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Potential of Cottonseed Oil as Fish Oil Replacer in European Sea Bass Feed Formulation

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Abstract

Triplicate groups of 20 European sea bass (35 g) were fed five diets in which the added lipid was 100% fish oil (FO), 40% (CSO40), 60% (CSO60), 80% (CSO80) and 100% (CSO100) refined cottonseed oil (CSO), for a period of 120 days. Overall fish growth, feed conversion ratio and protein utilization were unaffected by dietary treatment, but hepatosomatic and visceral fat indexes increased with increasing dietary CSO. Fillet fatty acid composition of total lipids reflected the fatty acids in the test diets. The monounsaturated fatty acids were significantly higher in fillet of fish fed diet FO, CSO40 and CSO60 compared to other treatments while saturated and polyunsaturated fatty acids (PUFA) were not affected by the dietary treatment. Some fatty acids (18:0, 18:1n-9, 20:5n-3 and 22:6n-3) were present in higher concentration in fillet lipid than in the CSO100 dietary lipid indicating accumulation in fillet relative to test diets. Retention of n-3 LC-PUFA within the fillet was increasingly inefficient among fish fed increasing levels of FO. Thus, this study suggests that CSO can be considered as a relatively effective substitute for fish oil in European sea bass (35 g) in terms of growth performances and feed efficiency as far as fish meal is present in the diet.

Keywords: *Dicentrarchus labrax*, linoleic acid, fish oil replacement, aquafeed, vegetable oils.

Avrupa Deniz Levreği Yem Formulasyonunda Pamuk Tohumu Yağının Potansiyel olarak Balık Yağına İkamesi

Özet

Yirmi adet Avrupa deniz levreği bireyi (35 g) 120 gün boyunca %100 balık yağı (BY), %40 (PTY40), %60 (PTY60), %80 (PTY80) ve %100 (PTY100) oranlarında rafine edilmiş pamuk tohumu yağı (PTY) ilave edilmiş beş yemle beslenmiştir. Balık büyümesi, yem çevirim oranı ve protein değerlendirme yem uygulamasıyla değişmezken, hepatosomatik ve visseral yağ indeksi yemdeki PTY'nin seviyesiyle artmıştır. Fileto toplam lipit yağ asidi kompozizyonu test yemlerinin yağ aside kompozisyonuna yansımıştır. BY, PTY40 ve PTY60 ile beslenen bireylerin fileto tekli doymamış yağ asidi miktarı diğer gruplara göre daha yüksekken doymuş ve çoklu doymamış yağ asitleri (ÇDYA) miktarı etkilenmemiştir. Test yemleri göz önüne alındığında, PTY100 grubu bireylerinin fileto yağlarında bazı yağ asitleri (18:0, 18:1n-9, 20:5n-3 ve 22:6n-3) yüksek oranda birikmiştir. Filetodaki uzun zincirli n-3 ÇDYA'lerin birikimi artan balık yağı oranı ile beslenen balıklarda yetersizdir. Bu çalışma, yemlerde balık unu kullanıldığı sürece, büyüme performansı ve yem değerlendirme açısından Avrupa deniz levreği (35 g) için PTY'nin balık yağını ikame edilebileceğini önermektedir.

Anahtar Kelimeler: *Dicentrarchus labrax*, linolenik asit, balık yağı değişimi, yem, bitkisel yağlar.

Introduction

Aquaculture is developing, expanding and intensifying in almost all countries and currently is regarded as the fastest growing food-producing sector globally (Subasinghe *et al.*, 2009). Yet, its dependence on marine derived raw materials for aquafeed production, and in particular fish oil, has been suggested to be one of the most stringent limits on its future sustainable expansion (Naylor *et al.*, 2009). Thus, in the last two decades, an intensive global research effort has focused on finding viable strategies to replace fish oil (Turchini *et al.*, 2009). Vegetable oils (VO) are envisaged to be the favoured candidates for fish oil replacement, as global VO availability is over two orders of magnitude greater than fish oil, and their production is constantly increasing (Gunstone, 2010). However, none of the

currently available VO contain long chain polyunsaturated fatty acids (LC-PUFA). Thus, when fish oil is substituted by VO, the reduction of final LC-PUFA content of fish fillet is unavoidable, which could be considered as a partial deterioration of the intrinsic nutritional quality of such products (Turchini *et al*., 2009).

It has been well established that fish oil can be replaced by a range of VO in salmonids and freshwater fish feeds with minimal/no impacts on fish performances (Bell *et al*., 2001; Rosenlund *et al.*, 2001; Torstensen *et al*., 2000; Mourente and Bell, 2006; Turchini *et al*., 2009). However, in feeds for marine carnivorous fish the use of VO as a sole lipid source is limited by the high dietary requirements of these species LC-PUFA (Tocher, 2010; Izquierdo *et al.*, 2005; Benedito-Palos *et al.*, 2009). The high requirements for LC-PUFA are due to the limited ability to these species of bioconvert C_{18} polyunsaturated fatty acids (PUFA; namely linoleic acid -18:2n-6, LA- and α-linolenic acid -18:3n-3, ALA-) into for LC-PUFA (namely arachidonic acid -20:4n-6, ARA-, eicosapentaenoic acid -20:5n-3, EPA- and docosahexaenoic acid -22:6n-3, DHA-) (Torstensen and Tocher, 2010).

European sea bass, *Dicentrarchus labrax*, is a strict carnivorous marine fish and it has been suggested to have extremely low rates of PUFA bioconversion (Mourente *et al*., 2005a, Mourente *et al.*, 2005b). Nevertheless, the enzyme Δ-6 desaturase was recently cloned and characterized in European sea bass (Santigosa *et al.*, 2011), and *in vivo* production of the products of all the enzymes involved in the elongation and desaturation of C_{18} PUFA (i.e. elongase, Δ-6 desaturase and Δ-5 desaturase) have been recorded, clearly indicating not just the presence, but also the activity of these enzymes (Mourente and Dick, 2002; Izquierdo *et al.*, 2003; Izquierdo *et al*., 2005; Mourente *et al*., 2005a; Turchini *et al*., 2009). Thus, European sea bass possesses the so called typical "marine fish pattern" in the metabolism of ALA to EPA and DHA, and it requires relatively high dietary levels of n-3 LC-PUFA (i.e. fish oil) in its diet (Mourente and Dick, 2002; Mourente *et al*., 2005b; Mourente *et al*., 2005a). However, independently from its PUFA bioconversion capability, the partial replacement of dietary fish oil with various VO in European sea bass feed has been reported to have limited effects on growth performances and feed efficiency (Yıldız and Şener, 2004; Izquierdo *et al*., 2003; Figueiredo-Silva *et al.*, 2005; Mourente *et al*., 2005b; Mourente and Bell, 2006; Martins *et al*., 2006a; Martins *et al.*, 2006b; Richard *et al.*, 2006).

With over 5 million tonnes produced in 2008, cottonseed oil (CSO) is the fifth largest produced VO, after palm, soybean, rapeseed, and sunflower oils and its annual production is roughly 5-fold that of fish oil (Gunstone 2010). CSO is an n-6 PUFA-rich VO, commonly containing 23% 16:0, 17% 18:1n-9 and 56% LA (n-6 PUFA), and has a relatively low level of ALA (Gunstone and Harwood, 2007). Thus, from nutritional and fatty acid composition viewpoints, CSO closely resembles soybean and corn oils. Various n-6 PUFA-rich VO have been reported to be high-quality sources of dietary energy during the grow-out cycle; but, while there are a considerable number of formal evaluations with soybean and corn oils, there are relatively few with CSO (Brown and Hart, 2010).

In light of the above, it is surprising that, to the best of our knowledge, so far only four studies have been published relative to CSO inclusion in fish feed; one on hybrid tilapia (*Oreochromis* spp.) reared in earthen ponds (Viola and Arieli, 1983), two on gilthead sea bream, *Sparus aurata*, in which CSO was blended with other VO (Wassef *et al.*, 2007; Wassef *et al.*, 2009), and one on European sea bass (Wassef *et al.*, 2004). In the latter, CSO was blended with linseed and sunflower oil (1:1, w:w) replacing 60% of dietary FO. This study reported a negative effect of such blend on fish performances, but no information about body and flesh fatty acid compositions was reported. Additionally, since CSO was used in a blend, it can be argued that no information on the actual potential or limits of CSO inclusion in feed for European sea bass is yet available. Therefore, the present study was undertaken to evaluate the efficacy of using CSO as partial (0%, 40%, 60% and 80%) or total (100%) substitute for fish oil in a practical diet for juvenile European sea bass.

Materials and Methods

Experimental Diets

Five isonitrogenous (~480 g crude protein kg^{-1} dry weight), isolipidic (~230 g lipid kg^{-1} dry weight) and isoenergetic (~20 MJ kg^{-1}) diets were formulated and manufactured. The diets had identical ingredient compositions, except from the lipid source, with refined cottonseed oil (CSO) replacing either 0%, 40%, 60%, 80% or 100% of the supplemental dietary lipid source (120 g kg^{-1} diet) with the remainder originating from FO. Thus, CSO comprised either 0% (FO), 40% (CSO40), 60% (CSO60), 80% (CSO80) or 100% (CSO100) of total added dietary lipid source (Table 1). All diets contained fish meal as the main protein source, thus some n-3 LC-PUFA was present in all diets (CSO100 included) as it was derived from the residual oil of fish meal. The fish meal used in the diets was anchovy meal containing crude protein, lipid and ash 70%, 15.7% and 12.1%, respectively. As fish meal itself contains some fish oil, the replacement of fish oil with cotton seed oil was maximized by reducing the level of fish meal in the diets by inclusion of corn gluten and wheat flour. The experimental diets were produced 5-kg batches at University of Cukurova using existing feed production equipment. All dry diet ingredients were

Table 1. Formulation and proximate composition of experimental diets (g kg^{-1})

	Diets (g kg air-dry $basis^{-1}$)[1]				
Ingredients	FO	CSO40	CSO60	CSO80	CSO100
Fish meal[2]	425	425	425	425	425
Corn gluten[3]	221	221	221	221	221
Wheat gluten[3]	90	90	90	90	90
Fish oil[2]	120	72	48	24	-
Cottonseed oil[3]	-	48	72	96	120
Carboxymethyl cellulose[4]	42	42	42	42	42
Di Calcium Phosphate[2]	27	27	27	27	27
Mineral premix[5]	20	20	20	20	20
Vitamin premix[5]	10	10	10	10	10
L-Lysine[6]	20	20	20	20	20
DL-Methionin[6]	25	25	25	25	25
Proximate composition (mg g^{-1})					
Moisture	87.8	86.6	86.1	85.8	84.8
Protein	486.5	485.7	489.9	487.9	483.1
Lipid	244.4	222.5	227.6	237.9	233.9
Nitrogen free extract[7]	174.7	198.6	189.3	182.2	189.8
Ash	94.4	94.3	94.2	93.0	94.3
Gross energy (kJ g^{-1})[8]	20.7	20.0	20.2	20.5	20.3
Protein:Energy (mg kJ^{-1})	23.5	24.3	23.2	23.7	23.8

[1] Numerical values after CSO refer to the percentage of CSO expressed in relation to the total dietary lipid content.
[2] Supplied by Sibal Black Sea Feed, İzmir, Turkey (Anchovy meal: crude protein, lipid and ash 70%, 15.7% and 12.1%, respectively). Anchovy oil supplemented with 200 mg kg^{-1} BHT.
[3] Çukorova Corporative Enterprise (Çukobirlik), Adana, Turkey.
[4] Interlab Laboratory Supplies, İstanbul, Turkey.
[5] Vitamin and mineral added minimum to NRC recommendations.
[6] Purified (99%) crystalline amino acids.
[7] Nitrogen free extract: 100-(protein+lipid+ash).
[8] Calculated based on the standard physiological fuel values: 19 kJ g^{-1} for protein, 36 kJ g^{-1} for lipid and 15 kJ g^{-1} for carbohydrate.

combined in a commercial baker's mixer and homogenized for 30 min until well mixed. Dietary oils added slowly to the running mixer to assist in an even application of lipid to dry ingredients. Lipid and dry ingredients were further homogenized for 10 min before addition of 0.8 L of water per 5 kg of diet and further mixing to attain a malleable doughy consistency. The dough was then screw-pressed though a 3 mm die and the strands of feed were air dried at room temperature over a 24-h period, and stored in airtight bags at 4 °C until use.

Fish Husbandry and Experimental Design

European sea bass (*Dicentrarchus labrax*), juveniles were obtained from Akuvatur Ltd. (Tuzla, Turkey) and were acclimatized to the new experimental culture conditions at the Cukurova University's marine station for 4 weeks before the beginning of the trial.

Subsequently, at the beginning of the experiment (day 0), 20 fish from the common pool of fish were sampled randomly and stored at -20°C for subsequent chemical analyses. Three hundred sea bass of initial mean weight 35.2±0.71 g were randomly distributed among 15 fiberglass tanks of 400 L with 20 fish $tank^{-1}$. Tanks were supplied with continuously aerated flow through sea water (40 g L^{-1}) at a flow rate of approximately 2 L min^{-1} (98% tank h^{-1}). Dissolved oxygen and pH were maintained above 7.0 mg L^{-1} and between 6.9 and 7.8, respectively. Water temperature (mean 23.2±0.9°C) was monitored daily. Individual fluorescent lighting was provided over each tank and was automatically controlled to provide12-h light/12-h dark (0700 h to 1900 h) photoperiod. The five dietary treatments were assigned using a randomized block design to triplicate groups of fish, and fish were fed by hand to apparent satiation two times daily (0900 h and 1800 h) for 120 days.

All fish in each group were anaesthetized (2-phenoxyethanol at 0.5 ml L^{-1}) (Sigma-Aldrich AS, MO, USA) and then weighed individually, after removal of excess surface moisture, to the nearest 0.01g at 10-day intervals during the study. At the end of experiment, out of the six fish each replicate tank, three fish per tank were dissected for biometric data and the fillet of these fish were finely chopped, pooled and homogenized before subsequent analysis. The remaining three fish (as whole fish) per tank were finely chopped, pooled and homogenized for subsequent chemical analyses. The chemical composition of biological samples was analyzed in triplicate on the pooled samples for each replicate (tank).

Chemical Analyses

Moisture content was determined by drying samples to constant weight at 103°C, and ash by burning the samples at 450°C for 5 h (AOAC 1990). The crude protein was determined by the Kjeldahl procedure (N x 6.25) using a Kjeltec 2200 (Foss

Tecator, Höganäs, Sweden). Lipids were extracted according to the procedure of Folch *et al*. (1957). Prior to fatty acid transmethylation, 0.8 mg per 50 mg of lipid of nonadecanoic acid (19:0) was added to the samples as internal standard for the subsequent quantification of fatty acids. Fatty acid methyl esters (FAME) were prepared according to Metcalfe and Schmitz (Metcalfe and Schmitz 1961) and analyzed as described previously by Czesny and Dabrowski (1998) with some modifications. Briefly, the FAME obtained were separated by gas chromatography (Agilent 6890 N, Santa Clara, USA), equipped with a flame ionization detector and fitted with a DB 23 capillary column (60 m, 0.25 mm i.d. and 0.25 μm; Agilent 6890 N, Santa Clara, USA). The carrier gas was hydrogen (2ml min^{-1}) and the split ratio was 30:1. The oven temperature program was 190°C for 35 min, than increasing at 30°C per min up to 220°C where it was maintained for 5 min. The individual fatty acids were identified by comparing their retention times with that of known standards.

Computations and Statistical Analyses

A series of parameters were used to assess the effects of dietary treatments on the growth performance of the fish and were computed by the following equations: i) Wet weight gain (WG) (g) = (final mean wet weight (FW) (g) − initial mean wet weight (IW) (g)); ii) Specific growth rate (SGR) (% body weight/day) = [(ln FW (g) − ln IW (g))/time (days)] × 100; iii) Feed conversion ratio (FCR) = total daily dry feed intake (g)/WG (g); iv) Protein efficiency ratio (PER) (g/g) = WG (g)/protein intake (g); v) Protein productive value (PPV) (%) = protein gain (g) x 100/protein intake (g); iv) the hepatosomatic index (HSI) and visceral fat index (VFI) (% body weight) = 100 x (liver or visceral fat weight (g wet weight)/total body weight (g, wet weight).

The fillet deposition efficiency of EPA -20:5n-3, DPA -22:5n-3 and DHA -22:6n-3 in the fish fillet was computed and expressed as % of total intake as previously described by Senadheera *et al.* (2010). Briefly, this was obtained by the following five equations: i) n-3 LC-PUFA total intake = (n-3 LC-PUFA content of diet) × (g of diet fed to fish); ii) Initial fillet n-3 LC-PUFA total content = (initial fillet n-3 LC-PUFA content) × (g of fillet in initial fish); iii) Final fillet n-3 LC-PUFA total content = (final fillet n-3 LC-PUFA content) × (g of fillet in final fish); iv) Fillet n-3 LC-PUFA deposition = (Final fillet n-3 LC-PUFA total content) – (Initial fillet n-3 LC-PUFA total content); v) n-3 LC-PUFA deposition efficiency = (Fillet n-3 LC-PUFA deposition) / (n-3 LC-PUFA total intake) × 100

All data are reported as mean±S.E.M. (n = 3; N = 15). Percentage values were arcsine square root transformed and after normality and homogeneity of variance were confirmed, one-way analysis of variance (ANOVA) was used to determine differences between means. Differences were considered statistically significant at $P<0.05$. Linear regression analysis was used to determine the coefficient of determination (R^2) and the slope values of linear plots of fatty acid concentration (% of total fatty acid) in fillet against fatty acid concentration in dietary total lipid. Curvilinear regression was also used to describe growth trends over time and the relationships between n-3 LC-PUFA content of the diet and the resultant n-3 LC-PUFA content in the fillet and n-3 LC-PUFA % retention. All statistical analyses were computed using SigmaStat 3.0, SPSS, Chicago, USA.

Results

The fatty acid composition of the two tested oils and the resulting five experimental diets is reported in Table 2. The major fatty acids of fish oil were, in decreasing order of abundance, 16:0 (20.2%), 18:1n-9 (18.5%), 22:6n-3 (15.4%) and 20:5n-3 (8.6%), whilst the most abundant fatty acids in cottonseed oil were 18:2n-6 (55%), 16:0 (23.4%) and 18:1n-9 (17.1%). The fish oil diet (FO) contained the highest levels of saturated fatty acids (SFA, 34.5%) and monounsaturated fatty acids (MUFA, 29.2%), whilst PUFA were increasing with the increasing inclusion cottonseed oil levels, up to 49.2% in CSO100. The n-3/n-6 PUFA ratio in the five diets decreased with the increasing inclusion of cottonseed oil, and it was 2.9, 0.7, 0.3, 0.2 and 0.1 in diets from FO to CSO100, respectively. As cottonseed oil did not contain any LC-PUFA, the content of n-3 LC-PUFA and n-6 LC-PUFA were decreasing from 23.5% and 2.3 % in FO to 6.5% and 1.1% in CSO100, respectively. Consistently, there were some small but noteworthy differences in proportions of specific fatty acids amongst the dietary treatments. The highest levels of EPA and DHA (7.7% and 13.7%, respectively) were measured in the FO diet.

During the 120 days of the feeding trial, fish doubled their weight, and mortality was less than 10% and independent of the dietary treatments. Fish were examined for health condition profile and health assessment according to Morgan and Iwama (1997) and no differences were noted among fish fed with different levels of CSO inclusion. All fish presented normal fins, eyes and gills, abundant mesenteric fat, hindgut and kidney, and liver presented a fatty or light brown color with dark green to blue green bile and full gall bladder. In the current study, the dietary treatment did not significantly affect the growth performance of European sea bass. No statistically significant differences on weight gain, SGR, FCR, PER and PPV at the end of 120-day feeding period were recorded (Table 3). However, statistically significant differences were noted for HSI and VFI, and in both cases with values increasing when moving from FO to CSO100 (Table 4). The chemical

Table 2. Fatty acids composition (% of total fatty acids) of the experimental diets and vegetable oils[1]

	Experimental Oil		Experimental Diets				
Fatty Acids Composition	Fish oil	Cotton seed oil	FO	CSO40	CSO60	CSO80	CSO100
14:0	6.0	0.8	6.0	3.9	3.2	2.3	1.5
15:0	1.0	nd	1.2	0.6	0.5	0.3	0.2
16:0	20.2	23.4	20.9	20.3	21.1	19.4	21.5
16:1n-7	5.1	nd	3.0	2.7	2.4	2.0	1.8
18:0	4.1	2.5	3.8	3.4	3.1	3.1	2.7
18:1n-9	18.5	17.1	18.2	17.9	17.1	18.7	18.2
18:1n-7	1.6	nd	2.2	2.0	1.9	1.8	2.0
18:2n-6	3.2	55.0	6.7	23.3	30.8	36.8	39.8
18:3n-3	1.3	0.4	1.3	1.0	0.8	0.7	0.4
18:4n-3	1.7	nd	2.1	1.9	1.9	1.5	1.3
20:0	0.8	0.4	1.3	0.8	0.6	0.4	0.2
20:1n-11	0.7	nd	0.2	0.1	0.1	0.1	0.1
20:1n-9	3.5	nd	2.9	2.5	2.3	2.0	1.9
20:2n-6	1.0	nd	1.3	1.0	1.0	0.9	0.8
20:4n-6	0.4	nd	0.5	0.4	0.3	0.1	nd
20:4n-3	0.3	nd	0.3	0.2	0.3	0.2	0.2
20:5n-3	8.6	nd	7.7	4.8	3.6	1.8	1.3
22:0	0.7	nd	1.0	0.5	0.2	0.1	nd
22:5n-3	1.3	nd	1.5	1.4	1.3	1.5	1.4
22:6n-3	15.4	nd	13.7	9.3	5.8	4.6	3.5
24:1n-9	0.1	nd	0.6	0.2	0.3	0.3	0.2
SFA[2]	33.5	27.1	34.5	29.4	28.4	25.6	26.2
MUFA[3]	31.5	17.1	29.2	26.6	25.3	25.9	24.7
PUFA[4]	35.0	55.6	36.3	43.9	46.1	48.5	49.2
n-3 PUFA	29.4	0.51	27.0	18.8	13.6	10.4	8.2
n-6 PUFA	5.6	55.2	9.2	25.1	32.6	38.1	40.9
n-3/n-6 ratio	5.2	0	2.9	0.7	0.3	0.2	0.1
n-3 LC-PUFA	26.3	nd	23.5	15.9	11.2	8.2	6.5
n-6 LC-PUFA	2.2	nd	2.3	1.7	1.6	1.2	1.1

[1] Values are means ±S.E.M., n=3. nd, not detected.
[2] includes 17:0.
[3] includes 14:1, 15:1, 17:1, 16:2n-4, 16:3-4, 22:1n-11, 22:1n-9.
[4] includes 18:3n-6, 20:3n-3, 20:3n-6, 22:2n-6, 22:4n-6.

Table 3. Growth performance and nutrient utilization in European sea bass fed with experimental diets over a 120 days period [1]

	FO	CSO40	CSO60	CSO80	CSO100
Initial Weight	35.4±0.33	35.3±0.45	35.0±0.86	35.4±0.80	35.2±1.30
Final Weight	68.2±0.63	69.4±3.06	67.4±4.14	70.7±2.47	69.1±1.86
SGR (% g day^{-1})	0.6±0.01	0.6±0.05	0.5±0.01	0.6±0.02	0.6±0.02
FCR	2.0±0.03	2.1±0.23	2.2±0.09	2.2±0.21	2.4±0.09
PER	1.0±0.01	1.0±0.11	0.9±0.04	0.9±0.08	0.9±0.03
PPV	38.6±0.02	38.7±0.02	42.0±0.02	38.0±0.01	38.5±0.02

[1] Values in the same row with different superscripts are significantly different (P<0.05) as determined by ANOVA.
Values are means ±error S.E.M. (n=3).

composition of fish whole body and fillet was significantly affected by the dietary treatment and results are shown in Table 4. Protein levels of whole body were lower in fish fed FO, CSO40 and CSO100 compared with fish fed CSO60 and CSO80. Conversely, the lipid content of fish body was significantly highest (P<0.05) in fish fed CSO100 compared to all the other treatments. The ash content of fish body increased with increasing levels of CSO inclusion. Fillet dry matter content was the highest in fish fed CSO80 diets, followed by CSO60 and CSO100, and significantly lower in FO and CSO40 fed fish. However, no significant differences in protein, lipid and ash content of fillet were observed among treatments over the course of the study (Table 4).

MUFA were significantly higher in fillet of fish fed diet FO, CSO40 and CSO60 (P<0.05), compared to other treatments, whilst, from a statistical viewpoint, SFA and PUFA were not affected by the dietary treatment (Table 5). LA content of fish fillets were significantly increased with increasing inclusion of CSO, whilst n-3 PUFA were highest in fish fed FO and CSO40 (P<0.05). The n-3/n-6 PUFA ratio was gradually reduced from 1 in fish fed FO to 0.4 in fish fed CSO100. In all treatments, n-3 PUFA were

Table 4. Proximate composition (mg g^{-1}) of whole-body and fillet of European sea bass fed different experimental diets over a 120 days period.

Proximate composition	Initial	FO	CSO40	CSO60	CSO80	CSO100
HSI		1.2±0.06[a]	1.3±0.13[b]	1.7±0.34[b]	1.5±0.36[b]	2.0±0.62[c]
VFI		2.0±0.52[a]	2.7±0.44[a]	3.3±0.17[b]	3.2±0.29[b]	3.4±0.38 [b]
Whole-body						
Protein	167.1±1.62	212.0±1.59[a]	207.4±2.07[a]	238.3±0.89[b]	233.0±1.99[b]	199.8±1.68[a]
Lipid	103.1±0.12	73.0±1.2[a]	77.3±1.1[a]	62.2±0.2[a]	74.0± 2.85[a]	118.0±0.9[b]
Dry Matter	351.7±0.15	369.7±1.85[a]	367.0±0.07[a]	395.7±1.56[a]	408.2±0.63[a]	413.0±0.90[b]
Ash	71.8±0.78	83.6±0.97[a]	81.6±0.74[a]	94.5±0.46[ab]	103.3±0.77[b]	94.2±1.94[ab]
Fillet						
Protein		192.1±0.23	201.0±0.78	195.1±1.15	202.3±0.90	210.6±0.92
Lipid		24.7±0.36	26.3±0.29	26.7±0.38	26.0±0.56	26.3±0.63
Dry Matter		257.3±0.29[ab]	255.3±0.66[a]	270.9±0.93[bc]	276.2±0.59[c]	253.9±0.37[b]
Ash		10.8±0.22	9.4±0.22	11.7±0.27	10.6±0.21	10.0±0.09

Values are means±S.E.M. (n= 3, N=15). Different letters in the same line show significant difference (P<0.05).

fundamentally comprised of n-3 LC-PUFA, and in particular EPA and DHA were the two most abundant. Total n-3 LC-PUFA in European sea bass fillets were gradually reduced with the increasing inclusion of cottonseed oil, from 18.3% in FO fed fish to 10.6% in CSO100.

The relationship between the fatty acids concentration (% of total fatty acid) in the diets and their concentration in fillet was investigated through a regression analysis. The correlation coefficients (R^2) and slopes of the lines for individual fatty acids are reported in Table 6. R^2 values greater than 0.60 were recorded only for the some of the main fatty acids (specifically 14:0, 18:0, 16:1n-7, 18:2n-6, 20:5n-3 and 22:6n-3). These showed a slope value varying from 0.19 to 0.49, indicating that the concentration in the fillet was proportionally lower than that of the diet. For the remaining fatty acids no clear relationship between dietary and fillet content was manifest. To more easily identify and understand the actual relationship between the fatty acid concentration in the diet and in the fillet, the numerical differences (delta value) between the concentration of individual fatty acids in diets and fillet for fish fed the two extreme treatments (FO and CSO100) was also computed and reported. In fillet of fish fed the FO diet, 18:1n-9 and 18:2n-6 were present in higher concentration (positive delta values), whilst 14:0, 16:0, 20:5n-3 and 22:6n-3 were present in lower concentration (negative delta values), than in the corresponding diet (Table 6). Conversely, 18:0, 18:1n-9, 20:5n-3 and 22:6n-3 were present in higher concentration, and 14:0 and 18:2n-6 in lower concentration, in the fillet of fish fed CSO100 than in the corresponding diets (Table 6).

The relationships between n-3 LC-PUFA (specifically, 20:5n-3, 22:5n-3 and 22:6n-3) content (% of total fatty acid) in the fillet of European sea bass relative to total dietary intake of n-3 LC-PUFA (g over the entire 120 day feeding trial) was properly described by a logarithmic curvilinear regression (R^2=0.95) (Figure 1). This showed that the n-3 LC-PUFA deposition in fish fillet was increasingly less efficient by increasing the dietary supply of these fatty acids. In fact, and interestingly, the relationships between total dietary intake of n-3 LC-PUFA and the actual deposition efficiency (% of total intake) in the fillet of European sea bass was properly described by a second order polynomial regression (Figure 1). This clearly highlighted the reduced efficiency in deposition of n-3 LC-PUFA fatty acid when supplied in excess. The actual n-3 LC-PUFA deposition efficiency was very limited, with values varying from a minimum of 0.1% in CSO100 to a maximum of 1.96% in fish fed CSO40. Fish fed FO recorded an n-3 LC-PUFA deposition efficiency of 1.33% (Figure 1).

Discussion

Prior to this study, the only information available on potential CSO inclusion in European sea bass diet was from Wassef *et al*. (2004), who reported that sea bass (1.9 g) fed for 24 weeks with a CSO, blended with linseed oil and sunflower (1:1:1, w/w), and replacing 60% of dietary fish oil, showed inferior growth performances than the control treatment fed a fish oil based diet. Thus, even though in the above study CSO was used in a blend, it was expected to see growth retardation, at least in the treatments containing the highest inclusion of CSO. For this reason, in the present study, fish growth was frequently monitored, but no significant effects were recorded. Admittedly, the overall growth performances of European sea bass in the present study were slightly lower, but generally comparable, to previously published data for this species (Izquierdo *et al*., 2003; Person-Le Ruyet *et al*., 2004; Montero *et al*., 2005). In the present study, daily growth rate (g d^{-1}) ranged from 0.27 g d^{-1} to 0.30 g d^{-1} which is consistent with the other short or long term studies conducted on European sea bass (Skalli and Robin 2004, Figueiredo-Silva *et al*., 2005). A possible cause for the relatively low performances recorded in

Table 5. Fatty acid composition (% of total fatty acids) of fillets of European Sea Bass fed diets containing increasing levels of cottonseed oil (CSO)[1].

	Diets				
	FO	CSO40	CSO60	CSO80	CSO100
FA (%)					
14:0	3.0±0.1^{b}	2.6±0.1^{ab}	2.4±0.0^{ab}	2.1±0.0^{a}	2.1±0.0^{a}
15:0	0.2±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0
16:0	16.9±0.1	17.0±0.2	17.3±0.1	17.3±0.3	18.3±0.0
16:1n-7	3.1±0.0	2.9±0.1	2.9±0.1	2.9±0.2	2.8±0.1
18:0	3.9±0.1^{a}	4.3±0.1^{a}	5.3±0.0^{b}	5.1±0.0^{b}	5.1±0.0^{b}
18:1n-9	26.1±0.6	25.0±0.2	26.1±0.1	23.8±0.2	24.4±0.3
18:1n-7	2.3±0.1	2.7±0.1	2.6±0.1	2.6±0.1	2.3±0.1
18:2n-6	16.1±0.4^{a}	18.4±0.2^{b}	20.3±0.2^{c}	22.8±0.5^{d}	25.3±0.1^{e}
18:3n-3	0.4±0.1	0.5±0.0	0.6±0.0	0.5±0.0	0.4±0.0
18:4n-3	0.4±0.1	0.5±0.0	0.4±0.0	0.5±0.0	0.4±0.1
20:0	2.2±0.2	2.5±0.1	2.4±0.1	2.4±0.0	2.2±0.0
20:1n-11	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
20:1n-9	2.4±0.2	2.2±0.2	2.1±0.1	2.4±0.1	2.3±0.2
20:2n-6	1.2±0.2	0.9±0.1	0.1±0.0	0.3±0.2	0.5±0.3
20:4n-6	0.5±0.1	0.5±0.0	0.4±0.0	0.4±0.1	0.4±0.0
20:4n-3	0.4±0.1	0.4±0.0	0.5±0.0	0.5±0.0	0.5±0.0
20:5n-3	4.3±0.1^{b}	3.5±0.1^{a}	3.3±0.1^{a}	3.2±0.0^{a}	2.7±0.2^{a}
22:0	0.9±0.2	1.4±0.1	1.2±0.1	1.2±0.1	1.0±0.1
22:5n-3	1.2±0.1^{b}	1.4±0.2^{b}	1.3±0.1^{b}	0.8±0.0^{a}	0.8±0.0^{a}
22:6n-3	12.0±0.4^{b}	11.0±0.1^{b}	8.5±0.1^{b}	8.9±0.8^{ab}	6.5±0.3^{a}
24:1n-9	0.3±0.1	0.2±0.1	0.3±0.0	0.4±0.1	0.4±0.0
SFA[2]	27.3±0.4	28.3±0.2	29.1±0.0	28.6±0.3	29.1±0.1
MUFA[3]	35.4±0.4^{b}	34.1±0.2^{b}	34.9±0.1^{b}	32.9±0.2^{a}	33.0±0.1^{a}
PUFA[4]	37.3±0.2	37.7±0.4	36.0±0.1	38.5±0.4	37.9±0.0
n3	19.1±0.4^{c}	17.5±0.5^{c}	14.9±0.2^{b}	14.6±0.8^{b}	11.4±0.4^{a}
n6	18.2±0.5^{a}	20.1±0.2^{b}	21.2±0.2^{b}	23.9±0.4^{c}	26.5±0.5^{d}
n3/n6	1.0±0.0^{b}	0.9±0.0^{b}	0.7±0.0^{a}	0.6±0.0^{a}	0.4±0.0^{a}
n-3 LC-PUFA	18.3 ± 0.4^{c}	16.6 ± 0.6^{b}	13.9 ± 0.2^{a}	13.7 ± 0.8^{a}	10.6 ± 0.4^{a}
n-6 LC-PUFA	2.0 ± 0.1^{c}	1.7 ± 0.1^{c}	0.7 ± 0.0^{a}	1.0 ± 0.2^{a}	1.2 ± 0.4^{b}

[1] Values in the same row with different superscripts are significantly different (P< 0.05) as determined by ANOVA. Values are means ±S.E.M., n=3.
[2] includes 17:0.
[3] includes 14:1, 15:1, 17:1, 16:2n-4, 16:3-4, 22:1n-11, 22:1n-9.
[4] includes 18:3n-6, 20:3n-3, 20:3n-6, 22:2n-6, 22:4n-6.

Table 6. Correlation coefficients (r) and slopes from plots of dietary fatty acid concentrations vs. fatty acid concentrations in fillet including the difference (Δ) between diet and fillet fatty acid values for FO and CSO100 groups[1].

Fatty acid	Correlation coefficient (r)	Slope	ΔFO[2]	ΔCSO[2]
14:0	0.98	0.22	-3.0	0.6
16:0	0.18	0.29	-4.0	-3.2
18:0	0.66	0.31	0.0	2.4
16:1n-7	0.71	0.19	0.1	1.0
18:1n-9	0.51	-1.24	8.0	6.3
18:1n-7	0.50	-0. 87	0.2	0.3
20:1n-9	0.08	0.11	-0.4	0.4
18:2n-6	0.89	0.26	9.4	-14.5
18:3n-3	0.00	0.00	-0.9	0.0
20:2n-6	0.31	1.23	-0.1	-0.2
20:3n-6	0.45	0.63	0.0	0.0
20:4n-6	0.48	0.13	0.0	0.4
20:4n-3	0.01	-0.04	0.1	0.3
20:5n-3	0.93	0.22	-3.4	1.4
22:5n-3	0.09	-0.99	-0.3	-0.6
22:6n-3	0.87	0.49	-1.7	3.0

[1] Fatty acid concentration are g fatty acid/100 g total fatty acids in fillet and test diets.
[2] Negative Δ values indicate lower values in fillet compared with diet, whereas positive values indicate accumulation in fillet relative to test diets.

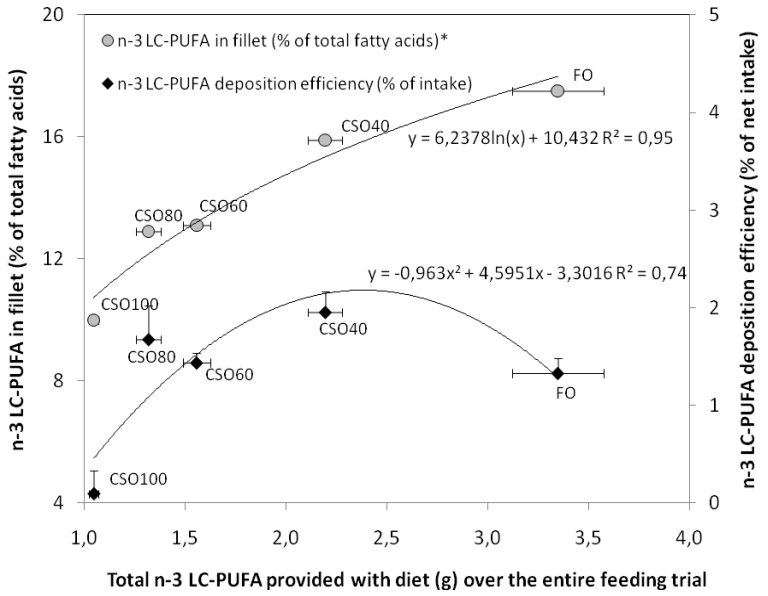

Figure 1. The relationships between n-3 LC-PUFA content (% of total fatty acid) and deposition efficiency (% of total intake) in the fillet of European sea bass relative to total dietary intake of n-3 LC-PUFA (g over the entire 120 day feeding trial) fed with the five different experimental diets. * S.E.M. values for n-3 PUFA in fillet (% of total fatty acids) were $P<0.01$.

the present study could be due to the frequent handling for monitoring fish growth over time. Mourente *et al*. (2005b) recorded better performances, but importantly in this study fish were fed with extruded diets which are reported to have higher digestibility. Nevertheless, even though cold-pressed diets were used, all groups in the present study exhibited excellent values for feed and protein utilization parameters; values close to the upper limits reported previously for European sea bass (Figueiredo-Silva *et al*., 2005; Mourente *et al*., 2005a). The present study indicated that diets containing CSO, in the range 40-100% of added oil, had no significant effect on growth rate, feed conversion ratio, PER and PPV compared with fish fed 100% marine fish oil (FO). These results are consistent with previous studies showing the viability of partially replacing dietary fish oil with a variety of VO, such as soybean, rapeseed, linseed, sunflower and olive oils in European sea bass (Izquierdo *et al*., 2003; Mourente *et al*., 2005b; Yıldız and Şener, 1997).

Interestingly, increasing CSO inclusion in the diet significantly increased hepatosomatic and mesenteric fat index. Increased hepatic lipid deposition is commonly associated with the morphological alteration known as steatosis, which is due to increased synthesis and deposition of triacylglycerols in hepatocytes vacuoles (Montero and Izquierdo 2010). In many instances, when n-6 PUFA rich alternative oils were used to replace fish oil, the modification of hepatic lipid content and hepatic histological/morphological alterations were recorded (Caballero *et al.*, 2004; Wassef *et al*., 2007; Benedito-Palos *et al.*, 2007; Piedecausa *et al.*, 2007; Leaver *et al.*, 2008). Conversely, Figueirdo-Silva *et al.* (2005) showed that replacing dietary fish oil with soybean oil was not responsible for any significant modification on hepatic lipid droplets accumulation in European sea bass. The results of the present study suggest that CSO, being a rich source of n-6 PUFA, may affect hepatocyte vacuolation and lipid infiltration, and this could be likely ascribable to the reported lipogenic effect of 18:2n-6, as previously suggested (Montero and Izquierdo 2010). Importantly, this alteration is reported to be only temporary and quickly reversible when fish shift back to a fish oil- based diet, but clearly it needs to be considered if fish had to be fed over a long period of time on CSO- based diets.

As well documented in almost all cultured finfish species, as reviewed by Turchini *et al*. (2009), the fatty acid compositions of fish fillets generally mirror that of the dietary treatments. However, not all dietary fatty acid showed the same, somewhat inert, deposition trend. For example, LA was more efficiently accumulated in fillet proportionally to its level in the diet when supplied in relatively low amounts (FO and CSO40), but it was less efficiently deposited in fish receiving larger dietary supplementation (CSO60 to CSO100). This result is consistent with a previous study on European sea bass (Montero *et al*., 2005) in which it was suggested that LA was quickly deposited into fish tissues, but when supplied in surplus, it was also readily utilized for β-oxidation for energy production. Similar observations were also made for different species (Bell *et al*., 2001; Regost *et al*., 2003; Caballero *et al*., 2004).

The content of 18:1n-9 in the two tested oils (FO and CSO) was almost identical. Therefore the five experimental diets contained a constant amount of OA

(~18%) and consistently, no differences were recorded in the resulting fish fillet. Interestingly, however, the fillet content of OA was consistently higher (~25%) than that of the diet, suggesting an active biosynthesis (liponeogenesis) of this fatty acid. This observation is highly consistent with the results of a previous study on the same species (Montero *et al*., 2005).

As mentioned above, in general, dietary fatty acids influence directly fillet fatty acids, but some specific fatty acids have been suggested to be selectively retained or deposited (Turchini and Francis, 2009). This has also been observed in marine carnivorous species such as red sea bream, *Pagrus major* (Huang *et al*., 2007); turbot, *Psetta maxima* (Bell *et al*., 1994; Regost *et al*., 2003) and European sea bass (Mourente *et al*., 2005a). Accordingly, in the present study some fillet fatty acid concentrations were very closely correlated with dietary fatty acid concentrations, recording R^2 values higher than 0.8 (such as 14:0, 18:2n-6, 20:5n-3 and 22:6n-3), as previously reported (Bell *et al.*, 1998; Bell *et al*., 2001; Bell *et al.*, 2002). This observation is particularly important for the two main n-3 LC-PUFA (namely 20:5n-3 –EPA- and 22:6n-3 –DHA), as they are responsible for the renown health benefit of eating fish, and are clearly important in determining the overall nutritional quality of aquaculture products. Thus, achieving a better understanding of n-3 LC-PUFA deposition into fish tissues is highly relevant. Even though in the present study juvenile specimens were used and final fish weight was not representative of fish actually found on the market for consumption, it is envisaged that the results obtained in this study could be useful to gain a better understanding of n-3 LC-PUFA deposition in European sea bass. The slope of the linear regression equation describing the relationship between dietary and fillet concentration of DHA was one of the highest, almost double than that of LA, suggesting a sort of preferential deposition. The possible mechanisms involved n-3 LC-PUFA deposition that should be considered include the high specificity of fatty acyl transferases for DHA and/or the relative resistance of DHA to β-oxidation, stemming from the complex catabolism pathway for this fatty acid (Bell *et al*., 2001, Mourente *et al*., 2005a). Bell *et al*. (2001) have shown similar deposition of DHA concentration of flesh lipid in Atlantic salmon, *Salmo salar*, where fish oil was replaced with rapeseed oil.

To further evaluate the *in vivo* n-3 LC-PUFA deposition, the computation of n-3 LC-PUFA deposition efficiency was implemented. Independently of the dietary treatment, European sea bass seemed to be quite inefficient in depositing or retaining dietary n-3 LC-PUFA into fillet. The percentage deposition values of n-3 LC-PUFA, which ranged from 0.1-1.96%, indicate that roughly 98% of dietary supplied n-3 LC-PUFA was catabolised or deposited in the non-edible portions of the fish, such as head, liver, perivisceral fat and other organs. These values are remarkably lower than what previously recorded using similar computations on Murray cod, *Maccullochella peelii peelii*, which varied from 5 to 12% (Senadheera *et al*. 2010, Torstensen and Tocher 2010). Similarly, a previous study on rainbow trout, *Oncorhynchus mykiss*, showed that up to 21.3% of dietary EPA and DHA provided with the feed was retained in fish fillet in trout fed a FO- based diet (Turchini *et al.*, 2011). This value was even higher than 200% in trout fed a linseed oil-based diet, clearly highlighting the efficient fatty acid bio-conversion capability of salmonid species (Turchini and Francis, 2009). It is important at this point to highlight that it has been previously outlined that during periods of weak growth, fatty acid retention would be low (Stubhaug *et al*., 2007). Accordingly, this can partially explain the results recorded in the present study as growth performances of European sea bass were, as mentioned and discussed above, not optimal.

In summary, the present study showed that cottonseed oil can be considered as a relatively effective substitute for fish oil in European sea bass in terms of growth performances and feed efficiency. However, inclusion of CSO affected the perivisceral and hepatic fat deposition and significantly modified the fatty acid composition of resultant fish fillet. Long-term effects of CSO as the main source of supplementary dietary lipid need to be assessed before definitive recommendation can be made.

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