment, but tanks stocked with app. 450 larvae tank $^{-1}$. From dph 30 to 80 juveniles pellet size given was 800 µm. Temp was gradually increased to 19-20 C. Welfare studies were carried out from dph 80.

Fast escape response

The fast escape performance was conducted in a white semi-translucent polyethylene circular tank with a diameter of 38 cm and a water depth of 5 cm, (slightly modified from Marras et al., 2011). The setup is placed on a light table illuminated by adjustable led light from below. Juveniles were transferred to the tank without air exposure and allowed to acclimatise to the tank for a period of 1 hour. The experimental setup is covered in black opaque plastic to prevent visual disturbance of the fish. Video recordings are made at a rate of 250 fps using a Casio high-speed camera (EX-FH100) mounted 80 cm above the water surface. The setup was illuminated from below using a 28W fluorescent light.
The escape response is triggered by mechanical stimulation by releasing an iron rod (ø 10mm, l 15mm) manually from a height of 90 cm above the water surface. To avoid visually stimulating the test subject, the iron rod fall inside a vertical PVC pipe suspended approximately 1 cm above the water surface. Fast/escape was determined for individual fish and only once per fish. Fish were subsequently anaesthetised and measured for standard and total length to the nearest half mm, blotted dry and weighed to the nearest mg or g. Escape responses was analysed using Tracker (v. 4.72, www.cabrillo.edu/~dbrown/tracker).

Metabolic rate and oxygen tolerance

Post stress oxygen consumption: standard metabolic rate was determined for 12 individuals from each dietary treatment. Individuals were subsequently subjected to a standardised stress (e.g. air exposure in net or manual chasing for 3 minutes), returned to metabolic chambers to determine oxygen debt from stress, peak oxygen consumption and time to recovery. Subsequently, hypoxia tolerance was established; PO2 was gradually brought down by bubbling with N2. The oxygen tension below which fish could no longer uphold their MO2 is determined. The chambers used for oxygen were of 6.4 ml. Tests were done by using Intermittent-flow respirometry: A recirculating pump ensures proper mixing of the water inside the respirometer and adequate flow past the oxygen probe.
A second pump swaps the water inside the respirometer with water from an ambient tank (temperature bath). During measurements of oxygen consumption, the flush pump was turned off and the systems operated like a closed respirometry setup.
Fish respiration rate was calculated from the decline in oxygen. During this time the recirculation pump was kept active to mix the water inside the respirometer and to ensure proper flow past the oxygen sensor. The measuring period was followed by a flush period, where the flush pump was actively pumping water

from the ambient temperature bath and into the respirometer and the oxygen curve will raise to approach the level of the ambient water.
Finally, the flush pump stops and the loop ends with a short Wait period before starting a new measuring period. This waiting period is necessary to account for a lag in the system response resulting in a non-linear oxygen curve. During the wait period the recirculation pump was active. SMR: The metabolic rate of a resting post-absorptive animal; MMR: Maximum (aerobic) metabolic rate (MMR) is defined here as the maximum rate of oxygen consumption (MO2max) that a fish can achieve. Aerobic scope: AS; the difference between an individual's maximum and SMR. Critical O2 tension was the oxygen level in which the juvenile could not maintain equilibrium.

Chasing stress

Juveniles were acutely exposed to a chasing stressor with a small net for 2 min. Fish (10/tank, n=30) were sampled at 0 (controls), 1h and 4h after the stressor. A battery of stress markers (cortisol, glucose, lactate, brain serotonergic activity) were then evaluated to obtain a stress response profile for each diet group.

Statistics

Percent data were arcsine transformed prior to analysis. Experimental data were subjected to a one-way or two-way analysis of variance (ANOVA) and all pairwise multiple comparison of means test for determining significance of differences among the four treatment groups where applicable. Linear regression was used to determine correlation of dietary FA; phosphoglyceride content and larval content as well as enzymatic activity. For larval survival, final weight, growth, FA, PL composition and enzymatic activity, the SigmaPlot 13.0 software was used and Holm Sidak test applied. The statistical comparison for plasma and brain stress markers in the acute stress challenge was performed with two-way ANOVA, with emergence fraction and exposure time as factors. For skeletal anomalies and related genes expression, the IBM SPSS Statistics 21 software was used and Tukey's test applied. For proteomics data, peptide sequencing and protein identification, the Statistica 5.5 software (StatSoft, Inc. 2000) was used and Tukey's test applied. Levene's test was used to check for homogeneity of variance within the treatment groups. Values throughout the text are expressed as the mean ± standard deviation. In all statistical tests used, $P<0.05$ was considered statistically different. All analyses were based on larval samples obtained from triplicate groups as described previously.

Results

Survival and growth (I)

Daily mortality assessed by the number of registered dead larvae increased from 15 dph showing a similar pattern between tanks and dietary codes. The registered mortality at the end of the study at 30 dph indicated a survival in the order 57 %- to 61% of start individual numbers (10 dph; subtracted larvae for sampling) and was not significantly different between dietary treatments ($P \geq 0.553$). Based on the remaining number of larvae at the end of the trial (30 dph), actual survival was much lower, i.e. 10-14%, however, not statistically different ($P \geq 0.633$).
Mean larval weight at 10 dph was 1.95 ± 0.31 (mg w.w.); (0.56±0.09 (mg d.w.) and ranged from 92-154 (mg ww) at 30 dph (**Fig. 12**). The figure illustrates an increase in final weight by an increase in dietary inclusion of phospholipids and a further increase by an additional supplementation of DHA (+EPA) in the form of Algatrium DHA 70. Larval weights were statistically significantly different. Larvae fed PL3H3 were significantly larger than larvae fed PL1 ($P<0.001$); PL2 ($P\leq0.01$) and PL1H1 ($P<0.001$). PL2H2 larvae were larger than larvae fed on PL1 ($P<0.03$) and PL1H1 ($P\leq0.001$). PL2 and PL 3 larvae were both larger than larvae on diet PL1H1, ($P\leq0.04$; $P\leq0.01$), respectively. Overall larval growth calculated as mean specific daily growth rate SGR, (% day^{-1}) (ln (final average body weight) – ln (initial average body weight)) x 100/feeding days was lowest for PL1H1 (21.2 % day^{-1}) and highest for PL3H3 (24.3 % day^{-1}) (**Fig. 12**).

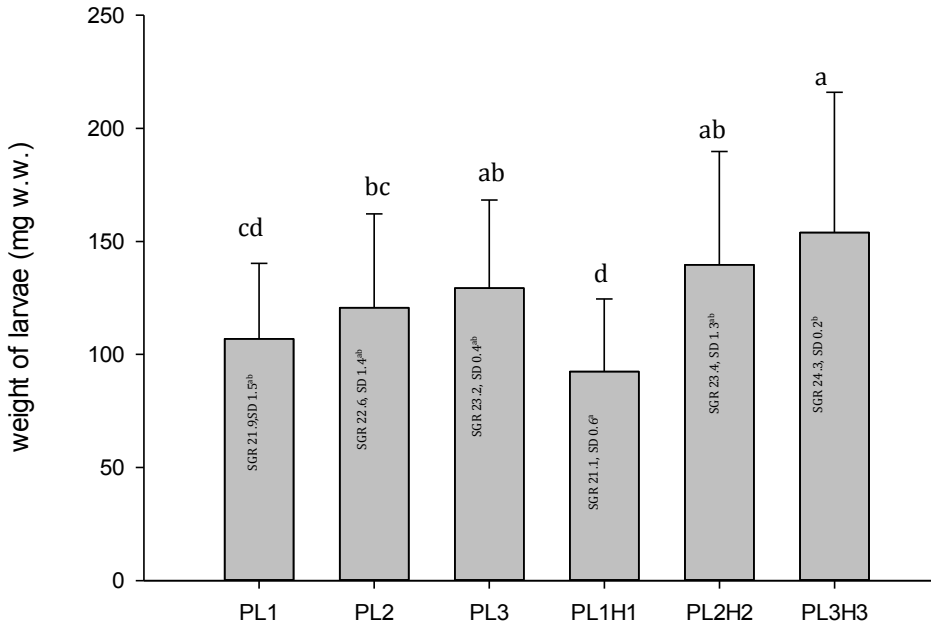

Figure 12. Mean larval weight (mg ww) (±SEM) at 30 dph and calculated SGR (±SEM (on each bar). Different letters denote statistically significant differences between treatments.

Dietary / Larval lipid class and fatty acid tissue composition

The dietary analytical content of the three main phospholipid classes identified increased as expected by dietary incorporation of soy lecithin (**Table 12**). Total phosphoglyceride content ranged from 3.7 % (% of lipids) for PL1 and PL1H1 to approximately 14.5 % for diets PL3 and PL3H3.

Phosphatidylcholine was the major phospholipid class in larval tissue (**Fig. 13**) and constituted between 68-73 % of the PL with no significant difference between groups, but with a trend towards an increase by increased dietary SBL inclusion. For phosphatidylethanolamine (PE) a similar trend was shown for larvae from PL1; PL2 and PL3 reflecting levels in the diets, but for larvae on PL1H1; PL2H2 and PL3H3 an opposite trend was found. Larvae on diet PL3H3 contained a significant lower amount of PE, than larvae given PL1, PL2 and PL3 ($P<0.05$, Fig. 13). For minor phospholipid classes analysed, there were no significant differences between larval groups.

There was a marked decrease in larval EPA and DHA tissue composition from 0 to 10 DPH and a corresponding increase in (18:0), (18:1) (18:3n-3) (**Table 14**). Larval FA content at 30 dphreflected well dietary formulation and the increase in supplementation of PL and Algatrium DHA70 at the expense of olive oil, the main contributor of oleic acid (**Table 14**). The highest inclusion of Algatrium DHA 70 caused DHA and EPA content in larvae of PL3H3 to increase to levels about 2.5 times higher and 1.5 times higher, respectively, that of PL3 fed larvae. An overall very significant tissue/dietary correlation was observed for all larval groups for both oleic acid ($P<0.001$, $R^2=0.99.6$) and for DHA and EPA ($P<0.001$, $R^2=0.98$), and $P=0.016$, $R^2=0.80$), respectively. Consequently, tissue DHA:EPA was significantly correlated to diet DHA:EPA ($P<0.001$, $R^2=0.97$).

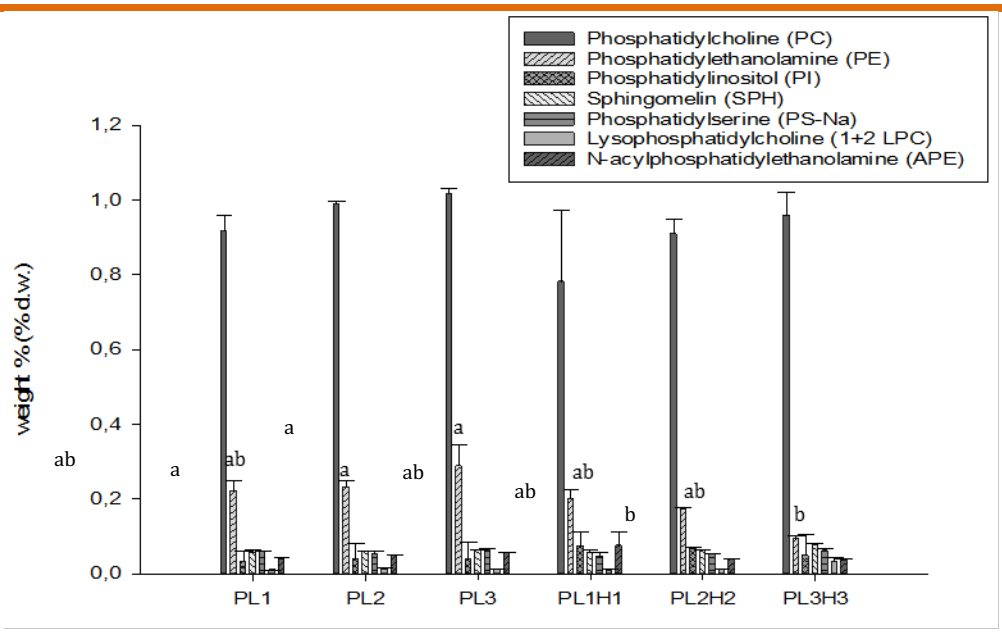

Figure 13. Larval phospholipid class tissue composition ± SD at 30 days post hatching (dph). Different letters denote statistically significant differences between treatments.

Table 14. Analysed TFA content and FA composition (% of TFA) in larvae at 0 dph. 10 dph and 30 dph (mg g^{-1} w.w.). Values (for larvae fed the experimental diets (30 dph) in a row followed by a different superscript are significantly different P<0.05

	dph 0	dph 10	dph 30					
			PL1	PL2	PL3	PL1H1	PL2H2	PL3H3
FA	109.9±27.5	139.4±18.4	41.5±7.8	69.2±36.1	64.1±9.5	73.1±19.7	69.1±14.5	38.2±15.6
FA								
16:0	11.9±0.9	13.5±0.6	13.5±0.1^{b}	14.5±1.5^{b}	18.3±0.4^{a}	13.1±0.3^{b}	13.9±0.5^{b}	14.8±0.6^{b}
18:0	3.3±0.2	7.2±0.2	4.4±0.0^{c}	4.2±0.1^{bc}	4.5±0.3^{c}	3.7±0.2^{b}	3.0±0.1^{a}	3.0±0.2^{a}
Total SFA	16.9±0.9	22.8±1.0	19.0±0.1	19.6±1.6	24.1±0.3	17.8±0.4	17.9±0.6	18.8±0.8
16:1 (n-7)	6.8±0.3	2.3±0.2	1.3±0.0	1.2±0.2	1.2±0.1	1.3±0.1	1.4±0.1	1.1±0.1
18:1 (n-9)	12.0±0.4	22.5±0.1	50.5±0.5^{e}	43.8±0.8^{d}	24.7±0.8^{b}	50.3±1.8^{e}	37.4±1.1^{c}	12.8±1.0^{a}
Total MUFAs	20.0±0.5	26.3±0.0	53.3±0.5^{e}	46.5±0.5^{d}	27.3±0.5^{b}	53.2±1.8^{e}	40.1±1.2^{c}	15.0±1.0^{a}
18:2 (n-6)	8.3±0.6	7.4±0.2	11.0±0.1^{a}	18.7±0.2^{b}	29.1±0.4^{c}	10.7±0.0^{a}	19.2±0.1^{b}	28.5±0.5^{c}
18:3 (n-6)	0.1±0.0	0.5±0.0	0.2±0.0^{ab}	0.3±0.0^{bc}	0.6±0.0^{d}	0.2±0.0^{a}	0.3±0.0^{c}	0.3±0.0^{c}
20:3 (n-6)	0.0±0.0	0.2±0.0	0.3±0.0^{b}	0.3±0.0^{b}	0.6±0.0^{d}	0.2±0.0^{c}	0.2±0.0^{c}	0.1±0.0^{a}
20:4 (n-6) ARA	1.2±0.0	0.9±0.0	0.5±0.0^{bc}	0.3±0.1^{a}	0.4±0.1^{ab}	0.4±0.0^{b}	0.4±0.0^{ab}	0.6±0.1^{c}
Total (n-6) PUFA	9.7±0.6	9.3±0.3	12.1±0.1^{a}	19.6±0.1^{b}	31.0±0.4^{e}	11.6±0.1^{a}	20.2±0.1^{c}	29.7±0.5^{d}
18:3 (n-3)	1.1±0.0	27.1±0.3	1.3±0.1^{ab}	1.3±0.0^{ab}	1.9±0.1^{c}	1.1±0.2^{a}	1.5±0.1^{b}	1.9±0.2^{c}
20:3 (n-3)	0.1±0.1	1.3±0.0	0.1±0.0^{c}	0.1±0.0^{a}	0.1±0.0^{b}	0.1±0.0^{abc}	0.1±0.0^{ab}	0.1±0.0^{ab}
20:5 (n-3) EPA	7.9±0.3	2.8±0.2	2.5±0.1^{a}	2.2±0.2^{a}	2.6±0.2^{a}	2.5±0.2^{a}	2.6±0.3^{a}	3.9±0.2^{b}
22:6 (n-3) DHA	37.1±0.3	8.5±0.8	9.4±0.3^{a}	8.6±0.8^{a}	10.6±0.4^{b}	11.2±0.9^{b}	14.6±1.1^{c}	25.4±0.8^{d}
Total (n-3) PUFA	46.1±0.5	39.7±0.7	13.3±0.3^{a}	12.2±1.0^{a}	15.3±0.7^{b}	14.8±1.2^{ab}	18.7±1.4^{c}	31.2±0.8^{d}
DHA/EPA	4.7±0.1	3.1±0.0	3.8±0.0^{a}	3.9±0.2^{ab}	4.2±0.0^{b}	4.5±0.0^{c}	5.7±0.3^{d}	6.6±0.2^{e}
(n-3) / (n-6)	4.8±0.3	4.3±0.1	1.1±0.0^{b}	0.6±0.1^{a}	0.5±0.0^{a}	1.3±0.1^{c}	0.9±0.1^{b}	1.1±0.0^{b}

Larval skeleton anomalies and gene expression

Overall there was a high incidence of severe anomalies, particularly those related with endochondral bones, such as cranium or dentary bones. The lowest incidence of severe anomalies was found in PL3H3 pikeperch, followed by PL3 (Fig. 14). An increase in dietary PL from PL1 to PL3 tended to reduce the incidence of severe anomalies. Moreover, a dietary increase in both PL and DHA from PL1H1 to PL3H3 significantly reduced the occurrence of severe anomalies. The incidence of maxillary anomalies was very low and only detected in larvae fed PL1H1 (Fig. 14). Dentary bones anomalies were significantly lowest in PL3 and PL3H3 larvae and highest in PL2 (Fig. 14). A similar trend was found in branchiostegal rays and cranial anomalies that were significantly lowest in larvae fed diet PL3H3 and PL3 and highest in PL1 and PL1H1. Indeed a highly significant inverse correlation was found between the dietary PL content or PC content and the incidence of cranial anomalies (r^2=0.98; P<0.001 or r^2= 0.98, P<0.001, respectively) or severe anomalies (r^2=0.89, P<0.01, r^2=0.88, P<0.01, respectively). No significant differences were found in any of the other anomalies studied.
Expression of bone morphogenesis related genes did not show a clear effect of the different treatments. In fact, no statistical differences were found between larvae fed the different diets for the four genes analysed.

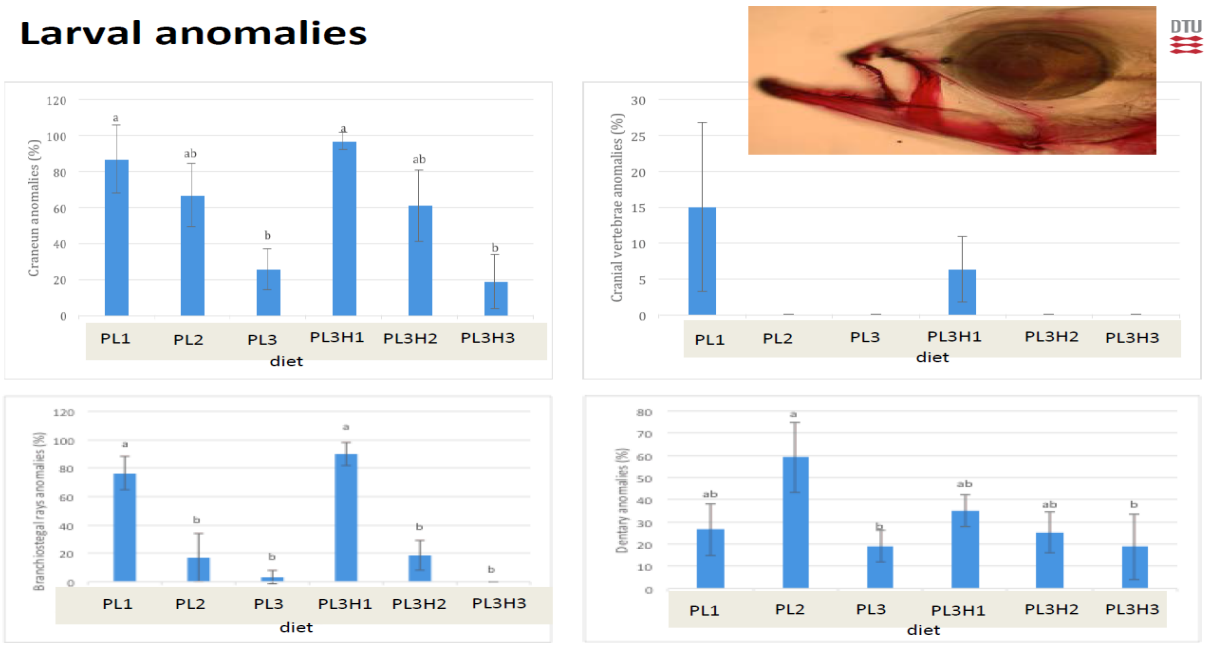

Figure 14. Craneum and Branchiostegal anomalies (%) in larvae fed one of 6 exp. diets. Different letters denote statistically significant differences between treatments.

Enzymatic activity

Results (**Table 15**) revealed some significant difference within trypsin activity, aminopeptidase activity and alkaline phosphatase activity between larval groups. Trypsin activity for PL1H1 was significantly higher than for PL2; PL3 and PL3H3, and PL2H2 trypsin activity was higher than for PL2, overall P=0.003. For amino peptidase activity PL2 was lowest followed by PL1H1, however only significantly different with PL3H3 (P=0.001). Alkaline phosphatase was significantly higher for PL3H3 than for PL1H1 (P≤0.038).

Table 15: Larval enzymatic activity of 30 dph pikeperch larvae fed different PL / n-3. *Values in a row followed by a different superscript are significantly different P<0.05*

Specific activity	PL1	PL2	PL3	PL1H1	PL2H2	PL3H3
Pepsin (mU mg protein^{-1})	48.0±7.9	49.8±9.0	56.3±7.4	51.8 ±7.9	55.2 ±2.9	63.0±3.2
Trypsin (mU mg protein^{-1})	10.1±3.1^{abc}	6.4±1.7^{a}	8.5±2.3^{ab}	16.4±3.9^{c}	14.2±1.7^{bc}	8.8±1.0^{ab}
Aminopeptidase N (mU mg protein^{-1})	5.9±0.8^{abc}	3.3±0.6^{a}	6.9±0.3^{bc}	5.3±0.7^{ab}	6.2±0.5^{bc}	8.0±1.9^{c}
Alkaline phosphatase AP (mU mg protein^{-1})	24.7±2.8^{ab}	20.8±4.8^{ab}	21.1±3.2^{ab}	18.7±1.0^{a}	20.5±3.7^{ab}	28.8±3.9^{b}
Leucine-ala (U mg protein^{-1})	376.2±39.0	350.9±39.9	395.2±36.2	371.0±52.3	364.4±14.0	361.1±58.9
Amylase (mU mg protein^{-1})	136.2±16.1	117.25±17.7	139.48±13.7	124.98±11.92	120.29±22.96	114.76±26.23

Proteomics

The mean number of spots detected per gel was 1917 ± 498. The one-way analysis of variance among the six experimental groups revealed 27 spots with differential intensity at p<0.05. These spots were selected for further statistical analyses and MS/MS identifications.
Of the 27 spots picked up for mass spectrometry analysis (**Fig.15**), 17 spots contained one protein identification per spot and this conducted to the identification of 15 different proteins, According to the Tukey post-hoc test (p <0.05), 8 proteins displayed a differential intensity between treatments (**Table 16**).

Among the identified proteins, fatty acid synthase (FAS) was significantly under-expressed in PL3H3 (14.51/27.9) compared to PL1(3.73/6.2), PL2 (8.19/7.7), and PL1H1(3.70/7.9) (4.36, 3.65, and 3.50-fold respectively, p<0.01), Further, for a given dietary PL content (14.4%), increase in dietary n-3 PUFA from 10.5 to 27.9% significantly affected FAS expression, this protein being over-expressed in PL3 (14.38/10.5) compared to PL3H3 (3.54-fold, p=0.002). However, the analysis showed that, for the same level of low n-3 PUFA (7.7%), the increase of dietary PL content did not affect significantly FAS expression (comparison between PL2, PL1H1, see table : dietary fatty acids content).

Another protein related to lipid metabolism, ATP-citrate synthase, was also under-expressed in PL3H3 compared to PL2 (2.60-fold, p<0.05). Equally, high PL and HUFA dietary content led to an under expression for spot 1633 in which two lipid transfer proteins were identified, the non-specific lipid transfer protein and the hydroxysteroid dehydrogenase-like protein 2 (HSDL2), as it appeared in larvae fed PL3H3 compared to PL1H1 (1.63-fold, p =0.03). On the other hand, compared to PL2, larvae fed PL3H3 displayed a decrease in abundance for two proteins involved in endoplasmic reticulum (ER) stress, the glucose regulated protein (Grp) and the glucose-regulated 94 (Grp94) (Hsp90) (1.70 –fold, p= 0.007and 1.52- fold, p=0.03, respectively). A similar pattern of protein expression occurred for Grp94 in PL1H1 larvae compared to PL2 (1.48-fold, p=0.03). In addition spot number 369 displayed a differential intensity between PL3H3 and PL1 treatment (fold 1.63, p=0.03) in which both Grp94 and a function ubiquitin carboxyl-terminal hydrolase 5 were identified. Two spots were identified as Protein disulfide-isomerase (PDI) (spots 1376 and 1947), according to the Tukey post-hoc test only spot 1947 display a differential intensity being lower in larvae fed PL1 compared to PL2H2 (8.32/15.9) and PL2 treatment (1.99 and 1.85 fold, p=0.005), larvae fed PL2 showed also an increase in its abundance compared to PL1H1 (1.67-fold, p=0.005).

Lastly, larvae fed PL1 treatment showed an increase in abundance for a spot identified as Keratin – a cytoskeletal and structural protein – in comparison with PL2H2 and PH3H3 larvae (2.27 and 2.33-fold respectively, p=0.03).

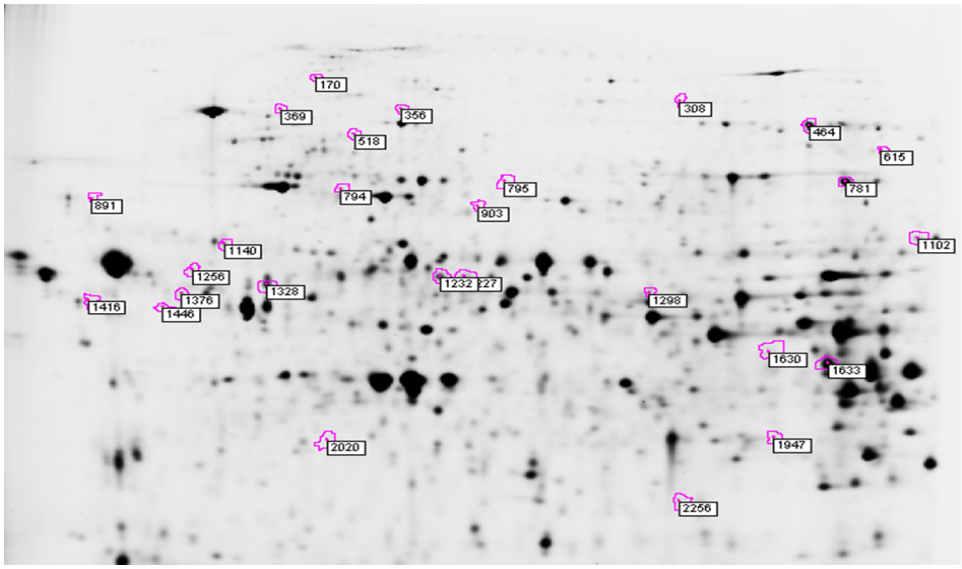

Figure 15. Representative two-dimensional gel electrophoresis of differential protein expression profile in pikeperch liver

Table 16: Protein differentially expressed in liver of 30 dph pikeperch larvae fed different PL / n-3 HUFA

Spot	accession	Protein identification	Species	peptide	PI/MW	p	Fold change
794	A0A0F8AHC2	Glucose-regulated	*Larimichthys crocea*	5	5.41/82	0.007	-1.70 in PL3H3/PL2
518	A0A0F8AWU1	Glucose-regulated protein (GRP94)	*Larimichthys crocea*	7	4.76/91	0.031	-1.48 in PL1H1/PL2
	UPI000557CE3B	Glucose-regulated protein (GRP94)	*Notothenia coriiceps*	6	4.73/92		-1.52 in PL3H3/PL2
369	A0A0F8AWU1	Glucose-regulated protein (GRP94)	*Larimichthys crocea*	6	4.76/91	0.033	-1.63 in PL3H3/PL1
	UPI000557CE3B	Glucose-regulated protein (GRP94)	*Notothenia coriiceps*	5	4.73/92		
	UPI00055340E4	Ubiquitin carboxyl-terminal hydrolase 5	*Notothenia coriiceps*	3	4.98/88		
795	UPI000556131D	fatty acid synthase-like	*Notothenia coriiceps*	4	5.66/54	0.002	4.36 in PL1/PL3H3 3.65 in PL2/PL3H3 3.54 in PL3/PL3H3 3.50 in PL1H1/PL3H3
1102	G3P216	ATP-citrate synthase	*Gasterosteus aculeatus*	3	7.80/120	0.036	-2.60 in PL3H3/PL2
1633	H2U634	non-specific lipid-transfer protein	*Takifugu rubripes*	3	8.11/59	0.042	-2.03 in PL3H3/PL1H1
	UPI000551760C	non-specific lipid-transfer protein	*Notothenia coriiceps*	2	6.58/58		
	H2SWA2	hydroxysteroid dehydrogenase-like protein 2	*Takifugu rubripes*	2	6.03/37		
1232	G8G8Y1	Keratin 8 (Fragment) n=2	*Epinephelus coioides*	9	4.72/41	0.035	-2.27 in PL2H2/PL1
	G3NI19	keratin, type II cytoskeletal 8-like	*Gasterosteus aculeatus*	9	5.22/60		-2.33 in PL3H3/PL1
	Q4QY72	type II keratin E3-like protein	*Sparus aurata*	4	4.89/39		
1376	UPI00054B498F	protein disulfide-isomerase	*Larimichthys crocea*	5	4.61 / 57	0.047	NS
1947	U3LRB6	Protein disulfide-isomerase	*Dicentrarchus labrax*	3	5.39 / 56	0.005	1.85 in PL2/PL1 1.99 in PL2H2/PL1 1.67 in PL2/PL1H1

Fast escape response

For the escape response study (II), fish on diets low in phospholipids; i.e. PL1 and PL1H1 grew much slower and had a higher mortality until DPH 80 than the 4 other remaining diets. Therefore juveniles from these diets were not included in studies on fast escape response, metabolic rate and stress response to chasing. On the fast escape response, there were no significantly differences in latency time between the tested diets (i.e. time to respond to a visual stimuli, velocity or in acceleration speed (**Fig. 16**)

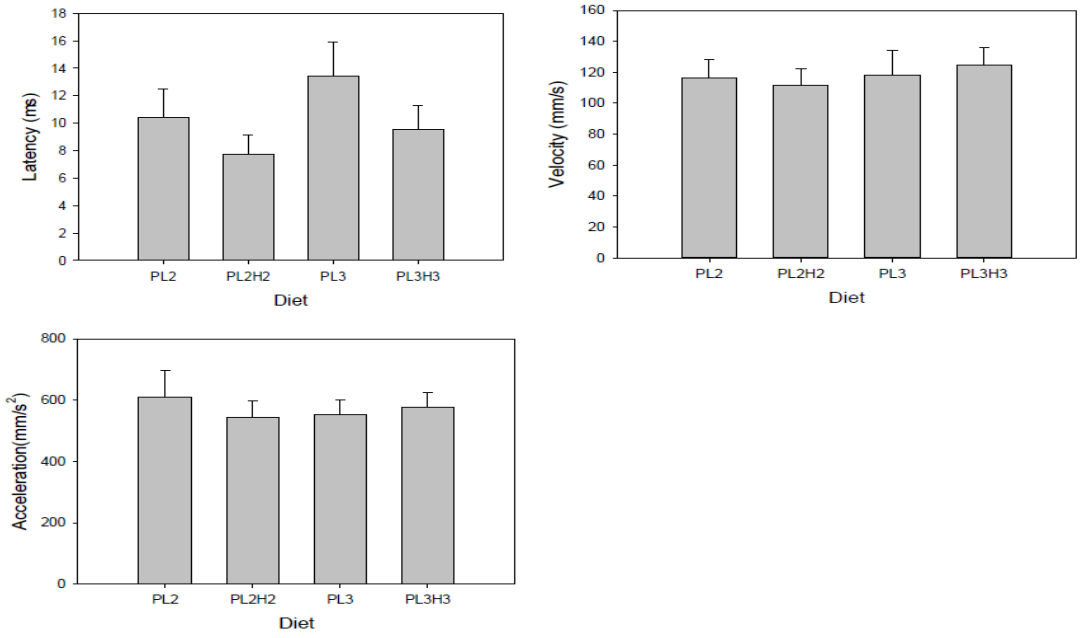

Figure 16 Latency time (ms), velocity (mm/s, and acceleration (mm/s^2) as well as exp. setup with illuminated test arena.

Metabolic rate and oxygen tolerance

As for the fast escape response, the results on metabolic rate showed no significantly differences between the diets tested **(Fig. 17**)

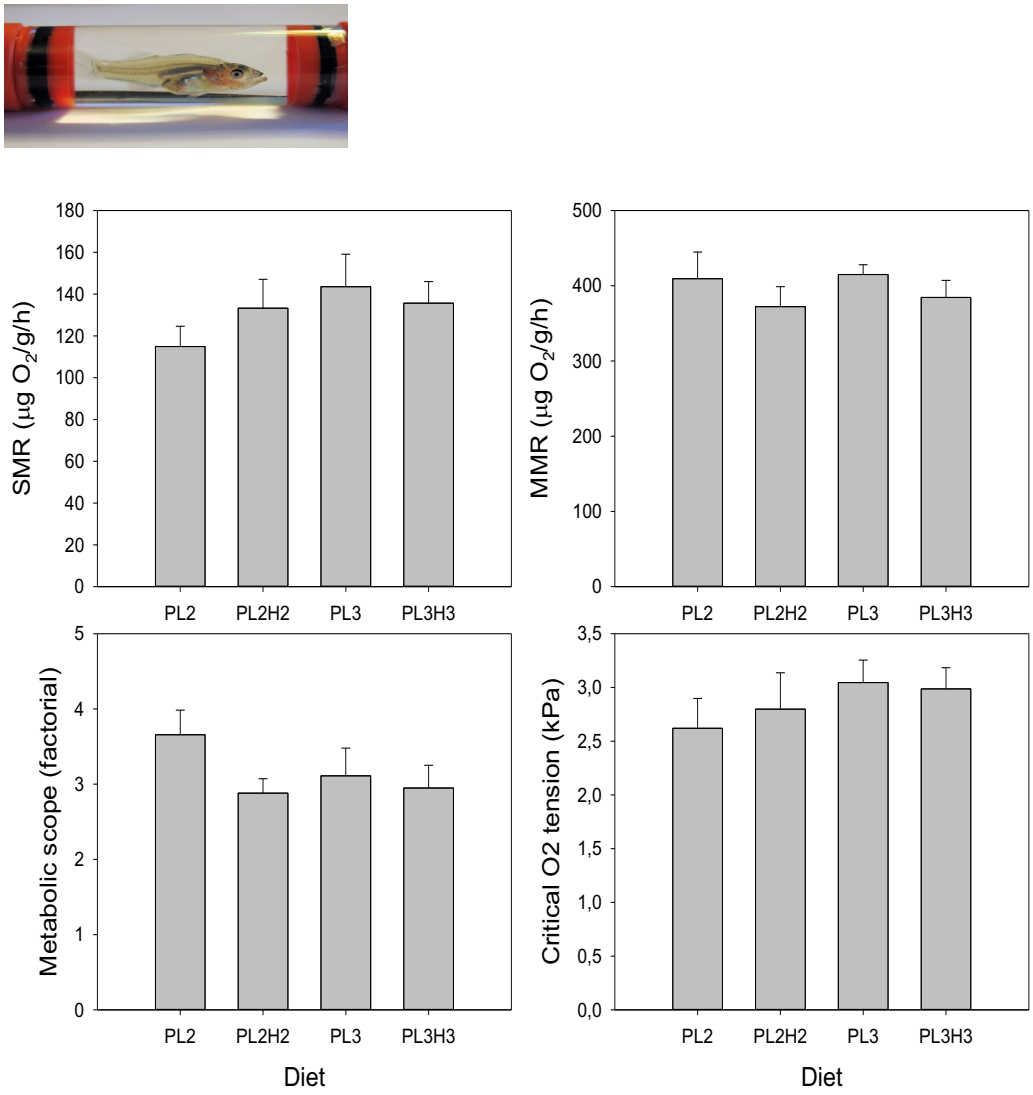

Figure 17. Fish in rep. chamber and analysed Standard metabolic rate (SMR); Maximum metabolic rate (MMR), metabolic scope and critical O_2 tension.

Chasing stress

Results did not show any significant differences in levels of body cortisol, glucose or lactate between the experimental diets tested (**Fig. 18**).

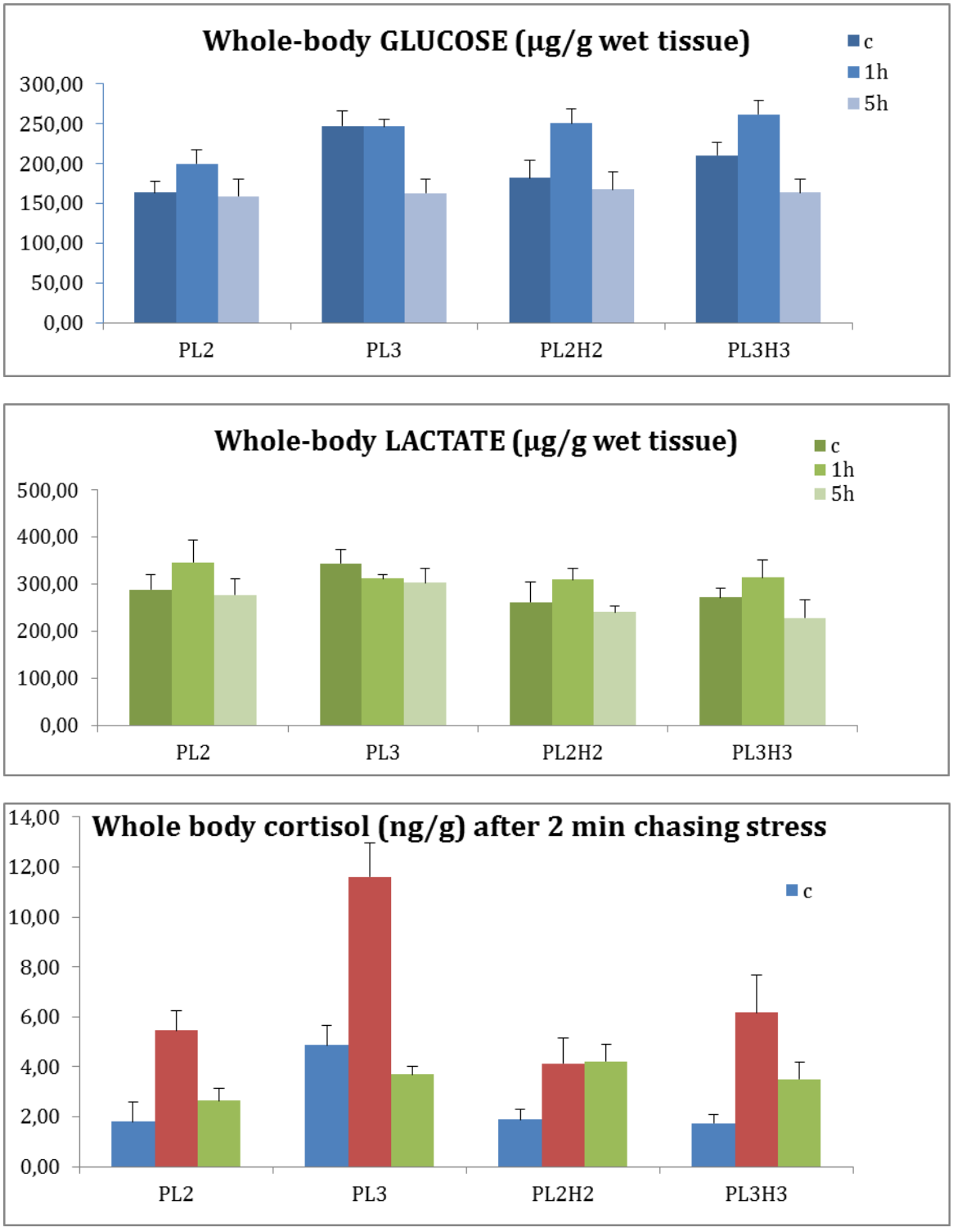

Figure 18. Tissue cortisol, glucose and lactate levels for juveniles from each diet following chasing stress (1, 5 h) as well as control values.

Discussion

Performance and growth of larvae

In order to optimize the culture of pike perch larvae a series of formulated diets with increasing PL and HUFA content were tested. We observed that survival in all tanks was affected by high cannibalism, that appeared around 16 DPH, and continued until the end of the experiment. A similar cannibalistic behavior has previously been described (Hamza et al., 2008; Lund & Steenfeldt 2011). All 6 microdiets supported good larval growth (SGR 21-24 % day $^{-1}$), even at the lowest PL, (soy lecithin) inclusion with no n-3 LC PUFA supplementation, indicating that the main dietary constituents i.e. fish meal ; soluble fish protein concentrate and krill meal may have provided the necessary nutrients required. Results, however, showed a clear effect on growth of larvae by an increase in PL supplementation, which was supported by results from a study on pike perch larval behavior with use of the same diets. In that study growth differences were more pronounced and survival much lower for larval groups reared on PL1 and PLH1 compared with the other diets (unpublished results). This strongly indicates the importance of early dietary PL supplementation, but also indicating batch related differences in larval requirement. Dietary PL inclusion levels above 9 % (i.e. 12 % and 15 % phoshopholipid supplement) have been reported in a few studies involving European sea bass and gilthead seabream larvae (Cahu et al., 2003) results reported an optimal inclusion level of 12 % and > 9%, respectively for growth and survival. In the present study up to 19 % soybean lecithin was supplemented as the maximum possible to keep diets isoproteinous and isoenergetic and resulted in PL levels of up to 14.5% ww for diet PL3 and PL3H3; (about 150 g PL /kg). This indicates, that technical formulation issues in controlled experiments may restrict the possible optimal range of PL levels to be tested in protein and energy dense formulated larval feeds. Although the highest PL level of 14.5 % ww gave the best growth result per se, precautions in concluding about optimal levels to support optimal larval growth should be taken. Thus, in a similar growth study on pike perch larvae fed soy lecithin a PL inclusion of 9.5% (95 g PL /kg) indicated a comparable larval growth as shown here until 34 DPH (Hamza et al., 2008).

The optimal levels of EPA and DHA in marine fish larvae has been estimated to about 3 % of dietary dry matter to support growth (Sargent et al., 2002). For pike perch larvae an optimal level of 1.2% DM has been suggested, based on experiments with soy bean lecithin without n-3 HUFAs and fish gonad lecithin with n-3 HUFAs (Hamza et al. 2012). In the present study a dietary supplementary inclusion of 2% and 3.4 % Algatrium DHA 70, tuna oil (i.e. composed of primarily DHA (660-700 mg/g) and EPA (60-75 mg/g)) resulting in EPA+DHA levels up to 6.6% DM indicated an additional positive growth effect (diet PL2H2 and PL3H3) as when compared with PL2 and PL3 without n-3 LC PUFA supplementation (Fig 1). Despite the apparent controversy with previous results suggesting a requirement of only 1.2 % LC PUFAs (Hamza et al. 2012) a direct comparison between studies may be of limited value, as it would require a common reference diet. Besides, quantitative or qualitative dietary PL requirements for fish larvae may be affected by factors such as fish larvae origin; rearing conditions; size; stage of ontogeny; PL source; as well as type of ingredients and dietary composition (Tocher et al. 2008).

In gilthead seabream, PL had a marked effect on lipid transport, while diets without lecithin accumulated lipid vacuoles in the basal zone of intestinal enterocytes and caused esteatosis in hepatic tissue; the addition of 2 % soybean lecithin reduced accumulation and similar results have been obtained in studies on sea bass larvae (Gisbert et al., 2005). It is believed, that the size and ultrastructural characteristics of lipid inclusions in the enterocytes (VLDL ;lipoprotein particles and lipid droplets) are valuable markers to evaluate lipid absorption; metabolism and lipid transport from the intestinal mucosa into body circulation. Moreover it is likely affecting the rate of lipoprotein synthesis and is possibly influenced by the amount and type of fat ingested besides the PL:TAG ratio (Izquierdo et al., 2000 ; Cahu et al., 2003). It has been further suggested, that a reduction in lipid accumulation may be compromised by dietary PL lipid class composition. Phosphatidylcholine (PC) is more predominant in fish lipoproteins (i.e. transport molecules involved in FA transport) than other

phosphoglycerides, consequently PLs composed of mainly PC may increase uptake and metabolism. For the soybean lecithin tested in this experiment each of the main phoshoglyceride classes were equally represented, suggesting instead a positive influence on growth by the overall increased supplementation and availability. For diets PL1 and PL1H1 the low level of PL and high level of NL (TAG) derived lipids was most likely the main factor in the relative reduction in growth of the larvae. This combined with differences in PUFA content of diet PL1; PL2 and PL3; as the gradually substitution of olive oil with soy lecithin caused a 3-fold increase in tissue linoleic acid, 18:2n-6 content and a concurrent two-fold decrease in tissue oleic acid, 18:1n-9 content. The 18:2n-6 is an essential FA for freshwater species and despite that C^{14} labelled FA analyses have indicated no elongation or desaturation capability to n-6 ARA, 20:4n-6 (unpublished results), 18:2n-6 may have had a growth promoting effect or a different metabolic utilization or fate compared to 18:1n-9. Apart from the possible improvement in lipid digestion and uptake by use of PL, the addition of soybean lecithin has shown to enhance micro diet ingestion rates in prawn and gilthead seabream (Teshima et al., 1986; Koven et al., 1993) and may cause growth advantages especially in the weaning transition phase from live feeding to micro diet acclimatization. The indication of an additional growth promoting effect by supplementation of triglyceride DHA (+ EPA) (diet PL2H2 and PL3H3) is interesting and in contradiction to previous studies on effects of PL and LC PUFAs in pike perch larvae (Hamza et al. 2008; 2012). Similarly, the general consensus is that main effects on growth in fish larvae is contributed to dietary n-3 LC PUFAs be contained in the polar lipid fraction (; Cahu et al., 2003); Gisbert et al., 2005; Tocher et al., 2008,). A pivotal difference between the referred studies and this experiment is that in the former different oils or proportions of marine lecithin, soybean lecithin and cod liver was used to obtain the different dietary levels of PL, NL and LC PUFAs. Instead diets PL1-PL3 and PL1H1- PL3H3 were formulated exactly similar to be able to isolate the effect of DHA concentrate supplementation in the latter. The supplementation of concentrated DHA even though provided as glycerides may have mitigated the forming of lipid vacuoles in the enterocytes with implications for metabolism, uptake, lipoprotein synthesis and transportation, but need further studies. The analysis of the content of EPA + DHA in the various PL and NL lipid fractions may have explained the results obtained and the pronounced difference in phosphatidylethanolamine tissue content between PL3 - and PL3H3 larvae.

Gene expression

Liver can be considered as the main metabolic reactor of the body, possessing a number of regulatory functions, including the storage of vitamins and minerals, as well as carbohydrate, fat, and protein metabolism and therefore chosen to access changes in protein expression profiles. In this study, FAS was under-expressed in larvae from PL3H3 compared to PL1 and PL1H1, suggesting a higher energy demand of the smallest larvae. Moreover, FAS seemed to be more regulated by LC PUFA content than by PL levels, which may indicate a positive effect of DHA supplementation. Indeed, regardless of PL levels, FAS expression changed between PL3H3 and PL3 (3.54-fold under-expressed in PL3H3), while no significant differences appeared between larvae fed the same n-3 LC- PUFA level (PL2 compared to PL1H1). Most previous studies of FAS regulation have focused on the control of gene expression, suggesting that HUFAs may decrease FAS expression through the inhibition of SREBP-1c (Yahagi et al, 1999; Hannah et al. 2001). On the other hand, FAS appears to participate in liver triglyceride metabolism by promoting β-oxidation of fatty acids through activation of peroxisome proliferator–activated receptors PPARα under nutrient-deficient conditions (Georgiadi & Kersten 2012). Indeed FAS is required for generating the phospholipid 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (16:0/18:1-GPC), an endogenous ligand for PPARα (Chakravarthy et al. 2009*)*. However, this interpretation should be considered carefully, taking into account the changes in FAS activity without corresponding changes in FAS protein levels. Equally, we also observed a low expression of ATP-citrate synthase in larvae fed the highest n-3 PUFA/PL diet (PL3H3 compared to PL2), which is the primary enzyme responsible for the synthesis of cytosolic acetyl-CoA considered as

the principal building block of fatty acids, since acetyl-CoA is converted to malonyl-CoA by acetyl CoA carboxylase (ACC). Fatty acid elongation is catalyzed by Elovl6 known to use malonyl-CoA.

In the present experiment we found another marked effect of dietary PL/n-3 LC PUFA in regulating proteins involved in lipid metabolism. Our data show that the high PL/n-3 LC PUFA content down-regulated the expression of proteins involved in transfer and exchange of phospholipids and cholesterol such as the non-specific lipid transfer protein, and hydroxysteroid dehydrogenase-like protein 2, that were both identified in the same spot (spot 1633, 1.63-fold, p =0.03). The non-specific lipid transfer protein more commonly denoted as sterol carrier protein 2 (SCP-2) is able to transfer all common phospholipids between membranes including sterols (cholesterol and glycolipids) (Bloj et al. 1977, 1981; Vahouny et al. 1987). This peroxisomal protein is able to bind fatty acyl-CoAs, where it is likely involved in the b-oxidation of fatty acids (Seedorf et al. 1994; Wouters et al. 1998; Kowalik et al. 2009). Furthermore, its association with the b-oxidation complex could be important for the protection of unsaturated fatty acid intermediates against oxidative attack (Dansen et al. 2004). It's well known that hydroxysteroid dehydrogenase-like protein 2 is the product of one of the sterol carrier protein 2 (SCP2) domain encoding genes (Edqvist et al. 2006), thus pointing to its potential involvement in the transport and/or metabolism of fatty acids. Similarly, the peroxisomal localization of human HSDL2 may suggest also an involvement in fatty-acid metabolism (Kowalik et al. 2009). Previous studies demonstrated that the promoter region for SCP2 encoded gene contains several regulatory domains including a peroxisomal proliferato41r response element PPRE which is the specific DNA region of target genes that bind with PPARs (Gallegos et al. 2001; Berger and Moller 2002). Therefore, the down-expression of SCP2 observed in this study in PL3H3 larvae might be explained by a deficient PPARα activation due to the decrease in FAS expression in these larvae as a response to the high dietary n-3 HUFA (explained above). Consistently with this hypothesis, in a recent study, SCP2 gene expression was down-regulated after colon cancer cells transplantation in mice fed high DHA (Zou et al. 2015). Besides, the same authors observed a decrease of SCP2 expression in cultured colon cancer cells HCT-15 after 48 h treatment with DHA.

Protein disulfide isomerase (PDI) is a multifunctional protein which acts as a catalyst of disulfide bond formation, reduction and isomerization of newly synthesized proteins (Noiva, 1999; Turano et al. 2002). In the present experiment, an increase in abundance of PDI was observed in the liver of larvae fed on PL2 and PL2H2 compared to PL1. A similar pattern of expression was reported in mice fed low and high n-3 LC PUFA diets suggesting an increase of the protein synthesis in high n-3 LC PUFA animals (Ahmed et al. 2014). On the other hand, in our experiment, a significant difference in PDI expression was recorded between larvae fed the same n-3 LC PUFA – PL2 compared to PL1HI. The over-expression in PDI in the biggest larvae may reflect an enhanced protein synthesis through the high energy mobilization for growth. In this sense Hamza et al. (2010) suggested the enhanced ability to allocate nutrient and energy into tissue formation in pikeperch larvae fed high PL. Moreover, PDI has a chaperone activity under stress, it is synthesized in response to the unfolded protein response pathway (UPR) in the ER lumen (Malhotra and Kaufman, 2007). This points to its important role in the maintenance of ER homeostasis (Okumura et al. 2015). In this respect, PDI over-expression in PL2 might indicate an ER stress (Dorner et al. 1990). Similarly, glucose regulated protein (Grp) and glucose-regulated 94 (Grp94) known by their major roles during UPR to maintain ER homeostasis (Zhu and Lee, 2014) were over-expressed in PL2. Glucose-regulated 94 (Grp94) is a hallmark of the UPR response defined as an HSP90 family member commonly denoted endoplasmin (Marzec et al 2012). This protein together with PDI and other folding factor components form a functional –folding-network under a coordinate transcriptional regulation (Marzec et al 2012). As mentioned above, both Grp and Grp94 were over-expressed in larval group PL2 compared to PL3H3 in the present study, suggesting a reduced sensitivity to stress thanks to the dietary PL supplementation (Kontara et al. 1997; Coutteau et al. 2000, Hamza et al. 2010). Interestingly, the same pattern of expression regarding the response to the dietary PL was observed in PL1 and PL3H3 treatments. Indeed, beside Grp94, another protein was identified, i.e. the ubiquitin carboxyl-terminal hydrolase L5, which is a cellular pathway responsible for the degradation of misfolded and damaged proteins involved in the ubiquitin proteasome system (Ciechanover and Brundin 2003). However, the over-expression of Grp94 in PL2

as compared to PL1H1 (same n-3 LC PUFA content) supports the importance of a balanced inclusion of both phospholipids and n-3 LC PUFA in the formulation of pikeperch diets.

In the present study, different isoforms of the keratin type II were detected in pikeperch liver with a 2.27 and 2.33-fold increase in larvae fed on PL1 compared to PL2H2 and PH3H3 larvae respectively. It has been demonstrated that keratin type II displays a differential expression pattern during the early ontogeny of fish, with a higher abundance in younger larvae (Sveinsdóttir et al. 2008). In consequence, the down-regulation of keratin type II together with the significantly higher growth in PL2H2 and PL3H3 indicate an advanced developmental stage in these latter larvae.

In addition to the improved growth results underpinned by proteolytic assessment; digestive enzymatic activity indicated a positive physiological response of high inclusion levels of PL and n-3 LC PUFAs. In the present study, activities of the brush border membrane enzymes, namely alkaline phosphatase and aminopeptidase were significantly higher in larvae fed high PL and DHA+EPA (treatments PL3H3 and PL2H2) compared to larvae receiving low levels of the latter fatty acids (treatments PH1H1(AP and N) and PL2 (N)). The latter result corroborates previous observations, that an increase in digestive enzyme activities was associated with increased dietary PL content (Gisbert et al. 2005; Hamza et al. 2008, 2012; Saleh et al. 2013). The improvement of growth observed in fish fed increased PL content has been related, by several authors, to a higher maturation of gut (Cahu and Zambonino 1994; Ribeiro et al 2002; MacQueen Leifson et al. 2003; Gisbert et al. 2005; Hamza et al. 2008; Saleh et al. 2013), and more precisely to a faster maturational process of the enterocytes (Hamza et al. 2015). Indeed, in fish larvae, enterocyte morphology is influenced by the developmental stage and dietary composition (MacQueen Leifson et al. 2003; Kjørsvik et al. 2011). In our study, the acceleration of gut developmental processes in larvae fed high PL was further supported by the advanced ontogenetic stage in larvae fed high PL suggested by proteomics finding of keratin type II expression

Trypsin activity displayed an opposite pattern to that of the brush border enzymes in the present study. The highest values were recorded in larvae fed PL1H1 characterized by high oleic acid content, while the lowest ones were measured in larvae fed PL3H3 and PL3 which contained low oleic acid. This result corroborates the hypothesis of Gisbert et al. (2005) that C18 fatty acids stimulate the regulation of pancreatic secretion (Shintani et al. 1995; Gisbert et al. 2005). Similar studies reported an increase in pancreatic secretion of trypsin and in fish larvae fed high PL diet, suggesting an efficient digestion resulted by the enhanced secretion of cholecystokinin CCK, the primary regulator of pancreatic secretion (Saleh et al. 2013, Zhao et al. 2013). Thus authors suggested that an increased PUFA content in bio-membrane of larval tissues and intestinal lumen could modulate CCK function (Saleh el al. 2013, Kamaszewsk et al. 2014). In this regard, an antagonistic feedback mechanism between CCK and tryptic activity was demonstrated in cod larvae (Drossou, 2006), and further supports the existence of a CCK-releasing factor (Cahu et al. 2004; Rønnestad et al. 2013). On the other hand, although no significant difference was recorded in pepsin activity, it should be noted that the lowest trypsin activity was associated with the higher pepsin activity. In fact, during the early larval ontogeny, a progressive shift in relative activity from alkaline to acid proteases was observed (Zambonino-Infante and Cahu, 2001, 2007; Pérez-Casanova et al., 2006; Lazo et al. 2007). As a consequence, the decrease in tryptic activity may be linked to the enhancement of acidic digestion in the stomach of the biggest pikeperch larvae. Precaution in the interpretation of enzymatic activity is the lack of digestive enzyme activity with a concomitant development of pike perch larvae ontogeny, and hence results presented may be due to differences in growth rate and gut maturation.

Larval anomalies and condition

As opposed to Hamza (Hamza et al., 2008) this study gave clear evidence for an effect of PL in preventing malformations and deformities Furthermore DHA and EPA seemed to have an additional but more limited effect.

Conclusion

In conclusion, we confirmed the importance of high PL levels of approximately 8 % in diets for pikeperch as well as the positive additional beneficiary effect of supplementation with DHA+ EPA in the form of concentrated TAG in otherwise identical formulated diets. Thus, combined supplementation of SBL up to 14.51% d.w. PL with n-3 LC-PUFA (1.00 % d.w. DHA and 0.16% d.w. EPA) in the form of triglycerides lead to the highest growth and lowest anomalies incidence, which improved digestive enzymes activities. . Data were supported by analyses of liver proteomics and revealed, that PL3H3 caused a down regulation of both FAS and ATP- citrate synthase involved in triglyceride and FA synthesis in comparison when fed PL3. A high dietary PL content reduced skeletal deformities indicating some additionally positive effects of LC PUFA inclusion. Diets with an adequate amount of PL (8%) caused no negative effect on stress behaviour /stress markers or escape response, but diets with low levels of PL and LC-PUFAs caused a reduced growth and therefore these larvae were not evaluated in terms of stress behaviour and escape reponse. Overall the results indicate, that essential fatty acids may not need to be incorporated in the PL fraction of the diet and could be supplemented as triglycerides to have a beneficial effect in pike perch larvae development. Additional future confirmatory larval studies in pikeperch larvae could involve effects of TAG and PL supplemented LC-PUFA and resultant FA composition in PL and NL tissue fractions.

6. Overall conclusions

The experiments conducted within WP 10.1 of the diversify project have gained new insight into the nutritional requirements of pike perch larvae and juveniles. As such diets with 8% phospholipids and about 1.0% LC PUFAs are recommended to obtain good performance and lower degree of malformations and can be supplemented as vegetable lecithin and in the form of triglycerides. Digestive enzymatic activity seem related to ontogenetic development more than nutritional composition, while live proteomic expression of some important proteins are clearly related to the availability and presence of LC – PUFAs especially DHA.
Ca/P, fatty acids and their interaction seem to be key nutritional factors influencing pikeperch larval development. In addition, it appears that total P % content should be considered in determining the optimal Ca/P level. Based on the gained information a test diet will be formulated and tested in a commercial farm (D10.3)

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