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Physiological and molecular responses to selected dietary nutrients during early development stages of pikeperch Sander lucioperca L

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Physiological and molecular responses to selected dietary nutrients during early development stages of pikeperch *Sander lucioperca* L.

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Abstract

Pikeperch (*Sander lucioperca* L.) is recognized as one of the main freshwater species with a great potential for the expansion of the EU aquaculture industry. Tailored commercial starter feeds do not exist for this species, and feeds used in hatcheries are likely developed for marine species. The lack of knowledge on the essential nutritional factors has been considered as a bottleneck in new emerging species farming, since nutrition is the significant criteria to be considered for economical and sustainable aquaculture. The present thesis focused primarily on the importance of PL and LC-PUFAs composition and dietary requirement of Ca/P, as well as their interactions in pikeperch larval development. The multi-nutritional approach allows a more precise estimates of nutritional requirements, since the relevant interaction among nutrients are considered. In the course of this thesis, four feeding experiments were carried out using skeletal anomaly incidence, molecular biomarkers and digestive enzymatic activity as main endpoints. Results confirmed the synergistic role of dietary PL and n-3 LC-PUFAs in pikeperch larvae. Dietary PL combined with supplementation of LC -PUFA in the form of TAG resulted in highest growth, lowest incidence of anomalies and improved digestive enzyme activity; and had a differential effect on liver proteomics. Essential FA may be directly supplemented as triglycerides to have a beneficial effect in pikeperch larval development. Results highlighted also the major role of Ca/P and EPA+DHA/ARA ratios as well as their interactions with vitamin C during the early post-weaning period. Lordosis and scoliosis seemed to be likely affected by dietary LC-PUFAs, especially DHA. Besides, a strong association is found for the larval tissue amount of ARA and DHA with eicosanoid metabolism, stress response and skeleton anomaly related genes, denoting the effects of dietary LC-PUFAs on immune/stress gene regulation and their potential implication in skeleton development. Interestingly, dietary ARA has likely a sensitive effect on the maturation process (especially intestinal development) in pikeperch larvae. Results showed that 0.6 Ca/P is efficient in reducing the incidence of kyphosis in pikeperch larvae. Meanwhile, the total P % content should be considered in determining the optimal Ca/P level. This provided new insights into the nutritional requirements of pikeperch larvae and contributed to the development of new pikeperch specific feed.

Résumé

Le sandre (*Sander lucioperca* L.) est reconnu comme l'une des principales espèces d'eau douce ayant un grand potentiel pour l'expansion de l'industrie aquacole de l'UE. Il n'existe pas d'aliments de démarrage commerciaux adaptés à cette espèce, et les aliments utilisés dans les écloseries sont généralement développés pour des espèces marines ou pour des salmonidés. Le manque de connaissances sur les facteurs nutritionnels essentiels a été considéré comme un goulot d'étranglement dans l'élevage des nouvelles espèces émergentes, puisque la nutrition est un critère important à prendre en compte pour une aquaculture économique et durable. La présente thèse s'est concentrée principalement sur l'importance de la composition en phospholipides (PL) et acides gras polyinsaturés à longue chaîne (AGPI-LC), ainis que sur les besoins alimentaires de Ca/P et les interactions de ces nutriments dans le développement larvaire du sandre. L'approche multi-factorielle permet une estimation plus précise des besoins nutritionnels, puisque les interactions pertinentes entre les nutriments sont considérées. Au cours de cette thèse, quatre expériences d'alimentation ont été menées en utilisant l'incidence des anomalies du squelette, des biomarqueurs moléculaires et l'analyse d'activités enzymatiques digestives comme principaux paramètres. Les résultats ont confirmé le rôle synergique des phospholipides (PL) alimentaires et des AGPI-LC n-3 chez les larves de sandre. Les PL alimentaires combinés à une supplémentation en AGPI-LC sous forme de triglycérides (TAG) ont permis d'obtenir la croissance la plus élevée et la plus faible incidence de différentes anomalies, d'améliorer l'activité des enzymes digestives et d'avoir un effet différentiel sur la protéomique du foie. Les AG essentiels peuvent être directement supplémentés sous forme de triglycérides pour avoir un effet bénéfique sur le développement des larves de sandre. Les résultats ont également mis en évidence le rôle majeur des ratios Ca/P et EPA+DHA/ARA ainsi que leurs interactions avec la vitamine C pendant la période de post-sevrage précoce. La lordose et la scoliose semblent être probablement affectées par les AGPI-LC alimentaires, en particulier le DHA. En outre, une forte association a été trouvée pour la quantité d'ARA et de DHA dans les tissus larvaires avec le métabolisme des eicosanoïdes, la réponse au stress et les gènes liés aux anomalies du squelette, indiquant les effets des AGPI-LC sur la régulation des gènes immunitaires/stress et leur implication potentielle dans le développement du squelette. Il est intéressant de noter que l'ARA alimentaire a probablement un effet sensible sur le processus de maturation (en particulier le développement intestinal) chez les larves de sandre. Les résultats ont montré qu'un rapport Ca/P de 0,6 est efficace pour réduire l'incidence de la cyphose chez les larves de sandre. Par ailleurs, la teneur totale en P % doit être prise en compte pour

déterminer le niveau optimal de Ca/P. Ce travail a fourni de nouvelles informations sur les besoins nutritionnels des larves de sandre et a contribué au développement de nouveaux aliments spécifiques pour cette espèce d'intérêt aquacole.

List of Abbreviations

ANOVA	Analysis of variance
Alp	Alkaline phosphatase
ARA	Arachidonic
Cat	Catalase
Cox2	Cytochrome c oxidase subunit
DHA	Docosahexaenoic acid
Elovl5	Elongation of very long chain fatty acids protein 5
EPA	Eicosapentaenoic acid
Fadsd6	Fatty acid desaturase 2/acyl-coa 6-desaturase 6
GC	Glucocorticoids
Gpx	Glutathione peroxidase
Gr	Glucocorticoid receptor
Hsl	Hormone-sensitive lipase
LC-PUFA	Long chain polyunsaturated fatty acids
Lox5	Arachidonate 5-lipoxygenase
Lta4h	Leukotriene A (4) hydrolase
Mef2c	Myocyte enhancer factor 2C
Pepck	Phosphoenolpyruvate carboxykinase
Pge2	Prostaglandin E synthase 2
PL	Phospholipid
Pla	Phospholipases
Pparα	Peroxisome proliferator-activated receptor
SGR	Specific growth rate
Sod	Superoxide dismutase
Sox9	Transcription factor Sox9
StAR	Steroidogenic acute regulatory protein

List of content

Chapter 1

General introduction

Chapter 2

Structure of thesis

*This chapter presents the general research approach, the objective and the thesis outline.

Structure of thesis

1. General research approach

To effectively study the effect of nutritional factors, it is necessary to adopt a global approach investigating the dietary responses at different organismic levels, including physiological, anatomical-histological, biochemical and molecular responses. Larval growth and survival are presented as the most stringent criteria to estimate the nutritional requirements and to evaluate the effects of a given nutrient factor and /or interaction. Digestive enzymes are considered to be reliable biochemical indicators for larval nutritional status due to their species and age specificity, sensitivity, and short latency (Lazo et al., 2011). One of the bottlenecks of finfish aquaculture production is the presence of morphological deformities. In this sense, the knowledge and the study of skeletal developmental pattern represent an important contribution for the optimization of larval rearing. Molecular approach is also an interesting level of investigation for defining nutritional conditions in fish. For instance, some nutrients (such as LC-PUFAs) are able to modulate the transcription of genes involved in skeletogenesis, stress response and lipid metabolism. Proteins are the principal mediators of metabolic activity and connect transcript expression to cellular metabolism. Both molecular and cellular indicators should be considered in order to obtain a more comprehensive information about the physiological status of fish larvae (Lazo et al., 2011). Therefore, combining transcriptome and proteome analyses could be a powerful tool for developing a comprehensive understanding of the molecular mechanisms regulating nutritional metabolism. Proteomics permits visualisation of the protein content of the cell under varying conditions. Proteome analysis can provide useful information about the effects of nutrients on metabolic pathways in targeted cells or tissues (Hamza et al., 2010). In this thesis, and comparing with fish database, the proteomic approach has been used to study the mechanisms that occur in the liver at the cellular level under the nutritional conditions studied. This approach helps to identify genes that may be affected by nutritional factors for further transcriptomic study. The several assays performed in the present thesis are described in details in material and methods section of the subsequent chapters.

2. Objectives

Pikeperch (*Sander lucioperca*) is recognized as one of the main freshwater species with a great potential for the expansion of the EU aquaculture industry, mainly because the good flesh quality and the high market value. Such as other new/ emerging species, pikeperch is fed with available diets designed for other well-established species, which may constraint the growth

performance, welfare and health. The main objective of this thesis is to improve the current knowledge of the nutritional requirements of pikeperch in order to develop species-specific feed that consider the nutritional needs at the early development stage. Moreover, larval fish diets provide a broad spectrum of nutrients, and the relative proportion of nutrients in the commercial feeds, plus how these nutrients are combined into the diet are important factors determining the fate of each nutrient. Therefore, this thesis has focused on understanding not only the effects of single nutrients, but also their interactions and relevance for physiological function and ultimately larval quality. To achieve the main goal of the present study, the following objectives were formulated:

1. Investigate the specific dietary requirements of phospholipids, fatty acids and minerals (Ca, P) and the effect on their interaction during early pikeperch ontogeny.
2. Determine optimal levels of SBL derived PL in formulated extruded starter feeds and the additional effects of n-3 LC-PUFA TAGs on performance and development of larval pikeperch.
3. Investigate the dietary effects of LC-PUFAs and their interactions with vitamins and minerals on larval development and performance, digestive capacity, and skeleton malformation.
4. 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, skeletal anomaly occurrence and lipid metabolism.
5. Understand how dietary DHA/EPA/ARA ratios affect tissue fatty acid profiles and antioxidant and stress response capacity, as well as the relationship between the skeletal anomaly occurrence and the stress status in pikeperch.
6. To assess the effect of the dietary Ca/P considering the dietary Ca and P levels with a view to determine their optimal levels.

3. Outline of thesis

The thesis starts with the introduction, which presents a general overview of the importance of nutrition in the aquaculture industry. The chapter (1) emphasises the importance of considering multinutrient interactions in nutritional studies, and ultimately current status of nutritional requirement knowledge in pikeperch larvae was reviewed.

Subsequently, the effect of selected nutrients and interactions at different larval organismic levels by anatomo-histological, biochemical and molecular biomarkers was studied. This was assessed by 4 experiments (chapters 3, 4, 5 and 6).

Chapter (3) is dedicated to investigate if an increase in dietary PL in the presence of supplemented DHA (as TAG) may increase both the *de novo* PL synthesis and the incorporation of LC-PUFAs (especially DHA) into the tissue molecules of both TAGs and phospholipids. Since a controversy exists about physiological and metabolic effects of dietary PL combined with LC-PUFAs supplemented as TAG.

Effect of LC-PUFAs combined with other macro and micro nutrients was studied in chapter (4). A multifactorial screening trial was conducted to determine the importance of various levels and interactions of major essential nutrients; LC-PUFAs (EPA+DHA and ARA), vitamins (Vit A, E, C, D) and minerals (Ca/P, Se). Results highlighted the major role of EPA+DHA and ARA interaction as well as Ca/P ratios during the early post-weaning period. In the subsequent chapters (5 and 6), further experiments were conducted as confirmatory experiments testing gradual levels of the dietary EPA+DHA/ARA (chapter 5) and Ca/P ratio (chapter 6).

In chapter (7) of this thesis, the general experimental design and findings are discussed. Conclusions are drawn from the findings of the preceding chapters. Ultimately, some perspectives for further studies are suggested.

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Chapter 3

Dietary phosphoglycerides and n-3 LC-PUFAs play important functions in the development of pikeperch larvae (*Sander lucioperca*). However, a controversy exists about physiological and metabolic effects of dietary PL combined with LC PUFAs supplemented as TAG. In the present study, we investigated if an increase in dietary levels of soy bean lecithin (SBL) derived phospholipids in the presence of supplemented DHA (as TAG) may increase both the *de novo* PL synthesis and the incorporation of LC-PUFAs (especially DHA) into the tissue molecules of both TAGs and phospholipids. Liver proteomic study can help to understand fish metabolism responses to dietary changes, to better understand the physiological mechanisms implied in the effect of PL and n-3 LC-PUFA on larval performance.

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The importance of phospholipids combined with long-chain PUFA in formulated diets for pikeperch (*Sander lucioperca*) larvae

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The importance of phospholipids combined with LC-PUFAs in formulated diets for pikeperch (*Sander lucioperca*) larvae.

Abstract

Dietary phosphoglycerides and n-3 long chain polyunsaturated fatty acids (LC-PUFAs) play important functions in the development of pikeperch (*Sander lucioperca*) larvae. The aim was to determine the optimal dietary levels of soy bean lecithin (SBL) derived phospholipids in formulated starter feeds for pikeperch larvae from 10 to 30 days post hatch (DPH) and examine larval performance and ontogeny by additional supplementation of n-3 LC-PUFAs in the form of Algatrium DHA 70 (glyceride product; 660-700 mg g^{-1} DHA; EPA 60-75mg g^{-1}). Six isoproteic and isoenergetic extruded diets were formulated with increasing levels of phospholipids (PL) i.e. (3.7%; 8.3 % or 14.5 % w.w., respectively). Three of the diets were additionally supplemented with three levels of Algatrium DHA 70 (0.6 %; 2.0% or 3.4%, respectively). Liver proteomic analyses of larvae at 30 DPH were included for effects of PL and primarily DHA on performance; physiological expression and interactions in larval proteins. Additionally, bone anomalies, digestive enzymatic activity, candidate gene expression and skeleton morphogenesis were examined. Results confirmed the importance of high dietary PL levels of at least 8.2 % w.w.; as well as a positive additional beneficiary effect of supplementation with DHA+ eicosapentaenoic (EPA). Thus, combined supplementation of SBL (up to 14.51% w.w. PL) with n-3 LC-PUFAs (1.004 % d.w. DHA and 0.169 % d.w. EPA) in the form of triacylglycerides (TAG) resulted in highest growth and lowest incidence of anomalies; improved digestive enzyme activity and had a differential effect on liver proteomics. The results denote essential fatty acids (EFA) be directly supplemented as triglycerides to have a beneficial effect in pikeperch larvae development.

Keywords: ***Sander lucioperca; Phospholipids; LC-PUFA and DHA, performance, proteomic expression, abnormalities*****.**

1. Introduction

Pikeperch (*Sander lucioperca*) is a freshwater species of high commercial interest in aquaculture with an increasing production in intensive recirculation systems[25]. Tailored commercial starter feeds have yet to be formulated for this species and among challenges in larval rearing are specific fatty acids (FA) dietary requirements during early ontogenetic development. Thus, dietary n-3 LC-PUFAs, such as eicosapentaenoic (EPA) and docosahexaenoic acids (DHA), play a critical role in performance, neurological development and stress resilience of pikeperch larvae and fry, dietary DHA deficiency provoking shock syndromes and large mortalities[40,62,63]. While other percid fishes, like the close relative walleye (*Sander vitreus*), seem to have a comparative lower LC-PUFA requirement[24] and Eurasian perch larvae (*Perca fluviatilis*) possess some capability to elongate and desaturate 18-C n-3 PUFAs to EPA and DHA[105], recent research has indicated no such ability in pikeperch larvae and the necessity of a dietary supplementation of DHA. Hence, recent *in vivo* [1-C-14] FA incubation studies in pikeperch larvae with use of labelled 18:3n-3; EPA concluded that most of n-3 elongation/desaturation products are consistent with the ELOVLS directly producing respectively 20:3n-3 from 18:3n-3 and 22:5n-3 and 24:5n-3 from EPA (unpublished data, pers. communication Rodriguez Covadonga; University of Las Palmas, Spain). An observed Δ6 desaturase enzyme activity was also consistent with a significant production of 18:4n-3 from 18:3n-3, however no further or any desaturation activity over EPA was evident, precluding the expression of Δ5 or even Δ4 desaturases in the larvae.

The essentiality of phosphoglycerides (phospholipids, PL) has been demonstrated since decades, exerting beneficial effects on growth, survival, stress resistance, enzymatic activity and performance of fish larvae or fry in a variety of marine and freshwater species [3,18,30,34,46,48,51,91,96, 98,111]. Also in pikeperch, increase in dietary PL improves growth and intestinal brush border membrane enzymatic activity, modulating expression of selected genes in liver [40,41]. The optimal dietary phospholipid levels for this species has been suggested to be at least 9.5 % D.W.[40], which is in the range of those reported for other freshwater and marine carnivorous species (5-12 % of dietary lipids)[16,48,98]. Dietary inclusion of phospholipids is typically in the form of soybean lecithin (SBL)[61,84], but also marine lecithins[83] and, occasionally, egg lecithin have been used[35]. Consequently, the content and proportion of lipid classes and LC-PUFAs may vary[47,95,98] and affect the optimal inclusion in the diet for a given species[16,35,36,86]. SBL contains typically 65-75% PL[89] with about 20-25 % each of the major phospholglycerides: phosphatidylcholine, phoshatidylethanolamine and phosphatidylinositol.

These are formed by L-glycerol with two FA esterified on positions 1 and 2, the second one typically with an unsaturated FA[98]. SBL contains no LC- PUFAs but up to 50-55% of 18:2n-6, and 4% of 18:3 n-3, together with 8-11 % each of 16:0, 16:1 and 18:1, depending on the commercial product(93).

Despite a general consensus about the positive influence of either dietary n-3 LC-PUFAs or phospholipids during pikeperch larval ontogeny, the optimal supplemented levels and the combined effects of dietary phospholipids and n-3 LC-PUFAs during exogenous feeding are still controversial. Thus, growth improvement of pikeperch larvae has been related to the positive effect of dietary levels of PL independently of LC-PUFA levels[40,42]. This observation is in agreement with studies on larvae of the freshwater carp (*Cyprinus carpio*) and the amphidromous ayu (*Plecoglossus altivelis*) where soybean lecithins, despite lacking n-3 LC-PUFAs, were as effective or even more effective than marine lecithins rich in n-3 LC-PUFAs[34,50]. In other studies, conducted with marine fish larvae, phospholipids were superior to neutral lipids (NL) as a source of EFA[47], and fish larvae reared on PLs rich in n-3 LC-PUFAs showed further enhanced growth, digestion and absorption as well as an increased lipid transport and uptake into larval membranes [37,46,86]. The provision of n-3 LC-PUFAs, as preformed intact PL (i.e. wild live preys such as copepods, marine lecithin, etc.) or as neutral lipids or TAG (i.e. enriched cultured live preys such as rotifers, microdiets, etc.) is likely a plausible explanation for the higher efficiency of the former in sustaining growth; survival; skeletal and ontogenetic development[4,16,37,54,55,76,82]. Ingested LC-PUFAs are hydrolysed and emulsified in bile salts and passively transported over the enterocytes as monoacylglycerol (MAG) or free fatty acid (FFA) and re-esterified or re-acylated to TAG or PL mainly on the sn-2 position. Although PL can be effectively synthesized by the enterocyte mainly by the glycerol-3-phosphate pathway, as well as by the monoacylglycerol pathway, the efficiency of these pathways can be markedly impaired by dietary lipids[12]. Moreover, fish larvae have a poor development of enterocytes and this may further reduce the ability to synthesize PLs *de novo*[21] and a low PL /TAG ratio is found to accumulate lipid droplets in the enterocytes[82] by inhibiting the assembly of lipoprotein particles (chylomicrons and VLDL) or transport mechanisms out of the enterocytes and consequently cause TAG to accumulate in the intestinal epithelium[61,71,76].

In the present study, we hypothesize that an increase in dietary PL in the presence of supplemented DHA (as TAG) may increase both the *de novo* PL synthesis and the incorporation

of LC-PUFAs (especially DHA) into the tissue molecules of both TAGs and phospholipids for optimal development and performance of pikeperch larvae during early larval development. Thus, the main objective was to determine optimal levels of SBL derived PL in formulated extruded starter feeds and the additional effects of n-3 LC-PUFA TAGs on performance and development of larval pikeperch. Since an increased dietary PL may reduce bone malformations[50,85,86] and liver proteomic studies can help to understand fish metabolism responses to dietary changes[1,80], to better understand the physiological mechanisms implied in the effect of PL and n-3 LC-PUFA on larval performance, studies on hepatic proteomics, expression of lipid metabolism related genes, digestive enzymes activity and skeleton morphogenesis were also conducted.

2. Experimental Methods

Experimental conditions

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 until hatching in upflow McDonald type incubators with a flow through system. Freshwater heated at 17.5°C from a temperature controlled 10 m^3 reservoir was constantly supplied. Hatched larvae were distributed into two 0.5 m^3 fibreglass tanks and kept until 10 DPH at 19.5-20.3°C and constant low intensity light. From 3-10 DPH larvae were fed newly hatched non-enriched *Artemia* nauplii (strain MC460, INVE Dendermonde, Belgium kept at 5° C after hatching) for 2 x 8 h through a peristaltic pump (Longer pumps BT300-2J) to secure that live *Artemia* nauplii were always present *ad libitum* (i.e. 1-3 mL^{-1}) 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. Pikeperch larvae were manually distributed into these tanks at density of 17 larvae/L and fed one of the 6 experimental dry diets until 30 DPH under a water temperature of 20-20.5°C and at dimmed light (30-40 lux at water surface) provided by electrical led bulbs. Daily, flow rate was adjusted at 15 L/h/tank and oxygen (7.8 ±0.4 mg/L^{-1}) and temperature (20.2±0.1 °C) were monitored by a hand-held oxymeter (Oxyguard, Birkerød, Denmark).

Six isonitrogenous and isoenergetic diets (Table 1) were formulated to contain 3 PL levels (PL1, PL2, PL3) adding 3,10 and 19 % SBL and these same PL levels combined with increased DHA levels (PL1H1, PL2H2, PL3H3) adding 0.55, 2, and 3.4% Algatrium DHA70. Experimental diets were manufactured by SPAROS (Olhão, Portugal). All powder ingredients were initially

mixed and grinded in a micropulverizer hammer mill (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 (P55, Italplast, Italy). The resulting pellets (0.8 mm) were dried in a convection oven for 3 h at 40ºC (OP 750-UF, LTE Scientifics, United Kingdom), crumbled (103, Neuero Farm, Germany) and mechanically sieved (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 the 6 experimental dry feeds together with newly non-enriched *Artemia* nauplii (INVE, Dendermonde, Belgium) from 8 a.m-22 p.m. During this period *Artemia* feeding was daily postponed by 1 hour. From 10-17 DPH larvae were fed 400-600 µm pellet size diet (initially further grinded in a mortar to 200-250 µm), gradually increased to 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 feeders (screw feeders, made by DTU Aqua), which allowed very small quantities to be fed at short specific time intervals (app. 15 min) during the entire feeding period. Dry feeds were administered at 25% of expected larval biomass in the first weeks and 10-15 % during the last week. Larvae were not fed 12 h prior to sampling for analytical purpose to ensure an empty gut. Tank bottom was daily vacuum cleaned to remove uneaten feed and to count dead larvae.

Table 1. Feed formulation and proximate analysis of the 6 experimental diets formulated to have several PL and DHA levels

Diet Ingredients (%)	PL1	PL2	PL3	PL1H1	PL2H2	PL3H3
MicroNorse fishmeal[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
Soybean 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 composition (% W.W.)						
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
EPA (% d.w. TFA[n])	0.164	0.097	0.08	0.30	0.174	0.169
DHA (% d.w. TFA)	0.39	0.228	0.174	0.977	0.815	1.004
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
(% 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
(% w.w.)						
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. 0.2 g ferric citrate. 5 mg potassium iodide. 15 mg manganese sulfate. 0.2 mg sodium selenite. 40 mg zinc sulfate. 0.6 g magnesium hydroxide. 1.1 g potassium chloride. 0.5 g sodium chloride. and 4 g calcium carbonate. [j] Soy lecithin powder: Lecico P700IPM. LECICO GmbH. Germany. [k] Binder (guar gum): HV109. SEAH International. France. [l]Antioxidant powder: Paramega PX. KEMIN EUROPE NV. Belgium. [m]Antioxidant liquid: NATUROX. KEMIN EUROPE NV. Belgium. [n]TFA: Total fatty acids, 34 analysed.

Larval sampling

A representative number of larvae (2 x 100) were sampled at hatching (0 DPH) and 10 DPH for FA analysis and 50 larvae sampled for initial weight measurement. At 30 DPH, 2 x 10 larvae per replicate tank were sampled for phospholipid and FA analysis. 10 larvae per tank were sampled for analysis of digestive enzymes at 30 DPH. In addition, 2 x 10 larvae per replicate were sampled for proteomics at 30 DPH. All larvae were sacrificed by an overdose of benzocaine and immediately frozen and kept at –80°C until analysis. At 30 DPH additionally 10 larvae per tank were sampled for larval organ and skeleton development for specific staining of bone and cartilage and stored in 10 % phosphate buffered formaldehyde. In order to quantify the gene expression involved in skeletal development 10 larvae per replicate were sampled and stored in RNA-later overnight and frozen at -80 °C until analysis.

Larval survival and growth

Daily mortality was assessed by the number of registered dead larvae, whereas the total number of alive larvae manually counted at the end of the trial was considered as the final survival in each tank. Growth was determined by individually weighing 100 randomly sampled larvae at 10 DPH and 35 larvae per replicate tank at 30 DPH. Each anaesthetized larvae were wiped for any excess water (Medical Care tissue cloth) and weighed to the

nearest 0.01 mg on a Mettler Toledo At 200. Mean larval weight at 10 DPH was 1.95 ± 0.31 mg w.w. and 0.56±0.09 mg d.w. Specific daily growth rate SGR (% day^{-1}), was calculated according to the equation; SGR: (ln (final average body weight of sampled larvae) – ln (initial average body weight of sampled larvae)) x 100/feeding days.

Biochemical analysis

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), crude lipid [7]. 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 1).

The fatty acid composition in diets (Table 2) and larvae (Table 3) was determined by lipid extraction with a chloroform/methanol mixture[31] and either sonicated in an ultrasound cleaner, model Branson, 2510, or homogenised (larger larvae) by a tissue-tearor probe diameter 4.5 mm (Biospec Products, Inc, Bartlesville, USA). Trans-esterification of the lipids was done by acetyl chloride in methanol at 95 °C. The fatty acid methyl esters were analysed 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 FA 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 FA were analysed, with the 20 most relevant FA shown (Table 2; Table 3). Total FA (TFA) differed between the diets and decreased by substitution of olive oil with SBL. The main differences in dietary FA profiles (% TFA) were related to oleic acid (18:1n-9), linoleic acid (18:2n-6), α-linolenic acid (18:3n-3), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and consequently total MUFAs, n-6 PUFAs and n-3 PUFAs (Table 2). 18:2n-6 and 18:3n-3 content increased by the increased supplementation of SBL, for which 18:2n-6 is the dominant FA (around 55 % TFA, data not shown) with the additional content of some 18:3n-3 (3% TFA). Diets PL1H1-PL3H3 contained significantly increased levels of EPA and DHA (as compared with diets (PL1-PL3) due to the supplement of Algatrium DHA 70. 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 ([69]). 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 an 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 corresponding 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. The integrated signals of the test substance and of the internal standard TPP (triphenylphosphate) were used for calculation. The dietary analytical content of the three main phospholipid classes identified increased as expected by dietary incorporation of SBL (Table 1). Total PL content ranged from 3.7 % (% of lipids) for PL1 and PL1H1 to approximately 14.4-14.5 % for diets PL3 and PL3H3

Table 2. 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 (Mean values and standard deviations, n=2)

Diet	PL1	SD	PL2	SD	PL3		PL1H1		PL2H2		PL3H3	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TFA	109.4	5.7	54.3	26.0	34.1	8.2	187.8	30.6	69.7	9.6	46.9	6.9
FA												
14:0	0.7	0.0	0.8	0.0	1.0	0.0	0.7	0.0	0.79	0.0	1.0	0.0
16:0	13.0	0.2	14.6	0.1	17.8	0.1	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	0.22	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

Table 3. Analysed TFA content and FA composition (% of TFA) of total lipids in pikeperch larvae at 0 DPH and 10 DPH, as well as in 30 DPH (mg g-1 w.w.), after 20 days of feeding experimental dry diets containing different PL and DHA levels. (Mean values and standard deviations, n= 3)

Larval age	DPH 0		DPH 10		DPH 30											
Diet					PL1		PL2		PL3		PL1H1		PL2H2		PL3H3	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TFA (mg g^{-1} w.w.)	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 (% TFA)																
14:0	0.9	0.0	0.5	0.0	0.4	0.0	0.3	0.2	0.6	0.1	0.4	0.0	0.5	0.1	0.6	0.1
16:0	11.9	0.9	13.5	0.6	13.5^{b}	0.1	14.5^{b}	1.5	18.3^{a}	0.4	13.1^{b}	0.3	13.9^{b}	0.5	14.8^{b}	0.6
18:0	3.3	0.2	7.2	0.2	4.4^{c}	0.0	4.2^{bc}	0.1	4.5^{c}	0.3	3.7^{b}	0.2	3.0^{a}	0.1	3.0^{a}	0.2
20:0	0.1	0.0	0.2	0.0	0.1^{b}	0.0	0.1^{ab}	0.0	0.1^{a}	0.0	0.1^{b}	0.0	0.1^{a}	0.0	0.1^{a}	0.0
22:0	0.2	0.1	0.4	0.0	0.1^{b}	0.0	0.1^{b}	0.0	0.1^{ab}	0.0	0.1^{b}	0.0	0.0^{a}	0.0	0.0^{a}	0.0
24:0	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
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^{e}	0.5	43.8^{d}	0.8	24.7^{b}	0.8	50.3^{e}	1.8	37.4^{c}	1.1	12.8^{a}	1.0
20:1 (n-9)	1.1	0.1	0.5	0.0	1.1^{c}	0.0	1.2^{c}	0.0	1.0^{b}	0.0	1.1^{c}	0.0	1.0^{b}	0.0	0.8^{a}	0.0
22:1(n-9)	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0
24:1(n-9)	7.3	0.7	1.9	0.1	2.3^{ab}	0.1	2.0^{a}	0.1	2.4^{b}	0.1	2.5^{b}	0.2	3.1^{c}	0.2	5.2^{d}	0.2
Total MUFAs	27.3	0.2	28.1	0.1	55.6^{e}	0.4	48.4^{d}	0.6	29.7^{b}	0.4	55.7^{e}	1.7	43.2^{c}	1.0	20.2^{a}	0.9
18:2(n-6)	8.3	0.6	7.4	0.2	11.0^{a}	0.1	18.7^{b}	0.2	29.1^{c}	0.4	10.7^{a}	0.0	19.2^{b}	0.1	28.5^{c}	0.5
18:3(n-6)	0.1	0.0	0.5	0.0	0.2^{ab}	0.0	0.3^{bc}	0.0	0.6^{d}	0.0	0.2^{a}	0.0	0.3^{c}	0.0	0.3^{c}	0.0
20:2 (n-6)	0.0	0.0	0.2	0.0	0.1^{a}	0.0	0.1^{a}	0.1	0.3^{b}	0.0	0.1^{a}	0.0	0.2^{a}	0.0	0.2^{ab}	0.0
20:3(n-6)	0.0	0.0	0.2	0.0	0.3^{b}	0.0	0.3^{b}	0.0	0.6^{d}	0.0	0.2^{c}	0.0	0.2^{c}	0.0	0.1^{a}	0.0
20:4(n-6) ARA	1.2	0.0	0.9	0.0	0.5^{bc}	0.0	0.3^{a}	0.1	0.4^{ab}	0.1	0.4^{b}	0.0	0.4^{ab}	0.0	0.6^{c}	0.1
Total (n-6) PUFA	9.7	0.6	9.3	0.3	12.1^{a}	0.1	19.6^{b}	0.1	31.0^{e}	0.4	11.6^{a}	0.1	20.2^{c}	0.1	29.7^{d}	0.5
18:3(n-3)	1.1	0.0	27.1	0.3	1.3^{ab}	0.1	1.3^{ab}	0.0	1.9^{c}	0.1	1.1^{a}	0.2	1.5^{b}	0.1	1.9^{c}	0.2
20:3(n-3)	0.1	0.1	1.3	0.0	0.1^{c}	0.0	0.1^{a}	0.0	0.1^{b}	0.0	0.1^{abc}	0.0	0.1^{ab}	0.0	0.1^{ab}	0.0
20:5(n-3) EPA	7.9	0.3	2.8	0.2	2.5^{a}	0.1	2.2^{a}	0.2	2.6^{a}	0.2	2.5^{a}	0.2	2.6^{a}	0.3	3.9^{b}	0.2
22:6(n-3) DHA	37.1	0.3	8.5	0.8	9.4^{a}	0.3	8.6^{a}	0.8	10.6^{b}	0.4	11.2^{b}	0.9	14.6^{c}	1.1	25.4^{d}	0.8
Total (n-3) PUFA	46.1	0.5	39.7	0.7	13.3^{a}	0.3	12.2^{a}	1.0	15.3^{b}	0.7	14.8^{ab}	1.2	18.7^{c}	1.4	31.2^{d}	0.8
DHA/EPA	4.7	0.1	3.1	0.0	3.8^{a}	0.0	3.9^{ab}	0.2	4.2^{b}	0.0	4.5^{c}	0.0	5.7^{d}	0.3	6.6^{e}	0.2
(n-3)/(n-6)	4.8	0.3	4.3	0.1	1.1^{b}	0.0	0.6^{a}	0.1	0.5^{a}	0.0	1.3^{c}	0.1	0.9^{b}	0.1	1.1^{b}	0.0

DPH: days post hatch. Values (for larvae fed the experimental diets (30 dph) in a row followed by a different superscript are significantly different P<0.05

Digestive enzymes activity

The heads and tails of pikeperch (*Sander lucioperca*) larvae were removed to isolate the digestive segment on a glass maintained on ice (0 °C), and the 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 & Kim[73] using leucine-alanine (Sigma-Aldrich, St Louis, MO, USA) as substrate. Alkaline phosphatase (AP) and aminopeptidase N (AN), two enzymes of brush border membrane, were assayed according to Bessey et al[6] and Maroux et al.[66] using p-nitrophenyl phosphate (Sigma-Aldrich) and L-leucine p-nitroanalide (Sigma-Aldrich) as substrates, respectively. Pepsin was assayed by the method of Worthington[103] modified by Cuvier-Péres and Kestemont[23]. Trypsin and amylase activities were assayed according to Holm et al.[45] and Metais and Bieth[70], respectively such as described by Gisbert *et al*.[38]. Protein was determined using the Bradford procedure[11]. Enzyme activities are expressed as specific activities (U or mU mg protein^{-1}).

Skeleton anomalies and related genes 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[49] modified from previous studies [101]. Classification of skeletal anomalies was conducted according to Boglione et al.[10]. 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). Total body tissue was homogenised 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% etanol 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). 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 β-actin 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 analysed 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 normalised 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). Detailed information on primer sequences and accession numbers is presented in Table 6.

Proteomics

Slightly thawed larvae (30 DPH) were dissected on a glass plate maintained at 0°C. The liver was extracted and immediately frozen in liquid nitrogen and kept at -80°C until analysis. 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 an appropriate volume of 50mM NaOH and protein concentration was evaluated using a Pierce 660 nm protein assay kit as described by the manufacturer. Minimally labelling of samples (containing 25 µg of solubilized 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 (75 µg) were added v:v reducing buffer (7M urea, 2M thiourea, 2% DTT, 2% CHAPS, 2% IPG 4–7 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) and 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).

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 [(53)] and the Protein Prophet algorithm[(72)], respectively. Proteins were functionally annotated using AgBase-Goanna[(68)] as described in Roland *et al.*[(79)]. The GO categories for biological processes were then sorted to determine the most common ones represented by the proteins identified. The mean number of proteomic spots detected per gel was 1917 ± 498. A one-way analysis of variance among the six experimental groups revealed 27 spots with differential intensity at $P<0.05$ (supplementary figure S1). These spots were selected for statistical analyses and MS/MS identifications. Of the 27 spots picked up for mass spectrometry analysis, 17 spots contained one protein identification per spot and this led to the identification of 15 different proteins.

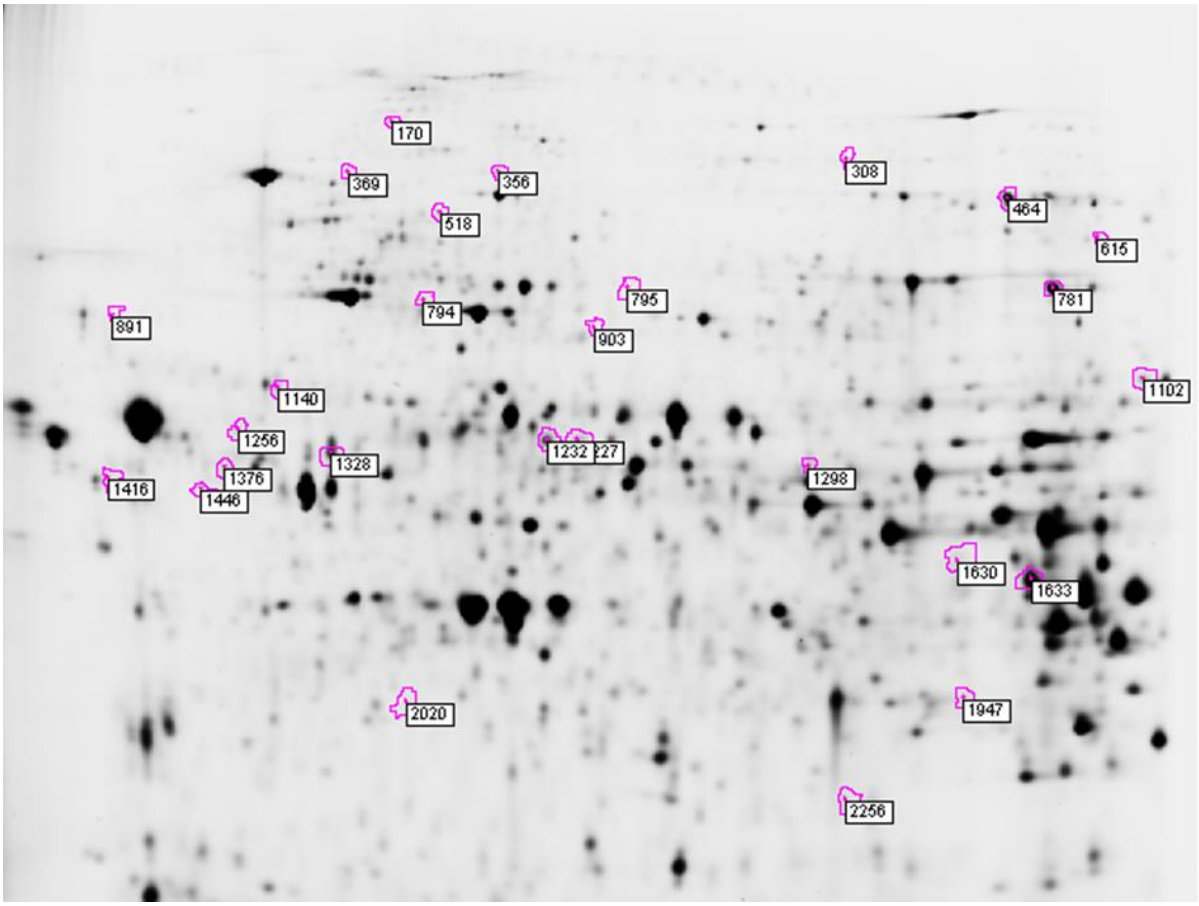

Fig. S1. Representative two-dimensional gel electrophoresis of differential protein expression profile in pikeperch liver

Statistics

Percent data were arcsine transformed prior to analysis. All experimental data were subjected to a one-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. 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.

3. Results

3.1. Larval survival and growth

From 15 DPH, there was an increase in daily larval mortality (data not shown), regardless tanks and dietary codes. At the end of the trial (30 DPH), the sum of daily registered mortality suggested a survival ranging 57-61% for the different tanks and without significant differences among dietary treatments ($P \geq 0.553$). However, based on the remaining number of alive larvae at the end of the trial, actual survival was much lower (10-14%) but neither statistically different ($P \geq 0.633$).

Mean final larval weight at 30 DPH ranged 92-154 mg w.w. (Fig.1). Dietary inclusion of phospholipids resulted in an increase in body weight and additional supplementation of DHA (+EPA) in the form of Algatrium DHA 70 further increased weight. Thus, larvae fed the highest DHA and PL levels (diet PL3H3) showed the highest mean body weight, being significantly larger than in those fed PL1 ($P<0.001$); PL2 ($P \leq 0.01$) and PL1H1 ($P<0.001$). PL2H2 larvae were larger than those fed PL1 ($P<0.03$) and PL1H1 ($P \leq 0.001$). Finally, PL2 and PL3 larvae were both larger than larvae fed PL1H1, ($P \leq 0.04$ and $P \leq 0.01$, respectively). Overall, larval growth calculated as SGR was lowest for PL1H1 (21.2% day^{-1}) and highest for PL3H3 (24.3% day^{-1}) (Fig. 1).

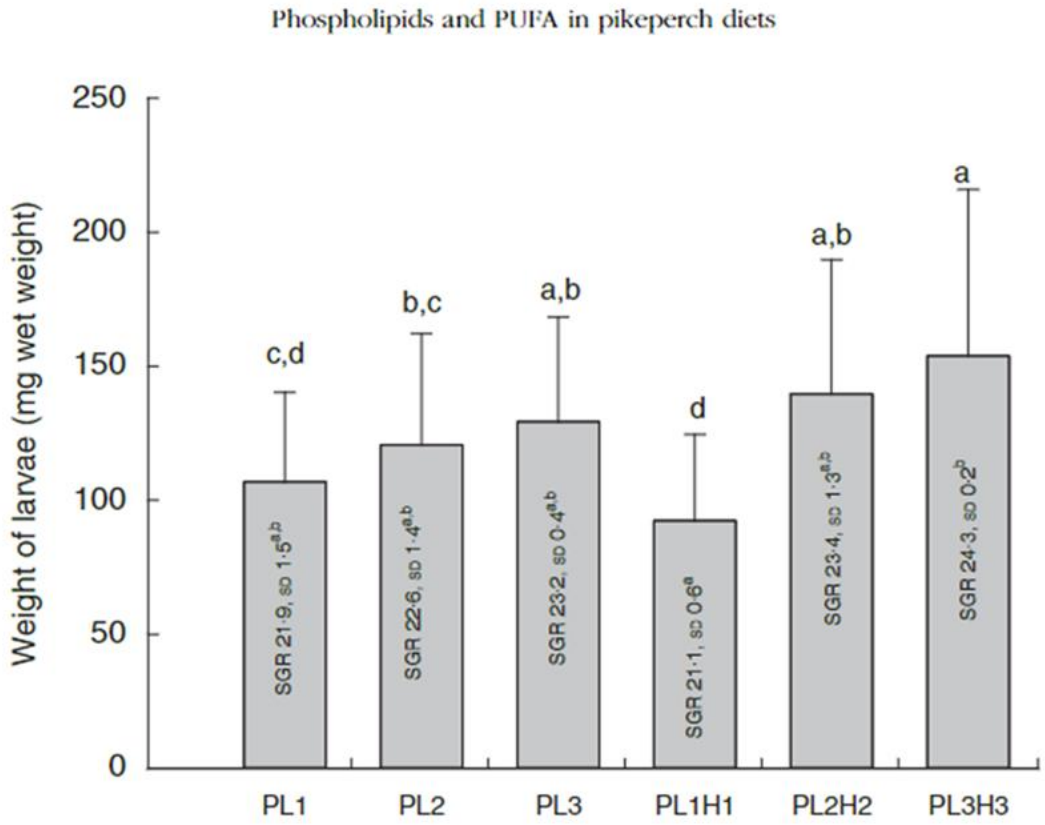

Fig.1. Mean final larval body weight (mg w.w.; bars) fed experimental diets with different PL and DHA levels for 20 days (10-30 DPH). Final body weight values are pooled means of 3 tank replicates (n 35 fish per tank) with standard deviations represented as vertical bars. SGR (% day^{-1}, values inside each bar, n 3 replicate tanks with standard deviations. Values for each diet group with a different superscript are significantly different ($P<0.05$).

3.2. Biochemical analysis

There was a marked decrease in larval EPA and DHA larval composition from 0 to 10 DPH and a corresponding increase in stearic acid (18:0), oleic acid (18:1) and 18:3n-3 (Table 3). Larval FA content at 30 DPH reflected 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 3). The highest inclusion of Algatrium DHA 70 caused an increase of DHA content in larvae of PL3H3 about 2.5 times higher that of PL3 fed larvae, while it was 1.5 times higher for EPA. An overall very significant larval/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, larval DHA: EPA was significantly correlated to diet DHA: EPA ($P<0.001$, $R^2=0.97$).

Phosphatidylcholine (PC) was the major phospholipid class in diets and larvae (Fig. 2). In larvae it constituted between 68-73 % of the PL with no significant differences between groups, but with a positive PC diet:PC larvae correlation by an increased dietary SBL inclusion (Fig.

3a). For phosphatidylethanolamine (PE) a similar positive PE diet: PE larval correlation for larvae from PL1 to PL2 and PL3 (Fig. 3b). However, for larvae reared on PL1H1, PL2H2 and PL3H3 a negative correlation with dietary content was observed (Fig. 3). Moreover, in larvae fed diet PL3H3 there was a significantly lower content of PE, than larvae fed PL1, PL2 and PL3 ($P<0.05$, Fig. 2). Similarly for phosphatidylinositol (PI) a slight positive PI diet: PI larval correlation for PL1-PL3, but a strong negative PI diet: PI larval correlation for PL1H1-PL3H3 (Fig.3c) with no significantly difference between dietary groups. For other minor phospholipid classes analysed, there were no significant differences among content in larvae between groups, while analysed content in all diets were below detection.

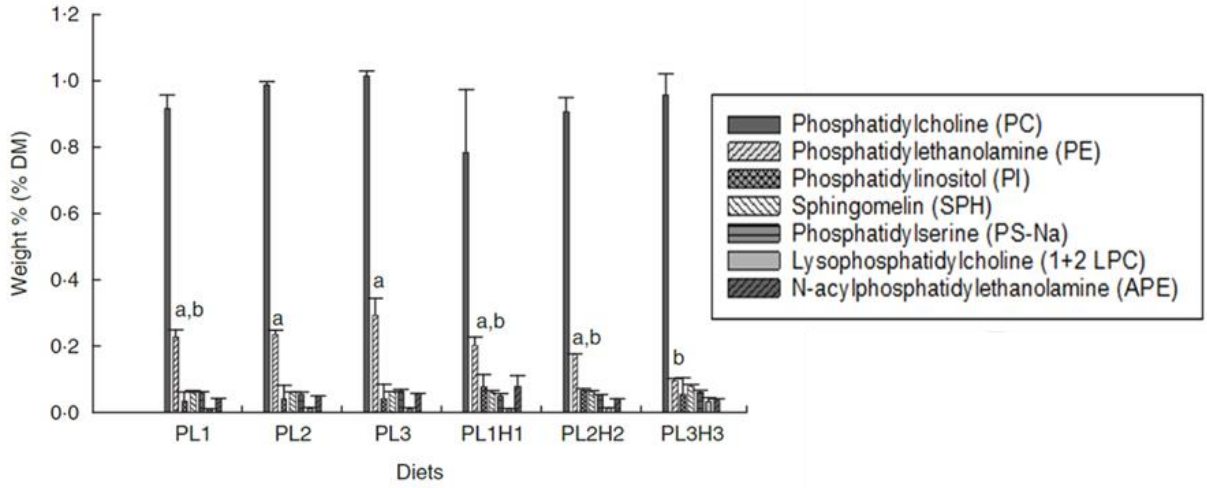

Fig.2. Phospholipid class composition in larvae (% DM mean values and standard deviations, n3 replicate tanks) fed experimental diets for 20d (10–30 DPH). PL1, PL2 and PL3 (soyabean lecithin (SBL): 3, 10 and 19 %). PL1H1, PL2H2 and PL1H3(SBL: 3, 10 and 19%) + Algatrium DHA 70: 0·55, 2·05 and 3·4%.

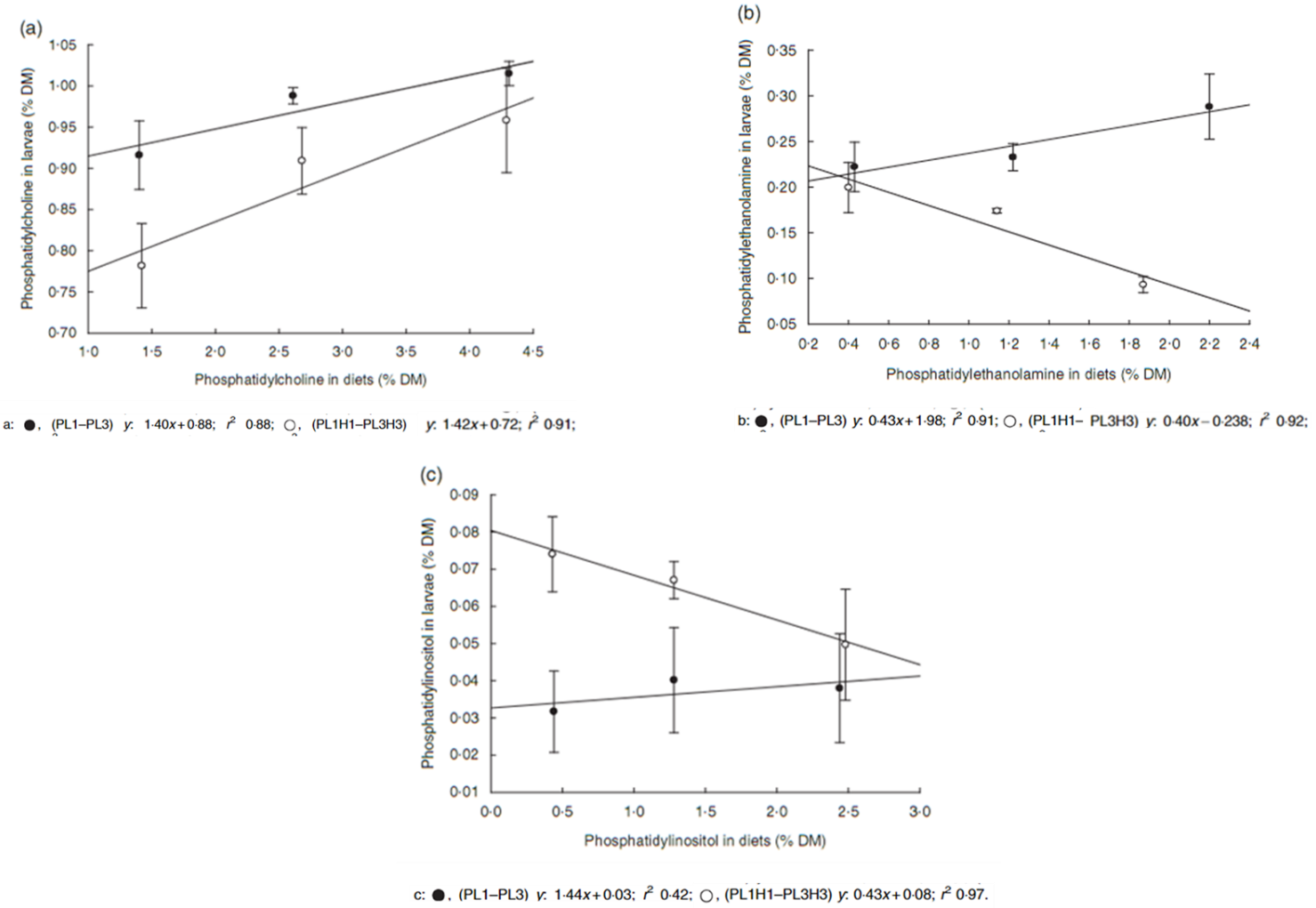

Fig. 3. (a–c) Dietary: larval tissue phospholipid class correlation when fed experimental diets for 20d (10–30 DPH). Values are means (n3) replicate tanks.

3.3. Digestive enzymes activity

No significant differences were found in pepsin activity (Table 4), despite a general trend for a higher activity in pikeperch fed increased PL and DHA contents. In this sense, a significant positive linear correlation was found among the two parameters (y=0.4971x+45.397; r^2=0.934) (Fig. 4). Trypsin activity, was significantly higher in fed PL1H1 than in those fed PL2, PL3 and PL3H3 (P=0.003; Table 4). Results showed that an increase in DHA, particularly, from PL2 to PL2H2 reduced significantly trypsin activity. In addition, a dietary increase in both PL and DHA from PL1H1 to PL3H3 significantly reduced trypsin activity (P=0.003). On the other hand, despite the increase in dietary PL from PL1 to PL3 it did not significantly affect larval trypsin activity (Table 4). Aminopeptidase activity was lowest in larvae fed PL2 compared to those fed PL3, PL2H2 and PL3H3 (P=0.001). An increase in dietary PL, particularly, from PL2 and PL1H1 to PL3H3 significantly increased aminopeptidase activity. Alkaline phosphatase was significantly highest for PL3H3 larvae than for PL1H1 larvae (P<0.038). Thus, despite the increase in dietary PL from PL1 to PL3 it did not significantly affect larval alkaline phosphatase activity, but a dietary increase in both PL and DHA from PL1H1 to PL3H3 significantly increased the activity of this enzyme.

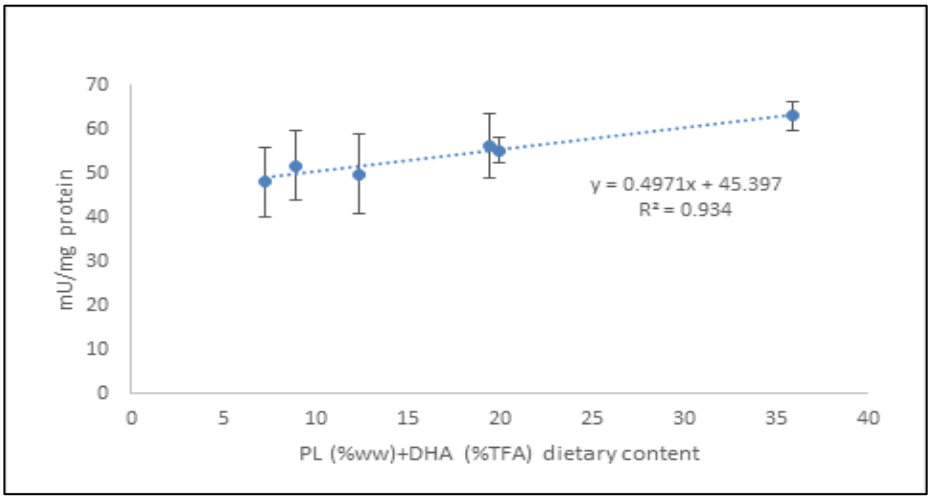

Fig.4. Effect of dietary PL (% w.w.) and DHA (%TFA) on pepsin specific activity in pikeperch larvae after 20 days of feeding experimental dry diets. Values are means (n 3) replicate tanks; standard deviation represented by vertical bars.

Table 4. Larval specific enzymatic activity (mU mg protein^{-1}) in 30 DPH pikeperch larvae after 20 days feeding experimental dry diets containing different PL and DHA levels. (Mean values and standard deviations, n=3)

Diet	PL1		PL2		PL3		PL1H1		PL2H2		PL3H3	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
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^{abc}	3.1	6.4^{a}	1.7	8.5^{ab}	2.3	16.4^{c}	3.9	14.2^{bc}	1.7	8.8^{ab}	1.0
Aminopeptidase N (mU mg protein^{-1})	5.9^{abc}	0.8	3.3^{a}	0.6	6.9^{bc}	0.3	5.3^{ab}	0.7	6.2^{bc}	0.5	8.0^{c}	1.9
Alkaline phosphatase AP (mU mg protein^{-1})	24.7^{ab}	2.8	20.8^{ab}	4.8	21.1^{ab}	3.2	18.7^{a}	1.0	20.5^{ab}	3.7	28.8^{b}	3.9
Leu-Ala (U mg protein^{-1})	376.2	39.0	350.9	39.9	395.22	36.2	371.0	52.3	364.4	14.0	361.1	58.9
Amylase (U mg protein^{-1})	5.2	0.6	4.4	0.7	5.4	0.5	4.70	0.5	4.5	0.9	4.3	1.0

Values in a row followed by a different superscript are significantly different P<0.05

3.4. Skeleton anomalies and related genes 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 (Table 5). 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 (Table 5). Dentary bones anomalies tend to be reduced by the increase in dietary PL and were significantly lowest in PL3 and PL3H3 pikeperch and highest in PL2 (Table 5). A similar trend was found in branchiostegal rays (Table 5) and cranial anomalies incidences (Table 5) 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.

Table 5. Incidence of different bone anomalies (%) in 30 DPH pikeperch larvae after 20 days feeding experimental dry diets containing different PL and DHA levels (Mean values and standard deviations, n= 3)

Diet	PL1		PL2		PL3		PL1H1		PL2H3		PL3H3	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Prevalence of Anomaly Type (%)												
Severe	86.7^{a}	18.9	81.5^{a}	13.9	54.2^{ab}	13.7	96.7^{a}	4.7	75.1^{ab}	11.4	35.2^{b}	10.9
Maxilary	0.0^{b}	0.0	0.0^{b}	0.0	0.0^{b}	0.0	6.7^{a}	4.7	0.0^{b}	0.0	0.0^{b}	0.0
Dentary	26.5^{ab}	11.6	59.1^{a}	15.8	19.1^{b}	7.1	35.2^{ab}	7.3	25.3^{ab}	9.1	18.8^{b}	14.9
Branchiostegal rays	76.3^{b}	8.2	17.0^{a}	11.9	3.3^{a}	3.3	90.0^{b}	5.8	18.6^{ab}	7.4	0.0^{a}	0.0
Cranium anomalies	86.7^{a}	18.9	66.5^{ab}	17.6	25.5^{b}	11.2	96.7^{a}	4.7	61.0^{ab}	19.8	18.8^{b}	14.9
Cephalic v. kyphosis	15.1	8.3	0.0	0.0	0.0	0.0	3.0	3.0	0.0	0.0	0.0	0.0
Pre-haemal v. lordosis	0.0	0.0	0.0	0.0	0.0	0.0	9.4	5.3	7.5	3.8	0.0	0.0
Pre-haemal v. anomalies	0.0	0.0	2.8	2.8	0.0	0.0	3.3	3.3	3.7	3.7	0.0	0.0
Haemal v. lordosis	10.3	5.2	6.5	3.3	0.0	0.0	9.7	5.8	0.0	0.0	3.3	3.3
Haemal v. scoliosis	0.0	0.0	5.6	5.6	3.3	3.3	12.7	6.4	3.3	3.3	0.0	0.0
Caudal v. fusion	6.7	6.7	0.0	0.0	16.4	12.1	0.0	0.0	8.3	8.3	13.0	3.5
Caudal v. anomaly	0.0	0.0	9.4	5.8	12.7	6.4	9.7	5.8	12.0	7.2	0.0	0.0
Caudal v. scoliosis	24.6	17.0	16.7	9.6	6.1	6.1	9.4	5.3	22.0	5.7	10.0	5.8

Values in a row followed by a different superscript are significantly different P<0.05

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 (Table 6).

Table 6. a) Gene expression in 30 DPH pikeperch larvae after 20 days feeding experimental dry diets containing different PL and DHA levels, b) Sequences of primers used for gene expression analysis. (Mean values and standard deviations, n=3)

a)

Diet	PL1		PL2		PL3		PL1H1		PL2H2		PL3H3	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Alp	2.03	2.70	0.81	0.28	1.05	0.85	3.02	2.08	0.68	0.79	1.28	1.47
Twist2	1.33	1.18	0.24	0.07	0.79	0.79	0.51	0.48	0.15	0.09	0.08	0.02
Mef2c	1.15	0.75	0.41	0.14	1.41	1.55	0.61	0.52	0.12	0.07	0.07	0.02
Sox9	1.11	0.64	0.53	0.13	1.08	0.78	1.52	1.93	0.27	0.19	0.14	0.01

b)

Gene	Nucleotide Sequence
Alp	F: 5'-GCTGTCCGATCCCAGTGTAA-3'
	R: 5'-CCAGTCTCTGTCCACACTGT-3'
Twist2	F: 5'-CCCCTGTGGATAGTCTGGTG-3'
	R: 5'-GACTGAGTCCGTTGCCTCTC-3'
Mef2c	F: 5'-GCGAAAGTTTGGCCTGATGA-3'
	R: 5'-TCAGAGTTGGTCCTGCTCTC-3'
Sox9	F: 5'-TCCCCACAACATGTCACCTA-3'
	R:5'-AGGTGGAGTACAGGCTGGAG-3'

3.5. Proteomics

Of 15 different identified proteins 9 proteins displayed a differential intensity between treatments (Tukey post-hoc test ($P<0.05$), Table 7). Fatty acid synthase (FAS) is primarily responsible for the synthesis of palmitate (16:0); (i.e. saturated FA with 12, 14 or 16 carbons can be synthesized), that can be further elongated and/or desaturated being a main reactor on lipogenesis and fatty acid synthesis. Among the identified proteins, FAS was significantly down-regulated in PL3H3 larvae compared to larvae of PL1, PL2, and PL1H1 (4.36, 3.65, and 3.50-fold respectively, $p<0.01$), suggesting a negative relation with DHA levels in larvae (Table 3). Besides, for a given dietary PL content (14.4%), an increase in total dietary n-3 LC-PUFA from 10.5 to 27.9% (i.e. Table 2) significantly down-regulated FAS expression, this protein being over-expressed in PL3 larvae compared to PL3H3 (3.54-fold, $p=0.002$). However, the analysis showed that, for the same level of low total n-3 PUFA content (7.7%), the increase in dietary PL content did not affect significantly FAS expression (comparison between PL2, PL1H1 larvae, Table 2).

Table 7. Protein differentially expressed in liver of 30 DPH pikeperch larvae after 20 days feeding experimental dry diets containing different PL and DHA levels

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

Another protein related to lipid metabolism, ATP-citrate synthase, was also under-expressed in PL3H3 larvae compared to PL2 larvae (2.60-fold, $p<0.05$). Similarly, a high PL and LC-PUFA dietary content (diet PL3H3) led to an under expression for spot 1633 in which two lipid transfer proteins were identified, the nonspecific lipid transfer protein and the hydroxysteroid

dehydrogenase-like protein 2 (HSDL2), as compared to PL1H1 (1.63-fold, p =0.03). In comparison to larvae reared on 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 larval group PL3H3 and PL1 (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 displaying a differential intensity being lower in larvae fed PL1 compared to PL2H2 and PL2 treatment (1.99- and 1.85-fold, p=0.005). Larvae fed on PL2 showed also an increase in its abundance compared to PL1H1 fed larvae (1.67-fold, p=0.005). Lastly, larvae reared on PL1 showed an increase in abundance for a spot identified as Keratin – a cytoskeletal and structural protein – in comparison with PL2H2 and PL3H3 larvae (2.27 and 2.33-fold respectively, p=0.03).

4. Discussion

Survival was reduced by cannibalism in all tanks, in agreement with previous observations [40,62]. Nevertheless, larval growth was generally high even when fish were fed the lowest PL and n-3 LC-PUFA dietary levels. Besides, an increase in dietary PL levels up to 8% markedly enhanced larval weight, in agreement with a recent unpublished study with these same diets, where pikeperch fed the lowest PL levels (3.70 and 3.73%) showed a significantly lower growth. However, elevation of dietary PL from 8- to 14.5% did not further increase fish growth significantly. These results are in agreement with the optimum PL requirement previously defined for pikeperch (9.5%) fed SBL [40]. Similarly, PL requirements for gilthead seabream (*Sparus aurata*) fed SBL were around 8%, when larvae were fed 5 dietary PL levels (4.9-9.6%)[84]. Pikeperch growth promotion by an increase in dietary PL may be related to a higher feed intake as shown in prawn (*Penaeus monodon*) and gilthead seabream[47,58,97]. Moreover, an increase in dietary PL tended to increase larval PC contents. This increase in PC at higher dietary PL contents would be in agreement with previous studies showing that PC is the main product of PL synthesis in fish enterocyte[12] and comprises up to 95% of the PL found in Very Low Density Lipoprotein (VLDL)[60]. Moreover, PC has been long considered the most effective PL to promote larval growth[48]. For instance, dietary supplementation with PC, but not PE, stimulated feeding activity in gilthead seabream larvae, probably through the ingestion

of the trimethyl group of the choline base of PC that binds to receptor gustatory cells[39]. Nevertheless, growth improvement in pikeperch larvae fed increased PL dietary levels could also be related to an improved lipid transport in agreement with previous studies. For instance, in gilthead seabream, fish fed diets without lecithin accumulated lipid vacuoles in the basal zone of intestinal enterocytes and caused steatosis in hepatic tissue, increase in SBL reduced accumulation[82] and enhanced lipoprotein synthesis[61].

An increase in dietary PL from 3.72 up to 8.19% did not significantly affect the incidence of anomalies in agreement with previous studies with pikeperch and similar PL levels (1.4-9.5%) supplementing SBL[40]. Similar results were found in gilthead seabream, where an increase in dietary SBL did not reduce bone anomalies.[86] However, in the present experiment a further dietary PL increase up to 14.38 significantly reduced the incidence of severe skeletal anomalies, particularly in fish fed increased DHA dietary levels. This was more evident on anomalies affecting endochondral bones, such as the cranium, where an increase in dietary PL reduced the prevalence of these anomalies. These results are in agreement with those obtained in European sea bass (*Dicentrarchus labrax*)[14], although the incidence of anomalies was much lower in seabass than in pikeperch. It should be noticed, that the seabass study used higher levels of phosphatidylcholine and phosphatidylinositol (35 and 16 mg/kg respectively). Bone anomalies reduction by PL was not related to the relative expression of the bone-development related genes studied such as *alp* (early mineralization indicator), *twist2*, *mef2c* or *sox9* (endochondral bone development-related genes). This lack of effect could be due to an earlier origin of the anomalies during the first part of the feeding trial, when cartilage was being developed and bones ossified. At the final sampling (30 DPH), bone mineralization in pikeperch was almost completed. Indeed, in gilthead seabream an increase in SBL up to 12% significantly up-regulated *alp*[84]. Reduction of bone anomalies by increased PL dietary contents has been related to the specific increase in dietary PI[87], as the second messenger inositol-3-phosphate, regulates calcium mobilization from the endoplasmic reticulum[98] and would stimulate osteocalcine production[48].

In addition to growth improvement, dietary SBL supplementation markedly affected pikeperch larvae fatty acid composition, elevating the 18:2n-6, an essential fatty acid for freshwater fish, as well as 18:3n-6 and 20:3n-6, products of delta-6- desaturase and elongase activity. However, 20:4n-6, a product of delta-5- desaturase activity was not increased, in agreement with the low LC-PUFA biosynthetic ability of pikeperch larvae (unpublished results).

Combined supplementation of SBL up to 14.51% d.w. PL with n-3 LC-PUFA (1.0% d.w. DHA and 0.16% d.w. EPA; i.e 1.17 % LC PUFA) in the form of triglycerides lead to the highest growth and lowest anomalies incidence, improving digestive enzymes activities and liver proteomics. This could be related to either a specific effect of the FA or a combined effect of PL and FA. The latter seems likely, as diets with lowest PL inclusion (3.70 and 3.73%) but different LC PUFA content (i.e. 0.17 % EPA+ 0.39% DHA vs. 0.3 % EPA + 0.98% DHA) both lead to a relatively lower growth. A negative effect on growth by a high inclusion level of olive oil; 18:1n-9 in both of these diets, however, cannot be excluded as the gradual substitution of olive oil with soy lecithin caused a 3-fold increase in tissue linoleic acid, 18:2n-6 levels and a concurrent two-fold decrease in tissue oleic acid, 18:1n-9 content. 18:2n-6 may have a growth promoting effect or a different metabolic fate as compared to 18:1n-9, despite C14 labelled FA analyses have indicated no elongation or desaturation capability in pikeperch larvae (unpublished results).The optimal levels of EPA and DHA in marine fish larvae has been estimated to be about 3 % of dietary dry matter[88], whereas for pikeperch larvae an optimal level of 1.2% DM has been suggested, based on experiments with SBL without n-3 LC-PUFA and fish gonad lecithin with n-3 LC-PUFA[42]. 1.2 % LC- PUFA is very similar to the 1.17% estimated to be optimal in this experiment; - in the presence of 14.5 % PL supplemented as SBL. In agreement with previous studies in marine fish larvae[14,37,47,48,82,98,102] , marine PL rich in n-3LC-PUFA could be more effective as a source of essential fatty acid than SBL supplemented with n-3 LC-PUFA. Izquierdo *et al.*[47] compared the effect of 4 isoenergetic, isoproteic and isolipidic diets with either 2 levels of SBL substituted by oleic acid and supplemented with EPA and DHA in a triglyceride form or by 2 levels of marine PL in 25 DPH larval seabream. These authors found that PL, regardless the SBL or marine origin, increased diet ingestion, but also, that there was a higher incorporation of n-3 LC-PUFA into the larval polar lipids when these fatty acids were provided in the form of marine phospholipids, that was related with growth improvement[47]. A combined increase in dietary PL and n-3 LC-PUFA reduced relative PE contents in the larvae, probably with a higher DHA content given the affinity of diacylglycerol ethanolamine phosphotransferase for DHA[12], what could have been related to the proportional increase in PC stimulated by PL and n-3 LC-PUFA[47].

In the present study, increased PL and n-3 LC-PUFA enhanced the activities of the brush border membrane enzymes, alkaline phosphatase and aminopeptidase, in agreement with previous studies [15,37,40,42,83]. This enhanced enzymatic activity is associated to a higher maturation of the gut followed by growth improvement[13,37,40,64,78,83], and more precisely to a faster

maturational process of the enterocytes[43]. Indeed, in fish larvae, enterocyte morphology is influenced by the developmental stage and dietary composition[54,55,64]. In our study, the acceleration of gut developmental processes in larvae fed high PL was further supported by the advanced ontogenetical 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, but also to the pepsin activity. Pancreatic secretion can be modulated by FA[37, 92], as well as by PL. Indeed it has been shown, that an improvement in digestion efficiency in high PL fed fish, resulted in an enhanced secretion of cholecystokinine CCK, the primary regulator of pancreatic secretion [84,108]. These authors suggested that an increased PUFA content in the biomembrane of larval tissues and intestinal lumen could modulate CCK function[52,84]. In this regard, an antagonistic feedback mechanism between CCK and trypsin activity was demonstrated in cod larvae [28], that further supports the existence of a CCK-releasing factor[81]. Despite 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 is observed[59,77,106,107]. As a consequence, the decrease in tryptic activity could be linked to the enhancement of acidic digestion in the stomach of the largest pikeperch larvae.

Liver proteomics were also affected by the combined elevation of PL and n-3 LC-PUFA. For instance, FAS expression was under-expressed in larvae fed 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, probably as a positive effect of DHA supplementation. Most of the previous studies of FAS regulation have focused on the control of gene expression, suggesting that LC-PUFA may decrease FAS expression through the inhibition of SREBP-1c[44,112]. Besides, FAS appears to participate in liver triglyceride metabolism by promoting β-oxidation of FA through activation of peroxisome proliferator–activated receptors PPARα under nutrient-deficient conditions[33]. 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[19]. 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, which is the primary enzyme responsible for the synthesis of cytosolic acetyl-CoA considered as the principal building block of FA, 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.

Another marked effect of dietary PL and n-3 LC-PUFA in pikeperch was the down-regulation of the expression of proteins involved in transfer and exchange of phospholipids and cholesterol such as the non-specific lipid transfer protein and the hydroxysteroid dehydrogenase-like protein 2. The non-specific lipid transfer protein, more commonly denoted as sterol carrier protein 2 (SCP-2), transfers phospholipids between membranes as well as sterols (cholesterol and glycolipids)[8,9,100]. This peroxisomal protein is able to bind fatty acyl-CoAs, where it is likely involved in the β -oxidation of FA[57,90,104]. Furthermore, its association with the β -oxidation complex could be important for the protection of unsaturated fatty acid intermediates against oxidative attack[26]. It is well known that hydroxysteroid dehydrogenase-like protein 2 is the product of one of the sterol carrier protein 2 (SCP2) domain encoding genes[29], thus pointing to its potential involvement in the transport and/or metabolism of FA. Similarly, the peroxisomal localization of human HSDL2 may suggest also an involvement in fatty-acid metabolism[57]. Previous studies demonstrated that the promoter region for SCP2 encoded gene contains several regulatory domains including a peroxisomal proliferator response element PPRE which is the specific DNA region of target genes that bind with PPARs[5,32]. Therefore, the down-regulation 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 LC-PUFA (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. Besides, the same authors observed a decrease of SCP2 expression in cultured colon cancer cells HCT-15 after 48 h treatment with DHA [110].

Protein disulfide isomerase (PDI) is a multifunctional protein that acts as a catalyst of disulfide bond formation, reduction and isomerization of newly synthesized proteins[74,99]. In this 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[2]. On the other hand, in this 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.[41] 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[65] pointing its important role in the maintenance of ER homeostasis[75]. In this respect, PDI over-expression in PL2 might indicate an ER stress[27]. Similarly, glucose regulated protein (Grp) and glucose-regulated 94 (Grp94) known by their major roles during UPR to maintain ER homeostasis[109] 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[67]. This protein together with PDI and other folding factor components form a functional –folding- network under a coordinate transcriptional regulation[67]. 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 [22,41,56]. Interestingly, the same pattern of expression regarding the response to the dietary PL was observed in PL1 and PL3H3 treatments. Indeed, apart from Grp94, another protein was identified, i.e. the ubiquitin carboxyl-terminal hydrolase L5, which participates in a cellular pathway responsible for the degradation of misfolded and damaged proteins involved in the ubiquitin proteasome system[20]. 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.

Regarding the reduction in the different isoforms of the keratin type II detected in liver of pikeperch fed increase PL and n-3 LC-PUFA, could indicate an advanced developmental stage in these larvae, since 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[94].

5. Conclusion

In conclusion, the present study confirms 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 and liver proteomics. Overall the results denote that essential FA may be directly supplemented as triglycerides to have a beneficial effect in pikeperch larvae development. Confirmatory larval studies in

pikeperch larvae should involve effects of TAG and PL supplemented LC-PUFA and resultant FA composition in PL and NL tissue fractions.

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Chapter 4

During the course of the study in Chapter 3, testing higher PL levels together with various levels of n-3 LC PUFAs, it was confirmed the synergistic role of dietary PL and n-3 LC PUFAs in pikeperch larvae for growth, digestive enzymatic activity and skeleton morphogenesis. Separately or in interaction with PL and n-3 LC PUFA levels, many other nutrients can influence the performance of pikeperch larval rearing. A fractional factorial experimental design including 8 nutritional variables (vitamins A, C, D, E; EPA + DHA, ARA, Ca/P ratio, Se) was conducted at URBE in Belgium, to identify the most influencing nutrients and their interactions, using digestive enzymatic activity, histology of the digestive tract and skeletal anomalies as endpoints.

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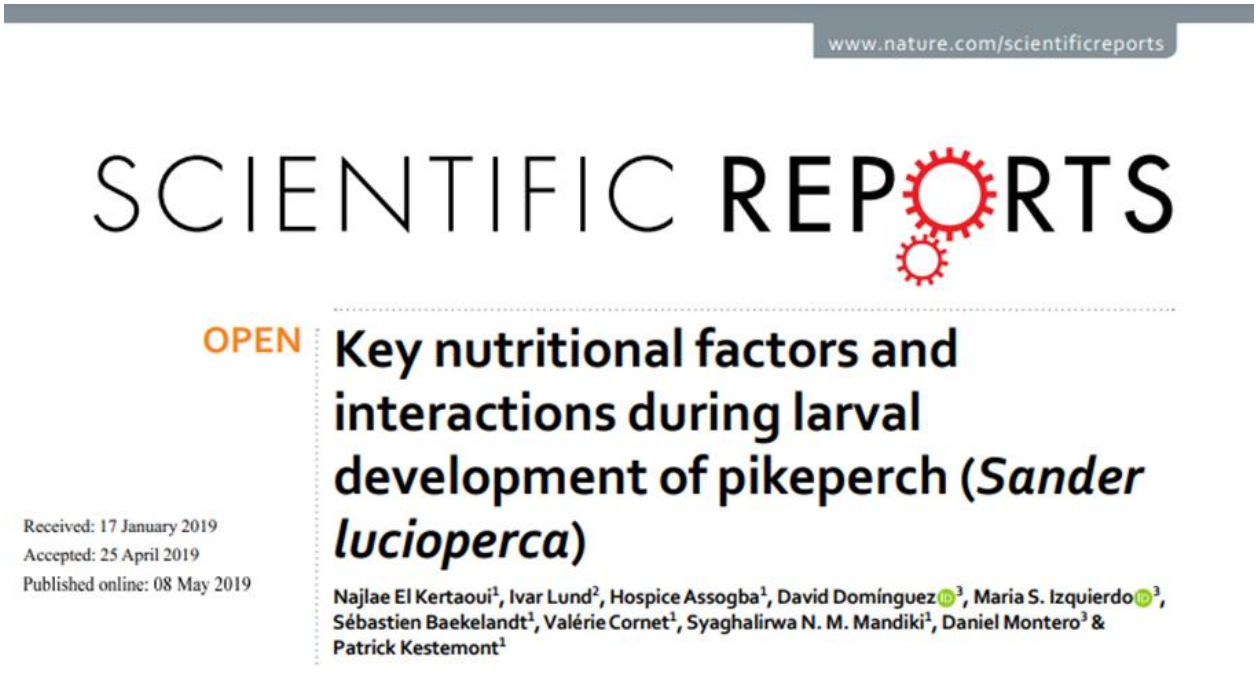

Key nutritional factors and interactions during larval development of pikeperch (*Sander lucioperca*)

Abstract

The effects of 8 nutritional variables (Ca/P, Eicosapentaenoic acid (20:5n-3) + Docosahexaenoic acid (22:6n-3) (EPA+DHA), Arachidonic acid (20:4n-6) (ARA), Se, vitamins E, C, D and A) were investigated to identify their respective importance and interactions in pikeperch larval development. In this respect, two modalities (low and high levels) of each variable were tested through a fractional factorial experimental design allowing a reduction from 256 (2^8) to 16 (2^{8-4}) experimental units. Survival was significantly higher in larvae fed a high Ca/P diet while larval growth was significantly lower in larvae fed the same diet variant, associated with a higher incidence of kyphosis and pectoral anomalies in these larvae. Lordosis and scoliosis seemed to be mostly affected by dietary long chain polyunsaturated fatty acids (LC-PUFAs). A significant interaction was shown between n-3 LC-PUFA and vitamin C on jaw anomalies, while myocyte-specific enhancer factor 2C (*mef2c*) gene expression correlated positively with dietary vitamin C increment. Results also demonstrated an effect of the different nutrients and their interactions on the activity levels of digestive enzymatic activities. The results of the present study highlight the importance of the interactions between Ca/P, LC-PUFAs and vitamins C and E, suggesting their essential roles as key nutritional factors influencing pikeperch larval development.

Keywords: ***Sander lucioperca; LC-PUFA; minerals, Ca/P; vitamin E, vitamin C, bone anomalies,*** **digestive enzymes.**

1. Introduction

Pikeperch (*Sander lucioperca*) has been identified as a candidate for diversification with a great potential in European aquaculture industry [1, 2, 3, 4, 5]. However, as for some other emerging species, weaning to dry diets remains a major bottleneck for the successful larval rearing, mainly due to the difficulties to valorize efficiently the compound diets, in terms of survival, growth performance and reduced skeletal anomalies. Indeed, the digestive system of early fish larvae is extremely immature, what may correlate with low enzymatic capacities [6, 7, 8, 9]. In pikeperch, it has been shown that the structure and functional ability of the digestive tract are affected by the larval developmental stage as well as by the diet composition [10]. In this respect, literature suggested a combined effect of weaning age/size and diets on skeletal anomalies and larval performance in pikeperch [11, 12, 10]. Tailored commercial starter feeds do not exist for this species, and feeds used in hatcheries are likely developed for marine species, such as Gemma Micro (Skretting, Norway) and Otohime (Reed Mariculture, California). Hence, during this critical life stage, adequate feeds are needed to fulfil the nutritional requirements of fish larvae.

Literature is scarce on the nutritional requirements of pikeperch; however, in the last decade, some studies have focused on the effects of long-chain polyunsaturated fatty acids (LC-PUFAs) and phospholipids (PLs) on early development of pikeperch larvae [13, 14, 15, 16, 17, 18]. Recently, Lund and El Kertaoui et al. [19] highlighted the beneficial effects of a combined supplementation of PL with n-3 LC-PUFA in the form of triglycerides on growth rate, reduced incidence of skeletal anomalies and increased digestive enzymatic capacity. Indeed, improvement in growth rate and digestive capacities have been observed in pikeperch larvae fed high PL levels [10, 14], while a deficiency in n-3 LC-PUFA, especially docosahexaenoic acid (DHA: 22:6n-3), resulted in a higher locomotor activity and alteration in swimming behavior [18]; a higher mortality rate and a decreased salinity tolerance [17, 20]. Authors suggested high requirements of pikeperch larvae similar to those of marine carnivorous fish larvae for both phospholipids and LC-PUFAs [10, 18].

Studies in marine fish larvae ranged the optimum levels of n-3 LC-PUFA from 0.05% to 4% on a dry matter basis, in live food or formulated diets [21]. This wide range is most likely due to species-specific requirements. However, literature data also reported different optimal LC-PUFA levels for arachidonic acid (ARA, 20:4n-6); eicosapentaenoic acid (EPA, 20:5n-3) and DHA for the same species and during the same life stage, most likely as a consequence of difference in experimental conditions, especially dietary composition, considering an additional

effect of interactions with and between different nutrients. Indeed, the interactions between LC-PUFAs and vitamins, especially vitamins E and/or C, have been reported in several freshwater species, such as walleye (*Sander vitreus*) [22], beluga (*Huso huso*) [23], Persian sturgeon (*Acipenser persicus*) [24, 25, 26] and marine fish larvae such as gilthead sea bream (*Sparus aurata*) [27, 28, 29], European sea bass (*Dicentrarchus labrax*) [30], yellowfin seabream (*Acanthopagrus latus*) [31], and meagre (*Argyrosomus regius*) [32]. 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 [33]. Eventually, being an efficient hydrogen donor, vitamin E – for which α-tocopherol has the highest biological activity- reacts as a chain breaking antioxidant [34] able to compete for peroxyl radicals much faster than any PUFAs. Vitamin C acts as an antioxidant able to regenerate α-tocopherol from tocopheroxyl radical [35, 36, 37]. In addition, vitamin C is a cofactor in many biological processes including collagen synthesis [38, 39]. Hence, skeletal malformations are common in vitamin C deficient fish [40]. Vitamin A whose antioxidant function has been also described [41] is among the nutritional causative factors of skeletal anomalies in reared fish together with vitamin D [42].

During the last years, more considerations have been given to mineral nutrition in fish larvae [43, 44, 45, 46, 47, 48]. Among the minerals studied, selenium (Se) has been investigated with respect to its antioxidant role in fish larvae [43, 45, 49], likewise, interactions between Se and vitamin E have previously been demonstrated [50, 51]. 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 [52], since the ratio between Ca and P affects the uptake of calcium [49]. As Ca and P exist in a constant ratio in fish bone [53], this suggests needs to be maintained in fish feeds [54].

In general, interactions among nutritional factors can yield antagonistic, additive or synergistic effects. Hence, to increase knowledge of the larval nutritional requirements, macro and micronutrients have to be considered together, such as described in case of interactions between pro and antioxidant nutrients. The fractional factorial design is a practical approach for studying the combined effects of the interactions between various nutrients. Therefore, the aim of the present study was to investigate simultaneously the dietary effect of LC-PUFAs, vitamins and minerals and their interactions using pikeperch larvae as a biological model.

2. Results

2.1. Evaluation of the combinations, based on the global score of interest

The highest final weight and SGR were recorded in larvae fed diet 11 (Table 1). Global score calculated by considering the results obtained from husbandry and skeletal anomalies results showed that the combination 3 (diet 3) resulted in the best larval performances (Table 1). High global scores of interest (>3) were also obtained for diets 5, 7, 11. All these experimental treatments contained the lowest dietary Ca/P ratio (0.6); in addition, these treatments (3, 7, 11) with the exception of diet 5 are characterized by the highest n-3 LC-PUFA content (EPA+DHA = 3.5%). The lowest weight and SGR were recorded in larvae fed diet 10 which combined the highest dietary Ca/P ratio and the lowest EPA+DHA, followed by treatments 8, 12, and 6 which were characterized by the highest dietary Ca/P ratio (Table 1). In addition, low global scores of interest were observed in treatments 15, 16, grouping the combination of high EPA+DHA, high vitamin E and high vitamin A.

Table 1: Husbandry and anomalies results of the different combinations tested in the experiment

Diet	Variables studied										Global score of interest	Rank of the global score
	Final weight (mg)	SGR (day^{-1})	Cannibalism (%)	Mortality (%)	Survival (%)	Anomaly abundance (%)						
						Pectoral elements	Kyphosis	Lordosis	Scoliosis	Jaws		
1	82.47	16.67	30.83	40.40	27.48	32.00	2.00	38.00	18.00	30.00	-0.49	9
2	71.40	15.56	35.86	20.32	42.35	28.57	4.08	46.94	28.57	24.49	0.04	7
3	101.02	18.23	46.11	26.35	27.60	20.00	2.00	26.00	4.00	18.00	6.92	1
4	74.26	15.86	32.66	16.87	49.51	45.83	10.42	47.92	25.00	39.58	-1.90	11
5	78.94	16.33	26.56	29.80	42.36	12.24	6.12	42.86	18.37	4.08	6.17	2
6	68.10	15.19	37.34	15.61	45.60	48.00	2.00	26.00	16.00	42.00	-0.62	10
7	90.72	17.40	53.28	20.62	25.66	20.41	0.00	44.90	12.24	18.37	3.00	4
8	64.19	14.74	26.52	24.85	47.11	22.00	14.00	46.00	20.00	22.00	0.31	6
9	86.35	17.02	42.19	29.43	27.96	41.51	0.00	54.72	16.98	37.74	-2.51	13
10	61.46	14.40	30.66	30.84	36.65	35.29	5.88	52.94	27.45	27.45	-4.58	14
11	103.52	18.42	32.47	31.09	35.58	35.29	1.96	25.49	9.80	35.29	5.30	3
12	67.92	15.17	40.53	22.89	35.74	28.00	14.00	50.00	16.00	24.00	-2.47	12
13	74.06	15.84	27.26	38.15	33.09	34.00	4.00	34.00	12.00	32.00	-0.37	8
14	82.18	16.64	25.33	43.59	28.65	34.00	6.00	24.00	10.00	28.00	1.08	5
15	71.38	15.56	14.14	50.31	33.75	34.62	15.38	59.62	25.00	26.92	-4.92	15
16	88.94	17.25	47.62	15.92	36.57	24.49	24.49	73.47	46.94	24.49	-4.94	16

2.2. Evaluation of the global effect of combination by PCA

The results of the principal component analyses indicate that the husbandry and deformity responses are related to the dietary content (Fig. 1). The plans 1–2 of the PCA explain 50.8% of the inertia (total variance). Indeed, the axis 1 (Fig. 1), characterized by the vectors scoliosis, lordosis, survival and kyphosis, and the modality 1.2 Ca/P, was especially represented by the dietary treatments 4, 8, 16 in which both kyphosis and survival rate were higher than the global average rate recorded (Table 1). On the opposite, on this axis, the treatments 3 and 11, in which a high final weight and SGR were recorded (Table 1) were characterized by the vectors final weight, SGR, aminopeptidase specific activity (N), and the modality 0.6 Ca/P. In addition, the increase in dietary vitamin E content up to 3000 mg kg^{-1} together with the use of low EPA+DHA (1.25%) dietary content significantly affected mortality rate and amylase activity.

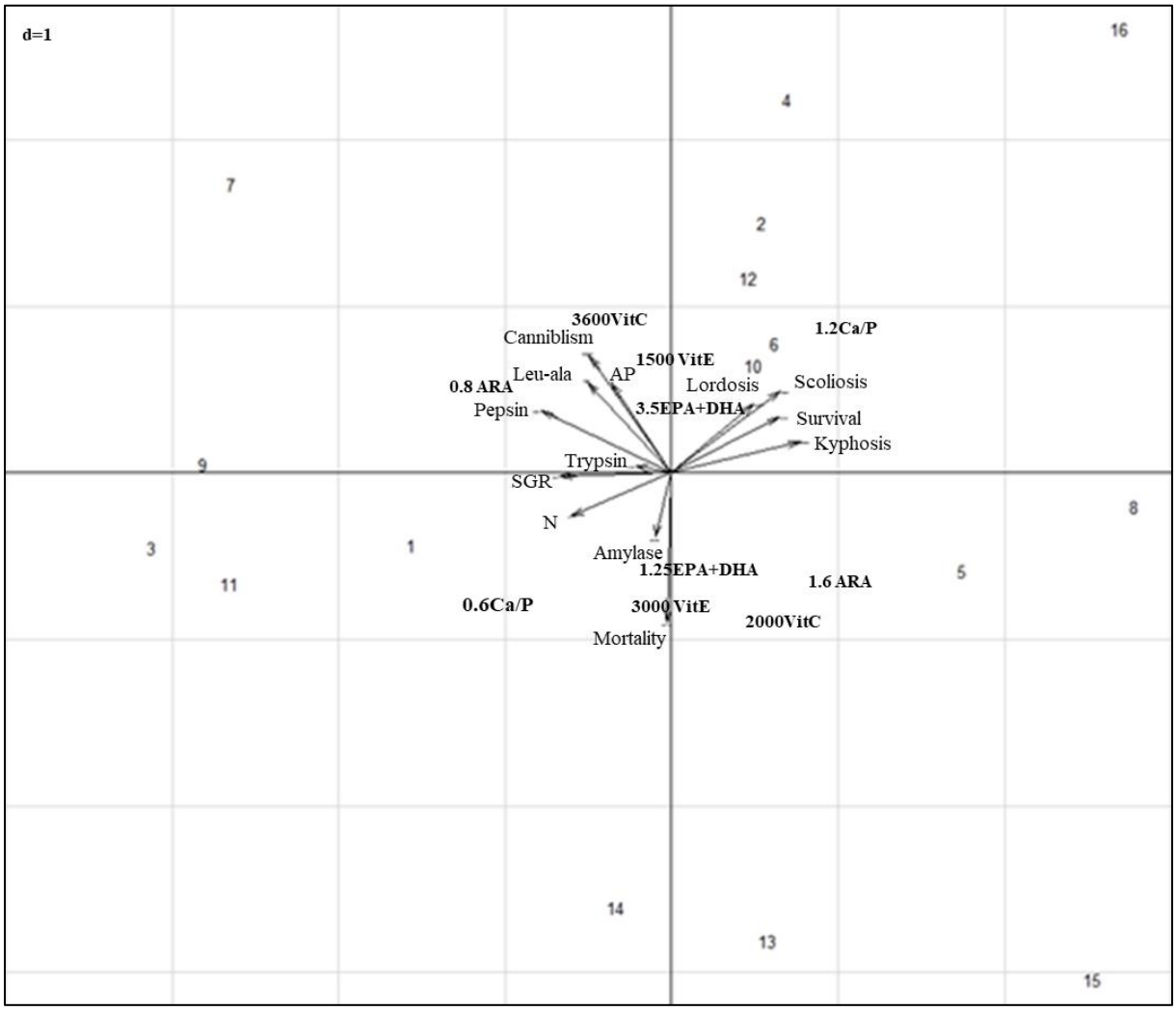

Fig. 1. Projections of outputs and combinations (1–16) on the two first axis of the principal component analysis. The plans 1 and 2 of the PCA explain 50.8% of the projected inertia. Axis 1 (inertia 29.2%), axis 2 (inertia 21.6%).

2.3. Results presented by factor effect

Husbandry and skeletal anomalies

The detection of the potentially active effects of the tested factors on the husbandry, and skeletal anomalies variables as given by Daniel graphics and followed by ANOVA showed significant effects of Ca/P ratio on several endpoints. Survival rate was higher in larvae fed high Ca/P (p= 0.017) (Fig. 2a). Contrary, final weight and specific growth rate (SGR) decreased significantly in larvae fed high Ca/P (p = 0.021 and 0.018 respectively) (Fig. 2b). In addition, the prevalence of kyphosis and pectoral element anomalies increased by the dietary Ca/P elevation (p = 0.0006) (Fig. 2c and 2d).

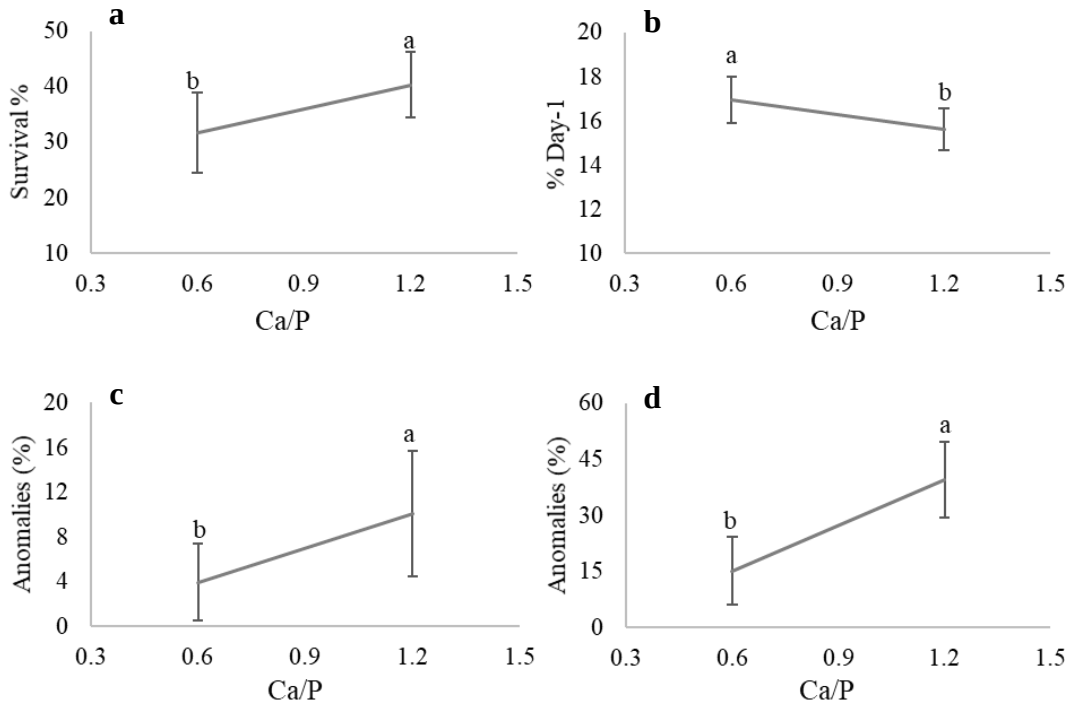

Fig. 2. Effect of dietary Ca/P on husbandry variables and anomalies: (a) larval survival rate (%); (b) specific growth rate (SGR, % day -1); (c) kyphosis rate (%); (d) pectoral element anomalies rate (%). Only graphs with significant effects are shown. Results are expressed as the mean ± SD (n=8). Different letters denote statistically significant differences between treatments.

Combined effects of n-3 LC-PUFA (EPA+DHA) and vitamin C had a clear direct incidence in jaw anomalies and cannibalism. The dietary increase in EPA+DHA reduced jaw anomalies in larvae fed 3600 mg vitamin C (p = 0.033) (Fig. 3b), while the same combination resulted in higher cannibalism rate (p = 0.05) (Fig. 3a).

Results of lordosis and pre-haemal scoliosis presented a significant interaction between EPA+DHA and ARA dietary contents (p = 0.0081 and 0.0071 respectively). The increase of

EPA+DHA (3.5%) seemed to reduce the prevalence of scoliosis in larvae fed 0.8% ARA (Fig. 3d), while there was no significant effect at high level of ARA (1.6%). Besides scoliosis, high levels of EPA+DHA and ARA increased lordosis (Fig. 3c), while the decrease in EPA+DHA with the high ARA level reduced skeletal anomalies (Fig. 3c and 3d). No statistically confirmed effects of the dietary nutrient factors on the normal specimen rate (larvae without severe anomalies) were detected, this latter ranging between 11.76% (larvae fed diet 10) and 56% (larvae fed diet 3).

Myocyte enhancer factor-2 (*mef2c2*) was overexpressed in larvae fed high vitamin C levels (Fig. 3e). In addition, *twist* expression was highest when pikeperch larvae were fed the combination low Se and high n-3 LC-PUFA; but dietary Se supplement resulted in a decrease of *twist2* expression in larvae fed high n-3 LC-PUFA (Fig. 3f).

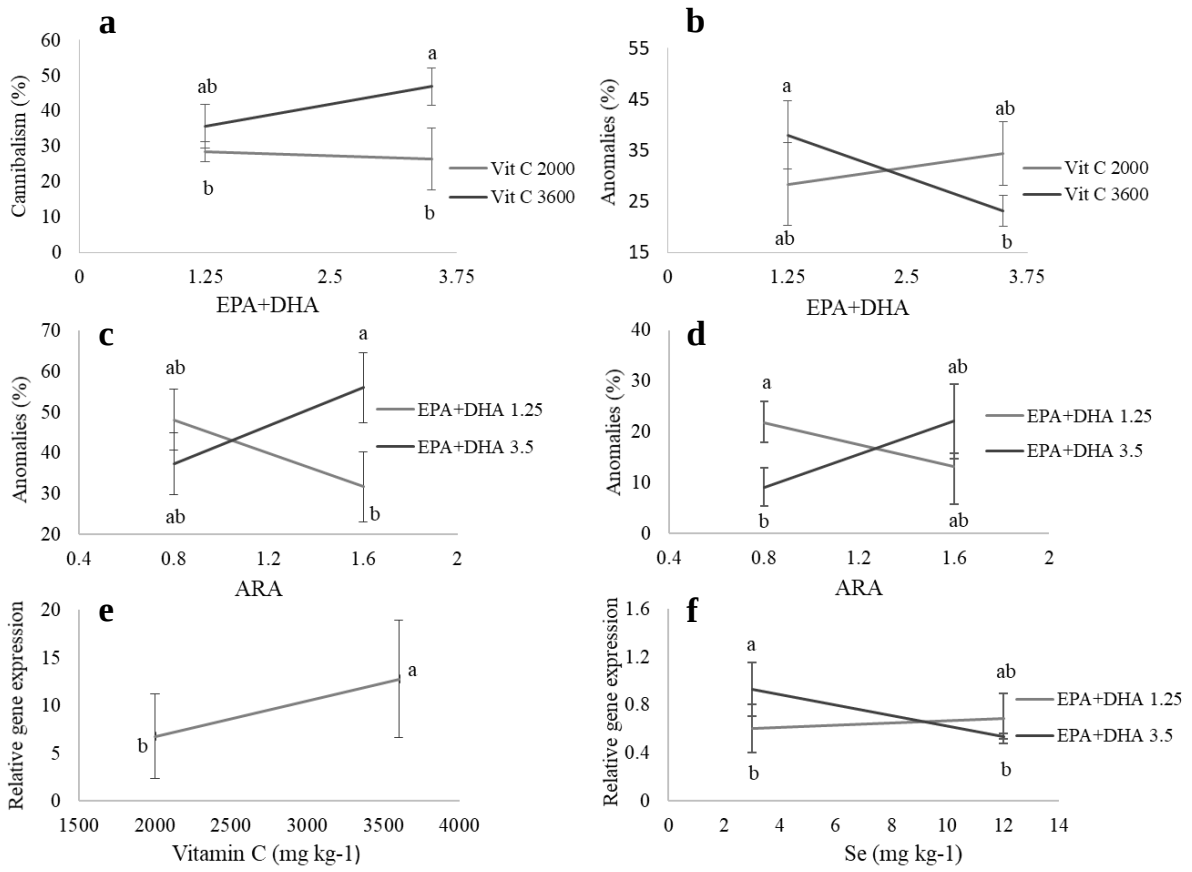

Fig. 3. Effect of dietary nutrient factors on cannibalism, larval anomalies and related gene expression: (a) EPA+DHA and vitamin C interaction effect on cannibalism rate; (b) EPA+DHA and vitamin C interaction effect on jaw anomalies; (c) EPA+DHA and ARA interaction effect on lordosis; (d) EPA+DHA and ARA interaction effect on pre-hemal scoliosis; (e) *mef2c2* and (f) *twist2* expression measured in pikeperch larvae. 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.

Activity of digestive enzymes

A differential pattern in the ontogenetic development of digestive enzymes was observed dependent on the dietary content. High dietary n-3 LC-PUFA content enhanced trypsin activity in larvae fed low Ca/P level (Fig. 4a). On the contrary, this elevation of dietary n-3 LC-PUFA resulted in a decrease in trypsin activity in larvae fed 1.6 Ca/P level ($p = 0.0044$) (Fig. 4a). A higher aminopeptidase activity (N) was also observed in larvae fed low Ca/P ($p = 0.026$) (Fig. 4b). Aminopeptidase activity was also significantly lower in larvae fed high ARA level ($p = 0.038$) (Fig. 4c). Similarly, leucine alanine peptidase (leu-ala), alkaline phosphatase (AP) and pepsin specific activities were negatively correlated with ARA levels ($p = 0.023$; 0.0017 and 0.0053; respectively) (Fig. 4d, 4e, and 4f), while specific activity of amylase increased with dietary vitamin E elevation ($p = 0.012$) (Fig. 4g).

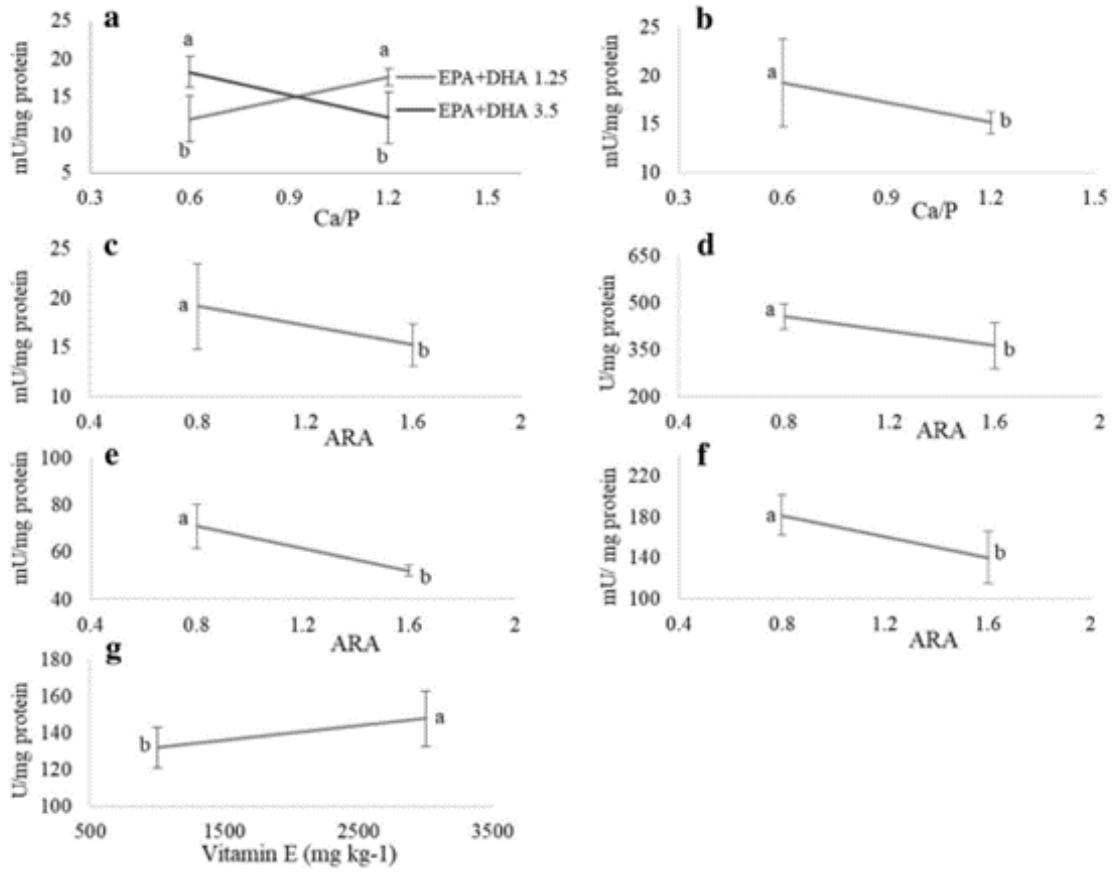

Fig. 4. Digestive enzymatic activity ((a) trypsin; (b) and (c) aminopeptidase; (d) leucine alanine; (e) alkaline phosphatase; (f) pepsin; (g) amylase) of 39 dph pikeperch larvae fed the different experimental diets. Only graphs with significant effects are shown. Results are expressed as the mean ± SD (single effect: n=8; interaction effect: n=4). Different letters denote statistically significant differences between treatments.

Larval content in fatty acids

In terms of fish fatty acid content, DHA, EPA and the total n-3 LC-PUFA were significantly higher in the group of larvae fed the high n-3 LC-PUFA treatments (3.5%) (Supplementary Table S6). On the other hand, the increase in dietary ARA seemed to reduce EPA content in larval tissues (Fig. 5a) as well as EPA/ARA ratio (Fig. 5b). No statically confirmed effects of vitamins or minerals on larval fatty acids content were detected.

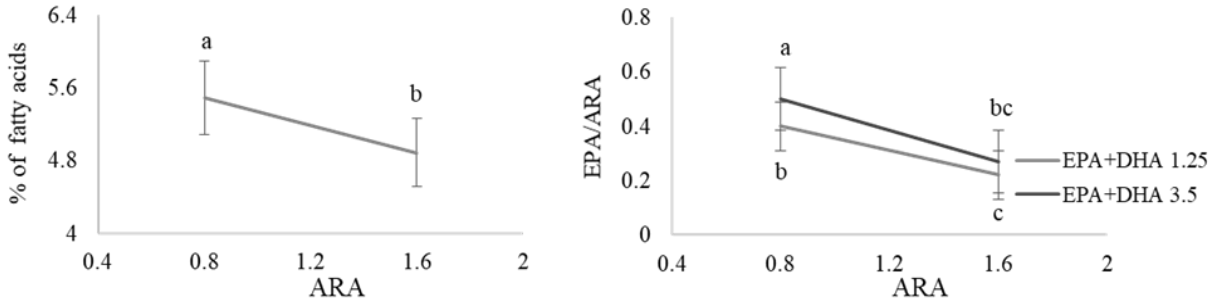

Fig. 5. Larval fatty acid content of 39 dph pikeperch. (a) EPA larval content and (b) EPA/ARA ratio in larvae fed different dietary ARA. Only graphs with significant effects are shown. Results are expressed as the mean ± SD (single effect: n=8; interaction effect: n=4). Different letters denote statistically significant differences between treatments.

Histological study

Histological changes were observed in the anterior intestine samples from 3000 mg kg^{-1} vitamin E diets group, that were characterized by an increased density of goblet cells (Fig. 6a) ($p = 0.0044$). Regarding the liver, high Ca/P resulted in an increase of lipid vacuole accumulation in larvae fed low n-3 LC-PUFA level (Fig. 6b) ($p= 0.0035$). 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 (Supplementary Fig. S1). On the contrary, the increase in Ca/P did not affect the lipid vacuole deposition in larvae fed higher LC-PUFA levels (Fig. 6b). No other histopathologies were observed in larval tissue.

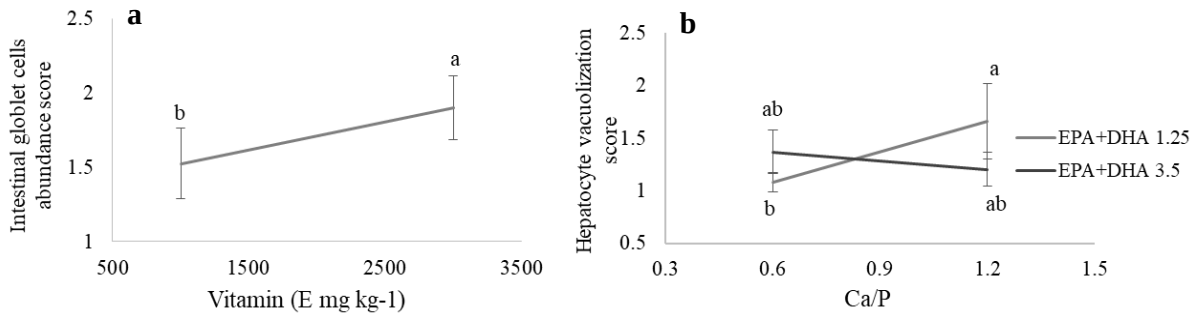

Fig. 6. (a) Intestinal goblet cells and (b) hepatocyte vacuolization observed in pikeperch larvae fed different diets. Score (1) not observed; (2) mild vacuolization or goblet cells presence; (3) severe vacuolization or goblet cells presence. Only graphs with significant effects are shown. Results are expressed as the mean ± SD (single effect: n=8; interaction effect: n=4). Different letters denote statistically significant differences between treatments.

3. Discussion

Although the fractional factorial design is considered as a practical approach permitting to identify the most influencing factors as well as the evaluation of the possible interactions between the tested factors [55, 56, 57, 58, 59, 60, 61], such design regrouping more than 3 nutrients concurrently are rarely used in fish [55, 62]. To our knowledge, the present study represents a first attempt to evaluate the simultaneous effects of selected fatty acids, vitamins and minerals using a multifactorial approach in fish larvae.

Our finding shows that specific endpoints describing larval performance are specifically influenced by different nutrients and doses. The evaluation of the combinations, based on the global score of interest, suggested a profound effect of Ca/P ratio, n-3 LC-PUFA and vitamin E. In fact, the four best dietary combinations are commonly characterized by a low dietary Ca/P ratio (0.6) and a high EPA+DHA content (3.5%), while the elevation of dietary vitamins E and A (3000 mg kg $^{-1}$ and 30000 IU respectively) and LC-PUFA (EPA+DHA and ARA) resulted in a lower global score of interest. In the present study, no potential main effect was assigned to vitamins D and A. Similarly, except its related effect to n-3 LC-PUFA on *twist* expression, no significant differences were recorded among larvae fed different Se levels. Minor effects of micronutrients on gross fish performance have also been suggested in Atlantic salmon (*Salmo salar, L.*) by Hamre et al. [55].

Our results vary according to the endpoint considered. Indeed, Ca/P ratio appears as a determining nutrient for pikeperch larvae considering the significant decline in growth as well as the increase in the incidence of skeletal anomalies (especially kyphosis and pectoral element anomalies) recorded in larvae fed high Ca/P. Kestemont et al. [11] already suggested the need

for a low Ca/P ratio in pikeperch larval diets, below those usually found in commercial feeds formulated for marine fish larvae. In this study, growth was probably reduced due to the higher incidence of bone anomalies, since skeletal deformation may affect various physiological and behavioral performances of fish larvae such as swimming, feed intake and feeding efficiency [63]. Taking into account the total phosphorus level, which was about two times higher in 0.6 Ca/P than in 1.2 Ca/P feeds, we can speculate that the increase in skeletal anomalies induced by the dietary Ca/P elevation indicates that the diets with high Ca/P ratio were not able to fulfil the phosphorus requirements of pikeperch larvae. In accordance with our findings, high incidence of kyphosis is often reported as a typical consequence of phosphorus deficiency in fish larvae [64]. Based on a rather large review of the literature (including 25 studies on different finfish species), Antony Jesu Prabhu et al. [65] reported that the estimated minimal dietary available P content is 3.5 g kg^{-1} DM. In our study dietary Ca/P ratio induced opposite effects on survival and on growth. The high survival rate in larvae fed the high Ca/P ratio could be associated to the increment of the efficiency of P utilization in low dietary available P feeds [66]. While the low survival rate governed also by the cannibalistic behavior of pikeperch larvae could be responsible for the higher growth rate recorded in low Ca/P groups.

PCA analysis also associated the increase in mortality with the high vitamin E intake (3000 mg kg^{-1}). High vitamin E levels have been suggested to act as pro-oxidant [67, 68], which explains the lowest score of interest recorded in larvae fed diet 16 combining high Ca/P and high vitamin E levels, taken into consideration the increased antioxidant defense ability by optimal dietary phosphorus [69]. Thus, contrary to what is observed in marine fish larvae in which a beneficial effect of dietary vitamin E elevation – up to 3000 mg kg^{-1}- has been recorded [27, 29, 30, 32], the vitamin E levels chosen in this study seem to be higher than the need of this species. Similarly, an increase in mortality and tissue lipid oxidation has been reported in Atlantic salmon and Atlantic halibut (*Hipoglossus hipoglossus*) juveniles fed high levels of vitamin E in the absence of a sufficient amount of vitamin C [33, 67]. High dietary vitamin E levels resulted in low growth and death in rainbow trout (*Oncorhynchus mykiss*) [70], equally a negative effect of high dietary vitamin E levels on growth was also observed in parrot fish (*Oplegnathus fasciatus*) [71]. Furthermore, the toxic effects have been reported in several species such as brook trout fry (*Salvelinus fontinalis*) [72], African catfish (*Clarias gariepinus*) [73], and rainbow trout [74].

In the present study, high vitamin C dietary content seemed to be efficient in reducing the incidence of jaw anomalies when high levels of n-3 LC-PUFA were included in the diets, pointing out the antioxidant function of this vitamin [30, 75]. In this sense, Izquierdo et al. [28]

highlighted the relationship between the appearance of anomalies in skeletal elements developed from a cartilaginous precursor and the increased oxidative status in seabream larvae fed high DHA levels. A possible explanation may be related to the dietary effect of ascorbic acid on the ossification of cartilaginous- origin bone process, since ascorbic acid affects collagen synthesis in structural organs such as cartilage and bone [76]. Hence, impaired biosynthesis of collagen resulted in skeletal malformations in ascorbic acid deficient fish [40]. Meanwhile, high vitamin C associated with high EPA+DHA level resulted in higher cannibalism rate, probably because of the decrease in the incidence of jaws anomalies. Kestemont et al. [11] also linked the incidence of jaw anomalies to the weaning age and the dietary intake, especially of vitamin C, being deficient in the non-enriched *Artemia* nauplii. Interestingly, in the present study, an increase in dietary vitamin C resulted in the overexpression of *mef2c2* (myocyte enhancer factor-2) expression, providing an additional support to the role of vitamin C intake in the ossification process and collagen synthesis in fish larvae. The role of *mef2c* in promoting precocious chondrocyte hypertrophy and ossification of endochondral bones has been reported by Arnold et al. [77] and Potthoff and Olson [78]. Actually, *mef2c* acts as an essential, early regulator of bone development by orchestrating transcriptional and cell-cell signaling events involved in chondrocyte hypertrophy which is necessary for bone vascularization, osteoblast differentiation and endochondral ossification. Selenium has also been considered to be related closely to endochondral ossification [79]. *Twist2* expression suggested that n-3 LC-PUFA and Se act synergistically, likewise, *twist2* down expression recorded in larvae fed high n-3 LC-PUFA with dietary Se supplement may reflect the antioxidant role of Se in pikeperch larvae, since the twist2 gene antagonizes osteoblast formation [80, 81]. Twist2 is a runx2 inhibitor and suppresses runx2 by interacting with its DNA binding domain, and the osteoblast differentiation occurs only after *twist2* gene expression decreases [82, 83]. Hence, authors suggested that the expression of *twist* during early fish ontogeny could be used as an indicator of skeleton malformation [84, 85]. Meanwhile, in the present study, lordosis and pre-haemal scoliosis were mainly affected by dietary LC-PUFAs. Feeding high dietary LC-PUFAs (diets combining high ARA and EPA+DHA levels) led to an increase in both anomalies (Fig. 3c and d). Excessive amounts of PUFA have been suggested to accelerate osteoblasts differentiation, causing supranumerary vertebrae in sea bass larvae [86].

In our study, the richness in LC-PUFA could result in a high level of lipid peroxidation. Furthermore, the increase in dietary ARA had a differential effect on skeletal anomalies depending on the EPA+DHA levels. In this regard, a possible explanation could be related to

EPA+DHA/ARA ratio, suggesting the importance of a balanced n-3 LC-PUFA/n-6 LC-PUFA ratio in this species. The importance of a balanced level of n-3 and n-6 PUFA for a proper skeletogenesis has been proven in other species [21, 87]. In flatfish, authors linked the dietary imbalances in EPA and ARA to the disruption of bone formation and osteoblast differentiation in skeletal tissues, bone remodeling problems in the cranial region and reduction of bone mineralization [88]. This sensitivity to dietary EFA ratios, in particular the balanced proportion among EPA and ARA acids, is related to the fact that both acids are precursors for highly bioactive eicosanoids – especially PGE2 and PGE3 – for which the effects on bone metabolism and osteoblasts regulation have been well reported [88, 89, 90, 91, 92]. Thus, although PGE levels were not measured in this study, it can be hypothesized that the anomalies observed indicate a disturbance of bone development, probably due to the prostaglandin imbalance especially the PGE2/PGE3 ratio, such as suggested in previous studies [88, 89, 90, 91, 92, 93]. This is in agreement with the fact that, in the present study, the increase in dietary ARA seemed to reduce EPA content in larval tissues; denoting a selective deposition and retention of LC-PUFAs in pikeperch larvae, likely due to the inhibition of EPA incorporation by dietary ARA [94], which may also result in an alteration of prostaglandin production as suggested above in favor of the more biologically active PGE3 involved in pro inflammatory properties. Actually, the relation among dietary EPA and ARA has been proposed to be a critical factor for larval performance due to competitive interaction among them [89, 95, 96].

Previous studies showed that n-3 LC-PUFAs are potent stimulators of cholecystokinine (CCK) secretion [97]. In our study, effects of EPA+DHA levels on trypsin activity may reflect the endocrine modulation of the pancreatic digestive function, which is regulated by CCK [98, 99, 100]. In fact, trypsin is secreted as a trypsinogen activated by an enterokinase requiring Ca ions [101]. Consistently, the better utilization of Ca under specific condition of LC-PUFA and Ca/P should be further investigated. The same interaction was found in hepatocyte vacuolization. Fatty liver has been considered as an expression of the impaired lipid metabolism [102]. High intake of LC-PUFAs (mainly EPA and DHA) prevents lipid accumulation [103]. Hence, we speculate that the decrease in dietary Ca/P may enhance lipid utilization in n-3 LC-PUFA deficient fish larvae. In mice, authors found that the dietary phosphorus restriction (in the form of mono-potassium phosphate KH_2PO_4) resulted in hepatic lipid accumulation [104]. Moreover, the opposite interaction found between EPA+DHA and Ca/P- on trypsin specific activity as well as the hepatic fat accumulation- could be linked to the effect of Ca/P on growth. On the other hand, the immature intestine of marine fish larvae, has been suggested as a risk for P

deficiency [49]. In this study, high Ca/P dietary level - which was also characterized by the low level of dietary P – led to a decrease in aminopeptidase specific activity. Considering the profound Ca/P effect on growth, we hypothesize an enhancement of gut maturation in larvae fed low Ca/P ratio, as indicated by the increase in brush border enzymatic activities, these latter reflecting the normal maturation process of enterocytes in fish larvae [12, 105]. Analysis of factor effects as well as principal component analysis linked the decrease in specific activity of pepsin, leucine alanine and brush border enzymes (alkaline phosphatase and aminopeptidase) to the dietary ARA elevation. In this respect, Yuan et al. [106] suggested that the use of a diet containing a moderate level of dietary ARA promotes the maturation of enterocytes in larval tongue sole. Therefore, the high ARA level used in our study may have delayed the enterocyte maturation, supporting its potential involvement in the regulation of the digestive tract development in pikeperch larvae. In addition, dietary vitamin E elevation resulted in an increase in amylase specific activity. Vitamin E supplementation resulted in significantly higher muscle glycogen levels in mammals [107]. Consistently, Ma et al. [108] attributed the increase in amylase activity to the high level in glycogen in copepods distributed to large yellow croaker (*Pseudosciaena crocea*) larvae, since amylase is stimulated by glycolytic chains, glycogen, and starch in fish larvae [109]. In addition, dietary vitamin E elevation resulted in an increased incidence of goblet cells secreting mucins. These results suggest a higher mucus production in fish fed high vitamin E level, that would contribute to explain the observed increase in amylase specific activity. Increased density of goblet cells has been also observed in fish fed immunestimulants such as mannan oligosaccharides [110, 111], whereas vitamin E is considered as a key immune stimulating factor in fish [112, 113].

From the different endpoints analyzed in this multifactorial fractional design, it appears that the directive factors for larval performance, as far as the husbandry aspects and the incidence of skeletal anomalies are concerned, are the Ca/P ratio and the LC-PUFAs dietary contents. Amongst vitamin nutrients, vitamins E and C appear as the major micronutrients of interest for pikeperch larvae.

4. Materials and methods

Ethical standards

All procedures and protocols were approved by the local Ethic Committee for Animal Research of the University of Namur, Belgium (Protocol number: 16271 KE) in accordance with national and international regulations for the use of animals in scientific experimentation.

Experimental design

Eight nutrient variables (Ca/P, EPA+DHA, ARA, Se, vitamins A, C, D and E) were tested at two levels of diet inclusion (Supplementary Table S1). With a full factorial design with 8 factors at 2 levels, 256 (2^8) treatments would be required for the calculation of the main effects and all the interactions. Therefore, the experiment was carried out as a $2^{(8-4)}$ reduced factorial design (Table 2). With such fractional factorial design, the number of combinations (treatments) is reduced from 256 (2^8) to 16 (2^4).

Table S1: Selected factors and modalities

Factors	Levels	References
Ca/P	0.6	- Good performance in pikeperch larvae at Ca/P: 0.5-0.6 even with a lower level of dietary protein compared with other diets [11]
	1.2	- Normal Ca/P for rainbow trout: 0.9-1 [132], but low: 0.5-06 in condition of low Ca availability [133, 134]
EPA+DHA (%)	1.25	- Best growth and development in pikeperch larvae fed 1.25% EPA+DHA (1.25% vs 2.64%) [10, 14]
	3.5	- Significant improvement in growth (body weight) in pikeperch larvae fed 2.67 and 3.79% EPA+DHA [19]
ARA (%)	0.8	- Best growth and development in pikeperch at 0.8% ARA vs higher level [14]
	1.6	- Good survival and SGR in sea bass: up to 1.2% [27]
Vitamin E (mg/kg)	1000	- Higher Vit E requirement (3000mg/kg) due to high LC-PUFA needs during larval stages [28, 135]
	3000	- In seabass larvae: 3000mg/kg adequate for good larval performance, avoid muscular lesions, and improved stress resistance [135, 136]
		- Requirement measurements depend to interactions of vitamin E with other nutrients [68]
Vitamin C (mg/kg)	2000	- 3600 mg/kg improved the protection against peroxidation, decreased TBARS contents, spared vitamin E, and reduced the incidence of muscular lesions [30]
	3600	
Vitamin A (IU/kg)	8000	- Optimum dietary vitamin A content (based on growth): around 8000–9000 IU VA/kg for Atlantic halibut and Japanese flounder juveniles respectively [137, 138]
	30000	- Vitamin A toxic level: maximum dose around 45000 IU vitamin A/kg dry diet [139]
Vitamin D (IU/kg)	2800	- 27600 IU vitamin D3/kg: best result of sea bass larval morphogenesis, and earlier maturation of digestive function [140]
	28000	- Vitamin D content in commercial diets used in larval rearing: 2500-2800 IU vitamin D3/kg diet, in agreement with the value recommended by NRC [53] (2400 IU VD3/kg).
Se (mg)	3	- Se concentration in fish around 2-3 mg/kg [46]
	12	- 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 [45]
		- Se toxicity has been demonstrated at dietary levels of 10-20 mg/kg [141]

To generate the experimental design, an alias structure was selected (Supplementary Table S2), determining which effects are confounded with others [55, 56, 57]. In this way, it is possible to calculate main effects separated from each other and from the effects of two-factor interactions [55, 57, 58,114].

Table S2: Aliasing structure considering the 8 dietary variables: Ca/P, EPA+DHA, ARA, vitamin E (vit E), vitamin C (vit C), vitamin D (vitD), vitamin A (vitA), and selenium (Se)

Group	Aliased effects
1	Ca/P*ARA; EPA+DHA*Se; vitE*vitD; vitC*vitA
2	Ca/P*vitE; EPA+DHA*vitC; ARA*vitD; vitA*Se
3	Ca/P*Se; EPA+DHA*ARA; vitE*vitA; vitD*vitC
4	Ca/P*EPA+DHA; ARA*Se; vitE*vitC; vitD*vitA
5	Ca/P*vitD; EPA+DHA*vitA; ARA*vitE; vitC*Se
6	Ca/P*vitA; EPA+DHA*vitD; ARA*vitC; vitE*Se
7	Ca/P*vitC; EPA+DHA*vitE; ARA*vitA; vitD*Se

The main advantage of this approach is that it considers simultaneously the impact of a large number of interrelated nutritional factors using a limited number of experimental units. With a $2(^{8-4})$ reduced factorial design, each of the 16 combinations was tested once but each level of every factor was repeated eight times (Table 2).

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

Diets	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

Rearing conditions

A batch of pikeperch larvae 3 dph (day post hatching) was obtained from breeders held in Viskweekcentrum Valkenswaard located in Leende, The Netherlands. The initial larval rearing was carried out in two tanks (500L rectangular tanks with a water depth of 30 cm) from 3 dph until the weaning period. Larvae were fed *Artemia* nauplii enriched with DHA Protein Selco®

(INVE, Dendermond, Belgium) each hour (from 8:00 am to 6:00 pm) until 17-day old. This first rearing phase was followed by a co-feeding period from 18 to 24 dph using *Artemia* nauplii and a mixture of the 16 experimental dry diets (200-400 µm pellets). The multifactorial experiment started on 25 dph with completely weaned larvae in order to avoid any bias due to habituation to dry feed. Sixty larvae were randomly sampled and weighed to estimate the initial body weight (9.44 ± 4.42 mg). The 25 dph larvae were randomly distributed into 16 experimental tanks (rectangular aquarium with a water volume of 90 L) with a density of 770 larvae $tank^{-1}$ and fed one of the experimental diets (mixture of 200-400 µm and 400-700 µm pellets) for 14 days. The system was based on flow through and all tanks were supplied with filtered fresh water at a rate of 8% h^{-1} to ensure water renewal and to maintain a high water quality during the experiment. Water was continuously aerated by using an airstone in each tank. Temperature and oxygen were daily measured; average water temperature was 19.5 ± 0.4 °C and dissolved O_2 averaged 7.8 ± 0.32 mg. Photoperiod was kept at 12h light: 12h dark. Tanks were manually cleaned once daily between 3:00-6:00 pm by siphoning allowing a daily counting of larval mortality.

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 S.A. (Portugal) as cold extruded feed pellets of 200-400 µm and 400-700 µm. Experimental diets were formulated (Supplementary Table S3) using a mix of oil as sources of EPA, DHA and ARA to reach the required fatty acid content and to equalize the lipid content in each diet. Rovimix A, Lutavit C, Rovimix D3 and Lutavit E, were used as vitamin sources of vitamins A, C, D and E 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 (Supplementary Table S3). The proximate composition of the main important nutrients and vitamins/minerals is shown in Supplementary Table S3**.** Diets were supplied manually every 45 min from 8:00 am to 6:00 pm. Dry feeds supplied was maintained at 25% of the expected larval biomass in the 1st week and 10–15% during the last week. The experimental diets were tested in the factor-modality design (Table 2) which represented a unique variant nutrient combination.

Table S3: Formulation and the proximate composition (%) of the experimental diets

	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
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	14.00	14.00	4.00	15.00	4.00	4.00	14.00	5.00	14.00	14.00	4.00	14.0	4.00	4.00	14.00
Vitamin A**	7794	7775	28933	28896	28866	28917	7825	7794	28877	28891	7816	7777	7800	7814	28891	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 analyses (larval performance)

Final survival was calculated by individually counting all living larvae at the end of the experiment, and expressed as the percentage of the initial numbers of fish. The apparent mortality was calculated by adding the daily counted dead larvae. Type I cannibalism (dead fish showing signs of cannibalism, i.e. fish partly consumed by a cannibal) was not observed. Thus, only missing larvae due to type II cannibalism (i.e. fish completely ingested, usually head first, by a cannibal) were considered to estimate the mortality rate due to cannibalism. All sampled larvae were sedated with tricaine methanesulfonate (MS-222). Growth was determined by measuring wet body weight of 40 larvae per tank at the beginning and the end of the experiment. Specific daily growth rate SGR (% day^{-1}) was calculated according to the equation: SGR = 100 (Ln (final average body weight of sampled larvae) – Ln (initial average body weight of sampled larvae))/number of feeding days.

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 and alcian blue were conducted to evaluate skeletal anomalies following a modified method from previous studies [(28)]. Classification of skeletal anomalies was conducted according to Boglione et al. [(115)]. Anomalies were expressed as frequency of total severe anomalies and specific anomalies, such as jaw anomalies, scoliosis, lordosis, kyphosis, pre-haemal and caudal vertebrae, within each dietary group (Fig. 7).

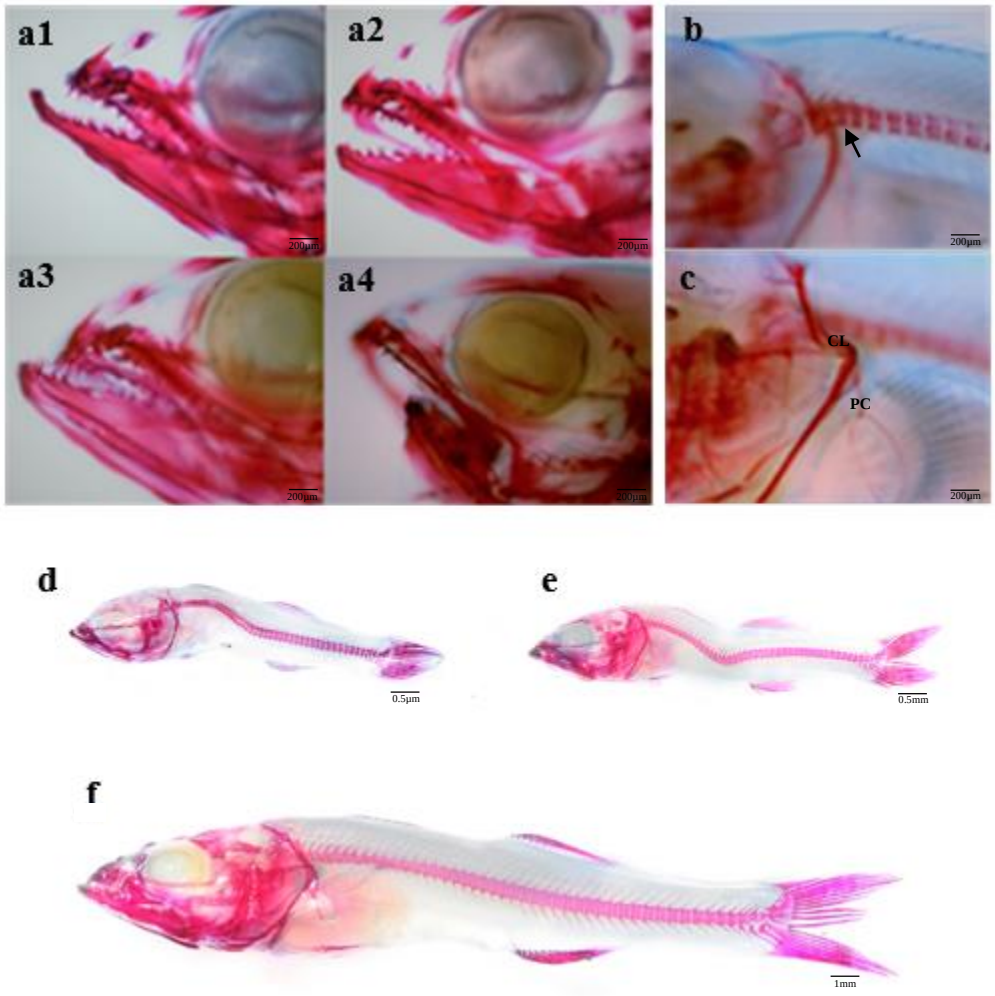

Fig. 7. Examples of some skeletal anomalies observed in 39 dph pikeperch *sander lucioperca* larvae. (a) larvae showing different jaw anomalies (a1: lower jaw increment, a2: normal jaws morphology, a3: upper jaw reduction, a4: lower jaw reduction). (b) larvae showing a cephalic kyphosis (arrow). (c) pectoral element anomalies (CL) cleithrum (PC) post-cleithrum. (d) larvae showing a severe scoliosis. (e) larvae showing a severe lordosis. (f) normal skeleton of pikeperch larvae.

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 analyzed 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. Primer design was carried out taking into account the needs of Real time PCR amplification: primer size between 18-22pb, GC content 50-60 %, product size 150-200 pb, avoiding harping or secondary structures, Cs or Gs in 3'extremity if it was possible and melting temperature of 60 °C ± 2 °C. Primers were designed using Primer3 (v. 0.4.0) software from the conserved regions of each gene obtained by the alignment of gene sequences available on The National Center for Biotechnology Information (NCBI) databases of different fish. Once the primers were designed, the set-up was performed and the correct amplification products were sequenced. Then, the sequences of amplicons were aligned with BLAST NCBI tools searching (Supplementary Fig. S1). Detailed information on primer sequences and accession numbers is presented in Supplementary Table S4.

Table S4: 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'			

a.

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Sl-mef2c                    GCGAAAGTTTGGCCTGATGAAGAAGGCGTATGAGCTGCAGTGTGCTGTGTGACTGTGAGA       60
XR_003213332.1-On-mef2cX1   GAGAAAGTTTGGCCTGATGAAGAAGGCGTATGAGCT-GAGCGTGCTGTGTGACTGTGAGA       59
XR_003213335.1-Onmef2cX4    GAGAAAGTTTGGCCTGATGAAGAAGGCGTATGAGCT-GAGCGTGCTGTGTGACTGTGAGA       59
XM_010782446.1-Nc-mef2cX5   GCGAAAGTTTGGCCTGATGAAGAAGGCGTATGAGCT-GAGTGTGCTGTGTGACTGTGAGA       59
XM_010782445.1-Nc-mef2cX4   GCGAAAGTTTGGCCTGATGAAGAAGGCGTATGAGCT-GAGTGTGCTGTGTGACTGTGAGA       59
XM_028578836.1-Pf-mef2cX7   GCGAAAGTTTGGCCTGATGAAGAAGGCGTATGAGCT-GAGTGTGCTGTGTGACTGTGAGA       59
XM_028578833.1-Pf-mef2cX4   GCGAAAGTTTGGCCTGATGAAGAAGGCGTATGAGCT-GAGTGTGCTGTGTGACTGTGAGA       59
                            * ********************************  ** ******************

Sl-mef2c                    TTGCCCTGATCATCTTCAATAGCACCAACAAGCTGTTCCAGTATGCCAGCACAGACATGG       120
XR_003213332.1-On-mef2cX1   TTGCCCTGATCATCTTCAACAGCACCAACAAGCTGTTCCAGTATGCCAGCACAGACATGG       119
XR_003213335.1-Onmef2cX4    TTGCCCTGATCATCTTCAACAGCACCAACAAGCTGTTCCAGTATGCCAGCACAGACATGG       119
XM_010782446.1-Nc-mef2cX5   TTGCCCTGATCATCTTCAATAGCACCAACAAGCTGTTCCAGTATGCCAGCACAGACATGG       119
XM_010782445.1-Nc-mef2cX4   TTGCCCTGATCATCTTCAATAGCACCAACAAGCTGTTCCAGTATGCCAGCACAGACATGG       119
XM_028578836.1-Pf-mef2cX7   TTGCCCTGATCATCTTCAATAGCACCAACAAGCTGTTCCAGTATGCCAGCACAGACATGG       119
XM_028578833.1-Pf-mef2cX4   TTGCCCTGATCATCTTCAATAGCACCAACAAGCTGTTCCAGTATGCCAGCACAGACATGG       119
                            ****************** ****************************************

Sl-mef2c                    ACAAGGTCCTGCTTAAATACACCGAGTACAACGAGCCCCATGAGAGCAGGACCAACTCTG       180
XR_003213332.1-On-mef2cX1   ACAAGGTCCTGCTTAAATACACCGAGTACAATGAGCCCCATGAGAGCAGGACCAACTCTG       179
XR_003213335.1-Onmef2cX4    ACAAGGTCCTGCTTAAATACACCGAGTACAATGAGCCCCATGAGAGCAGGACCAACTCTG       179
XM_010782446.1-Nc-mef2cX5   ACAAGGTCCTGCTTAAATACACCGAGTACAACGAGCCCCATGAGAGCAGGACCAACTCAC       179
XM_010782445.1-Nc-mef2cX4   ACAAGGTCCTGCTTAAATACACCGAGTACAACGAGCCCCATGAGAGCAGGACCAACTCAC       179
XM_028578836.1-Pf-mef2cX7   ACAAGGTCCTGCTTAAATACACAGAGTACAATGAGCCCCATGAGAGCAGGACCAACTCTG       179
XM_028578833.1-Pf-mef2cX4   ACAAGGTCCTGCTTAAATACACAGAGTACAATGAGCCCCATGAGAGCAGGACCAACTCTG       179
                            ********************** ******** *************************

Sl-mef2c                    A 181
XR_003213332.1-On-mef2cX1   A 180
XR_003213335.1-Onmef2cX4    A 180
XM_010782446.1-Nc-mef2cX5   C 180
XM_010782445.1-Nc-mef2cX4   C 180
XM_028578836.1-Pf-mef2cX7   A 179
XM_028578833.1-Pf-mef2cX4   A 180
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b.

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Sl-Twist2                   CCCCTGTGGATAGTCTGGTGACCAGCGAGGAGGAGCTGGACAGACAGCAGAAACGCTTCG       60
XM_028591816.1-Pf-Twist2    CCCCAGTGGATAGTCTGGTGACCAGCGAGGAGGAGCTGGACAGAAGGCAGAAACGCTTCG       60
XM_023292754.1-Ao-twist2    CCCCTGTGGATAGTCTGGTGACCAGCGAGGAGGAGCTGGACAGGCAGCAGAAGCGCTTCG       60
XM_005450442.4-On-Twist2    CCCCTGTGGATAGTCTGGTGACCAGCGAGGAGGAGCTGGACAGACAGCAGAAAAGGTTCG       60
                            **** ************************************   ******  * ****

Sl-Twist2                   CGGGGAAGAGGAGACAAAGCAAAAAGTCCAGCGAGGACAGCAGCGGCGGCAGCAGCCCGG       120
XM_028591816.1-Pf-Twist2    CGGGGAAGAGGAGACAAAGCAAAAAGTCCAGCGAGGACAGCAGCGGCGGCAGCAGCCCGG       120
XM_023292754.1-Ao-twist2    CGAGGAAGAGGAGACACAGTAAAAAGTCCAGCGAGGACAGCAGCG---GCAGCAGCCCGG       117
XM_005450442.4-On-Twist2    CGAGGAAGAGGAGGCACAGCAAAAAGTCCAGCGACGACAGCAGCG---GGAGCAGCCCGG       117
                            ** ********** ** ** ************** **********   * **********

Sl-Twist2                   GTCCGGTTAAACGGGTTAAAAAGGCGAGTCCGAGCAGCAATCAGTCGTACGAGGAGCTGC       180
XM_028591816.1-Pf-Twist2    GTCCGGTTAAACGGGTTAAAAAGGCGAGTCCGAGCAGCAATCAGTCGTACGAGGAGCTGC       180
XM_023292754.1-Ao-twist2    GGCCGGTGAAGCGGGGCAAGAAGCCGAGTCCGAGCAGCACTCAGTCGTACGAGGAGCTGC       177
XM_005450442.4-On-Twist2    GTCCGGTGAAACGGGGGAAGAAGCCGAGTCCGAGCAGCACTCAGTCGTACGAGGAGCTGC       177
                            * ***** ** ****  ** *** *************** ********************

Sl-Twist2                   AGAACCAGCGGGTCCTGGCCAACGTCCGGGAGAGGCAACGGACTCAGTC         229
XM_028591816.1-Pf-Twist2    AGAACCAGCGGGTCCTGGCCAACGTCCGGGAGAGGCAACGGACTCAGTC         229
XM_023292754.1-Ao-twist2    AGAACCAGCGGGTCCTGGCCAACGTCCGGGAGAGGCAACGGACTCAGTC         226
XM_005450442.4-On-Twist2    AGAACCAGCGGGTCCTGGCCAACGTCCGGGAGAGGCAACGGACTCAGTC         226
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Figure S2. (a) Sequence alignment of myocyte enhancer factor 2C (mef2c) genes from *Sander lucioperca*, *Oreochromis niloticus* mef2c variant X4 (XR_003213332.1) and X5 (XR_003213335.1), *Notothenia coriiceps* variant X5 (XM_010782446.1) and X5 XM_010782445.1, *Perca flavescens* variant X7 (XM_028578836.1) and X5 (XM_028578833.1). (b) Sequence alignment of twist-related protein 2-like genes from with *Sander luciopera*, *Perca flavescens* (XM_028591816.1), *Amphiprion ocellaris* (XM_023292754.1), *Oreochromis noliticus* (XM_005450442.4). Primer sequences used for qPCR amplification are underlined in the gene sequences of S. lucioperca,* represents the similarity of sequences.

Digestive enzyme activities

The head and tail of pikeperch larvae were dissected on a glass maintained on ice (0 °C) to isolate the digestive segment, and the stomach region was separated from 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 [116] using leucine-alanine (sigma-Aldrich, St LLuis, MO, USA) as substrate. Alkaline phosphatase (AP) and aminopeptidase (N), two enzymes of brush border membrane, were assayed according to Bessey et al. [117] and Maroux et al. [118] using p-nitrophenyl phosphate (Sigma-Aldrich) and L-leucine p-nitroanalide (Sigma-Aldrich) as substrates, respectively. Pepsin was assayed by the method of Worthington [119] modified by Cuvier-Péres and Kestemont [120]. Trypsin and amylase activities were assayed according to Holm et al. [121] and Metais and Bieth [122], respectively such as described by Gisbert et al. [123]. Protein was determined using the Bradford [124] procedure. Enzyme activities are expressed as specific activities (U or mU mg protein^{-1}).

Histological analysis

At the beginning and at the end of the experiment 10 pikeperch larvae from each tank were collected and fixed in 10% buffered formalin. For histological process larvae were dehydrated through graded alcohols (70-96º) using a Histokinette 2000 tissue processor (Leica, Nussloch, Germany), then xylene and finally embedded in paraffin wax (Jung Histoembedder, Leica, Nussloch, Germany). Paraffin blocks were sectioned at 5 μm, on a microtome (Leica, RM2135, Leica Instruments, Nussloch, Germany) and stained with hematoxylin, eosin, safran (HES) and examined using light microscopy using a Olympus CX41 binocular microscope (Olympus, Hamburg, Germany), in a range of magnifications (10-40x), connected to an Olympus XC30 camera (Olympus, Hamburg, Germany), which was linked to a computer using image capturing software (CellB®, Olympus, Hamburg, Germany). To assess hepatocyte vacuolization and presence of goblet cells, a three-point scoring system was used (Supplementary Fig. S2), based on a modified method from Betancor et al. [125]:

1. Score 1: no hepatocyte vacuolization or presence of goblet cells
2. Score 2: mild hepatic vacuolization or presence of goblet cells
3. Score 3: severe hepatocyte vacuolization or presence of goblet cells

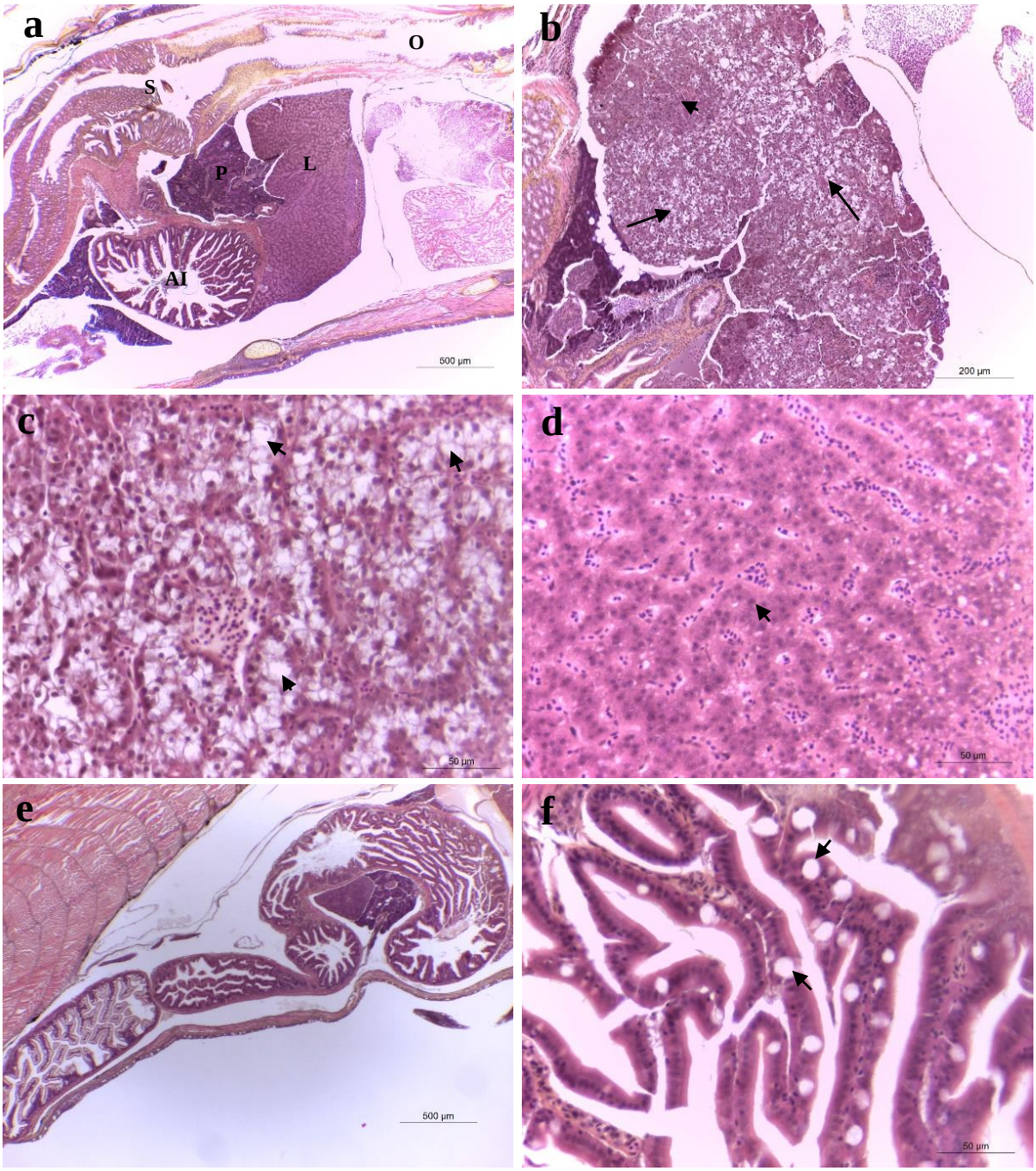

Figure S2. Sections of larvae intestine and liver of pikeperch *Sander lucioperca* from different treatments (10 larvae evaluated per treatment). HES stain. (a) longitudinal section of pikeperch larvae digestive tract: (AI) anterior intestine, (O) oesophagus, (L) liver, (P) pancreas, (S) stomach. (b) liver larvae section showing different degrees of hepatocyte vacuolization levels: very scarce hepatocyte vacuolization area (small arrow) and high lipid vacuoles deposition area (large arrows). (c) high magnification section showing cytoplasmic vacuolization of hepatocytes (arrows). (d) histological structure of hepatic lobule showing condensed hepatocytes with centred nucleus (arrow) and marked cytoplasm staining. (e) whole intestine of 39 dph pikeperch larvae. (f) abundance of goblet cells in the intestine sections (arrows).

Biochemical analysis

Representative samples of the experimental diets were analyzed for ash (NMKL, 1991), crude protein 245 (ISO 2005; crude protein; Kjeldahl N× 6·25) and crude lipid [126]. FA analysis of feeds and larvae was done according to previously described [18]. Lipids were extracted by a chloroform/methanol mixture, (2:1 (v/v) [126] and 40 µl (1 mg mL^{-1}) of an internal 23:0 FAME standard from Sigma Aldrich was added. A fixed amount of each feed (5 mg) was weighed and for larval samples (2x10 larvae per tank) were weighed and homogenized by a Tissue Tearor probe diameter 4.5 mm, Biospec Products, Inc; Bartlesville, USA. Samples were allowed standing for 24 h in -20°C followed by thawing and subsequent centrifugation. The supernatant was subsequently transferred to clean GC vials and allowed drying out in a Pierce, reacti-therm heating module at 60°C, under a continuous flow of nitrogen. Trans esterification of the lipids was done by addition of 1 mL of acetyl chloride in methanol (40:50:10, HPLC quality) 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 (St. Louis, MO, USA). 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 (MSD Chemstation Data Analysis, G1710FA) 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, but only the most relevant are shown (Supplementary Table S5 and S6).

Table S5: 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
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

Table S6: Larval fatty acids composition (% of TFA) of pikeperch larvae fed different experimental diets (a pool of 10 larvae per treatment)

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16
Total SFA	26.66	28.42	23.23	23.93	27.53	25.9	26.31	22.39	28.53	27.65	24.69	24.51	27.29	28.07	23.04	24.51
Total (n-9)	6.33	6.56	4.46	4.52	4.93	4.84	4.62	3.81	6.40	6.04	4.71	4.63	5.24	5.77	4.08	4.41
Total MUFAs	6.42	6.60	4.53	4.55	4.28	4.91	4.73	3.84	6.47	6.10	4.78	4.69	5.30	5.83	4.14	4.45
18:2 (n-6)	15.27	15.33	10.94	10.32	10.82	11.26	10.22	8.34	14.52	13.42	11.28	10.92	12.26	14.97	9.50	9.61
18:3 (n-6)	0.50	0.55	0.39	0.36	0.63	0.64	0.66	0.52	0.55	0.46	0.42	0.38	0.71	0.80	0.61	0.62
20:4 (n-6) ARA	12.01	12.61	10.99	11.06	21.41	20.48	19.97	17.39	13.73	13.62	12.32	11.06	21.01	20.85	19.17	20.14
Total (n-6)	28.36	29.12	22.78	22.74	33.69	33.22	31.77	26.94	29.60	28.16	24.53	23.41	35.03	37.68	30.09	31.28
Total (n-3)	36.02	33.11	46.26	45.19	31.27	33.42	34.23	44.50	32.92	35.04	42.42	44.47	29.86	26.01	29.98	37.45
18:3 (n-3)	6.43	5.79	0.78	0.82	1.97	2.09	0.71	0.72	5.23	5.26	0.83	1.23	2.39	2.95	0.68	0.71
20:5 (n-3) EPA	5.03	5.12	5.52	5.74	4.61	4.46	5.42	4.76	5.19	5.31	6.16	5.88	4.6	4.69	5.27	5.31
22:6 (n-3) DHA	24.35	21.94	39.57	38.28	24.28	26.58	27.65	38.39	22.08	24.12	35.19	37.08	22.45	17.83	33.71	30.92
Total (n-3) LC-PUFA	29.59	27.32	45.48	44.37	39.3	31.33	33.52	43.78	27.69	29.78	41.59	43.24	27.47	23.06	39.30	36.74
DHA/EPA	4.84	4.29	7.17	6.67	5.27	5.96	5.10	8.07	4.25	4.54	5.71	6.31	4.88	3.80	6.40	5.82
EPA/ARA	0.42	0.41	0.50	0.49	0.22	0.22	0.27	0.27	0.38	0.39	0.50	0.51	0.22	0.22	0.27	0.26

Ascorbil-2-monophosphate was extracted from feeds using a phosphate buffer and quantitated by reversed-phase HPLC with UV detection as developed by Roche Vitamins Ltd. Vitamin C concentrations were determined at a wavelength of 293 nm and quantification achieved by comparison with tris (cyclohexylammonium) ascorbic acid-2-phosphate (Sigma-Aldrich), used as a reference substance. Vitamin A (Retinoids) on diets was analyzed by HPLC using the method proposed by Takeuchi et al. [127]. Vitamins E (α-tocopherol) and D3 were determined using HPLC with UV detection at 293 nm and 254 nm, respectively, as described by McMurray et al. [128] and Takeuchi et al. [129].

Experimental feeds were analyzed for alkali and trace minerals in ICP-MS (iCapQ ICP-MS, Thermo Scientific, Waltham, USA) equipped with an auto sampler (FAST SC-4Q DX, Elemental Scientific, Omaha, USA) according to Julshamn and Brenna [130].

Statistics

To determine the best combinations of factors-modalities, each experimental unit was assigned to a global score of interest. This global score was calculated using results of husbandry output variables and was based on the transformation of each output in centered reduced output [58]. Principal component analyses (PCA) were also performed to analyze the global effect of combinations on husbandry output variables.

Main effects and two factor-interactions were then analyzed using Analys software [114]. This method is first based on the detection of potentially active effects using Daniel graphics [131]. It is followed by ANOVA to test these potentially active effects. Significant results ($p < 0.05$) were finally confirmed by ANOVA ($p < 0.05$) using Statistica software version 10 (StatSoft Inc., France, 2011), means were compared according to the Tukey post hoc test.

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Chapter 5

In light of the results obtained in chapter 4; EPA+DHA, ARA and their interaction seem to be key nutritional factors influencing pikeperch larval development. However, only two levels were tested in the multifactorial experiment. Therefore the 4th chapter presents a confirmatory study aiming to investigate the combined effect of graded levels of ARA with two DHA dietary levels (low and high) on larval performance, digestive capacity, biochemical composition, oxidative status and skeletal anomaly incidence in pikeperch larvae. The study was conducted at DTU Aqua in Denmark.

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Dietary DHA and ARA level and ratio affect the occurrence of skeletal anomalies in pikeperch larvae (*Sander lucioperca*) through a regulation of immunity and stress related gene expression

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Dietary DHA and ARA level and ratio affect the occurrence of skeletal anomalies in pikeperch larvae (*Sander lucioperca*) through a regulation of immunity and stress related gene expression

Abstract

Several causative factors have been proposed for the occurrence of skeletal anomalies in fish larvae, among which we quote nutritional factors, such as LC-PUFAs. This study aimed to investigate the effect of different dietary DHA and ARA level and ratio on pikeperch (*Sander lucioperca*) larval development and performance, digestive capacity, fatty acids composition, skeleton anomalies and molecular markers of oxidative stress status (*sod*, *gpx*, and *cat*), stress response (*StAR*, *gr*, *pparα, hsl* and *pepck*), fatty acid synthesis (*fadsd6*, *elovl5*), eicosanoids synthesis (*pla2*, *cox2*, *lox5*, *pge2*, and *lta4h*), and bone development (*twist*, *mef2c*, *sox9*, and *alp*). Pikeperch larvae were fed six microdiets containing two different dietary levels of DHA (0.5 % and 3.5 %) combined with three levels of ARA (1.2 %, 0.6 %, and 0.3 %). Dietary fatty acid changes did not affect growth performance but significantly influenced enzymatic activities. A significant increase in skeletal anomalies with DHA intake increment was recorded. *StAR*, *cox2*, *pla2* and *hsl* expression were significantly depressed in 2.5 % DHA larvae. An opposite effect of dietary DHA elevation was recorded in *gpx* expression. Both DHA and ARA had a significant effect on *pparα*, *gr*, and *pge2* expressions. Although no significant interactions were found, *pge2*, *gr*, and *pparα* displayed a differential pattern of expression between the different treatments. A strong association was found for the larval tissue amount of ARA and DHA with eicosanoid metabolism, stress response and skeleton anomaly related genes. These results denoted the effects of dietary LC-PUFAs on immune/stress gene regulation and their potential implication in skeleton development.

Keywords

Pikeperch, Skeletal anomalies, LC-PUFA, Stress gene, Larvae

1. Introduction

Pikeperch (*Sander lucioperca*) is recognized as one of the main freshwater species with a great potential for the expansion of the EU aquaculture industry mainly because the good flesh quality and the high market value (Alexi et al.,2018). The major bottlenecks for further expansion of pikeperch culture today include low larval survival and high incidence of skeletal anomalies (Kestemont et al., 2015). Pikeperch larvae are very stress sensitive to lack or low levels of n-3 dietary essential long chain polyunsaturated fatty acids (LC-PUFA, n-3) causing lower performance, higher mortality; deficiency syndroms and deformities (Lund and Steenfeldt 2011; Lund et al., 2014). Thus, recent studies suggested requirements similar to those of marine carnivorous fish larvae for both phospholipids and LC-PUFAs (Hamza et al., 2015; Lund et al., 2019). 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). Fish have an endogenous antioxidant defense system with a wide range of antioxidant mechanisms to maintain an adequate oxidative balance (Filho et al., 1993). Among them, various antioxidant enzymes such as catalase (cat), superoxide dismutase (sod) and glutathione peroxidase (gpx) (Bell et al., 1987).

Glucocorticoids (GCs) are central steroid hormones on endocrine stress response modulation and whole-body homeostasis in vertebrates, well known to affect glucose metabolism, immune system, reproduction as well as bone metabolism regulation (Subramaniam et al., 1992; Sapolsky et al., 2000; Suarez-Bregua et al., 2018). Endogenous GC hormones regulate the expression of target genes through glucocorticoid receptor (*gr*) signaling within bone cells, and affecting skeletal development and metabolism (Suarez-Bregua et al., 2018). Also, *gr* is considered as an indicator of lipid nutrition effect on stress response in fish (Alves Martins et al., 2012). In trout, it has been shown that unsaturated fatty-acids inhibit glucocorticoid receptor-binding of hepatic cytosol (Lee and Struve, 1992). Previous studies report possible regulation by *gr* of the transcription of hormone-sensitive lipase (*hsl*) (Alves Martins, et al. 2012; Le et al., 2005; Lampidonis et al., 2008). Furthermore, the gene expression of lipolytic enzymes such as *hsl* were regulated by dietary modifications (Turchini et al., 2003; Ma et al. 2013; Peng et al., 2014). In this respect, Alves Martins et al., (2012) suggested that fatty acids and their derivatives can-indirectly- modulate metabolic pathways related to energetic metabolism (*hsl* and phosphoenolpyruvate carboxykinase *pepck*).

LC-PUFAs are important ligands for nuclear receptors and transcription factors such as peroxisome proliferator-activated receptor (*ppar*) (Lin et al., 1999). Beside the regulation of the

expression of genes that participate in fatty acid oxidation, transcription factor *pparα* have been reported to modulate genes involved in cholesterol uptake and transport (Xie et al., 2002) which is central in steroidogenesis. Previous studies have reported the implications of LC-PUFAs and their derivatives in steroidogenesis in sea-bream (*Sparus aurata*) (Ganga et al., 2006; 2011). Interactions between Pparα and steroidogenic acute regulatory protein mitochondrial (StAR) have been addressed in Atlantic salmon (*Salmo salar*) (Pavlikova et al., 2010).

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 due to competitive interaction among them (Bell and Sargent, 2003; Izquierdo, 2005). Hence, regardless of the need to study the optimum absolute dietary values for LC-PUFAs in this species, optimum dietary ratios must be defined. In fact, LC-PUFAs (specially EPA and ARA) are precursors for highly bioactive eicosanoids. These PUFA-derived mediators (eicosanoids and resolvins), are recognized of high importance in signalling molecules playing roles in biological processes such as inflammation (Kremmyda et al., 2011). Eicosanoids are involved in a great variety of physiological functions and are produced in response to stressful situations. The major precursor of eicosanoids in fish is ARA, while eicosanoids formed from EPA are less biologically active than those formed from ARA (Tocher, 2003). Initially, eicosanoids production is catalyzed by phospholipases (pla), mainly cpla2. The free ARA can undergo several possible enzymatic pathways to create bioactive eicosanoids, among them cyclooxygenase – governed by cytochrome c oxidase subunit (coxs) such as cox2 that mediate the production of prostaglandins -including prostaglandin E synthase 2 (pge2); and lipoxygenase pathway which consists of arachidonate 5-lipoxygenase (lox5) enzymes as well as their products such as leukotrienes -including leukotriene A(4) hydrolase (lta4h) (Kremmyda et al., 2011; Hannah and Hafez, 2018). Furthermore, cox2 seems to play a key role in osteogenic differentiation (Kirkham and Cartmell, 2007).

Initially a multifactorial approach was used to investigate the effects of various dietary nutrients (fatty acids, vitamins and minerals). Results of this screening experiment showed a significant interaction between EPA+DHA and ARA in pikeperch larvae, especially on deformity occurrence, suggesting the importance of a balanced n-3 HUFA/n-6 HUFA ratio in this species (El Kertaoui and Lund et al., 2019). Based on this result, the present experiment was carried out in the facilities of DTU Aqua (Dannmark). The objective of the present study is to understand how dietary DHA/EPA/ARA ratios affect tissue fatty acid profiles and antioxidant and stress response capacity, as well as the relationship between the deformity occurrence and

the stress status in pikeperch. In this sense, the present data evaluated -particularly- larval development and performance, digestive capacity, skeleton deformities and molecular markers of oxidative stress status including: Sod, Gpx, and Cat; stress response including: StAR, Gr, Pparα, Hsl and Pepck; fatty acid synthesis such as fatty acid desaturase 2/acyl-coa 6-desaturase 6 (Fadsd6) and elongation of very long chain fatty acids protein 5 (Elovl5); eicosanoids synthesis such as Pla2, Cox2, Lox5, Pge2 and Lta4h: status and bone development such as twist related protein (Twist), myocyte enhancer factor 2C (Mef2c), transcription factor Sox9 (Sox9) and alkaline phosphatase (Alp).

2. Materials and methods

Ethical standards

The Animal Welfare Committee of DTU Aqua ensured, that protocols and all fish handling procedures employed in the study complied with Danish and EU legislation (2010/63/EU) on animal experimentation. All experiments were performed at the Technical University of Denmark (DTU Aqua) facilities in Hirtshals, Denmark. Fish larvae were not exposed to any surgery and sampled larvae for analyses were kept to an absolute minimum and euthanized by an overdose of clove oil. The dietary nutrient profiles provided were within the range that could reasonably be expected to be encountered in vivo

Larvae and rearing conditions

Newly hatched larvae were obtained from AQUPRI Innovation, Egtved, Denmark and transferred to DTU Aqua at North Sea Research Centre, Denmark, where the experiment was carried out. Larvae were distributed into conical tanks (0,7 m in height and a diameter of 0.3 m), and from 3 dph larvae were fed on unenriched *Artemia* nauplii (AF and EG strains) (INVE, Dendermond, Belgium) until they reached 14 dph, followed by a co-feeding period from 15 to 17 dph using *Artemia* nauplii and a mixture of the experimental diets. The experiment was carried out in a triplicate set-up with 3 tanks per diet. Pikeperch larvae (initial body weight 3.15±1.08 mg) were randomly distributed into 18 experimental conical tanks (50 L) at a density of 1300 larvae per tank in a flow through system with adjustable light and temperature control. Oxygen concentration and temperature were monitored daily by a hand-held Oxyguard meter from Oxyguard, Birkerød, Denmark. During the experiment, oxygen saturation was kept at a mean saturation of 74.8± 3.0 % for all tanks with no significantly difference between treatments ($P \geq 0.480$), and temperature was kept at 20.6±0.7°C. Larvae in each tank were fed with one of six experimental diets. Feed was administered by automatic feeders from 8 am to 6 pm. To

ensure feed availability, daily feed supply was maintained at app. 15-20 % of larval wet biomass per tank during the first week (particles of 200-400 µm /400-700 µm) and 10-15 % per tank biomass (particles of 400-700 µm) during the rest of the experimental period approximately every 20-30 min. Daily, bottom of tanks were vacuum cleaned to remove feed waste. Photoperiod was kept at 12h light: 12h dark.

Experimental diets

Two different dietary levels of DHA were formulated: 0.5 % (low) and 3.5 % (high) combined with three levels of ARA 1.2 %, 0.6 % and 0.3 % (Table 1). Therefore, six isonitrogenous and isolipidic diets were formulated and fabricated by SPAROS S.A. (Portugal) as cold extruded feed pellets of 200-400 µm and 400-700 µm. Experimental diets were formulated using a mix of oils as sources of EPA, DHA and ARA to reach the required fatty acid content and to equalize the lipid content in each diet. Moisture (A.O.A.C. 1995), crude protein (A.O.A.C. 1995) and crude lipid (Folch, Lees & Sloane-Stanley 1957) contents of diets were analyzed. The proximate composition of the main nutrients is shown in table 1. Feeds were tested according to DHA, ARA and DHA/ARA ratios respectively (See table 2).

Table 1. Formulation and the proximate composition (%) of the experimental diets.

	0.6% DHA			2.5% DHA		
	1.2%ARA	0.6%ARA	0.3%ARA	1.2%ARA	0.6%ARA	0.3%ARA
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Ingredients	5.00	5.00	5.00	5.00	5.00	5.00
MicroNorse	5.00	5.00	5.00	5.00	5.00	5.00
CPSP 90	5.00	5.00	5.00	5.00	5.00	5.00
Squid meal 80 ETOX	5.00	5.00	5.00	5.00	5.00	5.00
Krill meal (Low fat)	50.00	50.00	50.00	50.00	50.00	50.00
Fish gelatin	1.20	1.20	1.20	1.20	1.20	1.20
Wheat gluten	10.00	10.00	10.00	10.00	10.00	10.00
Potato starch gelatinised (Pregeflo)	9.50	9.50	9.50	9.55	9.55	9.55
Algatrium DHA70	0.00	0.00	0.00	2.85	2.85	2.85
VEVODAR	3.20	1.55	0.75	3.20	1.55	0.75
Krill oil	1.50	1.50	1.50	0.00	0.00	0.00
Vit & Min Premix PV01	1.00	1.00	1.00	1.00	1.00	1.00
Soy lecithin - Powder	6.20	6.20	6.20	4.80	4.80	4.80
Antioxidant powder (Paramega)	0.40	0.40	0.40	0.40	0.40	0.40
MAP (Monoammonium phosphate)	2.00	2.00	2.00	2.00	2.00	2.00
Proximate composition(%)						
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

Samplings, husbandry variables and analyses

Final survival was calculated by individually counting all living larvae in each tank at the end of the experiment, and expressed as the percentage of the initial numbers of larvae. Representative samples of pikeperch larvae were sampled at 27, 32 and 40 dph for wet weight, and digestive enzymatic assays. Specific growth rate (SGR) was calculated according to the formula (SGR= (ln w.w. f− ln w.w. i × 100) / t, Where ln w.w. f, i = the natural logarithm of the final and initial wet weight, t = time (days)). A random subsample of 10 larvae per replicate was used for FA composition at 32 and 40 dph. Additional 50 larvae per tank were also taken at the end of the experimental period for skeleton morphogenesis and mineralization by staining. These larvae were sedated by an overdosis of clove oil, fixed and stored in 10 % phosphate buffered formaldehyde until analysis. Finally, for the molecular study 10 larvae per replicate were similarly sedated and stored in RNA later overnight at 4 °C and then frozen at −80 °C until analysis.

Fatty acid analysis

FA analysis of feeds and larvae was done according to previously described method (Lund et al., 2014). Lipids were extracted by a chloroform/methanol mixture, (2:1 (v/v) (Folch et al., 1957) and 40 µl (1 mg mL^{-1}) of an internal 23:0 FAME standard from Sigma-Aldrich (Denmark A/S) was added. A fixed amount of each feed (2-3mg) was weighed and for larval samples (10 larvae per tank) were weighed and homogenized by a Tissue Tearor probe diameter 4.5 mm, Biospec Products, Inc; Bartlesville, USA. Samples were allowed standing for 24 h in -20°C followed by centrifugation. The supernatant was subsequently transferred to clean GC vials and allowed drying out in a Pierce, reacti-therm heating module at 60°C, under a continuous flow of nitrogen. Trans esterification of the lipids was done by addition of 1 mL of acetyl chloride in methanol (40:50:10, HPLC quality) 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-Aldrich (St. Louis, MO, USA). 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 (MSD Chemstation Data Analysis, G1710FA) 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}.

Table 2. Main fatty acid content (% TFA) of feeds

Diet	0.6%DHA			2.5%DHA		
	1.2%ARA	0.6%ARA	0.3%ARA	1.2%ARA	0.6%ARA	0.3%ARA
Σ Saturated	71.35	71.78	71.27	65.32	66.27	66.68
Σ Monoenes	7.41	9.20	10.57	6.33	9.83	9.69
Σ n-3	10.25	10.10	10.40	16.55	15.92	15.81
Σ n-6	11.19	8.35	7.28	11.28	7.47	7.32
Σ n-3 LC-PUFA	9.65	9.50	9.79	16.04	15.40	15.30
Σ n-6 LC-PUFA	4.94	2.49	1.35	5.61	2.16	2.08
18:1 n-9	4.94	7.44	8.83	4.68	8.17	8.03
18:2 n-6	6.00	5.71	5.84	5.40	5.18	5.13
18:3n-6	0.25	0.14	0.09	0.27	0.12	0.12
18:3 n-3	0.6	0.59	0.62	0.51	0.51	0.51
ARA	4.72	2.37	1.27	5.37	2.06	1.97
EPA	7.46	7.37	7.57	8.43	8.16	8.07
DHA	2.10	2.05	2.16	7.51	7.17	7.18
EPA/ARA	1.58	3.11	5.94	1.57	3.97	4.09
DHA/EPA	0.28	0.28	0.28	0.89	0.88	0.89
DHA/ARA	0.44	0.87	0.69	1.40	3.49	3.64
n-3/n-6	0.92	1.21	1.43	1.47	2.13	2.16
n-3 LC-PUFA/n-6 LC-PUFA	1.95	3.81	7.26	2.86	7.12	7.37

RNA extraction and reverse-transcriptase quantitative PCR

Samples were homogenized in 1 ml of TriReagent® (Sigma-Aldrich, Danmark A/S) RNA extraction buffer using a bead tissue disruptor (Bio Spec, Bartlesville, Oklahoma, USA). Total RNA was isolated following manufacturer's instructions and quantity and quality determined by spectrophotometry using a Nanodrop ND-1000 (Labtech Int., East Sussex, UK), and electrophoresis using 200 ng of total RNA in a 1 % agarose gel. cDNA was synthesized using 2 µg of total RNA and random primers in 20 µl reactions and the high-capacity reverse transcription kit without RNase inhibitor according to the manufacturer's protocol (Applied Biosystems, Warrington, UK). Gene expression was determined by qPCR of candidate genes: *pparα*, *fadsd6*, *elovl5*, *pepck*, *hsl*, *gr*, *StAR*, *pge2*, *pla2*, *lta4h*, *cox2*, *5-lox*, *gpx*, *sod*, *cat*, *twist*, *mef2c*, *sox9*, *alp*, and intestinal fatty-acid binding protein (*i-fabp*), Elongation factor-1α (*elflα*) and β-actin (*β actin*) were used as reference genes. The cDNA was diluted 20-fold with milliQ water. The efficiency of the primers for each gene was previously evaluated by serial dilutions of cDNA pooled from the samples to guarantee it was > 90 % for all primer pairs. qPCR was

performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicate 20 µl reaction volumes containing 10 µl of Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific, Hemel Hempstead, UK), 1 µl of the primer corresponding to the analyzed gene (10 pmol), 3 µl of molecular biology grade water and 5 µl of cDNA (1/20 diluted). In addition, amplifications were carried out with a systematic negative control (NTC, no template control) containing no cDNA. Standard amplification parameters contained an UDG pre-treatment at 50 °C for 2 min, an initial denaturation step at 95 °C for 10 min, followed by 35 cycles: 15 s at 95 °C, 30 s at the annealing Tm and 30 s at 72 °C. Primer sequences for genes are given in table 3. Data obtained were normalized and the Livak method (2–ΔΔCt) used to determine relative mRNA expression levels. Sequence alignment was done and conserved domains obtained were used to design primers with Primer3 (v. 0.4.0) program and subsequent sequencing of PCR products and BLAST of them. Sequences of genes encoding for *pparα, fadsd6, elovl5, pepck, hsl, gr, i-fabp, StAR, pge2, pla2, lta4h, cox2, 5-lox, gpx, sod* and *cat* were obtained by identifying the sequences from Sequence Read Archives (SRA) SRX1328344 and SRX1385650. The set of contiguous sequences were assembled using CAP3 (Huang and Madan, 1999) and identity of the deduced aa sequences confirmed using the BLASTp sequence analysis service of the NCBI. Sequences for *alp*, *twist22*, *mef2c* and *sox9* were available for the species of interest (Lund et al., 2019, Lund and El Kertaoui et al., 2018). Pikeperch specific gene primers were designed after searching the NCBI nucleotide database and using Primer3. Detailed information on primer sequences is presented in table 3.

Table 3. Sequences of primers used for gene expression analysis

Genes	Sens	Primer sequence (5'to 3')	Efficiency
5-lox	Forward	CAACACCAAGGCCAGAGAAC	0.89
	Reverse	AACTCTTGGTAGCCTCCCAC	
pla2	Forward	TGTGCTGTGGTTTGATCTGC	0.84
	Reverse	CACCTTCATGACCCCTGACT	
elovel5	Forward	CGAAGTATGTATGGCCGCAG	0.83
	Reverse	ATGCCCTGTGGTGGTACTAC	
cat	Forward	TACACTGAGGAGGGCAACTG	0.85
	Reverse	CTCCAGAAGTCCCACACCAT	
cox-2	Forward	GGAACATAACCGGGTGTGTG	0.88
	Reverse	ATGCGGTTCTGGTACTGGAA	
pge2	Forward	CTCGCGCACAATGTAGTCAA	0.84
	Reverse	CTGTGAACGAACGTGGGAAG	
gr	Forward	GTCCTTCAGTCTCGGTTGGA	0.85
	Reverse	TCTTCAGGCCTTCTTTCGGT	

lta4h	Forward	ATCCAGATGTTTGCGTACGG	0.88
	Reverse	GCGTCGTGTCGTACTGATTT	
gpx	Forward	ACACCCAGATGAACGAGCTT	0.93
	Reverse	TCCACTTTCTCCAGGAGCTG	
hsl	Forward	CAGTTCAGTCCAGGCATTCG	0.84
	Reverse	TTCTGCCCCTCTCAACTCTG	
pepck	Forward	CGAACACATGCTGATCCTGG	0.89
	Reverse	CGGGAGCAACACCAAAGAAA	
ppar	Forward	GCCCCAGTCAGAGAAGCTAA	0.87
	Reverse	TTTGCCACAAGTGTCTGCTC	
fadsd6	Forward	GGTCATTTGAAGGGAGCGTC	0.90
	Reverse	TGTTGGTGGTGATAGGGCAT	
sod	Forward	TGTGCTAACCAGGATCCACT	0.87
	Reverse	TCGCTCACATTCTCCCAGTT	
StAR	Forward	CTGGAGACTGTAGCCGCTAA	0.95
	Reverse	TGACGTTAGGGTTCCACTCC	
i-fabp	Forward	ATGTCAAGGAGAGCAGCAGT	0.89
	Reverse	TGCGTCCACACCTTCATAGT	
sox9	Forward	TCCCCACAACATGTCACCTA	0.95
	Reverse	AGGTGGAGTACAGGCTGGAG	
mef2c	Forward	GCGAAAGTTTGGCCTGATGA	0.91
	Reverse	TCAGAGTTGGTCCTGCTCTC	
alp	Forward	GCTGTCCGATCCCAGTGTAA	0.99
	Reverse	CCAGTCTCTGTCCACACTGT	
twist2	Forward	CCCCTGTGGATAGTCTGGTG	0.85
	Reverse	GACTGAGTCCGTTGCCTCTC	
elflα	Forward	TGATGACACCAACAGCCACT	0.81
	Reverse	AAGATTGACCGTCGTTCTGG	
b-actin	Forward	CGACATCCGTAAGGACCTGT	0.93
	Reverse	GCTGGAAGGTGGACAGAGAG	

Skeleton anomalies

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 and alcian blue were conducted to evaluate 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, cleithrum and branchiostegal rays within each dietary group (Fig. 1).

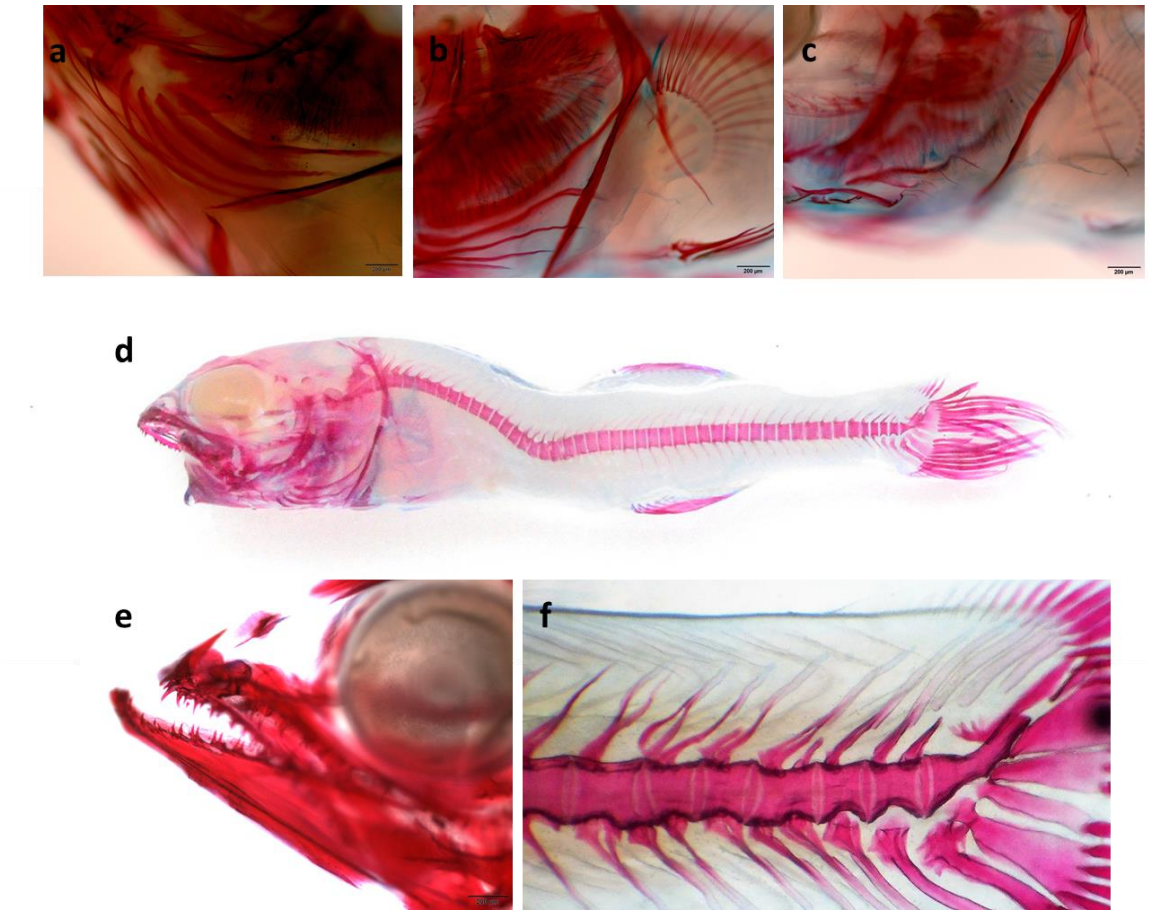


Fig.1. Examples of some skeletal anomalies observed in 40 dph pikeperch *sander lucioperca* larvae. (a) Larvae showing normal branchiostegal rays morphology. (b) Larvae showing a slightly deformed branchiostegal rays. (c) Twisted and fused branchiostegal rays. (d) Larvae showing a severe lordosis and cranium anomaly with marked lower jaw reduction. (e) Lower jaw increment. (f) Larvae showing vertebral body compression and fusion with neural spinal anomalies.

Digestive enzyme activities

The head and tail of 10 pikeperch larvae were dissected on a glass maintained on ice to isolate the digestive segment, and the stomach region was separated from the intestinal segments. Pooled samples from each tank were homogenized in 10 volumes (v/w) cold distilled water. Alkaline phosphatase (AP) and aminopeptidase (N), 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 activity was assayed according to Holm et al. (1988), 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 protein^{-1}).

Statistics

Data are expressed as the mean ± standard error (SEM). Kolmogorov and Smirnov's test was used to assess the normality of data sets ($p < 0.05$) and Bartlett's test was conducted to evaluate variance homogeneity ($p < 0.05$). Two-way ANOVA was used to compare the different endpoints using DHA and ARA dietary levels as fixed factors. The statistical analyses were performed using the JMP 12.1 software (SAS Institute Inc., North Carolina, USA). A Tukey HSD test was used to determine significance of mean differences ($P<0.05$) between the treatment groups where applicable. If no interaction between factors (DHA and ARA dietary levels) in the outcome of the two-way ANOVA, a further one-way ANOVA and Tukey's HSD test were used to determine any significant differences according to the DHA/ARA ratio effect. Data with no normality and/or homogeneity of variances were tested with Kruskall-Wallis tests and post-hoc pair-wise Wilcoxon comparison test. The relationship between the expression of the target genes and larval fatty acid profiles were performed using the R software; first association between paired samples was checked using one of Pearson's product moment correlation coefficient, the correlation matrix was generated using corrplot package and the significance levels (p-values) was generated using lattice package. Then, multivariate principal component analysis (PCA) combined with co-inertia analysis (CIA) were applied to the cross-platform comparison of gene-expression and fatty acid content datasets. Component scores were further clustered according to RVAideMemoire package. PCA and co-inertia analyses were performed with ADE-4 package. All statistical computations were considered significant when resulting p-values were: < 0.05.

3. Result

3.1. Growth and survival

The growth was similar in the different groups of larvae with no significant differences of individual body wet weight at 27, 32 and 40 dph (Table 4). Meanwhile, at the end of the experiment at dph 40, juveniles fed D4 exhibited a lower growth performance compared to the larvae fed D2. Specific Growth Rate (SGR) from 17–40 dph ranged between 12.45 ± 0.67 and 13.32 ±0.33 d^{-1}, and was not significantly different between treatments.

Overall survival at 40 dph was similar with a tendency for a better survival for D4. The apparent mortality (dead larvae siphoned and counted) and the total mortality (including lost larvae due to type II cannibalism) showed no significant differences between groups.

Table 4. Effects of dietary treatments on specific growth rate, individual weight, apparent mortality rate, cannibalism and survival rate. Data are presented as mean ± SEM (n = 3)

Diet	0.6% DHA			2.5% DHA			Two way ANOVA		
	1.2% ARA	0.6% ARA	0.3% ARA	1.2% ARA	0.6% ARA	0.3% ARA	DHA	ARA	DHA*ARA
SGR (% day^{-1})	13.05±0.35	13.32±0.33	13.02±0.45	12.45±0.67	12.99±0.75	12.82±0.55	ns	ns	ns
Apparent mortality (%)	25.62±1.63	27.10±9.40	31.28±5.19	35.03±8.88	34.31±3.91	31.85±7.86	ns	ns	ns
Survival (%)	22.69±4.46	21.13±3.07	22.46±3.09	26.64±4.10	21.15±5.87	19.54±6.02	ns	ns	ns
Cannibalism (%)	51.69±6.09	51.77±7.84	46.26±5.81	38.30±7.52	44.54±2.58	48.62±2.09	ns	ns	ns
Weight at 27dph (mg)	17.40±0.83	14.41±0.86	16.23±1.60	16.08±1.58	15.38±1.38	16.68±3.14	ns	ns	ns
Weight at 32 dph (mg)	23.10±1.77	21.64±2.21	24.74±3.12	22.42±0.99	23.47±1.14	22.25±4.49	ns	ns	ns
Weight at 40 dph (mg)	63.46±5.09	67.48±5.07	63.13±6.65	55.59±8.23	63.16±10.37	60.41±7.63	ns	ns	ns

3.2. Larval fatty acid composition and gene expression

Fatty acid compositions of 32 dph and 40 dph pikeperch larvae are presented in tables 5 and 6. Higher levels of DHA, 18: 3n-3, total n-3 LC-PUFA and total n-3 larval contents were found in 40 dph larvae fed diets 4, 5 and 6 (p: 0.0005, 0.0375, 0.0153 and 0.0219 respectively) as a consequence of higher dietary DHA levels, while no significant differences were detected at 32 dph except for 18: 3n-3 (p= 0.0001). The group of larvae fed a higher dietary ARA content showed an increase in their ARA body content and resulted also in a higher n-6 LC-PUFA and total n-6 at 32 dph as well as at 40 dph. In contrast, monounsaturated acid content was significantly higher in larvae fed 0.3 % ARA (p= 0.0442, 0.0005 respectively at 32 dph and 40 dph) principally due to a higher percentage of oleic acid (18: 1n-9) in these larvae (p= 0.0334 and 0.0087 respectively at 32 dph and 40 dph). Similarly, a decrease in dietary ARA resulted also in graded increase in EPA/ARA ratio (p=0.0001, 0.0005 at 32 dph and 40 dph respectively) and EPA larval content (p= 0.047, 0.0001 at 32 dph and 40 dph respectively) was negatively correlated with ARA larval content (Fig. 2; R= 0.9708 at 40dph). Significant interactions between DHA and ARA were found in DHA/ARA and n-3 LC-PUFA/ n-6 LC-PUFA ratios in 40 dph larvae (p = 0.00978 and 0.039 respectively), while the total content of saturated fatty acids (SFA) was similar among larvae fed the different experimental diets.

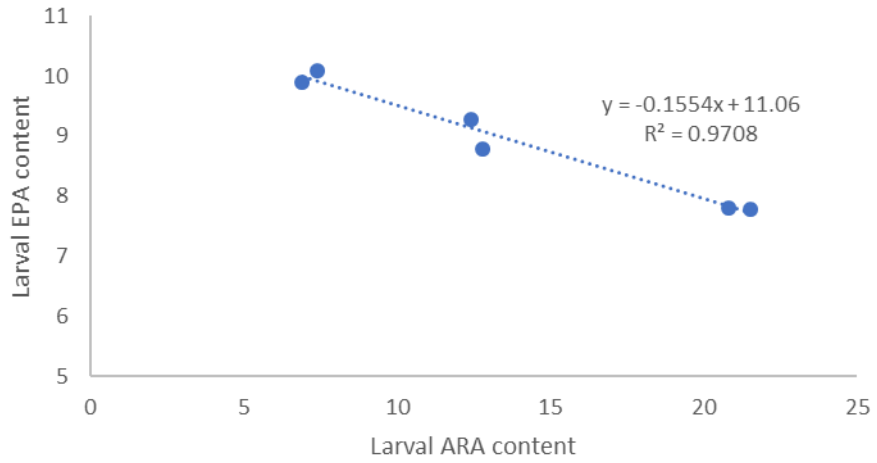


Fig. 2. Correlation between EPA and ARA levels (% total fatty acids) in the whole body of pikeperch larvae fed different experimental diets.

Table 5. Main fatty acid content of larvae (% TFA) at 32dph

Diet	0.6%DHA			2.5%DHA			Two way ANOVA		
	1.2%ARA	0.6%ARA	0.3%ARA	1.2%ARA	0.6%ARA	0.3%ARA	DHA	ARA	DHA*ARA
Σ Saturated	32.08±6.51	34.29±5.33	31.11±1.80	33.67±8.26	33.00±4.40	33.61±4.58	ns	ns	ns
Σ Monoenes	21.10±3.16^{b}	23.98±6.17^{ab}	25.66±1.07^{a}	18.72±3.50^{b}	24.17±3.52^{ab}	25.54±1.60^{a}	ns	*	ns
Σ n-3	15.87±7.28	13.44±4.64	19.48±3.06	17.79±8.99	18.30±6.21	21.77±5.40	ns	ns	ns
Σ n-6	30.60±3.70^{a}	27.93±6.73^{ab}	23.43±0.31^{b}	29.46±2.83^{a}	24.24±1.61^{ab}	18.79±0.79^{b}	ns	**	ns
Σ n-3 LC-PUFA	14.98±7.20	12.62±4.62	18.56±3.10^{a}	17.13±8.92	17.54±6.21	21.10±5.36	ns	ns	ns
Σ n-6 LC-PUFA	15.87±4.26^{a}	13.72±6.47^{ab}	8.27±0.02^{c}	17.76±2.89^{a}	11.73±1.61^{ab}	8.44±0.34^{c}	ns	**	ns
18:1 n-9	16.47±2.83^{b}	19.35±5.66^{ab}	21.18±1.12^{a}	14.13±3.30^{b}	19.38±3.55^{ab}	20.89±1.93^{a}	ns	*	ns
18:2 n-6	14.20±0.77^{a}	13.75±0.48^{ab}	14.83±0.31^{a}	11.16±0.05^{cd}	12.13±0.62^{bc}	10.13±1.10^{d}	***	ns	**
18:3n-6	0.53±0.11^{a}	0.47±0.12^{a}	0.33±0.01^{c}	0.54±0.01^{a}	0.38±0.03^{ab}	0.23±0.01^{c}	ns	**	ns
18:3 n-3	0.89±0.08^{a}	0.83±0.11^{ab}	0.92±0.04^{a}	0.66±0.06^{b}	0.76±0.03^{ab}	0.68±0.03^{b}	***	ns	ns
ARA	15.40±4.19^{a}	13.28±6.37^{ab}	7.97±0.05^{c}	17.28±2.90^{a}	11.41±1.61^{ab}	8.25±0.36^{c}	ns	**	ns
EPA	9.04±2.94	7.71±1.98	12.29±0.36	7.71±2.37	9.29±1.68	9.94±0.73	ns	*	ns
DHA	5.75±4.25	4.74±3.25	6.17±3.43	9.25±6.57	8.14±4.62	11.07±4.63	ns	ns	ns
EPA/ARA	0.61±0.27^{b}	0.62±0.17^{b}	1.54±0.05^{a}	0.44±0.07^{b}	0.81±0.03^{b}	1.21±0.14^{a}	ns	***	*
DHA/EPA	0.57±0.33	0.61±0.33	0.51±0.29	1.07±0.64	0.83±0.40	1.09±0.40	*	ns	ns
DHA/ARA	0.39±0.34^{b}	0.35±0.14^{ab}	0.77±0.43^{a}	0.50±0.33^{b}	0.68±0.34^{ab}	1.36±0.61^{a}	ns	*	ns
n-3/n-6	0.52±0.25^{b}	0.47±0.05^{b}	0.83±0.14^{ab}	0.59±0.26^{ab}	0.75±0.23^{ab}	1.15±0.25^{a}	*	**	ns
n-3 LC-PUFA/n-6 LC-PUFA	0.99±0.60^{bc}	0.95±0.09^{c}	2.24±0.37^{ab}	0.92±0.39^{c}	1.46±0.37^{bc}	2.52±0.72^{a}	ns	***	ns

Different superscript letters within a row denote significant differences among diets determined by one-way ANOVA with Tukey's comparison test ($p < 0.05$).
$*$ $p < 0.05$
$**$ $p < 0.01$
$**$ $p < 0.001$

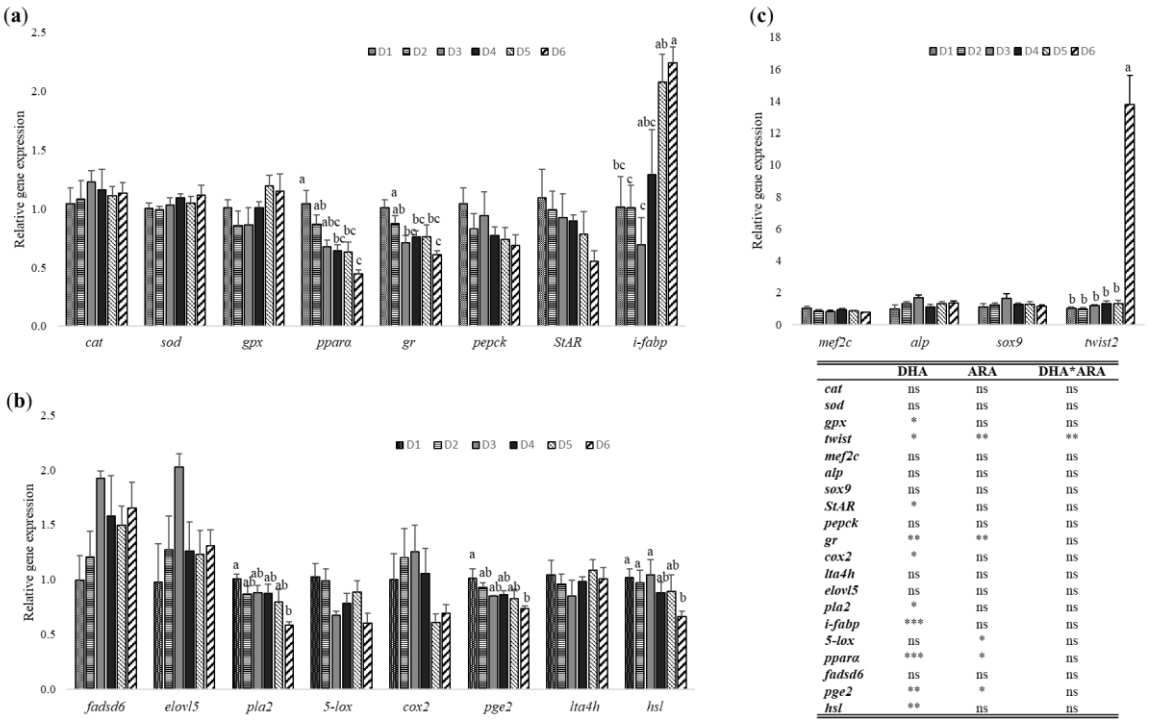
Table 6. Main fatty acid content of larvae (% TFA) at 40 dph

Diet	0.6%DHA			2.5%DHA			Two way ANOVA		
	1.2%ARA	0.6%ARA	0.3%ARA	1.2%ARA	0.6%ARA	0.3%ARA	DHA	ARA	DHA*ARA
Σ Saturated	32.68±0.39	34.94±2.55	36.27±2.92	37.03±5.01	33.03±10.20	33.18±5.75	ns	ns	ns
Σ Monoenes	18.03±1.29^{c}	24.21±1.26^{b}	27.08±0.71^{a}	16.33±0.50^{c}	24.65±2.04^{b}	28.20±1.96^{a}	ns	***	ns
Σ n-3	11.97±1.65	13.09±1.93	13.76±1.29	13.91±1.67	16.25±4.29	17.58±2.27	*	ns	ns
Σ n-6	37.01±0.23^{a}	27.48±1.31^{bc}	22.62±1.66^{c}	32.44±2.94^{ab}	25.85±4.53^{bc}	20.81±2.68^{c}	ns	***	ns
Σ n-3 LC-PUFA	11.18±1.60	12.28±1.87	12.97±1.23	13.29±1.58	15.55±4.16	16.84±2.18	*	ns	ns
Σ n-6 LC-PUFA	22.04±0.24^{a}	12.79±0.67^{b}	7.70±0.67^{c}	21.23±1.07^{a}	13.02±1.60^{b}	7.08±0.33^{c}	ns	***	ns
18:1 n-9	13.96±1.55^{b}	20.00±1.54^{a}	22.69±0.95^{a}	12.75±0.34^{b}	20.58±1.82^{a}	19.69±6.23^{a}	ns	**	ns
18:2 n-6	14.36±0.22	14.30±1.07	14.60±1.26	10.68±1.85	12.47±3.15	13.49±2.73	ns	ns	ns
18:3n-6	0.61±0.06^{a}	0.40±0.05^{bc}	0.33±0.01^{c}	0.53±0.08^{ab}	0.35±0.06^{cd}	0.25±0.02^{d}	*	***	ns
18:3 n-3	0.79±0.05	0.80±0.10	0.80±0.06	0.62±0.12	0.70±0.13	0.74±0.11	*	ns	ns
ARA	21.52±0.30^{a}	12.41±0.64^{b}	7.39±0.66^{c}	20.79±1.03^{a}	12.78±1.54^{b}	6.90±0.5^{c}	ns	***	ns
EPA	7.79±0.41^{b}	9.27±0.67^{ab}	10.09±0.65^{a}	7.81±0.88^{b}	8.79±0.85^{ab}	9.90±0.86^{a}	ns	***	ns
DHA	3.20±1.45	2.89±1.38	2.78±0.65	5.31±0.63	6.66±3.27	6.87±2.14	***	ns	ns
EPA/ARA	0.36±0.02^{c}	0.75±0.03^{b}	1.37±0.08^{a}	0.38±0.02^{c}	0.69±0.02^{b}	1.44±0.14^{a}	ns	***	ns
DHA/EPA	0.41±0.19	0.31±0.13	0.27±0.05	0.68±0.01	0.74±0.29	0.70±0.23	**	ns	ns
DHA/ARA	0.15±0.07^{c}	0.23±0.10^{bc}	0.37±0.05^{b}	0.25±0.02^{bc}	0.51±0.18^{b}	1.01±0.37^{a}	*	*	**
n-3/n-6	0.32±0.04^{c}	0.48±0.06^{bc}	0.61±0.03^{b}	0.43±0.02^{c}	0.62±0.07^{b}	0.85±0.11^{a}	***	***	ns
n-3 LC-PUFA/n-6 LC-PUFA	0.51±0.08^{e}	0.96±0.09^{cd}	1.68±0.05^{b}	0.62±0.04^{de}	1.18±0.17^{c}	2.39±0.42^{a}	ns	***	*

Different superscript letters within a row denote significant differences among diets determined by one-way ANOVA with Tukey's comparison test ($p < 0.05$).
$*$ $p < 0.05$
$**$ $p < 0.01$
$**$ $p < 0.001$

Among the 20 studied genes, 11 target genes showed significant differences in expression between the dietary treatments (Fig. 3). The transcription of *StAR*, *cox2*, *pla2* and *hsl* was significantly depressed in 2.5 % DHA larvae (p= 0.043; 0.030, 0.018 and 0.0076 respectively) while an opposite significant effect of dietary DHA elevation was recorded in *gpx* and *i-fabp* expression (p= 0.0218 and 0.0002). Besides the DHA effect, the results of one-way ANOVA indicated, that larvae fed D6 differed significantly in *hsl* and *i-fabp* expression from D1, D2 and D3 treatments (p = 0.0476 and 0.0014 respectively). *I-fabp* expression was significantly upregulated in larvae fed diet D5 compared to D2 and D3 groups (Fig. 3a) (p=0.0014), similarly *pla2* expression was higher in D6 than D1 treatment (p= 0.0186). Both DHA and ARA had a significant effect in *pparα*, *gr*, and *pge2* expressions. The transcription of these genes (*pparα, gr* and *pge2*) was significantly depressed with the dietary DHA increment (p= 0.0004; 0.0041; 0.003); a similar pattern of gene expression occurred in the lowest ARA-fed group compared to 1.2 % ARA group (p= 0.015; 0.0011; 0.0251). Although no significant interactions were found, *gr* and *pparα* transcript levels were higher in D1-fed larvae compared with D4, D5 and D6 groups (Fig. 3a) (One-way ANOVA p = 0.0083; 0.0004), while D2 differed significantly from D6 treatment. Compared to D6, larvae fed D1, D2 and D4 displayed an increased transcript level in *pge2* (Fig. 3b) (p = 0.0135). *Twist2* gene expression presented a significant interaction among DHA and ARA dietary content; pikeperch fed diet D6 showed the highest expression in *twist2* than larvae fed the other diets (p = 0.0079) (Fig. 3c). Furthermore, results from the two-way ANOVA regarding expression of *twist2* showed also a higher dietary effect of both DHA and ARA (p= 0.01 and 0.0043 respectively). Dietary ARA content had a clear effect on the expression of *5-lox* (p= 0.0345).

The large standard deviations in the expression of the rest of the genes studied (*fadsd6*, *elovl5*, *lta4h*, *cat*, *sod*, *sox9*, *mef2c*, *alp* and *pepck*) did not allow to find significant differences among the different treatments. However, larvae fed diet D3 showed approximately twice as high expression in lipid metabolism *elovl5* and *fadsd6* genes than D1 fed group (Fig. 3b). Likewise, *pepck* expression showed a tendency to up-regulation in larvae fed low DHA level (0.6 %) (p = 0.054). A trend for an increased expression of *mef2c* gene with the dietary ARA elevation was observed (p= 0.069), while *alp* gene expression tended to decrease gradually with the dietary ARA supply (Fig. 3c; p = 0.059). No significant differences or specific tendencies were found in *cat*, *sod*, *sox9* and *lta4h* gene expressions.



	DHA	ARA	DHA*ARA
cat	ns	ns	ns
sod	ns	ns	ns
gpx	*	ns	ns
twist	*	**	**
mef2c	ns	ns	ns
alp	ns	ns	ns
sox9	ns	ns	ns
StAR	*	ns	ns
pepck	ns	ns	ns
gr	**	**	ns
cox2	*	ns	ns
lta4h	ns	ns	ns
elovl5	ns	ns	ns
pla2	*	ns	ns
i-fabp	***	ns	ns
5-lox	ns	*	ns
pparα	***	*	ns
fadsd6	ns	ns	ns
pge2	**	*	ns
hsl	**	ns	ns

Fig. 3. Effects of dietary DHA and ARA on relative mRNA levels of genes involved in stress response (a), lipid metabolism pathways including LC-PUFA biosynthesis and eicosanoid metabolism (b), and skeleton anomaly related genes (c) in 40 dph pikeperch larvae as determined by qPCR. Results are normalised expression ratios (means±SEM; n=5). Different superscript letters denote differences among treatments identified by one-way ANOVA. The inset table presents p values for the effect of DHA, ARA and their interaction on the relative gene expression.
∗ $p < 0.05$
∗∗ $p < 0.01$
∗∗ $p < 0.001$
ns, not significant differences

3.3 Relationships between gene expression and larval fatty acid content

The relationships between the studied target genes and larval fatty acid profile explored through Pearson's correlation coefficient is illustrated in figure 4a. The strongest associations were found for the amount of ARA and DHA with eicosanoid metabolism, stress response and skeleton anomaly related genes ($p < 0.05$). Thus *mef2c*, *pparα, pla2*, *pge2* and *gr* were positively correlated with ARA, while negatively with the amounts of DHA. Significant correlation was found between *StAR*, *hsl* and *i-fabp* gene expressions and DHA. *Twist2* and *alp* showed a negative correlation with ARA level. Equally, 20:3n-3 and 18: 3n-6 displayed a similar correlation as ARA with *twist2* and *pparα*. The expression of specific antioxidant genes was significantly correlated with 18:3n-3 (*sod* and *gpx*) and 18:2n-6 (*gpx*). Those correlations were reinforced by principal component analysis (PCA) combined with co-inertia analysis and algorithm clustering results presented in figure 4b, which concomitantly illustrates the

segregation of two clusters in both genes and fatty acid profile. Except 20:3n-3; n-3 LC-PUFA (EPA and DHA) and oleic acid were clustered together and separately than the other figured fatty acids. Furthermore, all eicosanoid metabolism genes were clustered with *mef2c* and stress response genes (*StAR*, *gr* and *pepck*) in the opposite direction of DHA level while positively linked to ARA level.

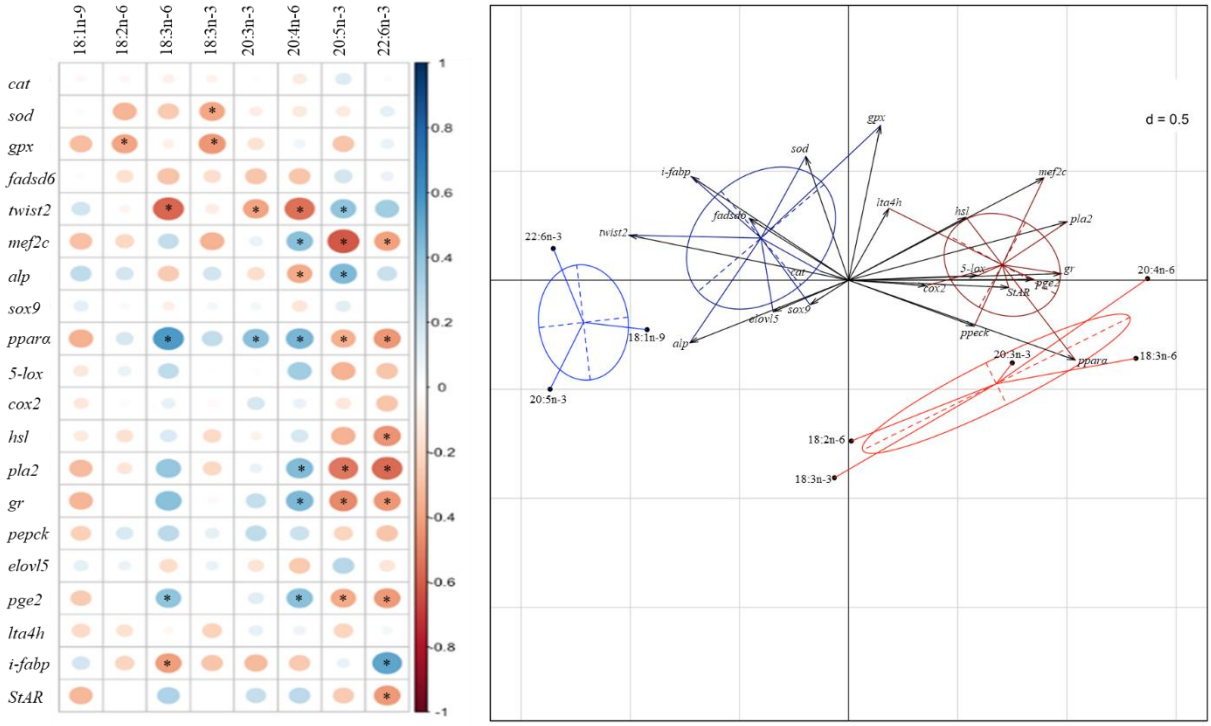


Fig. 4. Association between expression of target genes and selected larval fatty acid content. (a) Correlation matrix between gene expression and larval fatty acid content as presented by Pearson's product moment correlation coefficient. (b) Combined Principal component analysis (PCA) and co-inertia (CIA) of larval fatty acid data (%) and expression of target genes; the components scores were clustered according to RVAideMemoire package. Different color refers to the degree to which a pair of variables are linearly related as presented in the inset-colored axis. ∗ $p < 0.05$ Significative correlation.

3.4. Skeleton anomaly evaluation

Overall, high incidence of lordosis and cephalic anomalies were observed in the present study (Table 7). Two-way ANOVA results indicated a significant increase in different skeletal anomaly typologies with dietary DHA intake increment, in particular anomalies of bone formed by direct ossification ($p = 0.012$). Higher incidence of opercular deformities was observed in larvae fed high DHA ($p = 0.043$), mainly governed by the higher branchiostegal ray anomaly observed in these larvae ($p = 0.001$). Similarly, the increase in DHA led to a higher incidence of dentary bone anomalies ($p = 0.001$) and pectoral element deformities ($p = 0.007$), in particular cleithrum anomaly ($p = 0.006$). Furthermore, despite the lack of significant

interaction between DHA and ARA in the occurrence of deformities, one -way ANOVA results showed a significantly higher occurrence of maxillary bone and branchiostegal rays anomalies in D6 than in D2 fed-larvae ($p = 0.011$ and 0.008 respectively). No differences were found in the degree of mineralization according to the size of the larvae (data not shown).

Table 7. Occurrence of bone anomalies found at 40 dph in pikeperch fed the different experimental diets

Diet	0.6% DHA			2.5% DHA			Two way ANOVA		
	1.2% ARA	0.6% ARA	0.3% ARA	1.2% ARA	0.6% ARA	0.3% ARA	DHA	ARA	DHA*ARA
Severe	64.63±1.71	56.44±7.39	67.56±2.35	66.08±6.06	74.22±10.65	71.51±4.76	ns	ns	ns
Lordosis	35.22±5.97	37.23±3.62	41.65±7.30	38.23±3.53	38.02±11.32	25.83±0.83	ns	ns	ns
Scoliosis	8.01±5.03	5.80±5.80	8.64±2.93	7.21±2.46	26.87±14.25	6.07±2.78	ns	ns	ns
Branchiostegal rays	5.32±0.62^{b}	7.80±5.98^{b}	3.96±1.98^{b}	10.33±2.24^{ab}	17.17±2.64^{ab}	24.51±4.10^{a}	**	ns	ns
Dentary	22.69±1.85^{ab}	12.28±1.62^{b}	16.56±0.72^{ab}	26.10±5.42^{ab}	25.93±3.58^{ab}	31.11±3.09^{a}	**	ns	ns
Maxillary	12.68±2.45	9.86±5.94	3.97±2.31	14.56±10.72	11.38±7.49	11.94±6.74	ns	ns	ns
Jaws	28.06±3.27	20.81±8.09	18.56±1.84	27.38±4.45	35.06±7.53	39.85±1.23	*	ns	ns
Cleithrum	5.43±4.47	2.73±1.37	1.32±0.66	7.79±2.22	10.07±5.21	23.23±7.97	**	ns	ns
Opercular	11.44±5.50	10.70±8.85	10.58±5.43	14.18±4.13	20.76±2.76	28.15±4.24	*	ns	ns
Pectoral elements	6.79±5.82	3.37±1.74	1.32±0.66	8.44±2.78	10.07±5.21	23.23±7.97	**	ns	ns
Direct ossification	11.42±3.67	6.71±1.62	5.29±0.65	14.85±4.41	18.60±9.86	29.59±8.79	*	ns	ns
Other cephalic anomalies	41.96±2.74	31.66±8.20	42.50±8.52	36.367±3.34	54.42±13.91	48.14±2.87	ns	ns	ns

Different superscript letters within a row denote significant differences among diets determined by one-way ANOVA with Tukey's comparison test ($p < 0.05$).
$*$ $p < 0.05$
$**$ $p < 0.01$
$**$ $p < 0.001$

3.5. Specific enzymatic activities

Pepsin activity was higher in the high DHA-fed groups (Table 8) ($p = 0.0127$), while no differences were observed at 32 and 40 dph. Combined effect of DHA and ARA with significant interaction was found in trypsin activity at 27 and 40 dph ($p = 0.0003$ and 0.0017 respectively). Larvae fed diet 6 presented the highest trypsin activity at 27 dph ($p = 0.0003$); on the opposite, this treatment resulted in the lowest trypsin activity at 40 dph ($p = 0.0017$). Brush border enzymes (alkaline phosphatase and aminopeptidase) displayed significant differences among the different dietary ARA levels ($p = 0.0005$ and 0.001 respectively) at 40 dph. On the other hand, no differences of alkaline phosphatase and aminopeptidase activities were recorded between treatments at 27 and 32 dph.

Table 8. Specific enzymatic activity (mU mg protein^{-1}) in pikeperch larvae fed different experimental diets

Diet	0.6% DHA			2.5% DHA			Two way ANOVA		
	1.2% ARA	0.6 % ARA	0.3% ARA	1.2% ARA	0.6% ARA	0.3% ARA	DHA	ARA	DHA*ARA
Specific enzymatic activity at 27 dph									
Trypsin	13.63±1.1^{b}	11.13±1.4^{b}	7.73±1.6^{b}	7.67±2.9^{b}	14.07±2.4^{b}	27.46±1.9^{a}	**	*	***
Pepsin	4.50±1.6^{b}	3.08±0.8^{b}	4.30±0.9^{b}	5.07±1.5^{a}	5.80±1.1^{a}	14.57±3.0^{a}	*	ns	ns
Aminopeptidase	5.03±0.9	7.00±0.8	8.44±1.5	7.51±2.6	2.64±0.5	8.86±0.5	ns	ns	ns
Alkaline phosphatase	60.52±3.5	43.72±7.6	60.79±5.5	51.55±3.2	52.94±5.4	72.21±16.6	ns	ns	ns
Specific enzymatic activity at 32 dph									
Trypsin	12.33±1.0	10.67±1.2	15.76±2.2	11.62±1.4	11.81±1.5	9.62±1.1	ns	ns	ns
Pepsin	164.57±31.0	248.17±52.4	242.55±80.4	176.28±68.7	242.10±37.6	214.08±59.2	ns	ns	ns
Aminopeptidase	5.63±1.5	7.07±1.2	7.42±2.2	7.44±2.6	9.03±2.0	7.84±2.5	ns	ns	ns
Alkaline phosphatase	21.90±4.1	30.74±7.6	36.25±12.1	34.14±12.7	39.52±10.5	31.65±5.9	ns	ns	ns
Specific enzymatic activity at 40 dph									
Trypsin	10.88±0.4^{bcd}	20.0±2.0^{ab}	26.03±1.4a	8.43±1.6^{cd}	14.74±4.0^{bc}	3.25±1.2d	***	*	**
Pepsin	141.18±22.6	123.93±14.3	141.94±22.4	145.25±22.4	133.42±9.3	136.03±6.9	ns	ns	ns
Amonipeptidase	15.20±3.0^{a}	8.75±0.5^{b}	12.04±0.4^{a}	12.66±0.9^{a}	9.02±0.6^{b}	12.94±0.5^{a}	ns	**	ns
Alkaline phosphatase	56.52±0.5^{a}	39.32±2.6^{b}	45.85±1.3^{b}	66.39±6.7^{a}	40.71±3.1^{b}	50.29±2.3^{b}	ns	***	ns

Different superscript letters within a row denote significant differences among diets determined by one-way ANOVA with Tukey's comparison test ($p < 0.05$).

$*$ $p < 0.05$

$**$ $p < 0.01$

$**$ $p < 0.001$

4. Discussion

To the best of our knowledge, there are no report so far on the expression level of genes associated with eicosanoid synthesis, lipid metabolism and stress response during early development of pikeperch larvae. The present study represents the first investigation on how dietary LC-PUFAs (DHA and ARA) affect immune/stress gene regulation and their putative implication in skeleton development.

Although no significant growth differences were observed among the different treatments, molecular biomarkers, biochemical and osteological endpoints investigated in the present study highlight the influence of both DHA and ARA and their interaction on pikeperch larval development. The increased dietary DHA up to 2.5 %, led to the increment in incidence of skeletal deformities. This result is somewhat contradictory with the results of a recent study (Lund and El Kertaoui et al., 2018) in which a clear tendency towards decreasing prevalence of severe skeletal deformities was observed in pikeperch fed increased dietary levels of DHA. In fact, the positive effect of dietary DHA elevation recorded by Lund and El Kertaoui et al. (2018) on pikeperch skeletal anomalies was probably attributed to dietary phospholipid elevation applied in this experimental design, since the increased dietary PL reduced the prevalence of skeletal anomalies (Lund and El Kertaoui et al. 2018; Cahu et al., 2003; Boglione et al., 2013; Saleh et al., 2013). Thus, besides the fatty acid profile, lipid structure seems to be another important nutritional factor influencing the skeletal development in pikeperch larvae. In this respect Villeneuve et al. (2005) associated the increased skeletal anomaly occurrence with n-3 LC-PUFA (EPA and DHA) elevation in the neutral lipid fraction. Negative effects of excessive DHA intake on the occurrence of skeleton anomalies- especially dentary and maxillary deformities- were also reported in gilthead seabream *Sparus aurata* (Izquierdo et al., 2013). Same authors associated the increased oxidative stress with the endochondral bone anomalies. Consistently with this hypothesis, together with the increased oxidative status of pikeperch larvae – as presented by the higher expression of *gpx*- in the present study, the skull, especially the cranial structures such as dentary and operculum complex including the branchiostegal rays remind the most affected, when high DHA induced anomalies were detected. However, *sod* and *cat* expression showed no significant differences in transcription levels among the different groups. Jin et al. (2017) suggested no oxidative stress effects on antioxidant defense capability through Sod activation in juvenile black seabream (*Acanthopagrus schlegelii*) fed high DHA/EPA ratio. Interestingly, antioxidant enzyme mRNA expression levels increased concomitantly with the decrease of larval C18 fatty acid content, especially α-linolenic acid

(ALA; 18:3n-3) which correlated negatively with *sod* and *gpx* expression levels and linoleic acid (LA; 18:2n-6) negatively correlated with *gpx* expression levels. High dietary LA also negatively impacted nonspecific immunity and antioxidant capacity in juvenile large yellow croakers (*Larimichthys crocea*) (Zuo et al., 2015). Previous studies demonstrated that ALA tended to be more prone to β-oxidation or excretion rather than to elongation into EPA and DHA (Fu and Sinclair, 2000). In spite of the different dietary and larval fatty acid contents (including DHA, EPA, ARA and their precursors ALA and LA), the expression of genes involved in desaturation (*fadsd6*) and elongation (*elovl5*) were not influenced. Indeed, the present results likely reflected an adaptation as a result of a negative feedback, especially in fish fed higher DHA level (diets: D4, D5, D6) permitting to maintain LC-PUFA and their metabolites within the required physiological levels.

The differences in larval fatty acid profiles were not limited to ARA, and DHA. The results of gene expression may reflect the combined actions of other fatty acids (EPA, oleic acid, LA and ALA). Accordingly, differential pattern of gene expression was recorded depending on the fatty acid larval content. *Alp* expression showed a negative correlation with ARA content. Alp is recognized as a biomarker of osteoblast differentiation and direct formation of bone via the intra-membranous ossification pathway (Hessle et al., 2002). However, a significant increase in anomalies of bone formed by direct ossification was observed with dietary DHA increment. Increase in DHA in lower ARA-fed group (diet D6) resulted in higher branchiostegal rays and dentary deformities. These fish presented the highest expression of *twist2*, a gene involved in osteoblast inhibition, but also displayed an antioxidant activity being involved in the control of reactive oxygen species (ROS) (Floc'h et al., 2013). Recent results showed a differential effect of dietary ARA on skeletal deformities depending on the EPA+DHA levels (El Kertaoui and Lund et al., 2019) pointing out the need of a balanced dietary n-3/n-6 ratio in this species. This is well known that prostaglandins are potent regulators of bone formation and bone resorption (Meghji et al., 1988; Raisz, 1995). Thus, an imbalance of n-3/n-6 -especially EPA/ARA ratio- may result in the prostaglandin imbalance and consequently, affects the production of PGs which can lead to an imbalance of bone formation and resorption (Boglino et al., 2014), in particular, the PGE2 concentrations known to influence both bone formation and resorption (Berge et al., 2009). In Senegalese sole (*Solea senegalensis*) increased PGE2 production induced by dietary ARA supplementation resulted in the reduction in bone ossification (Boglino et al., 2013). ARA is the major precursor of eicosanoids in fish cells and usually considered as the major substrate for eicosanoid synthesis (Bell et al., 1994; Furuita et al., 2007). Thus,

increased amounts of ARA led to an increased amount of substrate available for synthesis of ARA-derived eicosanoids. In this sense, our finding showed a clear response to ARA intake with the expression of eicosanoid metabolism related genes. On the other hand, as expected, the larval body fatty acid composition reflected dietary fatty acid profiles, especially DHA and ARA, which increased in the higher DHA and higher ARA fed groups respectively. This explains the positive correlation ($p< 0.05$) found between the larval ARA content and the expression of the eicosanoid metabolism genes in particular *pge2* and *pla2*. *5-lox* expression was mainly governed by ARA level. The present results are in agreement with those found in gilthead sea-bream, where changes in the expression of these genes were associated with ARA intake (Alves Martins et al., 2012). Meanwhile, despite the similar EPA concentrations among the experimental diets, EPA larval content was reduced significantly with larval ARA increment, indicating a preferential EPA metabolism, especially with the increase in dietary ARA. The strong negative correlation between the two fatty acids in the tissues was reported in other studies, suggesting the competition between these latter for inclusion in the tissues (Alves Martins et al., 2012; Izquierdo, 2005; Sargent et al., 1999; Van Anholt et al., 2004). The major mechanism of action for n-3 LC-PUFAs (EPA and DHA) is thought to block the formation of pro-inflammatory mediators via substrate competition with ARA for enzymes that generate several inflammatory mediators (Lands, 1987; Massaro et al., 2008, Sears and Ricordi, 2012). Furthermore, the EPA: ARA ratio is considered as a major determinant of eicosanoid production. Nonetheless, genes related to eicosanoid production showed the higher expression in low DHA fed fish, in particular *pla2*, *cox2* and *pge2*. DHA has been suggested to affect eicosanoid production (Nablone et al., 1990). Long chain n-3 PUFAs such as DHA and EPA exert also an anti-inflammatory action by inhibiting production of ARA-derived eicosanoids (Huang et al., 2018). In concordance with this finding, we hypothesize that the production of ARA-derived eicosanoids was decreased due to DHA elevation in this species.

An imbalance in eicosanoid profiles due to dietary LC-PUFAs supplementation can affect various metabolic pathways, including the corticosteroid production and thus the stress response/tolerance in fish (Van Anholt et al., 2004; Wales, 1988; Bessonart et al. 1999; Koven et al., 2003). PCA performed in the present study clustered the genes involved in stress response together with eicosanoid metabolism and *mef2c* transcript level whose were positively correlated with ARA larval content, whereas all oxidative stress and the skeleton anomaly related genes other than *mef2c*, were clustered together and positively associated to DHA larval content. The potential of ARA in the modulation of genes involved in stress response has been

studied in gilthead sea-bream and Senegalese sole larvae (Alves Martins et al., 2012; 2013). Our results seemed to support this finding; in this respect, the *pla2* up regulation in the present study reflected the ARA abundance (Hughes-Fulford et al., 2005; Yoshida et al., 2007) since phospholipase A2 is mostly responsible for catalyzing the release of ARA from phospholipids in cell membranes (Burke and Dennis, 2009). In addition, in vitro results have already proved the marked participation of COX and LOX metabolites on cortisol release mechanism in fish (Ganga et al., 2006; 2011). Effects of dietary ARA on cortisol response have been clearly demonstrated in Senegalese sole post-larvae, accompanied by an up regulation of *gr* by ARA dietary supplement (Alves Martins et al., 2011; 2013). Equally, our finding indicated that the *gr* gene responded positively to ARA supply. In fact, cortisol is the main endogenous GC hormones that regulates the expression of target genes through Gr located in the cytoplasm, signaling within cells including bone cells (Suarez-Bregua et al., 2018). The skeleton is one of the target organs of the stress hormones and physiological levels of GCs are vital for normal skeletogenesis (Suarez-Bregua et al., 2018; Zhou et al., 2013). Considering the down regulation of *gr* and the higher anomaly occurrence in pikeperch fed high DHA diets, the endogenous glucocorticoids action on bone metabolism might have also been responsible for the above-mentioned differences observed in skeletal anomalies in these larvae. In addition, previous study on pikeperch larvae reported a positive effect of high levels of DHA supplementation on stress tolerance, while no such effects were observed by high levels of dietary ARA (Lund et al., 2012, 2014). The present molecular results confirm those previous findings on DHA effect on stress sensitivity in pikeperch. Since the lower transcript levels of genes involved in stress response such as *StAR*, *gr*, *pla2* and *hsl* likely reflect an adaptation to increasing dietary amounts of LC-PUFA (Alves Martins et al., 2012). DHA is involved in processes that increase stress tolerance through the regulation of *StAR*, a key rate-limiting enzyme in steroidogenesis. Indeed, DHA acts as an inhibitor of the oxoeicosanoid receptor (OXE-R) in steroidogenic cells, reducing StAR protein levels and steroidogenesis (Cooke et al., 2013). Hormone-sensitive lipase are important enzymes involved in lipolysis, that reported to be enhanced under stress conditions (Ma et al., 2013; Nielsen and Møller, 2014). This latter (hsl) catalyzes the hydrolysis of cholesteryl esters and plays an essential role in the regulation of Dibutyryl cyclic AMP (Bt2cAMP) - induced steroidogenic acute regulatory protein (*StAR*) expression, hence, steroid biosynthesis (Manna et al., 2013).

Besides, the relative expression of the above-mentioned stress response genes (*pla2* and *gr*) as well as *pge2* were highly correlated with *pparα* expression, likely pointing out a common

mechanism of dietary regulation in this case. Another mechanism by which LC-PUFAs and eicosanoids could be acting to regulate gene transcription is through ppar's pathway (Kresten et al., 2000), considering that PUFAs and their metabolites, in particular leukotriene B4 (LTB4), have been shown to activate pparα, being one of the main endogenous ligands (Lin et al., 1999, Choi et al., 2012). This provides an alternative explanation for the marked similarities observed in the expression pattern of these genes. *Pparα* has been suggested to regulate *gr* transcription, as one of the potent transcription factors adapting the expression of several genes involved in stress response and eicosanoid metabolism (Dichtl et al., 1999; Jia and Turek, 2005), and therefore we hypothesize that *pge2* and *pla2* were likely modulated by this transcription factor. The expression of *i-fabp* gene has been considered as an indicator for assessing nutrient supply and represents a useful marker for intestinal development functional and the digestive system function in fish larvae diets (Pierce et al., 2000; Andre et al., 2000; Yamamoto et al., 2007; Overland et al., 2009; Venold et al., 2013; Lin et al., 2018), due to its crucial role in intracellular fatty acid trafficking and metabolism in fish gut (Her et al., 2004). Thus, the resulted higher expression levels of *i-fabp* gene in high DHA fish group may indicate the enhancement of fatty acid transfer rate and absorption (Baier et al., 1996; Levy et al., 2001; Storch and Thumser, 2010). On the other hand, a recent study in pikeperch larvae highlighted the potential involvement of ARA but not n-3 LC-PUFAs in the development of the digestive tract (El Kertaoui and Lund et al., 2019). Within the duration of the present study, the intestinal brush border digestive capacity was not significantly affected by DHA dietary content, but was significantly increased in fish fed intermediate ARA level (0.6 %) at 40 dph. Such effect has been observed in tongue sole (*Cynoglossus semilaevis*) larvae (Yuan et al., 2015). The morphoanatomical development and maturation of the gut is known to be accompanied by an increase in activity of the brush border enzymes from the enterocytes (Zambonino-Infante and Cahu,2007; Lazo et al., 2010). Concurrently, larval ARA content was positively correlated with transcript level of myocyte enhancer factor 2c (*mef2c*), this latter regulates the final step of chondrocyte maturation- chondrocyte hypertrophy. And as a chondrogenic marker gene, *mef2c* has been used to characterize the maturation process in fish (Ytteborg et al., 2010). Taking together the present finding and the above referred studies, we suggest ARA-sensitive effect on the maturation process in pikeperch larvae.

In summary, considering the different endpoints investigated in the present study, our results suggest an antagonistic effect of ARA and DHA fatty acids on immune/stress response of pikeperch, and its influence on bone development and deformity occurrence.

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Chapter 6

The study conducted in chapter 4 has identified Ca/P ratio as one of the main key nutritional factors in pikeperch larvae without considering the importance of each mineral separately. In this respect, the present chapter aimed to achieve a better understanding of Ca/P effects, considering the dietary Ca and P levels with a view to determine their optimal levels; not only by varying one of the two minerals, but also varying both. Growth, survival, mineral content and incidence of skeletal abnormalities were chosen as key endpoints to assess the effects of Ca, P and Ca/P on pikeperch larvae, since Ca and P are closely related to the development and maintenance of the skeletal system. The study was conducted at URBE facilities in Belgium.

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Chapter 7

General discussion

General discussion

European freshwater percids, especially pikeperch (*Sander lucioperca*), have been identified as prime candidates for inland aquaculture diversification, with the potential to deliver products of high quality and value and associated market development (Overton et al. 2015; Alexi et al., 2018; Policar et al., 2019). Numerous studies have been addressed on its biology and culture over the last decades (Kestemont et al., 2015). These studies list several bottlenecks that limit the further expansion of pikeperch culture, such as the high sensitivity to stressors, low larval survival during larval and juvenile stages, high cannibalism and high incidence of skeletal anomalies that induce weak performances at the nursery level (Szkudlarek and Zakes, 2007; Dalsgaard et al., 2013; Policar et al., 2019; Colchen et al., 2020a). In addition, the lack of knowledge on the essential nutritional factors has been considered as a bottleneck in new emerging species farming since nutrition is the significant criteria to be considered for economical and sustainable aquaculture. In fact, growth performances and survival of aquatic organisms under aquaculture conditions can be influenced by the development of nutritionally balanced commercial diets (Tom and Van-Nostrand, 1989; Prabu eta al., 2017).

The challenges of a balanced diet for fish are multiple:

- To allow harmonious growth and optimal development by a nutritional intake adapted to species, age and growth
- To prevent certain medium- and long-term pathologies resulting from dietary deficiencies or imbalances
- To detect and remedy dietary disorders and deficiencies.

Therefore, research has focused on functional constituents in feeds to improve growth, feed efficiency, health status, stress tolerance and resistance to disease in order to reduce drugs and chemicals use in fish farms.

Experimental design

Nutritional studies are typically large-scale randomized controlled trials (RCTs) in which the goal is to confirm the superiority of a new developed diet over an existing one. For example, experiments using a commercial diet as a control. The new feed consists of a combination of many ingredients and nutrients (differing from the control diet), each of them potentially influencing the primary outcome variables. Thus, these trials do not provide direct information on which nutrient factors are active and whether they have been set at optimal levels. Therefore, when determining nutritional requirements for fish the objective is, theoretically, to reach the

maximum biological potential of the species under study. In other words, to determine the minimum level of a nutrient that guarantees the maximum performance of a species. This response is considered an important determinant of the cost-benefit ratio in fish feed composition since some ingredients are considered economically limiting factors in feed formulations. Hence, the 'broken-line model analysis', as performed by a linear regression procedure, was generally considered as simple, fast and reliable, easy to interpret and suitable for the analysis of fish performance data (Robbins, 1986). This model suggests the existence of a positive linear relationship between response criteria tested (such as weight gain) and the dietary levels of an essential nutrient, where the so-called "break-point" is determined. The break-point is considered to be the optimal level of a nutrient required for maximum beneficial effect.

However, literature data have reported different optimal levels for a nutrient factor (such as a LC-PUFA, or a vitamin) for the same species and during the same life stage, most likely as a consequence of dietary composition, and also considering an additional effect of interactions with and between different nutrients. Hence, to increase knowledge of the fish nutritional requirements, macro and micronutrients have to be considered together, thus the importance of studying the interaction between nutrients in the new diet.

Factorial designs can be more efficient and informative when several active interventions which may interact with each other are studied, than the other approaches of varying one factor at a time. These designs have considerable practical advantages, especially in early-stage experiments when a large number of factors must be screened.

The two-level full factorial design includes all the combinations and interaction factors which affect the product quality. For two-level full factorial design, the number of runs can identify on the basis of 2^K formula, where 2 indicates the level and K denotes the number of factors utilized during the experiment. With large number of variables, it is very difficult and tedious to perform such large number of experiments due to the limited resources and time. Subset of full factorial design may often give the desired information by carrying out only a fraction of the full design when K is not small (Gardeur et al., 2007). Scientific research in fish nutrition has also used the fractional factorial design in several screening studies (Blanchard et al., 2008; Hamre et al., 2004).

This approach has several strengths. A particular strength is that the fractional factorial design allowed us the possibility to assess the effects of key nutritional factors in diet. This approach

can address more than one question in one study and potentially lead to a reduction in animal use and savings in financial and scientific resources without loss of scientific validity. The main disadvantage of the fractional factorial designs is that some information may be lost or difficult to interpret when the number of the experimental runs is reduced, in such a way that the main effects become aliased with some low-order interactions (Nas et al., 2005, Vasilev et al., 2013). Eventually, an alias structure is generated to define the confounded effect and interaction using Planor (Baeklandt at al., 2018). In addition, interactions between 3 or more factors cannot be evaluated. Another limitation consists in limitation inherent to the monotonic responses resulted by testing only two levels of the selected nutrient factors.

In order to conduct a comprehensive study to determine and better understand the interactions between the most determining nutritional factors in pikeperch larvae, we have adopted for this study a global experimental design that consisted of three main steps.

The goal in the first phase was to "screen" a large set of potentially important treatments efficiently and to identify factors that are determining. This was done through a screening experiment in which the effects of selected fatty acids, vitamins and minerals were examined simultaneously (Chapter 4).

The second phase aimed at refining the understanding of the effects of the important factors and interactions identified in the first phase. Existing knowledge and working assumptions have been further examined and verified in follow-up experiments, which has untangled important effects, determine optimal "dosage" levels (appropriate levels of quantitative factors) via experiments with at least 3 graduate levels of nutrient factor (chapter 5 and 6). Furthermore, in order to optimize the overall design - based on a previous study (Hamza et al., 2015) - the dietary combination of phospholipids and LC-PUFAs was investigated in depth (chapter 3). An optimal treatment program could be formulated from the information gained from this phase.

The final phase consisted in a confirmation trial designed to compare the newly developed diet with the commercial standard diet and assess its advantages/limitations. Although this phase is similar to RCTs explained earlier, the multiphase approach allowed the inclusion of only important components at their optimized levels. Under the framework of the European Union Seventh Framework Programme project Diversify, an additional experiment was conducted under farm industrial conditions (Fish2Be) on the light of all information obtained from the nutritional experiments in the project. The experimental diet was produced and tested against a commercial Japanese high quality feed at a commercial hatchery on 20 dph pikeperch larvae

for 30 days. The primary results (unpublished) showed a better growth by use of the experimental diet at the end of the experiment. These results should be further validated and confirmed in upcoming experiments.

Experimental findings

The nutritional requirements studies for juveniles and sub-adults of freshwater and diadromous fish species indicate that the EFA requirements can generally be satisfied by the C18 PUFA,18:3n-3 and/or18:2n-6 (Tocher, 2003). Freshwater fish species are capable of producing biologically active LC-PUFA from C18 PUFA, and so must express all the biosynthetic activities necessary, whereas marine fish cannot (Tocher, 2003). In fact, the qualitative EFA requirement difference between marine fish and freshwater species likely expresses an evolutionary adaptation to the availability of fatty acids in the different environments (Tocher, 2010) since marine phytoplankton produce high levels of the n-3 LC-PUFA, EPA and DHA, whereas the freshwater phytoplankton are characterized by higher levels of 18:2n-6 and 18:3n-3, with a generally low DHA (Sargent et al., 1995; Ahlgren et al., 2009). Thus, the lower level of LC-PUFA, particularly DHA, has maintained the evolutionary pressure in freshwater to retain the ability to endogenously produce LC-PUFA. However, in recent studies, it has been proven that pikeperch larvae, as opposed to many freshwater species, are very stress sensitive to lack or low levels of dietary LC-PUFA n-3, causing lower performance, higher mortality; deficiency syndromes and anomalies. (Lund and Steenfeldt 2011; Lund et al., 2014). In chapter (5), regardless the dietary and the larval LC-PUFAs content, expression of genes involved in desaturation (*fadsd6*) and elongation (*elovl5*) were not influenced, pointing the low LC-PUFA biosynthetic ability of pikeperch larvae (Lund et al., 2019). In fact, C14 labelled FA analyses have indicated no elongation or desaturation capability in pikeperch larvae (Lund et al., 2019).

Previous study has suggested a requirement for phospholipids (Hamza et al., 2012), but optimal levels have not been determined, nor the effect of vegetable or marine phospholipids. In the present thesis, larval nutritional requirements of phospholipids, fatty acid (FA) as well as its combined effect have been elucidated (chapter 3). Our results showed that the combined supplementation of SBL up to 14.51% d.w. PL with n-3 LC-PUFA (1.0% d.w. DHA and 0.16% d.w. EPA; i.e 1.17 % LC PUFA) in the form of triglycerides lead to the highest growth and lowest anomalies incidence, improving digestive enzymes activities. This could be related to either a specific effect of the FA or a combined effect of PL and FA. The latter seems likely, as diets with the lowest PL inclusion (3.70 and 3.73%) but different LC PUFA content (i.e. 0.17 % EPA+ 0.39% DHA vs. 0.3 % EPA + 0.98% DHA) both led to a relatively lower growth rate.

In chapter (3) an increase of dietary PL levels up to 8% markedly enhanced larval weight. However, elevation of dietary PL from 8% to 14.5% did not further increase fish growth significantly. A clear tendency towards decreasing prevalence of severe skeletal deformities was observed in pikeperch fed increased dietary levels of PL and DHA dietary levels, contrary to the results obtained in chapter (5) in which the increased dietary DHA up to 2.5% led to the increment in incidence of skeletal anomalies. Concurrently to the combined effect of LC-PUFA and PL on the skeletal anomaly's occurrence, liver proteomic study pointed out a reduced sensitivity to stress thanks to the balanced dietary PL and LC-PUFA supplementation (chapter 3). Oxidative status disturbance effect on bone in pikeperch larvae, has been suggested (chapters 4 and 5). This detrimental effect on bone is achieved through direct and indirect mechanisms; directly by altering the mineralization process and indirectly by causing muscle damage that could lead to future deformities after repair in juvenile fish (Betancor et al., 2012). The molecular biomarker endpoints obtained in chapter (5) clearly linked the increase in oxidative status to the skull bone anomalies.

Besides, a common mechanism of dietary regulation of stress response genes, eicosanoid metabolism and pparα has been highlighted in chapter (5). The transcription of pparα, was significantly depressed with the dietary DHA increment (chapter 5). In agreement -based on SCP2 and FAS protein expression- liver proteomic study has previously suggested a deficient PPARα activation due to the decrease in FAS expression as a response to the high dietary n-3 LC-PUFA (chapter 3).

The expression of eicosanoid metabolism related genes was mainly governed by ARA intake, and the potential of ARA in the modulation of genes involved in stress response has been demonstrated (chapter 5). Furthermore, the study highlighted the importance of the interaction among ARA and n-3 LC-PUFA (especially DHA) (chapters 4 and 5). In fact, several studies have pointed out the competition between these latter fatty acids for inclusion in the tissues (Alves Martins et al., 2012; Izquierdo, 2005; Sargent et al., 1999; Van Anholt et al., 2004). Our results were consistent with the literature, over and above the antagonistic effect of ARA and DHA fatty acids on immune/stress response of pikeperch has been proven (chapter 5). In this sense, the present study suggests a decrease in ARA-derived eicosanoids production due to DHA elevation in this species. The imbalance in eicosanoid profiles due to dietary LC-PUFAs supplementation can affect various metabolic pathways, including the corticosteroid production and thus the stress response/tolerance in fish (Van Anholt et al., 2004; Wales, 1988; Bessonart et al. 1999; Koven et al., 2003).

Although the combined effect of dietary ARA and PL dietary was not investigated, the results of digestive enzyme activities highlighted the potential involvement of both ARA and PL in the development of the digestive tract (chapters 3, 4 and 5). Indeed, among the several nutrient factors studied in the experiments (including the screening experiment), no significant and/or clear effects were found on digestive enzymatic activities except of PL and ARA. These results were consistent with the literature, that previously reported a positive effect of dietary ARA and PL level in larval enterocyte maturation processes (Gisbert et al. 2005; Hamza et al. 2008, 2012; Saleh et al. 2013. Yuan et al., 2015).

The present study has highlighted the importance of both LC-PUFAs and PL effects on larval performance, especially the incidence of skeletal anomalies and digestive enzymatic capacity (chapters 3, 4 and 5). Dietary PL and LC-PUFAs changes have markedly affected the fatty acid composition in pikeperch larvae. Accordingly, differential pattern of gene expression was recorded depending on the fatty acid larval content (chapter 5). Thus, the results of gene expression may reflect the combined actions of other fatty acids (EPA, oleic acid, LA and ALA). Interestingly, the expression of genes involved in oxidative stress defense was not influenced by the dietary LC-PUFAs changes. While antioxidant enzyme mRNA expression levels increased concomitantly with the decrease of larval C18 fatty acid content, especially α-linolenic acid (ALA; 18:3n-3) which correlated negatively with *sod* and *gpx* expression levels and linoleic acid (LA; 18:2n-6) negatively correlated with *gpx* expression levels (chapter 5).

A part of the present study has been dedicated to identifying the most influencing micronutrients in pikeperch larval performance. Ca/P ratio appeared as a determining nutritional factor. The screening experiment results associated the good growth performance and the reduced incidence of kyphosis anomaly to the reduced dietary Ca/P ratio intake up to 0.6. However, kyphosis skeletal anomaly has been previously related to fish fed diets deficient in P (Fjelldal et al., 2016). The beneficial effect of 0.6 Ca/P ratio has been highlighted in chapter (6). In addition, larval performance and the incidence of skeletal anomalies responded significantly to both dietary P and Ca/P ratio changes, being lower in larvae fed 0.6 Ca/P and 2.01 P % total feed. An increase in the dietary P content above 2.01 % resulted in an increased incidence of kyphosis. A recent study has suggested that high rather than low dietary P content could be a causative factor for skeletal anomalies (Cotti et al., 2020).

A previous study has suggested a minor effect of micronutrients on gross fish performance (Hamre et al. 2004). However, the present study has identified vitamins E and C as the major

micronutrients of interest for pikeperch larvae (chapter 4). Indeed, besides the distinct metabolic functions of vitamin E and C in fish larvae, both vitamins constitute a part of larval defense against lipid oxidation especially in weaning diets for the fast-growing larvae besides other (Blazer 1992; Gatlin 2002; Halver 2002, Betancor et al., 2012, El Kertaoui et al. 2017). Vitamins C and E improved stress tolerance, immunological response and disease resistance in fish (Koshio 2007; Lim et al. 2008). Therefore, the importance of both vitamins may be explained by the high dietary n-3 LC-PUFAs demand in pikeperch larvae (as discussed above).

A common finding in all experiments was the elevated incidence of skeletal anomalies and the high mortality and cannibalism rate. In the present study, type I cannibalism was not observed; according to Kestemont et al. (2007), type I cannibalism includes dead fish showing signs of cannibalism (generally type I cannibalism, *i.e.* fish partly consumed by a cannibal). Only missing larvae were considered as type II cannibalism according to the same authors (type II cannibalism; *i.e.* fish completely ingested, usually head first, by a cannibal). In this study we presented the survival rate as the counting of all the larvae alive at the beginning and at the end of the experiment. It's well known that survival rates as well as growth performance in larvae are indicative of suitable rearing conditions (Canãvate and Fernandez-Diaz 1999). However, cannibalism is a known bottleneck in pikeperch larval rearing. Cannibalistic behaviour is very severe from 18 to 39 dph representing up to 80 % of all mortality at weaning stage (Kestemont et al., 2007; Szkudlarek and Zakęś, 2011; Colchen et al., 2020a). This coincides with the period in which feeding experiments were carried out. Thus, larval performance results, including larval growth, must be interpreted with caution, since the average growth of a fish population within a tank could be directly affected by the cannibalism rate as the cannibalized fish are usually the smallest ones. Another series of experiments were conducted concomitantly to our study under the DIVERSIFY project to study in depth the phenomenon of cannibalism in pikeperch larvae (see Colchen et al. 2017; 2019; 2020b,c). Effects of environmental, populational and nutritional factors on cannibalism have been investigated in pikeperch larvae (Colchen, 2017). For a such high cannibalistic behavioural species, we could hypothesize that fish larvae can remedy at least part of a nutrient imbalance through cannibalism. In fact, cannibalism may provide nutrients in proportions that are more optimal than heterospecific diets (Pfennig 2000; Fagan et al. 2002; Mayntz and Toft, 2006). In this sense, a recent study has linked a decrease in cannibalistic behaviour in pikeperch to crystalline L-tryptophan (TRP) dietary supplementation (Król and Zakęś, 2016).

Besides the nutritional factor, the effects of behaviour may explain the high incidence of skeletal anomalies recorded in the present study. The loss of locomotor function due to aggression and attacks by cannibals could have an impact on survival, growth but also on the incidence of skeletal deformities in the present study. This may partly explain the high rate of skeletal anomalies recorded. Skeletal anomalies could be caused by other environmental factors such water currents and temperature or also many risk factors that specifically relate to the behaviour of fish and how they interact with the rearing environment. Self-inflicted mechanical damage such as that caused by physical contact with tank walls. In addition, skeletal anomalies may be a result of some combination of genetic and environmental factors (broodstock nutrition, husbandry conditions). However, in the present study we only investigated the nutritional factors and endpoints, without any data related to behaviour aspect and the for each experiment, fish larvae were obtained from the same batch of eggs, and the rearing conditions were the same in all experimental tanks.

Chapter 8

Conclusions and perspectives

Conclusions

This thesis focusses on the importance of PL and LC-PUFAs' composition and dietary requirements of Ca/P, as well as their interactions. The results obtained highlight the importance of multinutrient interactions in nutritional studies. The thesis hypothesised that physiological functions of pikeperch larvae could be disturbed during the early life stage due to an imbalanced diet in n-3/n6 LC-PUFA ratio, with Ca/P ratio being much higher than those observed in common freshwater fish diets. Based on the results of this thesis, the ratio Ca/P appears as a key nutritional factor for pikeperch larval development. Our findings highlighted also the high requirements of PL and LC-PUFA in pikeperch larvae. Low amounts of LC-PUFAs and PL have detrimental effects on the early development of pikeperch, due to an altered oxidative status and reduced stress resistance capacity. In addition, this study demonstrated that essential fatty acids may be directly supplemented as triglycerides to benefit pikeperch larval development. DHA and ARA and their interaction are also influencing nutritional factors. The present study highlighted an antagonistic effect of ARA and DHA fatty acids on the immune/stress response of pikeperch. Imbalanced eicosanoid profiles due to dietary LC-PUFAs imbalance affected the corticosteroid production and the stress response, which was mainly reflected in high incidences of skeletal anomalies. Our results also showed that the skeleton is one of the target organs of the stress hormones. Further, our findings indicate the action of endogenous glucocorticoids on bone metabolism as responsible for skeletal anomalies in pikeperch larvae. The effects of dietary DHA and ARA on the occurrence of skeletal anomalies through the regulation of immunity and stress related gene expression have been highlighted.

Liver proteomics study indicated that the expression of some proteins is clearly related to the availability and presence of LC – PUFAs, especially DHA. Digestive enzymatic activities seem to be more related to ontogenetic development, while a potential involvement of both ARA and PL in the development of the digestive tract is suggested and further studies are needed.

In summary, starter feeds for pikeperch need to be adapted according to the insights gained here regarding the nutritional requirements of pikeperch larvae. Our recommendations are as follows:

- ✓ Ca/P ratio of 0.6 is recommended for a good growth performance in pikeperch larvae.
- ✓ Total P % content should be considered when determining the optimal Ca/P level in fish larvae.
- ✓ The optimal dietary total P level for pikeperch larvae is 2%.

- ✓ Diets with 8% phospholipids and about 1.0% LC PUFAs are recommended to achieve good growth performance.
- ✓ Combined supplementation of SBL up to 14.5% d.w.PL and n-3 LC-PUFA (1.00 % d.w. DHA and 0.16% d.w. EPA) in the form of triglycerides leads to the highest growth and lowest incidence of anomalies, improving the activity of digestive enzymes.

Perspectives

This thesis opens up new horizons in nutritional research in fish larvae. Nutritional interaction is the challenging bottleneck in the optimization of diets in larval fish culture. Nutritional interactions should be studied in detail following a nutritional ecology approach, that is the study of how animals relate their environment with their nutritional requirements (Simpson and Raubenheimer 2001; Raubenheimer et al. 2009). This approach includes the question how wild fish use a variety of food-related and metabolic signals to compose their diet from a range of food items that differ in nutrient composition (Simpson and Raubenheimer 2001). Similarly, combining physiological investigation with behavioural studies could identify chemicals to be added to feeds to stimulate olfactory (smell) and extraoral gustatory (taste) receptors and to induce fish to search for feed (Hara 1992, 1994, 2006; Kasumyan and Døving 2003).

Another interesting objective for future studies is the interaction of fish welfare and nutritional quality of feeds and its implications on the animal's performance. A previous study showed that dietary n-3 LC-PUFA levels affected the behaviour of pikeperch larvae, demonstrating long-term effects of early nutritional history in fish (Lund et al., 2014). A more anxious behavioural profile observed in fry fed low levels of DHA supports the hypothesis that long-term central effects, such as brain developmental patterns, are the cause of these behavioural effects (Lund et al., 2014). New insights could be gained by the in-depth study of effects of nutritional intake on the tolerance of physiological stressors (i.e. salinity, temperature or hypoxia) and on behavioural abnormalities (activity levels; cannibalism and the ability to escape predators) in pikeperch larvae.

Gut microbiota play a major role in host nutrition by providing essential vitamins to the host, by modulating the absorptive capacity of the intestinal epithelium and by fermenting indigestible polysaccharides into short-chain fatty acids (Martins dos Santos et al., 2010; Tremaroli and Backhed, 2012, Hacquard et al, 2015). Moreover, the diet of a host mainly drives its microbial community structure (Hacquard et al, 2015). In mammals, the evolution of gut

microbiota has been greatly influenced by the host diet. The microbiota plays a fundamental role in the induction, training and function of the host immune system and this immune system–microbiota alliance has been well reported (Belkaid and Hand, 2014). It would thus be interesting to study the immune system in relation to nutritional intake – microbiota interactions. This could advance the necessary knowledge to determine crucial physiological processes including immunity, metabolic functions and disease susceptibility and their relationship with nutritional requirements in fish. Feed absorption is the challenging bottleneck for new optimized diets in larval fish in new emerging species. Two main issues remain: the performance of the digestive system and the bioavailability of nutrients. Research should focus on the improvement of gut health, thus improving and increasing feed absorption (i.e studying supplementation of enzymes and additives).

In the present study, skeletal haemal and neural arche anomalies were often observed. These types of anomalies could be related to other nutritional factors and interactions such as the lipid classes, not tested here. The association of malformations and nutritional factors in pikeperch deserves further investigation.

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