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Dynamics of collagen indicating amino acids, in embryos and larvae of sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*), originated from broodstocks fed with different vitamin C content in the diet

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Abstract

The dynamics of hydroxyproline–proline (HYP–PRO) and hydroxylysine (HYL), as amino acids indicators of collagen, has been studied in fertilised eggs, embryos and fasting larvae of sea bass (*Dicentrarchus labrax* L.) and gilthead sea bream (*Sparus aurata* L.). Broodstocks of the two species were fed every second day with two types of diets: one containing sufficient ascorbate for a normal growth and the other with an extra addition of a very high dose (2000 mg kg^{-1} feed) of coated L-ascorbic acid (AA). The collagen hydroxylation expressed as the HYP-PRO ratio, hydroxylysine (HYL1 and HYL2) and total ascorbate (TAA) concentrations were analysed in several embryonic and larvae developmental stages. TAA concentrations in the newly fertilised eggs and larvae resulted significantly higher in offspring of broodstocks fed an additional dose of 2000 mg AA/kg feed than in those fed a normal diet ($P < 0.05$ or $P < 0.01$). The differences in HYP–PRO ratio values between the groups fed AA-supplemented and unsupplemented diets, were significant for all the analysed stages, being higher in the supplemented groups. The values of HYL were higher, although not always significantly, for all the analysed stages, in offspring of the supplemented group, compared to the offspring of the unsupplemented one. In conclusion, a vitamin C dose of 2000 mg kg^{-1} feed, delivered every second day to sea bass and gilthead sea bream broodstock, increases the collagen synthesis in embryos and fasting larvae, in comparison with a diet containing vitamin C at the recommended concentration for growth.

Keywords: Ascorbic acid; Broodstock; Collagen; Fertilised eggs; Hydroxyproline; Hydroxylysine; Sea bass (*Dicentrarchus labrax* L.); Gilthead sea bream (*Sparus aurata* L.)

1. Introduction

Collagen comprises 25–30% of all animal protein [4] and is involved in many basic physiological processes such as growth, development, various pathological conditions such as inflammation, tumour metastasis, burn wounds and some heritable connective tissue diseases [23]. It is a protein with three polypeptide chains, where each chain contains at least one stretch of the repeating amino acid sequence (glycine–hydroxyproline–proline) [14]. Collagen contains two unusual amino acids, hydroxyproline (HYP) and hydroxylysine (HYL) that apart from a small quantity of HYP in elastin, occur only rarely in other animal proteins [2]. HYL, known to be involved in the formation of intermolecular cross-links in collagen and HYP are formed by the enzymatic hydroxylation of specific proline (PRO) and lysine residues previously incorporated into peptide linkage during the process of ribosomal collagen polypeptide synthesis.

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A direct involvement of ascorbic acid (AA) in collagen metabolism has been recognised for many years [10]. Vitamin C acts as a cofactor in the enzymes prolyl and lysyl hydroxylase, which catalyse the hydroxylation of PRO and lysine in the procollagen molecule, contributing to bone and skin formation and thereby to growth [2].

Already several studies have reported the crucial importance of a dietary adequacy of vitamin C for optimum health and growth in fish [11,17,16,24]. The classical symptoms of dietary AA-deprived fish are similar to scurvy in upper vertebrates [15] and are expressed as deformation of skeletal and cartilaginous tissues, capillary fragility, slow wound repair, reduced growth rate and increased rate of mortality. Many of these vitamin C-deficient signs can be attributed to the impaired collagen and support cartilage formation in most tissues [12]. Signs of deficiency with severe spinal deformities, like scoliosis, lordosis and the resultant growth retardation, are reported also in fish juveniles obtained from female broodstock fed a diet devoid of ascorbic acid [24]. The broodstock dietary AA is known to be transferred to the eggs where it is stored for use during growth and development of the larvae until the first feed intake [20]. As the collagen synthesis begins during early embryogenesis [9,18], the deposition of AA in the growing oocytes is important for the hydroxylation of peptide-bound PRO and lysine to HYP and HYL to give optimum collagen strength through the embryonic stages [25].

Although the dietary AA transfer from broodfish to eggs and embryos has been studied in some fish species, a complete lack of data on this topic is observed with regard to sea bass (*Dicentrarchus labrax* L.) and gilthead sea bream (*Sparus aurata* L.), being among the most important ones in Mediterranean aquaculture. Also, a gap in the literature exists, concerning the influence of AA broodstock diet supplementation on collagen synthesis during early embryogenesis in these species.

The present experiment was designed to study and to compare the dynamics of some collagen indicating amino acids, in embryos and fasting larvae of sea bass and gilthead sea bream, originating from broodstocks fed, every second day, two types of diets: one containing sufficient ascorbate for a normal growth [22] and the other with an addition of a very high dose of L-ascorbic acid.

2. Materials and methods

2.1. Feeding protocol

The experiment was conducted in a commercial environment (Ittica Mediterranea, Marsala, Sicily). Marine water at 16.5–17°C, oxygenated to saturation was supplied and fish were exposed to a photoperiod of light:dark = 9:15 h.

Sea bass (*Dicentrarchus labrax*, L.) broodfish were divided into two groups of 94 fish each (17 male and 77 females). The average weight of the fish in group 1 was 4 kg, while in group 2 it was 3.8 kg. Broodstock of the other studied species, gilthead sea bream (*Sparus aurata*, L.), a protandrous hermaphrodite, was also divided into two groups: group 1 including 114 fish (37 males and 77 females), with an average individual weight of 1.8 kg (males, 0.4–0.6 kg) and group 2 of 148 fish (17 males and 131 females), with an average individual weight of 1.2 kg (males, 0.4–0.6 kg).

Broodfish do not generally show a constant interest for feed, so as usual in commercial mediterranean hatcheries, they were all fed manually every second day only, also in order to keep water cleaner and the ratio was adjusted each time according to appetite, interrupting feed distribution when fish let feed sink in the tanks. Feed was either a commercial extruded pellet for broodstock (Trouvit Europa Repro® from Nutreco-Hendrix), or fishery products (sardine and calamary), in continuous rotation. Starting from the beginning of December (3 months before the spawn), 2000 mg kg^{-1} of ethyl cellulose-coated L-ascorbic acid (Rovimix C-EC®, Roche), were added to each meal. Starting from 1 month before the expected spawning, vitamin C supplementation continued only with the group 2 of both species. The vitamin was first mixed with cod liver oil and then with either extruded pellets or chopped sardine and calamary, immediately before feeding the fish. All types of diet were sampled and analysed for ascorbate separately. Sea bass and gilthead sea bream groups 1, consumed every second day, a ratio ranging from 1.1 to 2.9% of body weight when fed with fishery products and 0.5–2.4% when fed with extruded pellets, containing an average of 364 ± 116 mg kg^{-1} of total ascorbic acid (TAA). Groups 2 of both species consumed every second day, the same basic diet as groups 1, in a ratio ranging from 0.7 to 3.4% of body weight when fed fishery products and 0.9–1.4% when fed extruded pellets, but enriched just before feeding with 2000 mg kg^{-1} ethyl cellulose-coated L-ascorbic acid.

2.2. Sampling procedure

Males and females were stocked together, so the fertilisation occurred naturally in the broodstock tanks. The utilised females belong to a selected well-known broodstock population and apparently all of them spawned naturally at the forecast dates, except for sea bass not supplemented group that delayed 5 days. Almost all the just fertilised eggs were collected between 08:00 and 10:00 h. A quantity of 20 g were immediately sampled and frozen, while the rest was incubated sepa-

Table 1
The total ascorbate (TAA) in embryos and larvae of sea bass and gilthead sea bream offspring of groups supplemented or not by L-ascorbic acid

Development stages	Sea bass (TAA μg g^{-1} wet weight)		Gilthead sea bream (TAA μg g^{-1} wet weight)	
	Supplemented (media (S.D.))	Unsupplemented (media (S.D.))	Supplemented (media (S.D.))	Unsupplemented (media (S.D.))
(I) Newly fertilised eggs (two to four cells)	219 (18)a	156 (7)e	122 (5)a	104 (4)c
(II) 24 h after spawning	201 (5)a	136 (5)d	140 (2)b	124 (5)d
(III) 48 h after spawning	188 (17)ace	127 (4)b	124 (4)a	102 (9)c
(IV) Hatching eggs (72 h after spawning)	186 (10)dc	164 (8)ef	209 (6)c	126 (6)d
(V) Yolk sac larvae (24 h after hatching)	163 (24)d	197 (25)f	185 (4)d	175 (7)a
(VI) Fasting larvae (144 and 96 h after hatching, respectively, for sea bass and gilthead sea bream)	173 (19)de	180 (21)ef	174 (14)d	147 (10)b

One-letter in common in the values along a row or a column, for each species, means that the difference is *not* significant ($P<0.01$)

rately in aerated marine water at 36 g l^{-1} of salinity, with the temperature ranging from 17 to 18°C, for successive development. The first collected sample was the newly fertilised egg stage (two to four cells, stage I), making it impossible to sample the unfertilised eggs. Successive samples collected from the four original groups were: embryos 24 h (stage II) and 48 h (stage III) after fertilisation, then hatching larvae (72 h after fertilisation, stage IV), pre-larval stage with yolk reserves (24 h after hatching, stage V) and fasting larvae ready for first feeding (stage VI, 144 and 96 h after hatching, respectively, for sea bass and gilthead sea bream). All the samples, a pool of 15–20 g each, were collected with a ring net, gently wiped, then immediately deep frozen and analysed for amino acids and vitamin C within few weeks from the day of sampling. To estimate gravimetrically the dry matter, subsamples were maintained at 105°C overnight.

2.3. Analytical procedures

The method used for the amino acid analysis was the Pico Tag method developed by Waters [5], which employs phenylisothiocyanate (PITC) to quantitatively derivatise amino acids with a simple in one-step reaction. All reagents are than easily removed via vacuum and the resulting phenylthiocarbamyl amino acids (PTC-AA) rapidly separated on a high-efficiency reversed-phase column. For hydrolysis, the method used was gas-phase hydrolysis. Proteins or peptides were subjected to 6 N HCl vapour in the presence of 0.5% phenol for 1 h at 150°C. Amino acid calibration standards for collagen hydrolysate, reagents and amino acid analysis column were purchased from Waters. External standards and relative amino acid quantitation was used, calculating the molar ratios of the components within a sample. Analyses were run in three replicates for each sample.

The concentrations of AA, dehydroascorbic acid (DAA) and TAA (AA + DAA) in feed, fertilised eggs and larvae were determined spectrophotometrically by the dinitrophenylhydrazine method [19], with correction aimed at subtracting interfering substances [8]. Analyses were run in five replicates for each sample.

2.4. Statistical analysis

The means of the experimental data for the diets supplemented or unsupplemented by AA, were compared using Student's t-test ($P < 0.05$ and $P < 0.01$). For comparison between more groups, one-way analysis of variance was performed.

3. Results

Total ascorbate (TAA) values for both species, were higher (significantly for the stage I and VI) in offspring of broodstocks fed an additional dose of 2 g ethyl cellulose-coated AA/kg feed, than in those fed a normal diet, except stages V and VI of sea bass groups (Table 1). The percentages of dry matter in embryos and larvae of both species are shown in Table 2. Percent dry matter increased at hatching (stage IV), then decreased with larval growth, showing a trend with lower values in the unsupplemented groups than the supplemented ones.

The patterns of HYP (% of protein) in fertilised eggs and larvae of both species, indicated significant differences between offspring of groups 2, fed with an additional dose of 2 g ethyl cellulose-coated AA/kg feed and those of groups 1 fed a normal diet (Fig. 1c,d).

In sea bass (Fig. 1c) the HYP (% of protein) value in offspring of group 2 was 0.116 ± 0.027 in the newly fertilised eggs (stage I). This value was maintained almost constant until hatching (stage IV), then increased at stages V and VI, at the last stage being significantly higher ($P < 0.01$) compared to stage IV. The HYP (% of protein) value at stage VI (144 h after spawning and just before receiving first feeding) was 0.43 ± 0.015.

Table 2
Dry matter in embryos and larvae of sea bass and gilthead sea bream groups supplemented or not by L-ascorbic acid

Development stages[a]	Sea bass (% dry matter (S.D.))		Gilthead sea bream (% dry matter (S.D.))	
	Supplemented	Unsupplemented	Supplemented	Unsupplemented
I	9.15 (0.17)	9.66 (0.29)	8.11 (0.65)	7.33 (0.18)
II	9.74 (0.10)	9.48 (0.23)	7.33 (0.09)	7.37 (0.42)
III	9.68 (0.05)	9.21 (0.09)	7.66 (0.21)	7.78 (0.44)
IV	12.08 (0.69)	10.50 (0.04)	9.05 (0.21)	7.96 (0.94)
V	9.80 (0.20)	10.15 (0.34)	10.07 (0.29)	9.52 (0.18)
VI	7.50 (0.31)	6.66 (0.20)	9.53 (0.13)	8.02 (0.48)

[a] As in Table 1.

In the offspring of group 1, HYP (% of protein) value in the newly fertilised eggs stage (I) was 0.037 ± 0.004. This value was maintained almost constant until yolk sac larval stage, except for a slope down at the stage before hatching. Subsequently, a highly significant increase ($P < 0.01$) was observed through stage VI compared to stage V. The differences in HYP values between offspring of the two groups, was significant for all stages, always being higher in group 2.

In gilthead sea bream (Fig. 1d), the HYP (% of protein) trend was quite the same as in sea bass. The value of HYP in offspring of groups 2 and 1 were, respectively, 0.086 ± 0.018 and 0.031 ± 0.007 in the just fertilised eggs (stage I). These values were maintained almost constant until the stage before hatching and then increased at the moment of hatching and through the following stages, at the last stage (VI) always being significantly higher ($P < 0.01$) compared to stages III–V. The HYP values at the larval stage (96 h after spawning and just before receiving first feeding) in the AA-supplemented and unsupplemented group were, respectively, 0.367 ± 0.01 and 0.260 ± 0.039.

The differences in HYP between offspring of groups 2 and 1, were significant for all stages, except I and II, always being higher in offspring of group 2.

The patterns of HYP–PRO (% of protein) ratio in fertilised eggs and larvae indicated significant differences among offspring of groups fed a normal diet and of groups fed with an additional dose of 2000 mg AA/kg feed (Fig. 1a,b). In sea bass (Fig. 1a) the differences in the HYP–PRO ratio between the two groups were significant for stages I, V and VI, while in gilthead sea bream (Fig. 1b) they were significant for stages I, IV, V and VI, always being higher in the supplemented group.

The patterns of the two diastereomeric forms of HYL: HYL 1 (% of protein) and HYL 2 (% of protein) in offspring of the two groups of sea bass are shown in Fig. 2a,c. The HYL 1 and HYL 2 values in the newly fertilised eggs (stage I) were, respectively, 0.106 ± 0.019 and 0.188 ± 0.025 in offspring of group 2 and, respectively, 0.006 ± 0.001 and 0.025 ± 0.005 in offspring of group 1. In the unsupplemented group the HYL1 and HYL2 values were maintained almost constant until hatching, and then increased although not significantly at the following two stages, while in offspring of the AA-supplemented one, the trend was quite the same as in the other group, except for a significant decrease of HYL1 and HYL2 at stage II compared to stage I. The values of HYL1 and HYL2 at the fasting larval stage (VI) were, respectively, 0.064 ± 0.014 and 0.075 ± 0.016 in offspring of group 2 and 0.050 ± 0.012 and 0.056 ± 0.015 in offspring of group 1. The differences between groups 1 and 2 HYL1 values were significant only for the newly fertilised eggs (stage I), while those of HYL2 were significant for the stages I and IV, being higher in offspring of group 2. In the other stages the values of both hydroxylated amino acids were always higher, although not significantly, in offspring of group 2, compared to the offspring of group 1.

In gilthead sea bream (Fig. 2b,d) offspring of group 2, the HYL1 and HYL2 values in the newly fertilised eggs stage were, respectively, 0.015 ± 0.001 and 0.073 ± 0.008, while in offspring of group 1 these values were, respectively, 0.007 ± 0.001 and 0.028 ± 0.006.

The differences between offspring of the two groups were significant in all the analysed stages for both HYL1 and HYL2, always being higher in offspring of group 2.

4. Discussion

In sea bass, vitellogenic activity starts when oocytes reach a size of 120 μm, and the most active vitellogenesis can be observed when the size reaches 250–700 μm, a few days before spawning [1]. The rate is temperature dependent and, at the environmental conditions operated by 'Ittica Mediterranea' in winter time, a time-span of 30–45 days occurs from the 250-μm stage and the spawn. The same may be observed on gilthead sea bream (Polo and De Maria, personal communication). The 4-week period during which two different diets were delivered to the broodstocks, fits with the

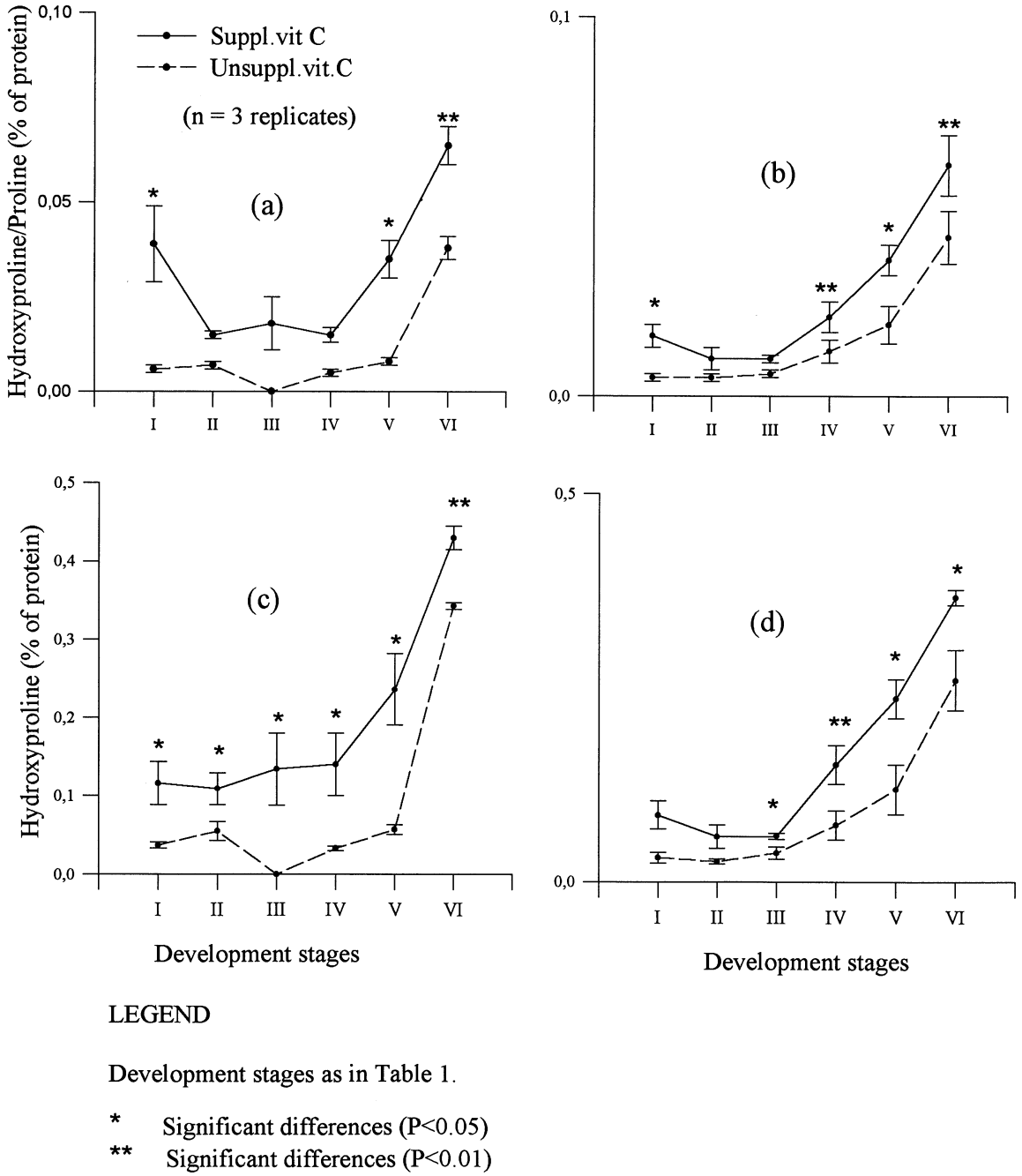

Fig. 1. Hydroxyproline–proline (% of protein) ratio and hydroxyproline (% of protein) in embryos and larvae of sea bass (a,c) and gilthead sea bram (b,d), offspring of broodstocks fed diets supplemented or not by L-ascorbic acid.

period of maximum vitellogenic activity in sea bass as well as in gilthead sea bream. The ethyl cellulose-coated L-ascorbic acid was added to the feed immediately before manual feeding, then by considering that the feed distribution was stopped as soon as fish ceased the 'fast capture' behaviour, we may reasonably assume that the water leaching was transcurable. In fact, in an earlier experiment [21], unprotected L-ascorbic acid in feed pellets resulted in a decay of 7% after 15 min contact with marine water. The availability of ascorbic acid (AA) in an adequate amount in the diet throughout the gonad maturation period, even before the vitellogenesis is at its most active, was than reflected in the total ascorbate (TAA) concentration, which was significantly higher ($P < 0.01$) in embryos and larvae originated from broodstocks fed AA-supplemented diets, compared to those originated from the unsupplemented ones.

The concentration of TAA increased between the third and the fourth stages, in correspondence with an

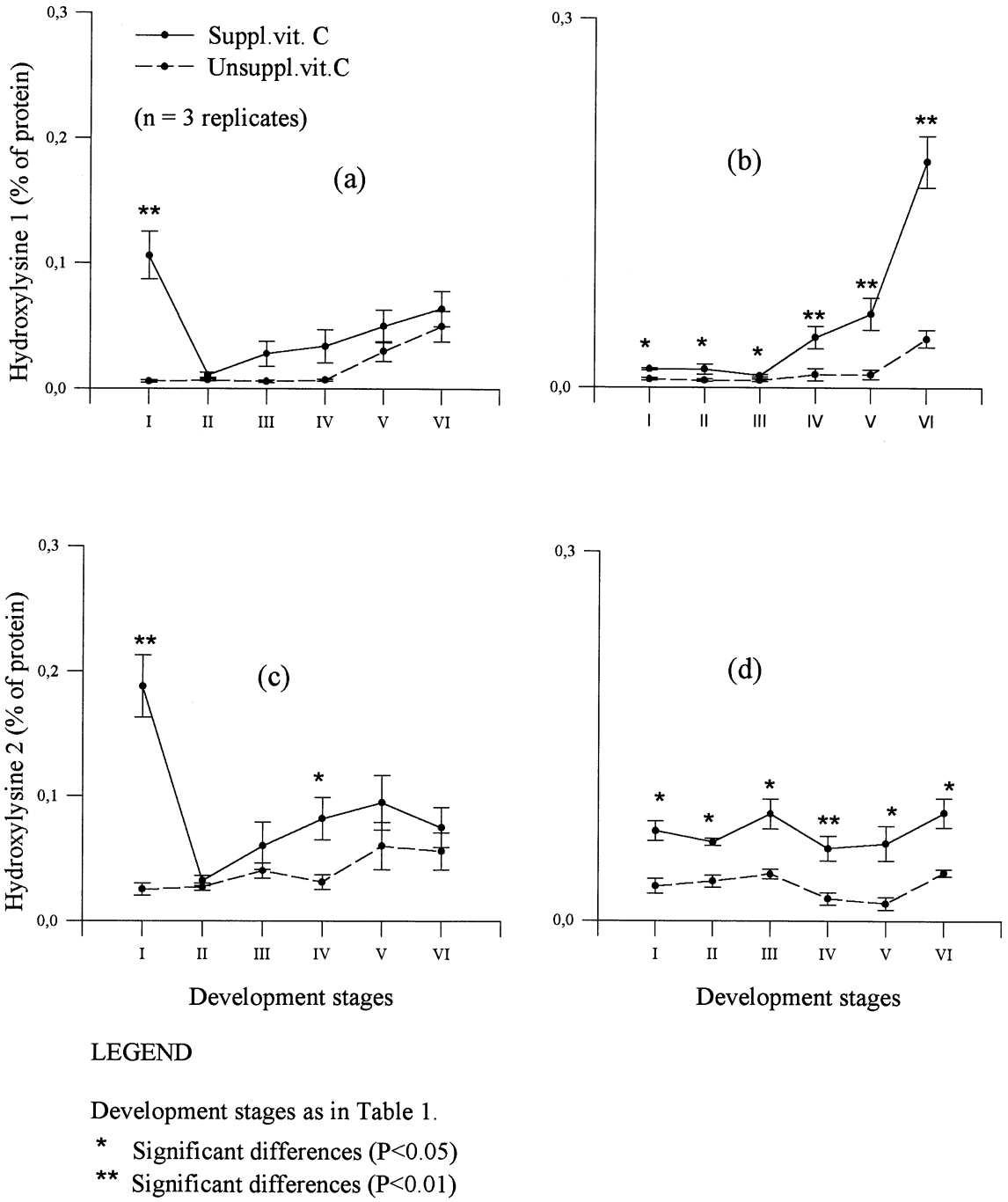

Fig. 2. Hydroxylysine 1 (% of protein) and hydroxylysine 2 (% of protein) in embryos and larvae of sea bass (a,c) and gilthead sea bram (b,d), offspring of broodstocks fed diets supplemented or not by L-ascorbic acid.

increase in the dry matter. This occurred at the moment of hatching and the increase of TAA was significant ($P < 0.01$) in all groups, except for the sea bass supplemented one, where only a flex was reported in the diagram. The loss of chorion and perivitelline fluid during the hatching stage, which are considered as parts of the egg not containing vitamin C [6], assumed to be located in the embryo and yolk, may explain the apparent increase of the total ascorbate concentration in the hatched larvae of both our species. Also the mechanical aspect of hatching might account for a relative increase of vitamin C between eggs and larvae [13].

A trend with a decrease in the TAA concentration from the yolk sac larvae stage (24 h after hatching), through the last examined larval stage, was found in all the groups, although it was less evident in the group originating from sea bass broodstock fed vitamin C-supplemented diet and was significant ($P < 0.05$) only for the group originating from gilthead sea bream broodstock fed unsupplemented diet. The depletion of

the yolk sac reserves of vitamin C to meet the nutritional demands of the larvae before first feeding [7] may be a reasonable explanation for this. It is not clear how the TAA may show an increasing trend, although not significant, in the last stage of the group originating from sea bass stock 2, unless we assume a possible uncontrolled incorporation from water, of some vitamin C, originated from phytoplankton; nevertheless, such an occurrence may eventually be related to the last sampling only, when larvae were potentially ready to capture feed.

It has been demonstrated with in vitro studies that ascorbic acid has two roles in the formation of collagen HYP: (1) it acts as a cofactor of prolyl and lysyl hydroxylase; and (2) it is involved in the conversion of a precursor of prolyl and lysyl hydroxylase to active enzyme [2]. The higher values of HYP–PRO ratio and HYL1 and HYL 2, we found in embryos and larvae originating from groups fed with an additional dose of 2000 mg AA/kg feed in both species, compared to offspring of the unsupplemented ones, may be explained on the basis of a decline in such a dual action of ascorbic acid arising from the less availability of it in two of the four groups.

According to Golob et al. [9], the production of collagen begins during the early embryogenesis when a group of cells acquire the capacity to produce specific proteins. This protein can be identified easily due to the existence of a built-in marker, HYP, that apart from a small quantity in elastin occurs only rarely in other animal proteins [2]. Since PRO is incorporated into both collagen and non-collagen proteins, while HYP is incorporated only into collagen, the observed increases in HYP (%), suggests that the increases in the HYP–PRO ratio were caused primarily by increases in the rate of collagen synthesis, rather than, for example, decreases in the rate of non-collagen protein synthesis. In their work, Golob et al. [9], studying two sea urchin species with tritium-labelled PRO, showed that collagen synthesis begins during gastrulation, increases rapidly between 20 and 40 h following a sigmoidal curve and then levels off at about 50 h, showing the HYP-PRO ratio to be 0.46%, which represents a 7-fold increase in collagen synthesis from the earliest observation. The same authors reported a maximum HYP–PRO ratio of 2.6% in *Xenopus laevis*, concluding that vertebrates may produce higher relative quantities of collagen than invertebrates.

Little information is available with regard to HYP–PRO ratio in fish and on the role that AA plays on it. The ratio of molar concentrations of HYP to PRO found in an in vitro study by Yoshinaka et al. [26], on the skin of rainbow trout (*Oncorhynchus mykiss*), fed an AA-deficient diet (0.58), was lower than the ratio (0.76) for samples of the control fish, and the HYP content of the collagen fraction was about 25% lower in the skin of the deficient fish, compared with the control fish. In our case, the whole embryos and larvae were analysed, so a dilution of collagen in the whole body may be assumed.

Maximum ratio of HYP–PRO (% of protein), found in the fasting larval stage of sea bass, was 0.038 in larvae originating from broodstocks fed AA-supplemented diet and 0.065 in those originating from broodstocks fed unsupplemented diets, representing, respectively, a 6.3- and 1.6-fold increase in collagen synthesis from the newly fertilised eggs stage, while in gilthead sea bream the maximum ratio of HYP–PRO (% of protein), was 0.042 in larvae originating from broodstocks fed AA-supplemented diet and 0.061 in those originating from broodstocks fed unsupplemented diets, representing, respectively, an 8.4- and 3.8-fold increase in collagen synthesis from the newly fertilised eggs stage.

The increase of HYP–PRO ratio started at hatching (stage IV) in sea bass and at stage III in gilthead sea bream (Fig. 1a,b), then continued to increase through stage VI (just before first feeding). The fact that we stopped the experiment at this stage, does not allow to include in the figures a successive dynamics, eventually affected by larval nutrition.

Also the higher values of HYL, together with those of HYP and HYP–PRO ratio, in embryos and larvae offspring of broodstocks fed AA-supplemented diets, compared to offspring of the unsupplemented ones, contribute to provide evidence for the higher rate of synthesis of collagen, in the groups supplemented by AA.

The beneficial effects of the addition of ascorbic acid in the broodstock diet on egg hatchability, found by Soliman et al. [24], were not so evident in our experiments. Neither did we find significant differences in egg quality and size, as in the study of Blom and Dabrowski [3]. The explanation may be found in the fact that in the present study none of the broodstock fish were fed a diet devoid of ascorbic acid. So, even eggs from the stocks fed without any AA addition had an ascorbate concentration apparently sufficient to ensure the minimum needs. One cannot estimate from our data the precise level of supplementation of ascorbic acid to obtain a desirable level of collagen synthesis, as no 'plateau' effects were investigated. Nevertheless, it is clear that a supplementation as high as 2000 mg kg^{-1} feed significantly increases collagen synthesis, when compared to the basic diet. So the supplementation we experienced may so far be recommended when sea bass and gilthead sea bream broodstocks are fed every second day, throughout the vitellogenic period.

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