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Effect of L-Hyp supplementation on collagen muscle histology, gene expression, growth performance, body composition and fillet texture on big size European sea bass (*Dicentrarchux labrax*)

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ABSTRACT

Hydroxyproline (L-Hyp) is amply present in fishmeal but limited in plant-protein sources. This study was conducted to evaluate the effects of supplementation with dietary L-Hyp on the distribution of collagen types in the muscle, and on the texture, survival rate, growth rate, feed utilization, body composition as well as the expression of the gene that encodes the pro-alpha2 chains of type I collagen (Col Iα2) of large European sea bass (initial body weight 609.21 ± 75.39 g) from high plant-protein diets. Four isoproteic (42 % crude protein) and isolipidic (20 % crude lipid) experimental diets were formulated adding 0.6 (HL diet), 1.2 (HM diet), and 2% (HH diet) L-Hyp, respectively. Three periods of feeding of 45, 99 and 143 days were studied. L-Hyp supplementation at 1.2 % and 2 % significantly improved specific growth rate (SGR) and feed conversion ratio (FCR). In the white muscle, type I, IV collagen and trichromic stain were significantly higher in HH feed than the control diet. In the red muscle, only type I collagen was higher. HH diet, also increases Col Iα2 mRNA levels in muscle significantly. It can be concluded that the addition of crystalline L-Hyp at 1.2 % or 2 % in high plant-protein diets indicates positive effects on growth performance of adult European sea bass and increase in muscle total collagen deposition.

1. Introduction

Collagen is the main component of the connective tissue in fish (Suárez et al., 2005; Moreno et al., 2012), essential not only to maintain the normal structure and strength (Li and Wu., 2018) but also responsible of biological processes involving growth and differentiation of cells (Gordon and Hahn, 2010). In the muscle, although most of the collagen constitutes the myocommata separating the myotomes, collagenous sheets form both the endomysium and the perimysium (Hagen et al., 2007) with type I collagen being the main component (Nishimoto, 2004; Moreno et al., 2012). Type I and V collagens form fibrous networks of endomysium, although other collagens are also present in the endomysial region as a fibrous structure in the interstice or non-fibrous network surrounding individual muscle fibres at the myotendinous junction of the myocommata (type IV) (Bruggemann and Lawson, 2005). Collagen molecules consist of three polypeptide chains coiled into a left-handed helix and are then wound around a common axis to form a triple helix (Myllyharju and Kivirikko, 2004). In this context, the hydroxyproline (L-Hyp) plays an important role in the stabilization of the helical structure (Krane, 2008) due to its hydrogen bonding ability through its hydroxyl group (Moreno et al., 2012).

L-Hyp is a conditionally essential amino acid (Li et al., 2009) synthesized normally in suitable amounts by organisms. It must be provided from diet to meet optimal needs under conditions where the rate of utilization is greater than the rate of synthesis (Wu, 2009). Such is the case for fish, the rate of endogenous synthesis of L-Hyp are inadequate (Wu et al., 2011) to guarantee maximal growth, collagen production or feed efficiency (Li and Wu, 2018). An important quality trait, such as flesh firmer texture, is associated with collagen and L-Hyp content in the muscle, and the varying fibre density (Hagen et al., 2007) is dependent on the fish origin, farmed or from the wild (Periago et al., 2005) or subjected to long-term exercise (Bugeon et al., 2003). Low collagen

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content, found in farmed Atlantic salmon (*Salmo salar*) muscle, results in tender meat, vulnerable to gaping (Aidos et al., 1999).

Ingredients from animal origin such as fishmeal, containing connective tissue, are excellent sources of L-Hyp (Wu, 2009; Kousoulaki et al., 2009, 2012). L-Hyp content in fishmeal is superior to that in plants proteins (Li et al., 2009), especially those used in aquaculture (Aksnes et al., 2008) as plant feed stuffs have highly variable protein content (Oliva-Teles et al., 2015). However, reducing aquafeed dependency on fishmeal and fish oil is of utmost economic and environmental importance. Dietary complementation, hence, is particularly necessary for fishmeal-replaced diet that exceed a certain high level (Liu et al., 2014) to maximize survival and growth rate of aquatic animals (Li et al., 2009). The addition of L-Hyp in high plant-protein based diets significantly increase the muscle collagen in juvenile turbot (Zhang et al., 2013, 2015; Liu et al., 2014), as well as improves growth parameters (Dabrowski et al., 2010) with no significant effect for feed intake or feed efficiency (Aksnes et al., 2008). Nevertheless, feed supplementation increases the final cost of diet. An opportunity to rationalize cost is to limit the concluding period of growth on fish species with a high market value, such as farmed European sea bass (*Dicentrarchux labrax*). This is important for general aquaculture production but especially for the objective of this study, large-sized seabass, those that weigh around or more than one kilogram.

Given that L-Hyp is a conditionally essential amino acid being European sea bass able to synthesizes to cover their needs, the aim of this work was to evaluate how the addition of different levels of crystalline L-Hyp in diets with high fishmeal substitution, increase the rates of L-Hyp utilization on large size European sea bass in terms of collagen construction, gene expression of type I collagen and texture.

2. Material and methods

2.1. Experimental conditions

In this study, 300 commercially European sea bass provided by AQUANARIA (Gran Canaria, Spain), were maintained in stocking tanks and fed with a commercial extruded diet for 3 weeks at the experimental facilities of Scientific and Technological Park Foundation of the University of Las Palmas de Gran Canaria (Gran Canaria, Spain). Afterwards, these fish were randomly distributed into 12 indoor cylindroconical 1 m^3 fibreglass tanks. The fish average initial weight was 609.21 ± 75.39 g. Tanks were supplied with filtered seawater in a flow-through system, at a temperature of 22.1–23.0 °C and under natural photoperiod with water dissolved oxygen ranging between 7.5 and 7.7 ppm. The fish were manually fed until apparent satiation 2 times a day, 6 days a week for three experimental periods of 45, 99 and 143 days. After 24 h of starvation, six fish per tank (eighteen fish per diet) were sampled for proximate, histology, gene expression, and texture studies. The animal experiments description complies with the guidelines of the European Union Council (2010/63/EU) for use in experimental animals and was approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria, Spain.

2.2. Experimental diets

Four isonitrogenous (45 % protein) and isolipidic (20 % lipid) diets were used (Table 1). A control diet (Control) based on a standard fattening composition, provided by the company DIBAQ (Segovia, Spain) were supplemented with crystalline L-Hyp: 0.6 % (HL diet), 1.2 % (HM diet), and 2.0 % (HH diet) (NutriScience Innovations LLC, Connecticut, USA) with a 99,6% of purity (Table 1). The feed was distributed in two shots of 8:30 and 14:30 to apparent satiation. The determination of total hydroxyproline in the diet were done with HPLC (method: ISO 13903:200) by Eurofins Steins Laboratorium (Vejen Denmark).

Table 1
Ingredients (g/kg of feed) and chemical composition of the experimental diets.

Ingredients	Diets			
	Control	HL	HM	HH
Fish meal[a]	230	227	224	220
Soya protein concentrate[b]	260	257	254	250
Blood meal (spray-dried)[c]	50	50	50	50
Corn gluten meal[d]	160	160	160	160
Wheat[e]	170	170	170	170
Fish oil[f]	60	60	60	60
Soya oil[b]	60	60	60	60
Vitamin and mineral premix[g]	10	10	10	10
L-hydroxyproline[h]	0	6	12	20
Crude protein	44.49	45.15	45.97	45.89
Crude lipids	20.07	19.79	19.43	19.56
Ash	8.35	8.42	8.35	8.54
Moisture	6.44	6.35	6.70	6.44
Total Hydroxyproline	5,70	11,14	18,21	24,83

[a] South-American, Superprime-Feed Service Bremen, Germany.
[b] Svane Shipping, Denmark.
[c] Daka, Denmark.
[d] Gargill, Netherlands.
[e] Hedegaard, Denmark.
[f] South American fish oil, LDN Fish Oil, Denmark.
[g] Supplied the following (mg/kg feed) Vitamins: A 3.8, D 0.05, E 102.4, K3 9.8, thiamin 2.7, riboflavin 8.3, B6 4.8, B12 0.25, niacin 24.8, pantothenic acid 17.2, folate 2.8, biotin 0.14, C 80; Minerals: cobolt 0.94, iodine 0.7, selenium 0.2, iron 32.6. manganese 12, copper 3.2, zinc 67; Other (g/kg): taurine: 2.45, methionine: 0.5; histidine: 1.36, cholesterol: 1.13 DSM, (Netherlands), Evonik Industries (Germany), Deutsche Lanolin Gesellschaft (Germany).
[h] NutriScience Innovations LLC, Connecticut, USA.

2.3. Proximate composition

To determine muscle proximate composition, each unskinned fillet (right side) was homogenized and immediately devoted to proximal analysis by a Food Scan™ (FOSS, Hillerød, Denmark). Ash was determined gravimetrically after combustion for 24 h at 450 °C (AOAC, 1995).

2.4. Histochemistry and immunohistochemistry

5mm thick cross-sections of muscle were taken from the posterior third of the fish body including both white and red muscle. Muscle samples were fixed in 10 % buffered formalin for 24−48 h, dehydrated and finally embedded in paraffin wax. Five serial sections (4 μm) were cut and processed for haematoxylin and eosin, Masson's trichrome (MT) staining (Martoja and Martoja-Pierson, 1970) and immunohistochemistry. For immunostaining, after deparaffinization and rehydration, the slides were heated for antigen retrieval in 0.01 mol/L Tris-EDTA buffer (pH 9.0). Endogenous peroxidase activity and nonspecific binding were blocked. Rabbit collagen polyclonal antibodies of type I, IV (BP 8005, BP5031 Acris GmbH, Germany) were used as the primary antibody at a dilution of 1:100. After overnight incubation at 4 °C, the slides were incubated with an anti-rabbit, horseradish peroxydase labelled polymer secondary antibody from the DAKO Envision+™ System (Dako Corporation). Immunoreactivity was visualized with 3,3′-diaminobenzidine (Dako Corporation, Carpinteria, CA). Finally, the sections were counterstained with haematoxylin and mounted permanently. The sections were evaluated under light microscopy (Olympus CX41 microscope). Micrographs were taken using an Olympus DP50 (Olympus Optical, Shinjuku-ku, Tokyo, Japan) camera, with a resolution of 5.8 million pixels, and CellSens Digital Imaging software (version 1.9, Olympus, Hamburg, Germany). Images, supported in TIFF format (2588 × 1960 pixels) were converted to RGB stacks providing grayscale pictures for separated channels). The relative percentage of positive trichromic staining was quantified by calculating the ratio of positive staining area to total area with an analySIS® (Image Pro Plus®, Media Cybernetics,

Silver Spring, MD, USA).

2.5. RNA extraction and real-time quantitative PCR analysis

Muscle samples were collected and placed on ice, snap-frozen in liquid N2 immediately after sampling and stored at –80 °C until RNA extraction. Type I collagen (Col Iα2) gene expression level was measured by absolute quantification. Total RNA from tissue samples (100 mg) was extracted using the RNeasyMiniKit (Qiagen) and quantified by measuring absorbance at 260 nm in a spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific Inc., USA). Electrophoresis of total RNA was also conducted in a 1% agarose gel. Synthesis of cDNA was done using the iScriptcDNA Synthesis Kit (Bio-Rad). Absolute quantification PCR was performed in QX200 Droplet Digital and C100 Touch PCR (Bio-Rad, Hercules, CA, USA) in a final volume of 20 μL and the kit ddPCR Eva Green Supermix. Then PCR plate was transferred to the QX200 Droplet Reader and the data analysed with QuantaSoft (Bio-Rad, Hercules, CA, USA). Primer design was carried out using Primer3 (v. 0.4.0) software from the gene sequences available on The National Center for Biotechnology Information (NCBI) databases of *D. labrax*. The primers sequence for Col Iα2 (GenBank: CX660451.1) assay was: forward primer 5′- ATTTCTCTCTGCGCATCCAC -3′; reverse primer 5′-TGGTGCTCAGGCAGTGC-3′;

2.6. Texture studies

For fillet texture studies, fish were kept whole without evisceration on ice for 24 h after harvest. After this period, the skin was removed and three-square pieces (cranial, central and caudal, 2.5 × 2.5 × 1.5 cm) were collected from the left fillets, above the lateral line. The force–deformation curve was analysed using a Stable Micro System texture analyser (TA.XT2, Surrey, England) to determine texture parameters (hardness, springiness, cohesiveness, gumminess, chewiness, resilience and fracturability). Compression plate and speed were 100 mm Ø and 0.8 mm/s, respectively. The deformation was 60 % of the original thickness (Ginés et al., 2004). Cooked fillet samples were prepared as above and cooked in an air-heated oven (Compact; Eurofred, Barcelona, Spain) for 10 min at 115 °C, packed in lidded aluminium boxes. In cooked fillet, deformation was 80 % of the original length and the fracturability was not determined.

2.7. Statistical analysis

Data were analysed with IBM SPSS Statistics for Windows, Version 26.0 (Armonk, NY: IBM Corp.). All variables were checked for normality and homogeneity of variance, using the Kolmogorov–Smirnoff and the Levene's test respectively. A one-way ANOVA was used to determine the diet effect. Significant differences between the means were evaluated by Duncan's multiple range tests.

3. Results

3.1. Growth performance

Fish fed with L-Hyp rich diets, HM and HH, showed a significant growth increase ($P<0.05$) comparing with diets Control and HL (Table 2). After 45 days, adding 3.8 % of extra weight. This gain was constant throughout the experimental period with a gain of 5.7 % after 99 and 6.2 % after 143 days at the end of the experimental period. SGR was lower feeding Control or HL than with HM and HH diets, after 45 and 99 days of experimentation. Similarly, for FCR, the values were higher in fish fed with diets Control and HL than those fed with diets HM and HH from 45 until 99 experimental days.

Table 2
Growth performance and feed utilization of European sea bass fed experimental diets. Values expressed in mean ± SD (n = 75 fish per diet; n = 3 tanks per diet).

		Diets			
		Control	HL	HM	HH
Initial	Body weight (g)	611.80 ± 87.76	607.71 ± 68.15	609.79 ± 75.47	607.56 ± 70.10
45 days	Body weight (g)	672.67 ± 93.86^{b}	667.76 ± 71.85^{b}	706.83 ± 83.60^{a}	691.88 ± 80.75^{ab}
	SGR[a]	0.19 ± 0.09^{b}	0.21 ± 0.07^{b}	0.31 ± 0.07^{a}	0.28 ± 0.08^{ab}
	FCR[b]	2.38 ± 0.46^{b}	1.93 ± 0.56^{b}	1.36 ± 0.19^{a}	1.47 ± 0.19^{a}
99 days	Body weight (g)	720.35 ± 97.41^{b}	711.86 ± 81.2^{b}	759.81 ± 99.76^{a}	757.33 ± 94.23^{a}
	SGR[a]	0.15 ± 0.04^{b}	0.16 ± 0.02^{b}	0.22 ± 0.05^{ab}	0.24 ± 0.06^{a}
	FCR[b]	2.17 ± 0.45^{c}	1.96 ± 0.29^{bc}	1.58 ± 0.26^{ab}	1.42 ± 0.07^{a}
143 days	Body weight (g)	766.57 ± 98.70^{b}	751.178 ± 77.23^{b}	809.22 ± 94.45^{a}	809.95 ± 93.36^{a}
	SGR[a]	0.16 ± 0.02	0.15 ± 0.02	0.20 ± 0.03	0.19 ± 0.04
	FCR[b]	1.97 ± 0.24	2.02 ± 0.35	1.68 ± 0.20	1.83 ± 0.27

Different letters within a line denotes significant differences ($P < 0.05$).
[a] Specific growth rate (SGR) = [(ln final weight − ln initial weight)/number of days] × 100.
[b] Feed conversion rate (FCR) = Feed consumption (g) / weight gain (g).

3.2. Proximate composition

Table 3 shows the muscle proximate composition of European sea bass fed with experimental diets. No significant differences were found despite the low standard deviation in each diet.

3.3. Histochemistry and immunohistochemistry

Antibodies anti-collagen I and IV and Masson's trichromic stain, allowed evaluating differences into the organizational structure of muscular collagen on European sea bass fast and slow-twitch muscle fibres. The quantitative morphological studies on white fast-twitch muscle sections showed an increase ($P<0.05$) of the proportion of type I collagen on the European sea bass fed with HM and HH diets (Fig. 1). In the samples from the Control diet, no clear definition of the muscle fibre bundles was present. The grouping of the fibres was less noticeable (Fig. 2) than feeding from rich L-Hyp diets. Anti-type IV collagen antibody increased ($P<0.05$) the immunolabelled area (Figs. 1 and 2) in the samples from fish fed with HH rich L-Hyp diet. The trichromic stain displayed similar results than that of the type I collagen. The area stained in blue (Fig. 3) was found to be larger ($P<0.05$) by studying the samples of the fish fed with HM and HH diet (Fig. 1). On the red slow-twitch fibres a significant increase ($P < 0.05$) was recorded, exclusively from HH feed diet, on the formation of type I collagen (Fig. 4) where the muscle fibres were gathered with a notable sheath of collagen (Fig. 5). A preferential distribution pattern was found where type IV was specially demarcated with the endomysium (Fig. 6).

Table 3
Muscle proximate composition (wet-weight basis) of European sea bass fed experimental diets. Values expressed in mean ± SD (n = 18 fish per diet).

	Diets			
	Control	HL	HM	HH
Protein	21.89 ± 0.78	21.98 ± 0.72	21.98 ± 0.63	22.31 ± 0.97
Lipids	3.45 ± 1.21	3.68 ± 1.18	4.07 ± 0.95	3.33 ± 0.99
Ash	1.13 ± 0.16	1.19 ± 0.11	1.17 ± 0.11	1.12 ± 0.13
Moisture	72.53 ± 1.42	72.13 ± 0.88	71.67 ± 0.75	72.45 ± 0.78

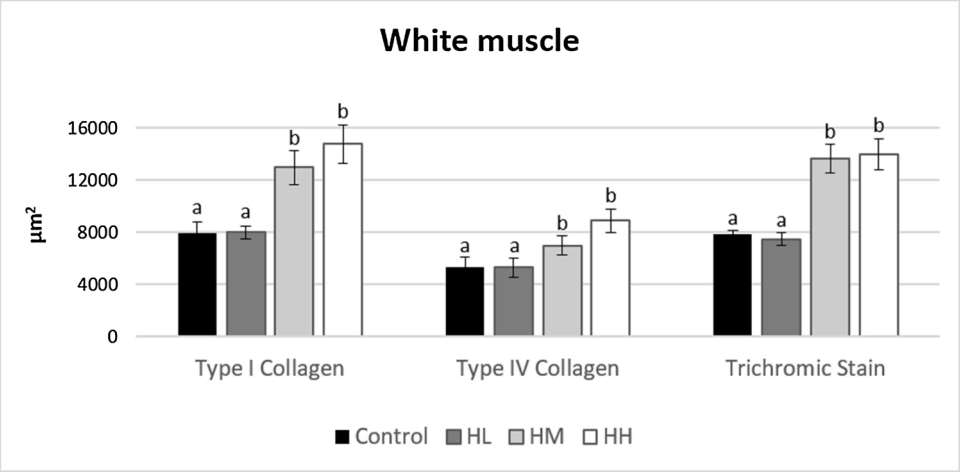

Fig. 1. Collagen area (μm^2) in the white, fast twitch muscle of European seabass fed experimental diets labelled with type I and type IV collagen and Masson trichrome stain (different letters within a type denotes significant differences; $P < 0.05$). Values expressed in mean ± SD (n=18 fish per diet).

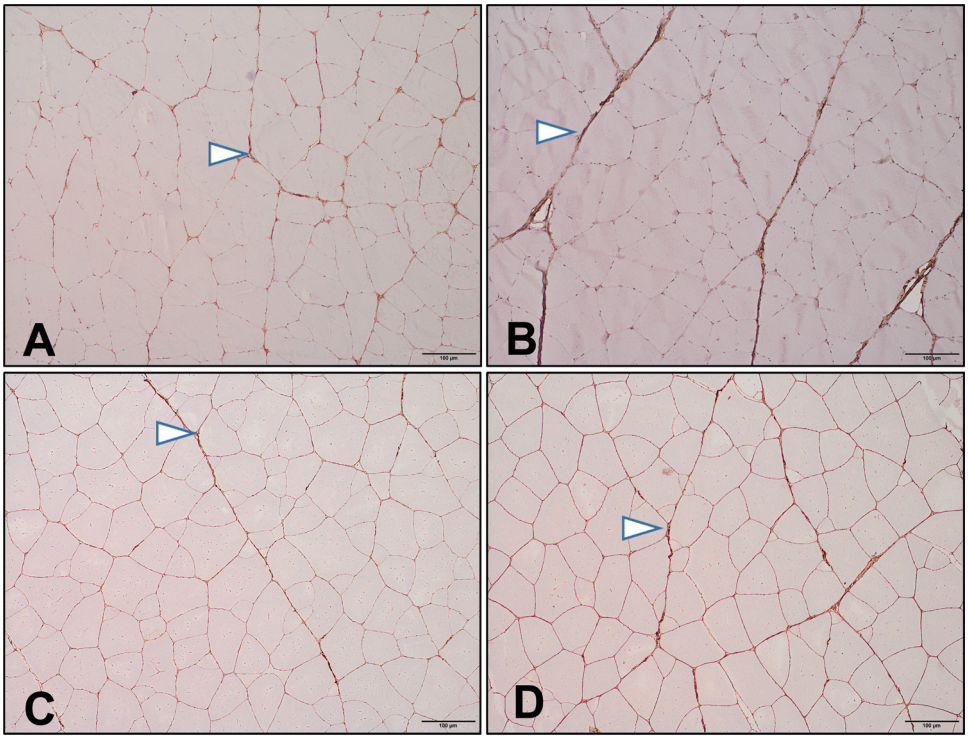

Fig. 2. White, fast twitch muscle fibres from European seabass fed experimental diets stained with type I and IV. A: type I collagen control diet; B: type I collagen HH diet. C: type IV collagen control diet; D: type IV collagen HH diet. Collagen (white arrowheads), immunostaining 10x (bar 100 µm).

3.4. Gene expression

The expression level of type I collagen (Col Iα2) in muscle was significantly affected by dietary L-Hyp content ($P < 0.05$; Fig. 7). Thus, fish fed with diet HH and HM showed higher absolute expression levels of cDNA copies/µl (331.55 ± 120.24 and 288.12 ± 101.42, respectively) than those fed with diet HL and Control (214.06 ± 58.85 and 197.68 ± 73.98 cDNA copies/µl, respectively).

3.5. Fillet texture

During the continuous compression of the raw fillet with the texturometer, significant differences were not recorded, neither the force required to break up the muscular structure fracturability, nor the maximum force upon reaching a deformation of 60 % of fillet thickness and hardness (Table 4). In any case, the fracturability force was around half of the hardness force with all experimental diets. The other parameters related to hardness, such as the fillet capacity to maintain its cohesiveness and structure after the deformation, resistance to the disintegration of the fillet and gumminess, did not show effect in diet. Similarly, the shape recovered after the first compression cycle, springiness, and the muscle structure recuperation, resilience, were not affected by diet. On the cooked fillet, after the application of a deformation of 80 % on fillet thickness, no significant differences were found (Table 4).

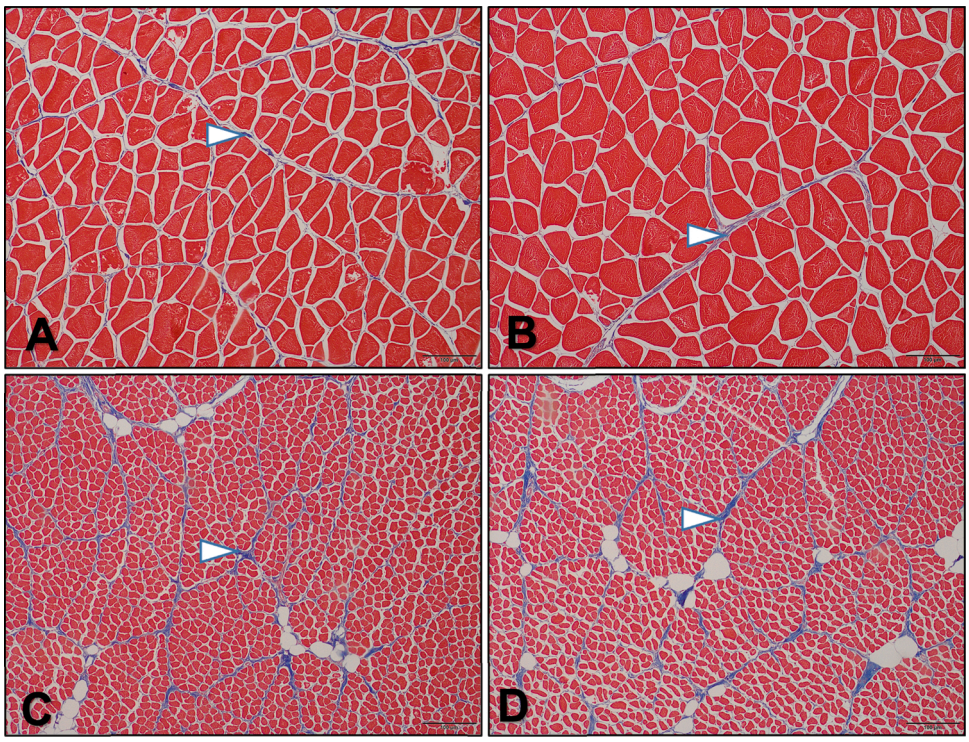

Fig. 3. White and red muscle fibres from European seabass fed experimental diets stained with Masson trichromic stain. A: white, fast twitch muscle, control diet; B: white, fast twitch muscle, HH diet; C: red, slow twitch muscle, control diet; D: red, slow twitch muscle, HH diet. Collagen (white arrowheads), Masson trichromic stain, 10x, (bar 100 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

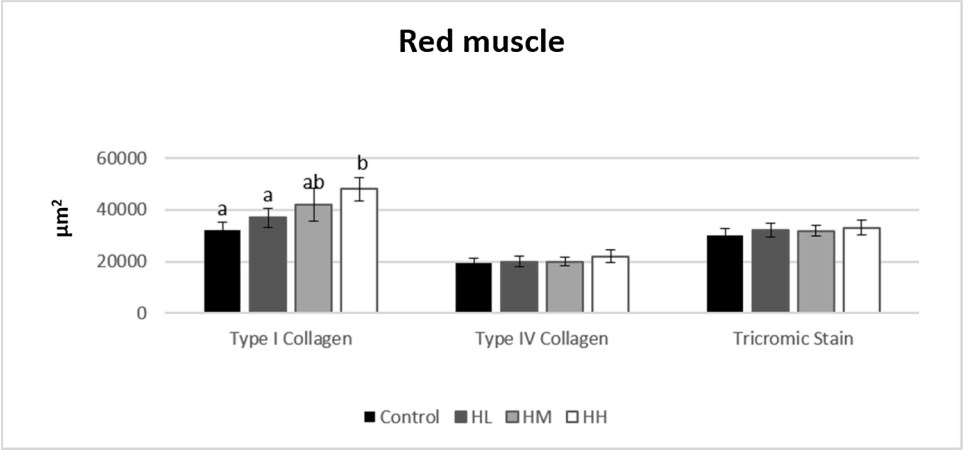

Fig. 4. Collagen area (μm^2) in the red, slow twitch muscle of European seabass fed experimental diets labelled with type I and type IV collagen and Masson trichrome stain (different letters within a type denotes significant differences; $P < 0.05$). Values expressed in mean ± SD (n=18 fish per diet). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

4. Discussion

4.1. Growth performance

L-Hyp inclusion effectively increased European sea bass growth performance and feed utilization. Supplementation levels up to 1.2 or 2.0 % of L-Hyp in the diets formula recover a 6% increase of the final weight, proving a positive effect on this economically relevant fish species. These results were reached only after 43 experimental days, indicating the interest to supply amino acids considered as conditionally essential. Traditionally, L-Hyp has been considered to be provide from diet under juvenile conditions where rates of utilization are greater than rates of synthesis (Li et al., 2009), showing this study that this dependency can be extended. Performance indicators such as SGR and FCR were also enhanced in diet with L-Hyp rich levels, although the significant differences between treatments were only maintained until the second experimental period, after 99 days. These results suggest additional insights to accurate supplementation practices subject to growth rate, season, or commercial size, noting the constraint that complements price could imply.

L-Hyp supplementation gains relevance in fish fed with high plant-protein based diets, as sources normally used in aquaculture contains naturally low levels or no L-Hyp precursors (Aksnes et al., 2008). Though, there is lack of experience with the use of L-Hyp supplementation on European sea bass, muscle free L-Hyp was considered an indicator for feed conversion efficiency and growth rate in Atlantic salmon

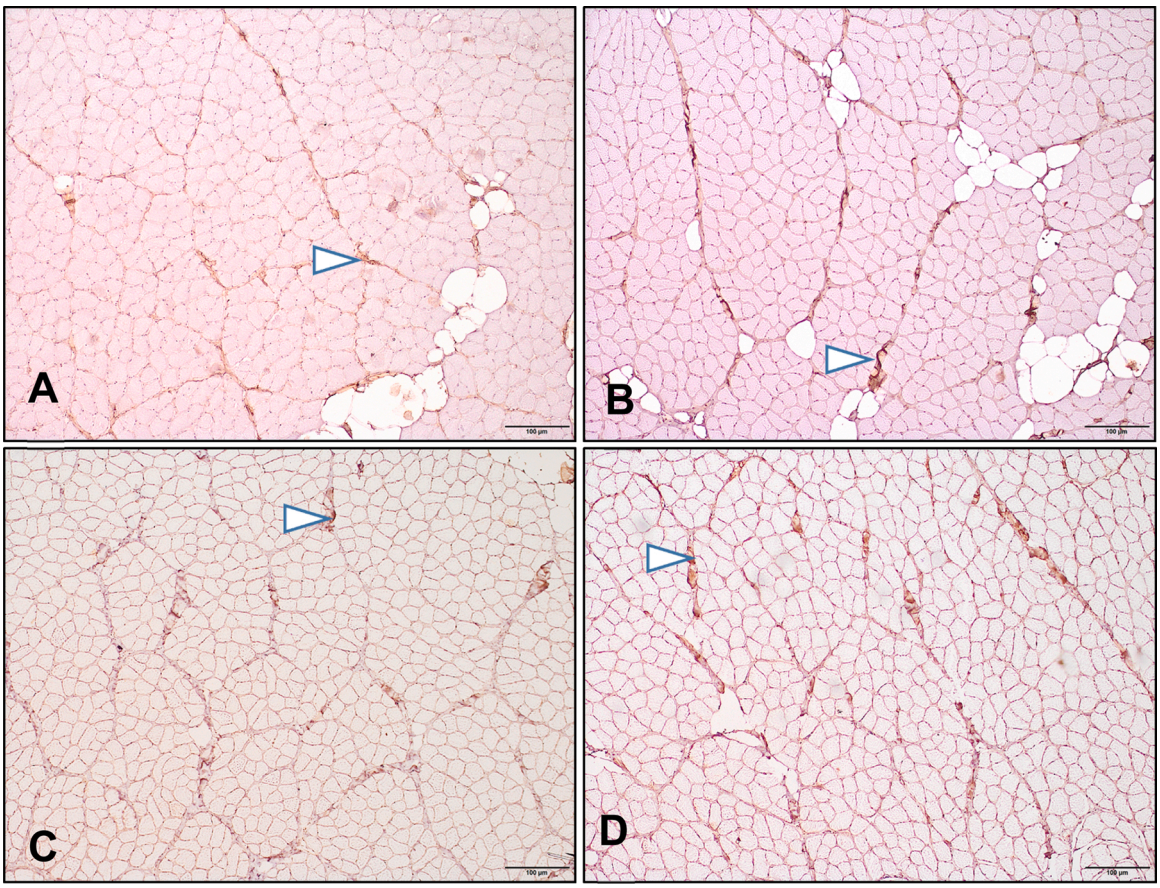

Fig. 5. Red, slow twitch muscle fibres of European seabass fed experimental diets labelled with type I and IV Collagen. A: type I collagen control diet; B: type I collagen HH diet; C: type IV collagen control diet; D: type IV collagen HH diet. Collagen (white arrowheads), immunostaining 10x (bar 100 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

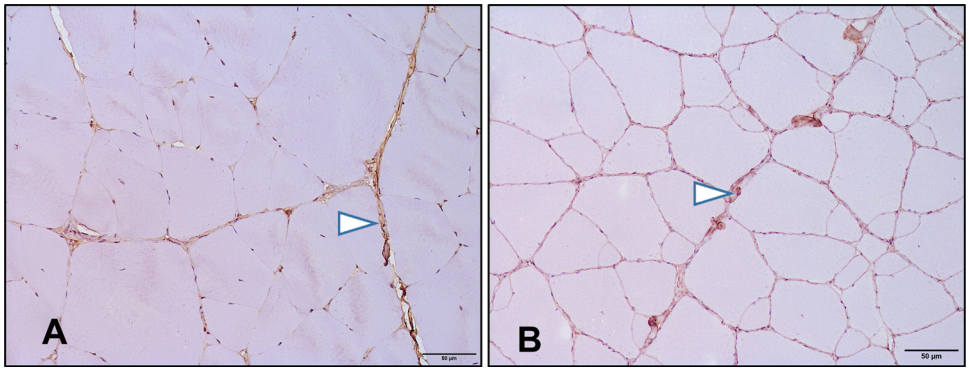

Fig. 6. Different distribution of the collagen in white, fast-twitch muscle fibres in European sea bass muscle fish fed HH diet. A: type I collagen B: type IV collagen. Collagen (white arrowheads), immunostaining 20x, (bar 50 μm).

(Sunde et al., 2001). Likewise, several results in different species during growing period support the positive effect of free L-Hyp addition on plant-protein based diets (Aksnes et al., 2008; Dabrowski et al., 2010; Liu et al., 2014; Rong et al., 2020; Wei et al., 2016).

4.2. Proximate composition

The muscular proximal composition of the European sea bass was unaltered by L-Hyp inclusion in isonitrogenous and isolipidic diets, as it has been shown in other fish species, such as turbot, from analysis of the whole body composition (Zhang et al., 2013; Liu et al., 2014). In this fish species though, L-Hyp added to plus proline produced a significant variation in the ash content, as both amino acids combination effect bone mineralization (Zhang et al., 2015). Regarding the effect of the dietary supply of amino acids low in fish meal diets, no significant effect in whole body composition was determined in European sea bass (Coutinho et al., 2017), red seabream (Gunathilaka et al., 2019), white seabream (Magalhães et al., 2019) or meagre (de Moura et al., 2018), in all cases concerning commercial size fish. In larger size fish, the use of L-Hyp supplemented ending feeds with a slight variation of ingredients will hardly change the proximate composition. The protein content is considered constant in species, for instance European sea bass studied from different origin and season (Grigorakis, 2007). Similar results from feeding with L-Hyp supplemented diets were recorded in turbot, having

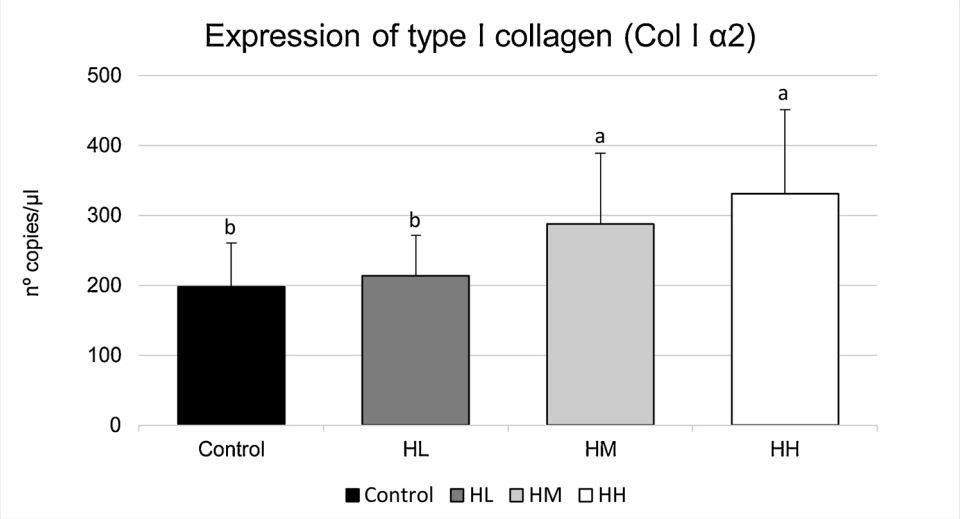

Fig. 7. Relative expression of type I collagen (Col Iα2) gene in European sea bass muscle fish fed with experimental diets. nº copies/µl obtained from 200 ng cDNA (100 mg muscle). Different letters within a type denotes significant differences; $P < 0.05$. Values expressed in mean ± SD (n=18 fish per diet).

Table 4
Texture parameters in raw and cooked fillet of European sea bass fed experimental diets. Values expressed as mean ± SD (n = 18 fish per diet).

	Diets			
	Control	HL	HM	HH
Raw fillet				
Hardness (N)	18.45 ± 3.25	17.30 ± 1.63	16.24 ± 2.31	17.48 ± 2.95
Springiness	0.42 ± 0.05	0.41 ± 0.07	0.43 ± 0.06	0.42 ± 0.04
Cohesiveness	0.27 ± 0.04	0.29 ± 0.04	0.29 ± 0.03	0.28 ± 0.03
Gumminess (N)	5.06 ± 1.29	4.91 ± 0.81	4.70 ± 1.03	4.95 ± 1.09
Chewiness (N)	2.21 ± 0.65	2.02 ± 0.57	2.16 ± 0.74	2.06 ± 0.60
Resilience	0.22 ± 0.06	0.20 ± 0.03	0.19 ± 0.04	0.20 ± 0.03
Fracturability (N)	8.35 ± 1.78	8.94 ± 1.77	8.22 ± 2.32	8.32 ± 2.89
Cooked fillet				
Hardness (N)	11.14 ± 3.04	10.71 ± 3.20	9.87 ± 3.63	9.64 ± 3.31
Springiness	0.29 ± 0.04	0.31 ± 0.03	0.29 ± 0.03	0.29 ± 0.02
Cohesiveness	0.50 ± 0.05	0.49 ± 0.04	0.49 ± 0.05	0.50 ± 0.04
Gumminess (N)	5.66 ± 2.41	5.37 ± 1.86	4.87 ± 2.16	4.87 ± 1.95
Chewiness (N)	1.62 ± 0.66	1.69 ± 0.61	1.72 ± 1.09	1.41 ± 0.56
Resilience	0.12 ± 0.02	0.10 ± 0.03	0.10 ± 0.03	0.11 ± 0.03

highest collagen content with no significant differences in protein level (Zhang et al., 2013; Liu et al., 2014). The lipid content could vary after the inclusion of amino acids and affects lipid metabolism, but could be associated with hyperplasia in the process, in smaller fish (Martins et al., 2018; Garcia-Organista et al., 2019).

4.3. Histochemistry and immunohistochemistry

Dietary supplementation with L-Hyp leads to a significant increase in collagenous fibrils of the myocommatal connective tissue sheets attached to the muscle fibres. From the samples stained with anti-type I collagen, the more abundant collagen type in the fish muscle were also positive in similar magnitude with the Masson trichromic stain, as the technique primarily stain type I collagen (Cheng et al., 2014). Masson's trichrome staining demonstrated collagen fibres retained within the composite scaffold in a dark blue, being an election stain to quantify collagen. Type IV collagen displayed a characteristic non-fibrous network surrounding individual muscle fibres which are coated by a layer of the extracellular matrix material. Type IV collagen, which is less abundant than type I, is found in the endomysium (Light and Champion, 1984; Nakajima et al., 1998) next to the muscle cell contours (Bruggemann and Lawson, 2005), which agrees with this study results. Type IV collagen is concentrated in the basal lamina, the inner layer of the basement membrane, (Sanes, 2003) at the myotendinous junction of the myocommata (Bruggemann and Lawson, 2005). The fact that in the red muscle were found no differences for collagen IV by immunohistochemical means and with trichromic stain could be related to a lesser diameter of the fibres and a less capacity of recognition as the program work in pixels. Also, a different proportion of the collagen fibres in the red, slow twitch muscle is also feasible.

L-Hyp is located almost exclusively in collagen and is essential to stabilize the triple helical structure of collagen (Brinckmann, 2005). The changes of L-Hyp contents in the muscle of fish in feed diets with graded levels of L-Hyp indicate metabolic changes in response to dietary L-Hyp levels, especially the metabolism of collagen. Orally ingested collagen undergoes degradation to small di- or tripeptides, which are detected in circulating blood (Ohara et al., 2010). Accordingly, most of these measurements have been made for plasma, while in fish the white musculature is quantitatively the most important site for protein accretion (Carter and Houlihan, 2001; Albrektsen et al., 2010). The results from Kousoulaki et al. (2009) also indicated that free L-Hyp content in the fillets of the fish fed with diet containing the highest amounts of total L-Hyp was significantly higher, except in the fish fed with fishmeal of control diet.

4.4. Gene expression

A strong correlation between muscle firmness and genes responsible for extracellular matrix deposition has been established (Yu et al., 2014a). In this sense, the higher type I collagen content in the muscle of crisp grass carp (*Ctenopharyngodon idellus*) compared with those of grass carp (*Ctenopharyngodon idella*) was linked to a higher quantitative expression of Col Iα1 and Col Iα2 (Yu et al., 2014b). Specifically, on the influence of dietary L-Hyp on collagen content in muscle, dietary L-Hyp may spare proline by reducing proline catabolism or stimulate tissue protein synthesis (Wu, 2010). Wei et al. (2018) concluded that it promotes an enhancement on the ability of biosynthesis and reduces degradation, while Zhang et al. (2013) limited their effect to suppression of collagen degradation. In both cases, increasing L-Hyp content in (high-plant) fish feeds extend collagen content in the muscle, which is in accordance with the results from this study.

4.5. Fillet texture

Regarding quality, the texture is one of the most important parameters not only for producers but also for consumers (Hyldig and Nielsen, 2001), since the change in firmness throughout the shelf life is closely associated with acceptability (Cheng et al., 2014). The increase in collagen deposition in the muscle of commercial size European sea bass fed with diets based on plant-protein ingredients and supplemented with L-Hyp, revealed no differences on the parameters defining fish muscle texture, both raw and cooked. These results differ from those in turbot of 40 g body weight (Liu et al., 2014) or yellow croaker of 270 g body weight (Wei et al., 2016). Thus, this occasioned the recording of differences in hardness, springiness and chewiness in the muscle of the raw fish depending on the L-Hyp level in diets. Age-size is a decisive factor that regulates the processes of hyperplasia-hypertrophy throughout growth (Silva et al., 2008), by increasing the amount of collagen in relation to the proportion of muscle fibre (Periago et al., 2005), and therefore establish a relationship between fibre size and fillet texture, and decreasing firmness as the fibre cross-section increases (Hurling et al., 1996). The age and the incremented size of the muscle structure of European sea bass fed with finishing diets including L-Hyp indicated that, despite having fostered collagen increases in the muscular level as histological studies have revealed, texture parameters were not modified. In juvenile fish, the emergent muscular structure is built based on an ample number of small girth fibres per unit area. Consequently, higher collagen content implies a noticeable effect, surrounding more fibres than on big size fish with large fewer fibres. This effect can be graded by evaluating the proportion of the total force required for maximum compression (hardness) for the fracture to appear after crumbling of the muscular structure (fracturability). In juvenile fish, the fracture force represents more than 50 % of the hardness but reaches up to 65 % when fillets are from wild fish with a higher fibre density (Rincón et al., 2016). Unlike the above, commercial size European sea bass muscle was fractured at 45 % of maximum compression force.

Flesh texture, apart from the muscular structure and the distribution of collagen, is also influenced by the lipid content and its distribution (Lie, 2001). In this work, the only source of variation on the formulation was the different proportions of L-Hyp, hence no differences in the fillet lipid content impacting texture were recorded. Regarding cooked fillet, after cooking, the collagen presents in the fillet muscle responsible for maintaining the structure of the fillet was gelatinized by thermal action, reducing the influence of connective tissue proteins on fillet texture parameters (Castro et al., 2015).

In summary, supplementation with crystalline L-Hyp in high plant-protein diets indicates positive effects on growth performance of European sea bass including large size samples. Type I collagen content was increased significantly as dietary L-Hyp increased in the muscle of European seabream while type IV collagen content increase was limited to fast-twitch muscle. These effects later enable collagen antibodies use, in fish muscle as an alternative procedure to understand the dynamics of the connective tissue in fish. The expression of Col Iα2 gene in muscle was increased significantly as dietary L-Hyp increased.

CRediT authorship contribution statement

Pedro Castro: Conceptualization, Methodology, Funding acquisition, Supervision, Writing - Reviewing and Editing. **Sergio Plasencia:** Data curation, Writing- Original draft preparation, Visualization, Investigation. **Mª Jesus Zamorano:** Gene expression, Validation. **Luis Guerrero:** Data curation, Visualization. **Anna Claret:** Data curation, Visualization. **José A. Beltrán:** Data curation, Visualization. **Juan Calanche:** Data curation, Visualization. **Rafael Ginés:**Conceptualization, Methodology, Funding acquisition, Data curation, Statistic, Supervision.

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Declaration of Competing Interest

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