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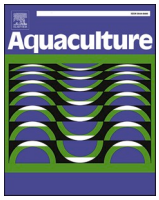

Effect of early peptide diets on European sea bass (*Dicentrarchus labrax*) skeletal development

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ABSTRACT

The mechanisms behind the beneficial effects of dietary peptides on fish skeletal development remain unrevealed. In the present study, we evaluated the effect of 0% (C, Control), 6% (P6) and 12% (P12) levels of small peptide incorporation on European sea bass (*Dicentrarchus labrax*) early larval skeletal development and post-larval skeletal integrity against a swimming challenge test. Survival was not affected by the peptide diets, whereas P6 presented the lowest growth rate. Larval quality control underlined the advantageous effect of P12 on reducing the frequency of cephalic deformities (e.g., branchiostegal rays, operculum and cross-bite), pre-haemal lordosis and vertebrae bone loss. Simultaneously, individuals from P12 group exhibited an earlier mineralization of the vertebral column and were less prone to develop swimming-induced haemal lordosis (16.0 ± 0.1%) and scoliosis (3.3 ± 0.6%). Expression analysis of genes involved in digestive function, protein transport, muscle ontogeny and bone mineralization revealed a peptide-enhanced larvae development of the P12 group. An early nutritional programming of the post-larval musculoskeletal system is proposed. Limitations induced by the differential free amino-acid profiles are discussed. A potential developmental-stage-specific incorporation of peptide diets in European sea bass rearing is suggested.

1. Introduction

The harmonious development of the marine larvae skeletal development is under environmental and genetic control (Boglione et al., 2013). Early fish life stages are characterized by rapid and continuous changes in terms of physiology, morphology, functional abilities and structure following the progressive character of ontogeny. Skeletal deformities appearance reflects the possible disconformities between the applied rearing conditions and fish optimal requirements (species and ontogeny dependent; Koumoundouros, 2010). Skeletal deformities develop either as deviations of the early ontogenetic pattern (Georga et al., 2011) or as post-larval bone adaptations (Divanach et al., 1997). Depending on the intensity and the implicated anatomical region (skull, vertebral column and fins), skeletal deformities affect the total fish performance (e.g., reduced growth or survival; Koumoundouros et al., 2002a, 2002b). Therefore, the presence of malformation undermines the welfare and quality of finfish aquaculture products. The standardization of the operating rearing procedures, the optimization of the husbandry techniques and the advanced quality controls nowadays highlight the lack of appropriate early nutrition as crucial for the normal skeletal development of the individuals.

Skeletogenesis can be affected by various nutrients (Boglione et al., 2013). Based on the late larval stomach differentiation and pancreas maturation (Zambonino-Infante and Cahu, 2010), studies utilizing inert diets from the first days of exogenous feeding report the species and age specific nature of larvae nutritional requirements for harmonious skeletogenesis (Holt, 2011). A direct link exists between nutrient mineral deficiency and decreased bone mineral contents in fish, which under muscular loads can lead to deformities (Lall, 2022). By the same token, calcium metabolism, synthesis of plasma and bone proteins (Sivagurunathan et al., 2022) and formation of structural bone components (Halver, 2003) have been found regulated by dietary vitamin availabilities (Lall and Lewis-McCrea, 2007; Mazurais et al., 2008). Macronutrients can influence bone formation and resorption at early life stages through the regulation of the expression of genes involved in osteoblast differentiation (Darias et al., 2011; Villeneuve et al., 2006).

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Furthermore, even after its original formation, bone responds to mechanical stimuli often induced by the adjacent muscle (Suniaga et al., 2018). Skeletal muscles are also under nutritional control (Kjørsvik et al., 2011) in terms of both regulation of myogenic factors (Hamade et al., 2006) at larval stages and regulation of fiber differentiation and contraction (Sáez-Arteaga et al., 2022) on later stages at the gene expression level.

Taking into consideration the rapid growth rate of fish larvae, a high muscle protein deposition is required during the early developmental stages. Protein fraction of various sources (e.g., yeast or pig blood, (Gisbert et al., 2012) and hydrolysis levels (Delcroix et al., 2015; Kotzamanis et al., 2007) has been proven advantageous for larval growth, survival and antioxidative response. Incorporation of protein compounds with low molecular weight such as peptides, has been correlated with enhanced proteolytic activity and earlier development of the digestive system in the intestine of European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) larvae (Gisbert et al., 2012; Zambonino Infante et al., 1997). Amylase (*amy*) and trypsin (*prss1*), beyond their differential regulation of expression during ontogeny (Péres et al., 1998), can be valuable indicators of exocrine pancreas maturation responding to the protein dietary conditions (Péres et al., 1996). Entry of small peptides directly on the apical membrane of the enterocytes allows their hydrolysis by the peptidases, ensuring the early protein absorption and potentially the fulfillment of the amino acid requirements and transporters (Dabrowski et al., 2005; Zambonino-Infante and Cahu, 2010). Simultaneously, an effect of peptides on the harmonious skeletal development has been observed (Cahu et al., 1999; Printzi et al., 2023; Zambonino Infante et al., 1997). In zebrafish early juveniles, even after the complete formation and ossification of the vertebral column, individuals fed with early peptide diets presented increased resistance against mechanical loads induced by swimming (Printzi et al., 2023). Based on the gained knowledge, it is now necessary to raise the awareness of the beneficial effects of early dietary peptides on the skeletal development and robustness of a farmed marine fish species, by further examination of the molecular mechanisms underlying those effects.

The purpose of the present study is to evaluate the effect of three experimental diets with partial incorporation of 0% (Control), 6% (P6) and 12% (P12) small peptides on a) the skeletal development of European sea bass larvae and b) the resistance of their vertebral column against swimming induced lordosis. Based on the beneficial effects of small di- and tri-peptide incorporation for zebrafish skeletal development presented by Printzi et al. (2023), the transcript levels of genes involved in digestive maturation, peptide transport in the intestine, bone mineralization and muscle differentiation were investigated during the ontogeny and/or after a swimming challenge test (SCT) in the present study.

2. Materials and methods

2.1. Experimental diets and rearing

Three experimental diets were designed to be isonitrogenous with partial incorporation of 0% (C, Control), 6% (P6) and 12% (P12) ("Aquaculture feed composition, for farmed fishes selected from marine species, suitable for preventing skeletal malformations", patent application no. 23307040.8, filed on November 23, 2023. Refs: CH613EP DAD/JF/KB) of their protein portion (fishmeal, CPSP 90) by small shrimp di and tri-peptides. The experimental levels of peptide incorporation were selected based on previous studies, highlighting approximately a 10% of peptide inclusion as optimal for the species (Cahu et al., 1999; Zambonino Infante et al., 1997). The total diet composition, peptides and free amino-acid analysis are described in detail by Printzi et al. (2023) (Tables 1, 2, Fig. 1). The control diet (C) was designed following the one highlighted by Cahu et al. (2003a, 2003b) for the species. The manufacturing of the diets took place at Ifremer - Center of Brittany (Plouzané, France), whereas Symrise Aqua Feed (Diana Aqua, Symrise group, Elven, France) provided the shrimp protein hydrolysate required. The CPSP 90 used was provided by Sopropêche (ZI de la Trésorerie, 62,126, Wimille, France).

Fertilized eggs of European sea bass (*Dicentrarchus labrax*) were obtained by Ifremer station in Palavas (France). Following an acclimation on a common 300 L tank, larvae of 1dph (days post hatching) were randomly placed in twelve 35 L fiberglass tanks (1300 larvae tank^{-1}, 37 larvae L^{-1}) to constitute the experimental populations. Four replicates of each experimental diet were formed. All tanks were equipped with a continuous seawater flow (30% of water replacement/tank/h, filtered at 10 μm) and common abiotic conditions were applied up to 42dph (Supplementary Table S1). At 42dph, the four replicates of each experimental diet were transferred in one common 600 L tank per group

Table 1
Formulation and proximate composition of the experimental diets C, P6, P12 (Printzi et al., 2023).

Ingredients (g kg^{-1}, dry matter basis)	C	P6	P12
Fish meal[a] (protein 74.7%, lipids 7.5%, EPA + DHA 20.9%)	640	670	620
Shrimp peptides [b] (protein 68.4%, lipids 8.6%, EPA + DHA 1.1%)	0	50	100
CP SP 90 [c] (protein 88.2%, lipids 2.5%, EPA + DHA 18.1%)	130	50	50
Fish oil[d]	10	10	10
Rape seed lecithin[e]	130	130	130
Starch	50	50	50
Vitamin mix[f]	30	30	30
Mineral mix[g]	10	10	10
Theoretical composition (%)			
Protein	54.7	53.2	53.0
Peptide/protein ratio	0.0	6.1	12.3
Lipids	16.7	17.1	17.9
EPA + DHA	1.1	1.2	1.1

a, d Fish meal and oil were provided from La Lorientaise (Lorient, France).
b Actipal Shrimp hydrolysate, Symrise Aqua Feed, ZA du gohelis, 56,250 Elven, France.
c SOPROPÊCHE, ZI de la Trésorerie, 62,126 Wimille, France.
e SAIPOL, Grand Couronne, France.
f Composition per kg of vitamin mix: retinyl acetate, 340 mg; cholecalciferol, 2.5 mg; all-rac-α-tocopherol acetate, 4 g; menadione, 0.1 g; thiamin, 1 g; riboflavine, 2.5 g; D -calcium pantothenate, 5 g; pyridoxine HCl, 1 g; cyanocobalamin, 0.006 g; niacin, 10 mg; folic acid, 0.5 g; biotine, 0.1 g; meso-inositol, 100 g.
g Composition per kg of mineral mix: KCl, 90 g; KI, 40 mg; $CaHPO_4{\cdot}2H_2O$, 500 g; NaCl, 40 g; $CuSO_4{\cdot}5H_2O$, 3 g; $ZnSO_4{\cdot}7H_2O$, 4 g; $CoSO_4{\cdot}7H_2O$, 20 mg; $FeSO_4{\cdot}7H_2O$, 20 g; $MnSO_4{\cdot}H_2O$, 3 g; $CaCO_3$, 215 g; $MgSO_4{\cdot}7H_2O$, 124 g; NaF, 1 g.

Table 2
Free amino-acid composition of the experimental diets C, P6, P12 (%). EAA, essential amino acids (Printzi et al., 2023). Sum free AA, summary of free amino acids.

Amino acids (% feed)		C	P6	P12
EAA	Threonine	0.04	0.1	0.14
	Valine	0.05	0.13	0.19
	Methionine	0.02	0.06	0.09
	Isoleucine	0.04	0.11	0.16
	Leucine	0.08	0.2	0.29
	Phenylalanine	0.04	0.13	0.2
	Histidine	<0.02	0.03	0.05
	Lysine	0.05	0.19	0.29
non EAA	Aspartic acid	0.03	0.09	0.13
	Serine	0.02	0.06	0.09
	Glutamic acid	0.07	0.16	0.22
	Proline	0.03	0.11	0.18
	Glycine	0.05	0.13	0.19
	Alanine	0.1	0.2	0.27
	Cystine	<0.02	<0.02	<0.02
	Tyrosine	0.03	0.11	0.18
	Arginine	0.04	0.19	0.32
Sum free AA		0.69	2.00	2.99

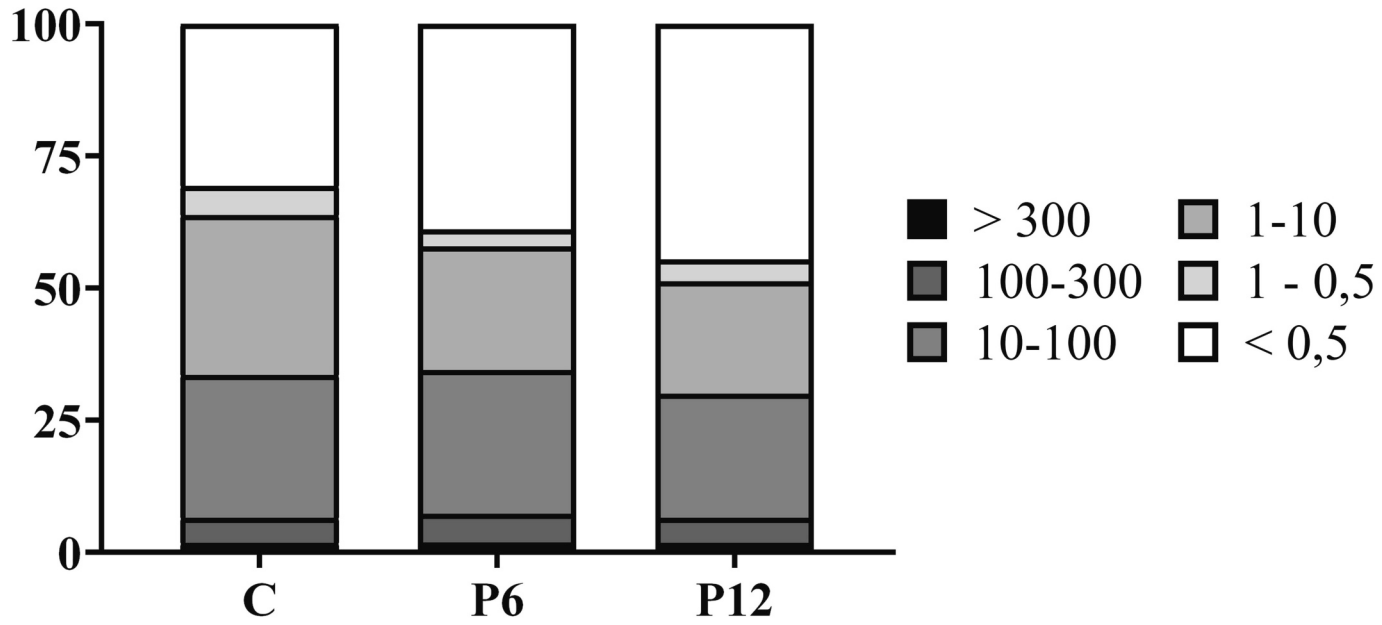

Fig. 1. Peptide profile (%) of the experimental diets (C, P6, P12). Differences in molecular weight are presented in classes of kDa.

(100% of water replacement/tank/h) and the abiotic parameters were followed in each tank individually (Supplementary Table S1) up to the swimming challenge test (SCT). Larvae feeding included exclusive provision of Artemia nauplii (VNBS Brine Shrimp eggs Artemia cysts, Viepearl,Vietnam) between 6 and 12dph followed by a gradual a co-feeding with the microdiets and Artemia (Instar-II enriched with Larviva multigrain, BioMar, Nersac, France) up to 20dph, before entering the exclusive dry feed provision (Supplementary Table S2). A belt feeder enabled the distribution of the diets throughout the experiment. Verification of food consumption was performed on a regular basis by observing the digestive tract of a random larval sample of each tank, under a binocular microscope approximately 1 h after food distribution. Food quantities were adjusted to the number of individuals and the uneaten food on the bottom of the tanks. Siphoning of the tank bottom was performed on a weekly basis during live food inclusion and every second day on the initiation of dry feed. Surface cleaning was applied on a daily basis during microdiets provision.

2.2. Sampling for growth, survival and early skeletal quality

Thirty five to forty five larvae were collected randomly at 28dph (per replicate and experimental diet, Sampling 1 – S1), at 42dph (per replicate and experimental diet, before the transfer in common tanks per diet, Sampling 2 – S2), at 64 (per experimental diet, Sampling 3 – S3) and at 92dph (per experimental diet, Sampling 4 – S4), euthanized in MS222 tricaine solution (2 g/L) and photographed under a camera (Nikon, D7200, Japan). Subsequently samples were fixed in phosphate buffered formalin and stained for bone and cartilage (acid-free method, Walker and Kimmel, 2007). Stained individuals were photographed under a stereoscope (Olympus SZX16). Growth measurements on the images obtained were performed in ImageJ software (Schneider et al., 2012) and expressed as total length (TL). Survival was estimated by counting the individuals before the pooling of the replicates (42dph).

2.3. Micro-CT scanning

Three stained individuals with the characteristic abnormal mineralization of the vertebral centra observed in their vertebral column after the deformities examination at 92dph (S4), were scanned with a Skyscan 1172 (resolution 1–1.3, total rotation 180°, exposure time 970 ms, voltage 40 kV, 250 μA). Reconstruction of the images obtained was performed in NRecon software (Skyscan). Images were transformed in cross-sections as Tiff files. The Amira v.5.2 (Visage Imaging, Berlin, Germany, and Burlington, USA) was used to enable the transformation of the cross-sections in two- and three- dimensional pictures.

2.4. Swimming challenge test (SCT)

When reaching 24-28 mm TL, 25–30 individuals per experimental group (C, P6, P12) with inflated swimbladder and lacking axial deformities on their external phenotype were subjected to a swimming challenge test. At the selected TL, all vertebrae are formed and all fins have complete ray counts (Darias et al., 2010). A continuous four-day swimming test against a velocity of 4.0 TL·s^{-1} was performed according to (Printzi et al., 2021) with the necessary modifications for the species. The selected water velocity corresponds to approximately 50% of the species' critical swimming speed for juveniles developed under control conditions at 20 °C (Koumoundouros et al., 2002a, 2002b). The swimming apparatus utilized, consists of two swimming tunnels (50 cm length, 15 cm width, 20 cm depth) connecting two separate tanks (250 L). Use of external water pumps with a stack of flow tubes, adjustable valves and plastic straws on the edges of the tunnels maintained the laminar flow through this closed-recirculation system. Abiotic conditions were monitored in an everyday basis during the swimming tests (Supplementary Table S3). Prior to the initiation of each test, individuals were let for acclimation inside the swimming tunnels (4 h). A gradual increase up to the selected velocity (1 TL/h up to 4.0 TL·s^{-1}) was applied to minimize the stress induced. The same procedure was followed to allow the feeding of the fish in a daily basis (food quantity adjusted to the number of individuals according their regime, Supplementary Table S4). Two groups (25–30 individuals/group) of each experimental diet were exercised. At the end of the exercise, fish were euthanized with MS222 tricaine solution (2 g/L). A categorization into normal (N) or lordotic (L) according to the external morphology took place, based on the presence or not of the characteristic dorsal shift of the caudal peduncle respectively. The categorized individuals were sampled randomly either for RNA analysis or for whole mount staining (Sakata-Haga et al., 2018).

2.5. Sampling and analysis of RNA samples

Samples for the RNA analysis were collected before and after the

swimming challenge test. To evaluate the effect of the diets on the maturation of the larval digestive tract, muscle structure and ossification progress, 3 samples per replicate and experimental group were collected at 28 (RNA Sampling 1 – rS1) and 42dph (RNA Sampling 2 – rS2). Similarly, 12 samples per diet were collected at 55dph (RNA Sampling 3 – rS3). Each sample consisted of 3 larvae collected randomly, euthanized with MS222 tricaine solution (2 g/L) and preserved in RNA Stabilization Reagent (RNAlater, Qiagen, Hilden, Germany). After SCT, 3–6 individuals per external phenotype, exercise group and diet were collected, euthanized with MS222 tricaine solution (2 g/L) and preserved in RNA Stabilization Reagent. The haemal parts of the exercised individuals, including the tissues located between the cross-section at the level of the anterior anal-fin ray and the cross-section at the base of caudal-fin rays, were dissected according to (Printzi et al., 2022). Total RNA extraction was performed with Extract-All reagent (Eurobio, Courtaboeuf, Essonne, France) and Nucleospin RNA column (Macherey–Nagel, Düren, Germany). The concentration/purity and integrity of the samples were verified by a ND-1000 NanoDrop® spectrophotometer (Thermo Scientific Inc., Waltham, MA, USA) and a Tapestation 4150 (Agilent Technologies Inc., Santa Clara, CA, USA). The RNA integrity scores (RIN) were higher than 9 for all the samples. A reverse transcription was followed by quantitative PCR analyses (iScript cDNA Synthesis Kit; Bio-Rad Laboratories, Hercules, CA) for each selected gene under the conditions described by Mazurais et al. (2008). Supplementary Table S contains the primers specifically designed for the examined genes (amylase alpha - *amy2*, trypsin precursor - *prss1*, solute carrier family 6 member 19 - *slc6a19a*, eIF-2-alpha kinase or general control nonderepressible 2 - *gcn2*, peptide transporter 1 - *pept1*, peptide transporter 2 - *pept2*, myogenic factor 5 - *myf5*, myogenic differentiation factor - *myod*, myogenin - *myog*, osteonectin - *sparc*, osteocalcin - *bglap*, collagen type 1 - *col1*, forkhead box O1 a - *foxo1*, peroxisome proliferator-activated receptor gamma - *pparg*, transforming growth factor beta - *tgfb*, troponin I type 2 - *tnni2*) and the reference ones (ribosomal protein L13 - *rpl13*, glyceraldehyde-3-phosphate dehydrogenase – *gapdh* and eukaryotic translation elongation factor 1 – *ef1*). *Rpl13* and *ef1* were used as reference genes for the samplings before the SCT, whereas *rpl13*, *ef1* and *gapdh* were used to normalize data after the SCT since their expression did not present significant differences within the groups of interest ($M < 0.05$).

2.6. Statistical analysis

The statistically significant differences in survival between the experimental diets were tested by means of Kruskal–Wallis and Mann–Whitney U-statistic. Non-parametric tests (Kruskal–Wallis, Mann–Whitney U-statistic) were also used to test the differences in gene expression data before the SCT among the tested groups and to determine possible replicate effects. Differences in deformities frequencies between the diets, before and after the SCT, were tested by G-test (Sokal and Rohlf, 1981). Comparison between the growth rates on logarithm-transformed data was performed with ANCOVA (Sokal and Rohlf, 1981), followed by Bonferroni-adjusted post hoc tests. One-way ANOVA determined the statistical differences in the mineralization progress of the vertebral column. Lastly, a general linear model with the phenotypic categorization (Normal - N, Lordotic - L) and replicate as nested factors within each diet, was built to determine the differences in gene expression data after the SCT. A significance level of $\alpha = 0.05$ was used in all statistical tests.

2.7. Ethical statement

All the experimental procedures followed the applicable national and international guidelines for care and use of animals according to the EU Directive 2010/63/EU for animal experiments. The specific experimental procedures of the current study were approved by the French Ministry, Ministère de l'Enseignement Supérieur, de la Recherché et de l'Innovation (Authorization APAFIS 34131, permit number 202111251455139 v5).

3. Results

3.1. Growth and survival

No significant effect ($p > 0.05$) of the diets on the survival rate was observed before 42dph (Kruskal–Wallis test, Fig. 2). A significant difference in growth rates was only indicated in the case of P6 group, which presented a significantly lower growth rate compared to C and P12 dietary groups up to 92 dph (ANCOVA, $F(2, 798) = 9.16$, $p < 0.05$, Fig. 2).

3.2. Peptide diets reduces the total larval abnormalities rates

The increased ontogenetic variability within each sample of the first two sampling points (S1 and S2) did not allow their inclusion in the present skeletal quality assessment. Following the evolution of the

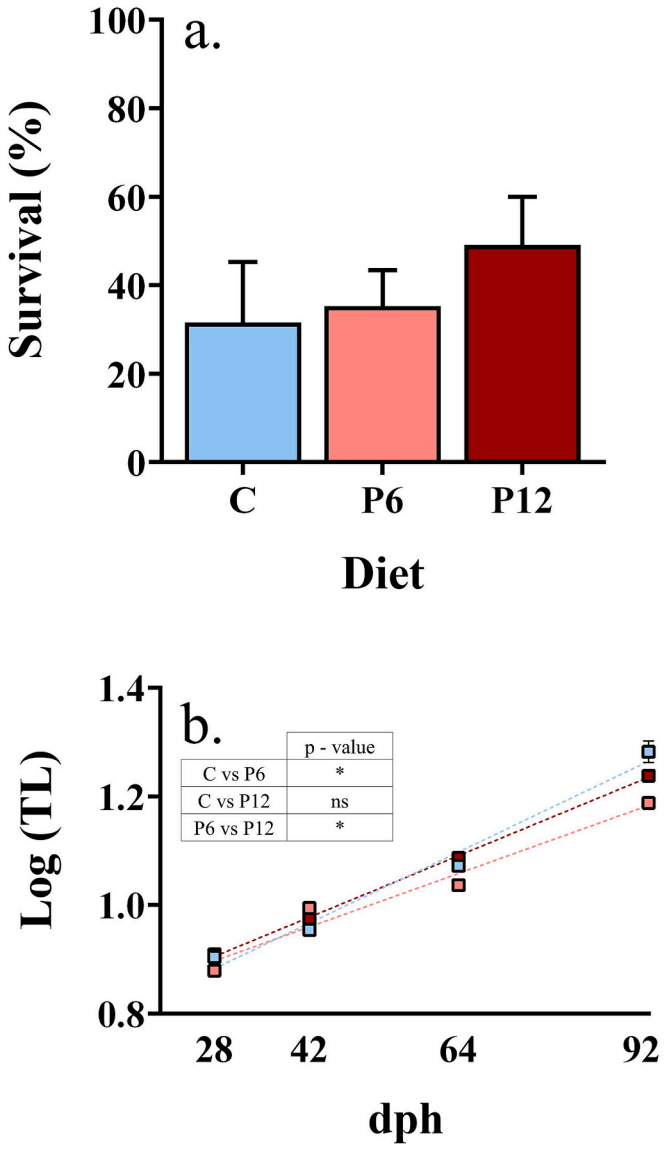

Fig. 2. Effect of experimental diets on mean survival (±SE) up to 42dph (a) and growth rate (±SE) from 28 up to 92dph (b) of European sea bass larvae. The table indicates the *p*-values between the regression lines (the significant values are indicated with an asterisk). dph, days post hatching. TL, total length. ns, non-significant.

observed deformity frequencies from 64 (Sampling 3- S3) to 92dph (Sampling 4- S4) enabled the identification of their induction period and the clarification of the diet effect. The most frequent skeletal abnormalities detected on the axial skeleton of our samples included the kyphosis of the pre-haemal vertebrae (Fig. 3a), the abnormal mineralization of the vertebral centra (Fig. 3b) and the scoliosis of the caudal peduncle (Fig. 3c). The characteristic lacunae due to disruption of the regular mineralization process of the vertebrae centra were also confirmed by a micro-CT scan of a double-stained larva (Fig. 4a-b). On the skull, deformed branchiostegal rays (shape and orientation, Fig. 3d) along with an inwards folding of the gill-cover (Fig. 3e) presented the highest rates. Cross-bite (Fig. 3f, lateral displacement of the lower jaw) and misalignment of the dentary bones on the lower jaw (Fig. 3g) were also scored. Haemal lordosis (Fig. 3h) was only observed at low frequencies in the last sampling (S4, 92dph).

A common pattern of a significant beneficial effect of P12 ($p < 0.05$) was highlighted in the case of branchiostegal rays (23.7% – Fig. 5a, 22.9% – Fig. 5b), left (31.6% – Fig. 5a, 22.9% – Fig. 5b) and right (26.3% – Fig. 5a, 22.9% – Fig. 5b) operculum deformities and cross-bite cases (21.1% – Fig. 5a, 10.4% – Fig. 5b) against the other groups. In total, results of the early quality control revealed that deformity frequencies between experimental diets C and P6 did not significantly differ ($p > 0.05$), except in the case of pre-haemal kyphosis ($p < 0.05$). Experimental diet P6 presented the lowest frequency of pre-haemal kyphosis (26.9%, Fig. 5b), being significantly differentiated with C (50.0%, $p < 0.05$) but not P12 (35.4%, $p > 0.05$) and. Individuals with abnormal mineralized vertebral centra were found reduced in P12 group (18.4% – Fig. 5a, 37.5% – Fig. 5b) compared to P6 (30.0% – Fig. 5a, 42.3% – Fig. 5b, $p > 0.05$) and C (41.7% – Fig. 5a, 60.4% – Fig. 5b, $p < 0.05$). Meanwhile, scoliosis of the caudal peduncle ranged between 41.6 and 52.1% regardless the diet (Fig. 5b). In all groups, lower jaw misalignment frequencies did not exceed 26.3% (Fig. 5a, b) whereas haemal lordosis remained under 21.1% (Fig. 5b).

3.3. P12 enhances the mineralization of the vertebral column

Counting the number of mineralized centra from their first appearance up to the complete mineralization of the vertebral column (25 vertebral centra), revealed a peptide-enhanced mineralization rate (Fig. 6). Between larvae of the same length, 8-10 mm TL, P12 individuals presented a significantly increased ($p < 0.05$) mean number of mineralized vertebrae (12.0 ± 0.5) compared to C (2.4 ± 0.6) and P6 (5.3 ± 0.7). Similarly, within 10-12 mm TL larvae, C (4.4 ± 1.2) and P6 (5.2 ± 1.0) presented a delayed mineralization of the vertebral column against P12 (18.3 ± 0.7).

3.4. P12 enhances an earlier larval development

Gene expression analysis following the development of European sea bass larvae at 28 (a, rS1), 42 (b, rS2) and 55dph (c, rS3) revealed a significant effect of the diets on the selected genes ($p < 0.05$, Fig. 7). The replicate effect was significant only in two cases belonging to rS2 (*slc6a19a* expression in P12 and *pept2* expression in C, $p < 0.05$, Supplementary Table S5). Experimental diets C and P6 presented no significant differences in their expression levels of various genes, regardless the sampling age ($p > 0.05$). On the contrary, P12 individuals exhibited the highest *amy2* (1.07 ± 0.08 - rS1, 0.89 ± 0.07 - rS2, 1.26 ± 0.07 - rS3) and *prss1* (1.40 ± 0.12 - rS1, 1.07 ± 0.04 - rS2, 0.82 ± 0.05 - rS3) mRNA levels compared to C and P6. A significant elevation of *sparc* expression in rS1, rS3 was also noted in the P12 group (1.25 ± 0.07 - rS1, 1.00 ± 0.04 - rS3, $p < 0.05$) against the other two dietary groups. However, in rS2, the opposite result was observed with P12 presenting significantly lower *sparc* expression (0.60 ± 0.05) compared to the other diets ($p < 0.05$). Moreover, a pronounced difference ($p < 0.05$) was spotted in *bglap* transcript levels in the last sampling (rS3), with P12 reaching the highest levels (1.14 ± 0.19) compared to C (0.71 ± 0.08) and P6 (0.61 ± 0.07). Concerning *pept1* and *pept2*, significant differences were only noted at rS3 ($p < 0.05$), between C (1.08 ± 0.07, *pept1* and 0.63 ± 0.08, *pept2*) and P12 (1.36 ± 0.11, *pept1* and 0.89 ± 0.07, *pept2*) groups.

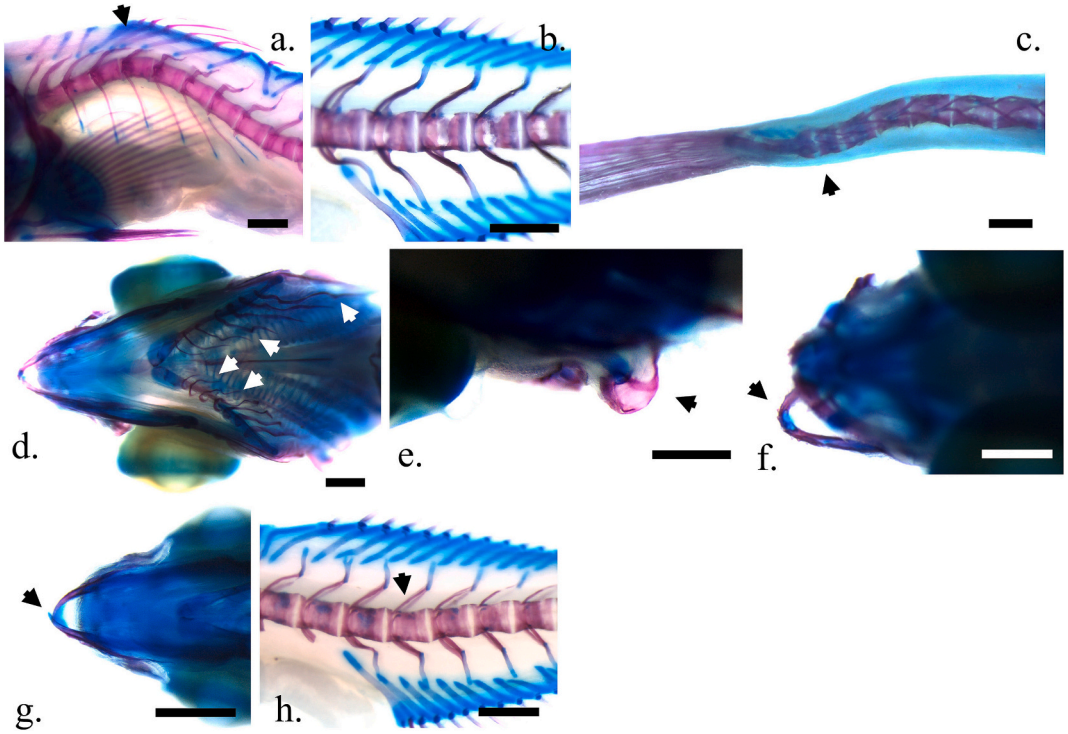

Fig. 3. Characteristic images of the main types of skeletal abnormalities observed in European sea bass larvae at 64-92dph. (a) Pre-haemal kyphosis. (b) Abnormal mineralized vertebral centra. (c) Scoliosis of the caudal peduncle. (d) Disorientated and malformed branchiostegal rays. (e) Folded operculum. (f) Crossbite. (g) Misalignment of the dentary bones of the lower jaw. (h) Haemal lordosis. Scale bars equal to 0.5 mm.

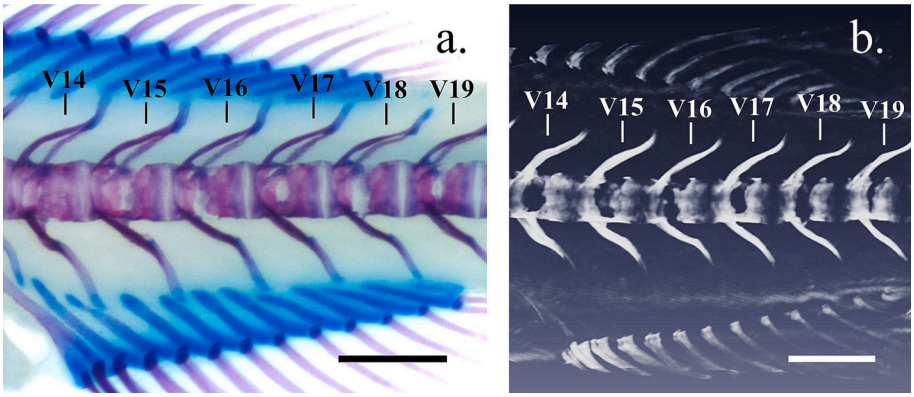

Fig. 4. Micro-CT scan of the abnormally mineralized vertebral centra of a double stained European sea bass larva. V14-V19, 14th to 19th vertebrae respectively. Scale bars equal to 0.5 mm.

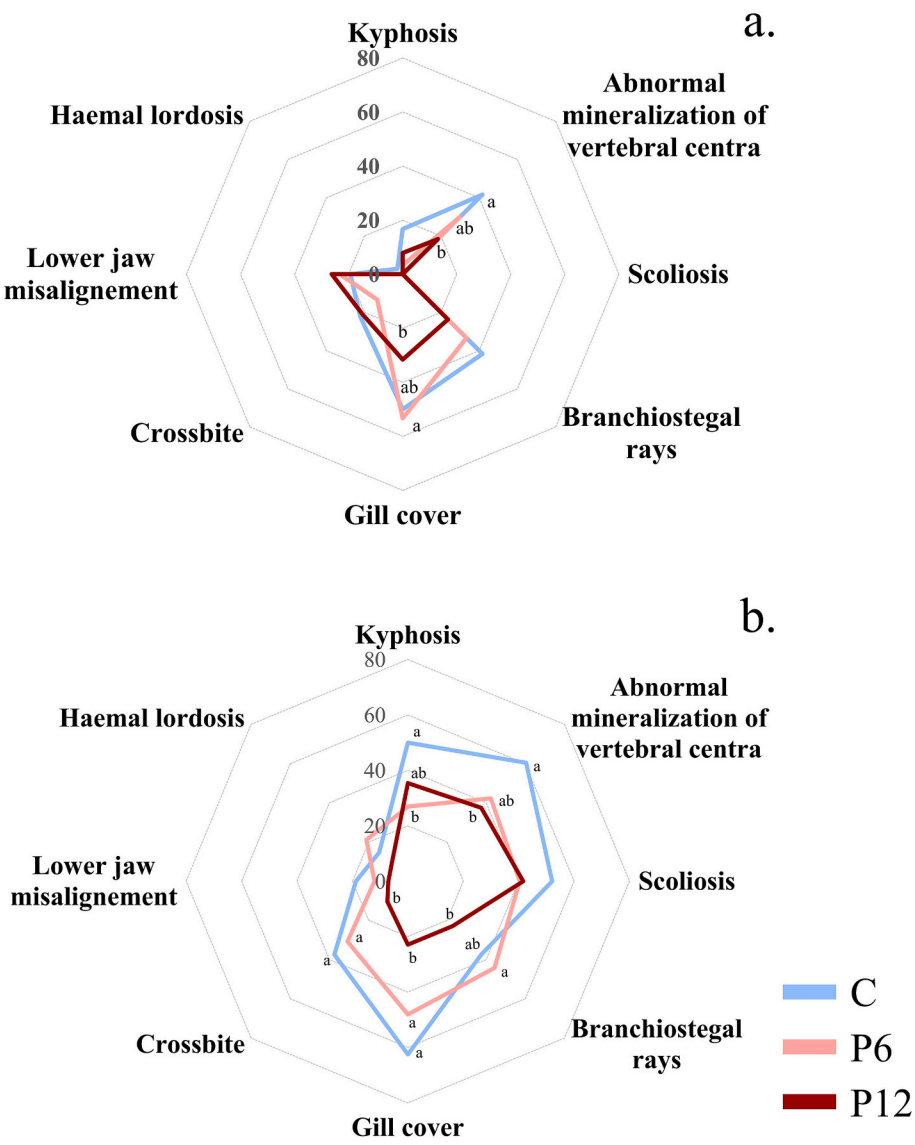

Fig. 5. Effect of the experimental diets (C, P6, P12) on the skeletal abnormalities frequencies in European sea bass larvae of 64 (a, S3) and 92dph (b, S4). The main abnormalities included are pre-haemal kyphosis, abnormal mineralization of the vertebral centra, scoliosis of the caudal peduncle, disorientated and malformed branchiostegal rays, folded opercula, crossbite, misalignment of the dentary bones of the lower jaw and haemal lordosis. $n = 35–45$ individuals per diet. Significant differences are indicated by the absence of a common letter ($p < 0.05$).

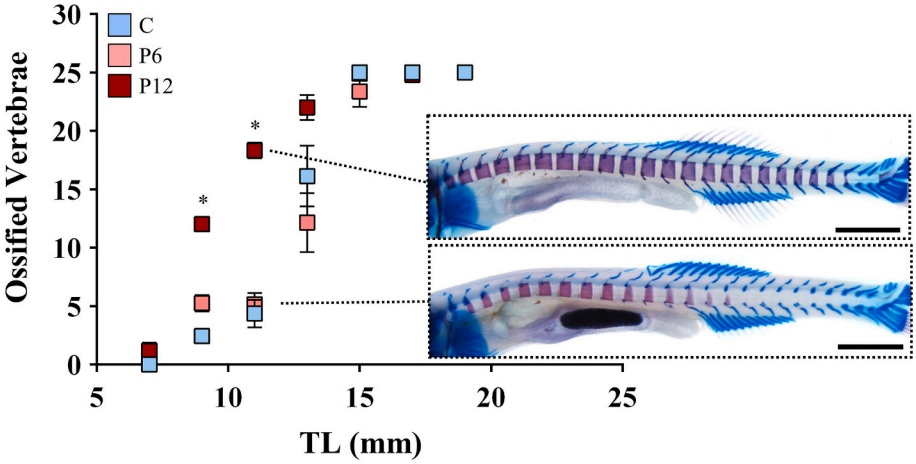

Fig. 6. Mean number of mineralized centra (±SE) in European sea bass larvae vertebral column under the three experimental diets (C, P6, P12). Significant differences are indicated by the asterisk (*). n = 150–180 samples per diet. Scale bars equal to 1 mm. TL, Total Length.

Similar is the case of *gcn2*, where its expression in rS3 was significantly higher in P12 (1.15 ± 0.04) compared to C (0.93 ± 0.06) group ($p < 0.05$). Also at rS3, *myod* (0.86 ± 0.05) and *myog* (1.09 ± 0.06) expression levels in P12 group diverged significantly with the ones of P6 (0.68 ± 0.03 for *myod* and 0.80 ± 0.03 for *myog*, $p < 0.05$). *Myf5* presented increased mRNA levels ($p < 0.05$) at P12 diet (1.49 ± 0.10 – rS1, 1.11 ± 0.06 – rS3) compared to C (1.12 ± 0.10 – rS1, 0.85 ± 0.04 – rS3) and P6 (0.79 ± 0.08 – rS3). The levels of *slc6a19a* transcripts were also significantly higher in P12 ($p < 0.05$) of both rS2 (0.88 ± 0.07) and rS3 (1.67 ± 0.10) against the two other dietary groups (0.47 ± 0.06 - rS2, 0.95 ± 0.12 - rS3 for C and 0.42 ± 0.06 - rS2, 0.92 ± 0.08 - rS3 for P6).

3.5. P12 increases the individual's resistance against swimming induced lordosis

Results after the SCT highlight the significantly increased resistance of the P12 group against swimming induced lordosis ($p < 0.05$). Only 16.0 ± 0.1% of the exercised individuals developed lordosis in the P12 diet, compared to 54.4 ± 0.2% in the C and 46.4 ± 2.0% in the P6 group (Fig. 8a). Haemal lordosis presented the characteristic V-shaped angle on the haemal vertebrae and upward shift of the caudal peduncle (Fig. 8d) compared to a normal vertebral column (Fig. 8c). An inverted orientation, facing rostrad, of the distal parts belonging to the haemal and neural spines in the lordotic area was also spotted. Simultaneously, a significantly increased frequency of caudal peduncle scoliosis in the C group was marked (31.9 ± 1.0%) in comparison with the peptide groups (10.7 ± 0.7% in P6 and 3.3 ± 0.6% in P12) ($p < 0.05$, Fig. 8b).

3.6. Peptide incorporation can modify the expression of genes involved in the musculoskeletal changes during the swimming test

Results of rt-qPCR assays underlined a significant effect of the experimental diets on the expression of selected genes after the SCT ($p < 0.05$, Fig. 9). Specifically, the expression of *bglap* was increased on the peptide groups (0.88 ± 0.12 in P6 and 0.71 ± 0.12 in P12) compared with the C (0.36 ± 0.03, $p < 0.05$). In opposition, the expression of *foxo* was significantly lower in the peptide groups (0.31 ± 0.06 in P6 and 0.27 ± 0.04 in P12) compared to C (0.67 ± 0.08, $p < 0.05$). *Myf5* expression was significantly elevated ($p < 0.05$) in P6 group (1.33 ± 0.09) compared to both C (0.43 ± 0.04) and P12 (0.90 ± 0.09). Lastly, *myod* transcript levels were significantly lower ($p < 0.05$) in P12 group (0.54 ± 0.05) compared to both C (0.76 ± 0.04) and P6 (0.76 ± 0.04). No effect of the phenotype (N,*L*) was observed within each diet and gene ($p > 0.05$), whereas the factor replicate had only a significant effect on the C group in *foxo* and *tgfb* expression ($p < 0.05$, Supplementary Table S6).

4. Discussion

The molecular size of dietary protein fractions and their reliant solubility have been underlined as critical for the optimum larval skeletal development in several species (European sea bass, (Zambonino Infante et al., 1997); gilthead sea bream, (Gisbert et al., 2012); largemouth sea bass, (Molinari et al., 2023); white sea bream, (De Vareilles et al., 2012). In line with this observation, the early skeletal quality results of our study report the beneficial effect of P12 favoring the normal development of the jaws, the first pre-haemal vertebrae and the gill cover complex. Incorporation of approximately 10% of smaller dietary peptides previously highlighted as ideal for European sea bass (Cahu et al., 1999; Zambonino Infante et al., 1997) is confirmed. The lack of significant benefit in terms of deformity rates observed in the P6 group indicates that the peptide diet levels should not be too far below 10%. An association between the deformities of the gill cover, the branchiostegal rays and pre-haemal kyphosis has already been documented in European sea bass and seabream larvae (Koumoundouros et al., 2002a, 2002b; Koumoundouros et al., 1997). Specifically, the association between pre-haemal kyphosis and abnormal branchiostegal rays was attributed to the common developmental period of the implicated skeletal structures (7.5–8.5 mm TL, (Gluckmann et al., 1999). In the meantime, jaw deformities as crossbite, developed during the early developmental stages, have been related with nutritional and mechanical factors (Cobcroft et al., 2001). The advantageous effect of the P12 on the normal development of the early bone structures (bones of the cephalic area and pre-haemal vertebrae) combined with the earlier mineralization of the vertebra centra in the P12 individuals, suggest a peptide-enhanced mineralization pattern. Indeed, we found a higher expression level of *bglap*, a marker gene for osteoblast differentiation associated with the mineralization of the extracellular matrix (Lian and Stein, 1995), in P12 group compared to P6 and C at rS3 (55dph). During the same developmental period, which corresponds to the TL range of 8-15 mm TL, the greatest differences in mean number of mineralized vertebrae were also observed between the groups. A link between dietary hydrolysates and *bglap* expression has already been described in European sea bass larvae (Delcroix et al., 2015). Moreover, *sparc* transcript levels in P12 samples maintained higher than in the two other groups throughout the development (rS1-rS3), providing additional information of a differential bone mineralization process from the early stages. Interestingly, a twisted pattern of bone mineralization was observed in all groups. X-ray images of the affected area allowed the clear observation of the non-mineralized areas of the vertebrae centra. This observation could be

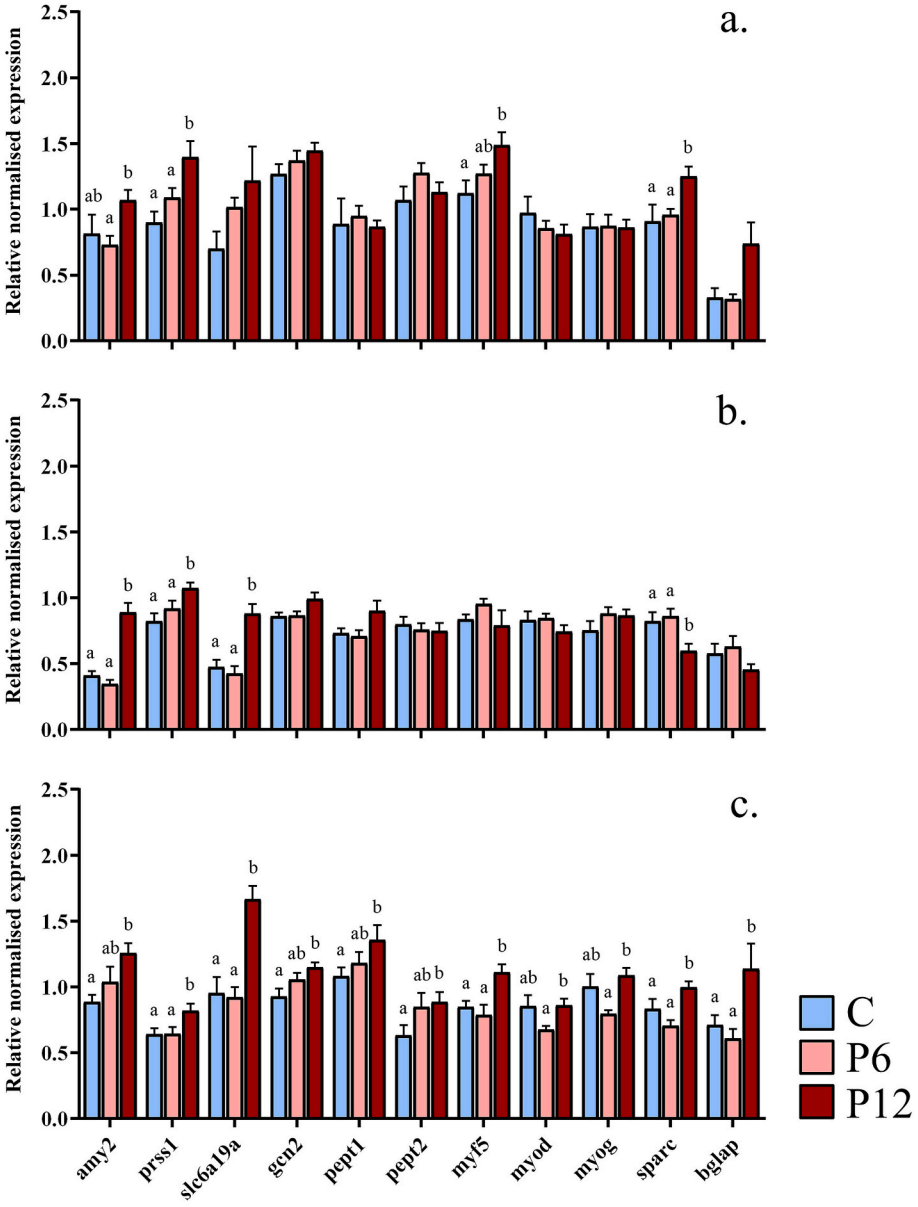

Fig. 7. Mean relative expression (±SE) of amylase alpha 2a (*amy2*), trypsin precursor (*prss1*), solute carrier family 6 member 19 (*slc6a19a*), eIF-2-alpha kinase - general control nonderepressible 2 (*gcn2*), peptide transporter 1 (*pept1*), peptide transporter 2 (*pept2*), myogenic factor 5 (*myf5*), myogenic differentiation factor (*myod*), myogenin (*myog*), osteonectin (*sparc*) and osteocalcin (*bglap*) among the diets (C, P6, P12). a, rS1 - RNA sampling 1 at 28 dph. b, rS2 - RNA sampling 2 at 42 dph. c, rS3 - RNA sampling 3 at 55 dph. $n = 8–12$ samples per diet per sampling. Significant differences are indicated by the absence of a common letter ($p < 0.05$).

potentially attributed to nutrient deficiencies beyond the critical larval period (>42dph). Several studies have already reported a direct linkage between nutrients deficiencies and vertebral centra mineralization not only in sea bass (Mazurais et al., 2009), but also in Atlantic salmon (Drábiková et al., 2021). Since teleost vertebral centra lack cartilaginous precursors, their formation is achieved through the segmental mineralization of the notochord sheath and the following intramembranous bone formation around the notochord (Witten et al., 2019). Based on the previously described uncoupling of bone formation and mineralization in salmon (Witten et al., 2016), potential mineralization defects in the vertebral bodies of the individuals are not necessarily linked with disruptions on their following bone ossification. The existing data allow us to assume a nutrient effect in bone mineralization only. Further investigation including the targeting of osteoblast markers (e.g., receptor activator of NF-κB ligand - Rankl and osteoprotegerin - OPG) would be necessary to conclude a peptide effect on bone matrix abundancy (To et al., 2012). Furthermore, the observed abnormally mineralized vertebral centra, could be also resembling the osteoporotic models already recorded in fish (To et al., 2012). Increased presence of bone resorption lacunae in zebrafish scales were previously attributed to a high-fat dietary (Carnovali et al., 2018), whereas a phosphorus deficiency was able to regulate bone resorption in haddock (Roy et al., 2002). Targeting osteoclast related gene markers, such as tartrate-resistant acid phosphatase (*trap)* (Witten and Huysseune, 2009), could potentially verify this scenario of increased bone resorption. Although P12 presented the lowest cases of fish with abnormal mineralized vertebral centra, the progressive frequency of this abnormality between the samplings (S3-S4), may be attributed to the inappropriateness of the diets beyond a developmental threshold.

An early effect of P12 diet on digestive tract maturation was also revealed through the increasing expression levels of *amy* and *prss1*. Such positive regulation suggests an earlier maturation of pancreatic digestive system in fish fed P12 (Rønnestad et al., 2013). Indeed, high peptidases activity at 20dph European sea bass larvae has been attributed to

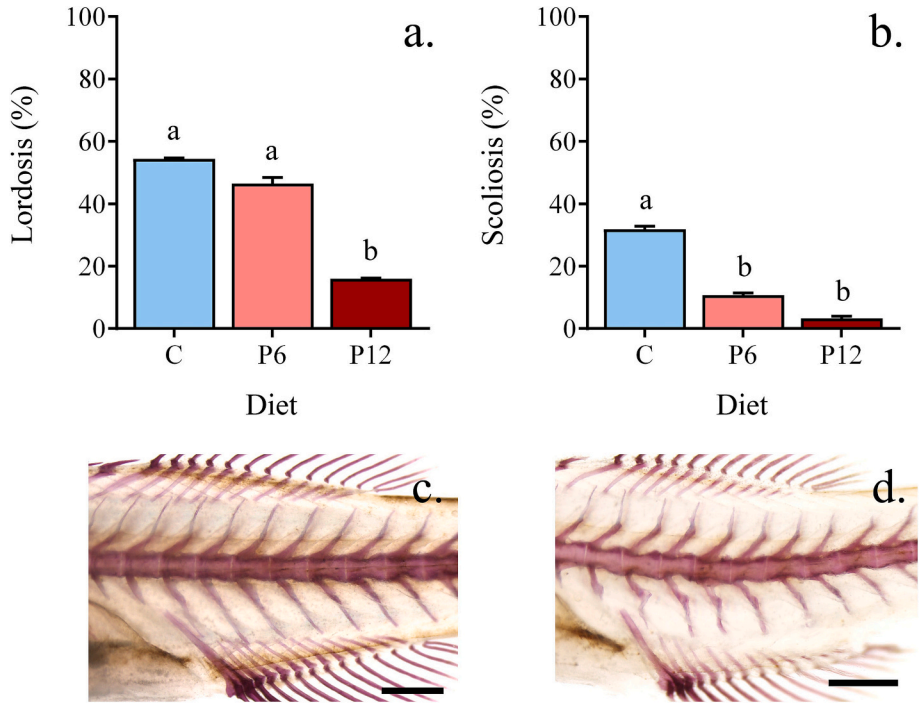

Fig. 8. Effect of the control (C) and the peptide diets (P6, P12) on the frequency of the swimming-induced haemal lordosis (a) and swimming-induced caudal scoliosis (b). Values are presented as means (±SE). Characteristic images of European sea bass individuals with a normal (c) and a lordotic (d) haemal vertebral area. Significant differences are indicated by the absence of a common letter ($p < 0.05$). Scale bars equal to 1 mm.

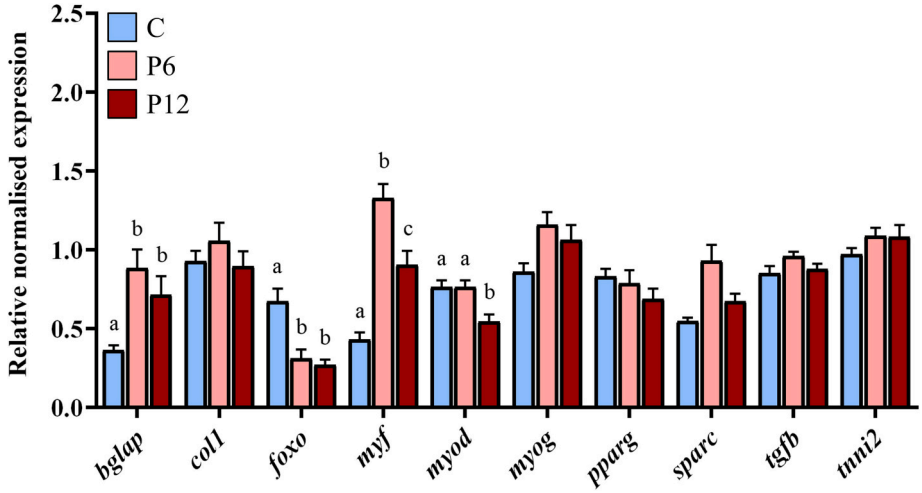

Fig. 9. Relative expression of osteocalcin (*bglap*), collagen type 1 (*col1*), forkhead box O1 a (*foxo1*), myogenic factor 5 (*myf*), myogenic differentiation factor (*myod*), myogenin (*myog*), peroxisome proliferator-activated receptor gamma (*pparg*), osteonectin (*sparc*), transforming growth factor beta (*tgfb*) and troponin I type 2 (*tnni2*) after the swimming challenge test (SCT) among the diets (C, P6, P12). Values are presented as means (±SE). Significant differences are indicated by the absence of a common letter ($p < 0.05$). $n = 16–22$ individuals per diet.

an efficient digestion of the protein hydrolysates (Cahu and Infante, 1995). Increased protein ingestion in the peptide groups could elevate the levels of di- and tri-peptides availability in the intestinal lumen which can necessitate amino acid and peptide translocation into the intestinal epithelia cells (Dabrowski et al., 2010). mRNA levels of *pept1*, a low-affinity and high-capacity peptide transporter, have been correlated with the nutritional status of the larvae and their feeding regime (Terova et al., 2009; Verri et al., 2011). In our case, the transcript levels of *pept1* and *pept2* in P12 were significantly higher than in the C group at rS3 indicating an increased absorption of protein smaller fractions ($p < 0.05$). A link between proteases and protein degradation products transporters has already been proposed (Verri et al., 2017). Similarly, the expression of the neutral amino acid transporter *slc6a19a*, usually expressed in the intestine and related with the dietary protein source (Margheritis et al., 2016), was significantly elevated in P12 group compared to the two others in rS2–3 ($p < 0.05$). In agreement with the differential free amino acid availability observed in our diets, we assume that P12 individuals present a peptide-enhanced digestive maturation through increased absorption of free amino acids and peptides from the early stages. On rS3, we detected also increased *gcn2* mRNA transcripts in P12 compared to C diets. A possible activation of the amino acid response pathway (AAR) acting in opposition of the nutrient-sensing target of rapamycin pathway (TOR), with increased phosphorylation of the *gcn2* aimed to reduce protein synthesis and cope with a dietary

stress as a result of imbalanced amino acid absorption could potentially explain this result (Dai et al., 2020; Sheng et al., 2022). Even though increased peptide absorption and free amino acid availability can be beneficial for the larvae during the early stages, possible excess or imbalance could lead to a fast flow within the digestive system (Cahu et al., 2003a, 2003b). Surprisingly, although the beneficial effect of similar peptide incorporation for the species on survival and growth has been already reported (Cahu and Infante, 1995), the earlier larval maturation (digestive function and mineralization) of P12 group was not accompanied by neither increased survival nor growth compared to the C group on our study.

The effect of the peptide diets on the haemal musculoskeletal response against the swimming challenge test was also significant ($p < 0.05$), with <20% of the P12 exercised individuals developing haemal lordosis. Swimming challenge test is a valuable means of testing the integrity of the haemal vertebrae and muscle against increased mechanical loads induced by intense swimming conditions (Antinero et al., 2023; Sfakianakis et al., 2006). Increased resistance of P12 individuals could be related with the earlier mineralization of the haemal vertebral bodies, resulting potentially in enhanced mineralization of the vertebral end plates which are known to experience the highest muscular forces (Printzi et al., 2021; Suniaga et al., 2018) and therefore reinforcing their stiffness (Kranenbarg et al., 2005). The higher expression of *bglap* after the SCT in the peptide groups can support this assumption. Moreover, the increased presence of osteoporotic cases in the C group, could also be related with this *bglap* expression pattern and may result in weaker bone structures eager to fragility fractures (Rosa et al., 2021). Interestingly, except lordosis, a high frequency of swimming-induced scoliosis of the caudal peduncle was induced in C group. The beneficial effect of the peptide diets on this specific deformity could be related with the differential amino acid profile compared to the C group. In salmonids, increased scoliosis individuals were also reported under a tryptophan deficiency (Akiyama et al., 1986). Surprisingly, utilization of the same diets in zebrafish did not result in the induction of scoliosis after the SCT (Printzi et al., 2023). This difference could be attributed to the species-specific musculoskeletal structure and remodeling.

Lordosis induction is suggested to involve morphological and physiological changes in the adjacent skeletal muscle too (Printzi et al., 2021; Sfakianakis et al., 2006). Expression of *myod* and *myog*, known myogenic regulatory factors, was significantly different between P6 and P12 diets in rS3 ($p < 0.05$) suggesting a differential myoblast regulation, muscle differentiation and growth pattern (Rescan, 2001). *Myf5* transcript levels were found elevated in the P12 group in our study, suggesting a potential peptide increased myogenic proliferation and differentiation. The myogenesis key genes are known to be affected by amino acid profiles (Canada et al., 2016). Experiments in trout reported that early dietary protein can program the activity of muscle precursor cells (Alami-Durante et al., 2014). *Myf5* and *myod* presented also differential expression patterns after the SCT among the groups, indicating a differential regulation of myogenic cell differentiation in a highly complex process involving cell cycle arrest and multinucleated myotube formation (Perelló-Amorós et al., 2021; Printzi et al., 2022; Sáez-Arteaga et al., 2022). The significantly reduced *foxo* mRNA levels reported in peptide groups after the SCT ($p < 0.05$), could be related with the activation of the protein kinase B pathway (Akt). Increased lysine supplementation in juvenile largemouth bass increased the mRNA expression levels of AKT inhibiting the transcriptional activity of FOXO (Huang et al., 2022). Furthermore, *foxo* expression has been linked with the dietary protein hydrolysis level in turbot (Wei et al., 2019).

5. Conclusions

The results of the present study reveal the beneficial effect of the early dietary di- and tri-peptides on the introduction of the larval inert diets in European sea bass. Specifically, a 12% small peptide incorporation enhanced the total sea bass larval maturation in terms of digestive function and ossification process. Within the same ontogenetic window, a beneficial peptide-driven effect on skeletal development was also observed. Prolonged dietary peptide incorporation up to four-month-old European sea bass, led to increased resistance against swimming induced deformities. Those insights suggest a potential utilization of di- and tri-peptides in inert larval diets aiming not only in enhanced survival and growth, but also in optimum larval and post-larval skeletal development.

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CRediT authorship contribution statement

A. Printzi: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **S. Jodet:** Formal analysis. **V. Fournier:** Resources, Methodology. **S. Collet:** Methodology. **L. Madec:** Methodology. **V. Simon:** Methodology. **J.-L. Zambonino-Infante:** Writing – review & editing, Supervision, Conceptualization. **G. Koumoundouros:** Writing – review & editing, Supervision, Conceptualization. **D. Mazurais:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

Zambonino Infante J.-L., Mazurais D., Koumoudouros G., Printzi A. have patent “Aquaculture feed composition, for farmed fishes selected from marine species, suitable for preventing skeletal malformations”, pending to European patent application no. 23307040.8, filed on November 23, 2023 (Refs: CH613EP DAD/JF/KB). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.740657.

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