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ARTICLE

Effects of Dietary α-Linolenic Acid (18:3n-3)/Linoleic Acid (18:2n-6) Ratio on Fatty Acid Metabolism in Murray Cod (*Maccullochella peelii peelii*)

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Global shortages in fish oil are forcing the aquaculture feed industry to use alternative oil sources, the use of which negatively affects the final fatty acid makeup of cultured fish. Thus, the modulation of fatty acid metabolism in cultured fish is the core of an intensive global research effort. The present study aimed to evaluate the effects of various dietary α-linolenic acid (ALA, 18:3n-3)/linoleic acid (LA, 18:2n-6) ratios in cultured fish. A feeding trial was implemented on the freshwater finfish Murray cod, in which fish were fed either a fish oil-based control diet or one of five fish oil-deprived experimental diets formulated to contain an ALA/LA ratio ranging from 0.3 to 2.9, but with a constant total C_{18} PUFA (ALA+LA) content. The whole-body fatty acid balance method was used to evaluate fish in vivo fatty acid metabolism. The results indicate that dietary ALA was more actively β-oxidized and bioconverted, whereas LA appears to be more efficiently deposited. LA was β-oxidized at a constant level (~36% of net intake) independent of dietary availability, whereas ALA was oxidized proportionally to dietary supply. The in vivo apparent Δ-6 desaturase activity on n-3 and n-6 PUFA exhibited an increasing and decreasing trend, respectively, in conjunction with the increasing dietary ALA/LA ratio, clearly indicating that this enzymatic activity is substrate dependent. However, the maximum Δ-6 desaturase activity acting on ALA peaked at the substrate level of 3.2186 (μmol g fish^{-1} day^{-1}), suggesting that additional inclusion of ALA is not only wasteful but counterproductive in terms of n-3 LC-PUFA production. Despite a constant total supply of ALA+LA, the recorded total in vivo apparent Δ-6 desaturase activity on both substrates (ALA and LA) increased in synchrony with the ALA/LA ratio, peaking at 1.54, and a 3.2-fold greater Δ-6 desaturase affinity toward ALA over LA was recorded.

KEYWORDS: Aquaculture; aquafeed; fish oil replacement; fatty acid; elongase; desaturase; β-oxidation; feed; vegetable oil; EPA; DHA

INTRODUCTION

To address the escalating issue of global fish oil shortages, the aquaculture industry has focused on increasing the utilization of readily available alternative oils for fish feed (aquafeed) production. However, the fatty acid composition of the resultant edible fish portion mirrors that of the diet (*1*). Thus, a great deal of attention has been placed on understanding fish lipid and fatty acid metabolism and, specifically, the capability of bioconverting polyunsaturated fatty acids with 18 carbon atoms (C_{18} PUFA), commonly found in most alternative terrestrial oils, to long-chain polyunsaturated fatty acids (LC-PUFA) which are typically present in oils of marine origin (*2*).

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Fish, like all other vertebrates, are incapable of de novo production of linoleic acid (LA, 18:2n-6) or α-linolenic acid (ALA, 18:3n-3) (*3*). Yet, if supplied with the aforementioned essential fatty acids, fish are capable, to a greater or lesser extent, of producing longer chained and more unsaturated counterparts (namely, arachidonic acid, ARA, 20:4n-6; eicosapentaenoic acid, EPA, 20:5n-3; and docosahexaenoic acid, DHA, 22:6n-3) via an alternating succession of desaturation and elongation steps regulated by desaturase and elongase enzymes (*2*−*4*). The extent of this innate ability is known to vary between species, to be modulated by the diet, particularly the dietary fatty acid composition, and reported also to be dormant or inhibited as a result of generally sufficient dietary levels of the above enzyme products (LC-PUFA) (*1*−*3*).

The renowned health benefits of fish consumption are derived majorly by their content of n-3 LC-PUFA (*5*, *6*). Thus, over the past two decades, an understanding of the capabilities of cultured

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fish to bioconvert ALA into n-3 LC-PUFA has attracted significant research focus (*1*,*4*) and produced promising results (*7*,*8*). On the other hand, n-6 PUFA, in particular LA, are reported to be excessively consumed in Western societies and to have detrimental impacts on human health (*9*). Unfortunately, the majority of readily available alternative oils used to replace fish oil contain large amounts of LA, which is also known to be abundantly deposited into fish tissues (*1*). Therefore, it is obvious that a balanced ratio of n-3/n-6 PUFA is paramount in determining the final nutritional quality of aquaculture products. Additionally, both ALA and LA are known to compete for the same desaturase and elongase enzymes and, therefore, the conversion of ALA to n-3 LC-PUFA is profoundly affected by the total dietary supply of LA (*2*−*4*).

Murray cod (*Maccullochella peelii peelii* Mitchell) is an Australian freshwater carnivorous finfish species of increasing commercial importance. Previous studies have revealed that Murray cod can bioconvert C_{18} PUFA to LC-PUFA when dietary fish oil is substituted with vegetable oil sources (*10*,*11*). A recent study on this species evaluated the effects of total dietary C_{18} PUFA (ALA+LA, at a constant ALA/LA ratio of 1:1) on the LC-PUFA bioconversion pathway, revealing that the absolute quantitative production of Δ-6 desaturated fatty acids was proportional to substrate availability, but the maximal efficiency of the Δ-6 desaturase enzyme was reached at an average substrate availability (*12*). In line with the above study, the present study was specifically designed to gain a better understanding of the role of dietary ALA and LA on LC-PUFA bioconversion metabolism in Murray cod. Specifically, the present study aimed to evaluate the effects of different ALA to LA ratios while maintaining a constant level of C_{18} PUFA (ALA+LA). This information is envisaged to further expand knowledge relative to the existing relationships between dietary fatty acids and the resultant in vivo fatty acid metabolism, ultimately providing new insights into more efficient fish oil replacement strategies for implementation in aquaculture nutrition.

MATERIALS AND METHODS

Fish Husbandry and Experimental Diets. Captive bred, 6-month-old juvenile Murray cod were sourced from the resident stock of the Aquaculture Research Facility at Deakin University (Warrnambool, Australia) and used for the present study. All procedures used in the trial were approved by the Deakin University Animal Welfare Committee. The experimental diets and experimental rearing conditions have been described previously in detail (*6*). Briefly, the experiment was conducted indoors, and fish were housed in a 24 tank (160 L) recirculating aquaculture system capable of complete environmental control on a 12 h light/12 h dark cycle at 24.2 ± 1.2 °C, a mean pH of 7.0, and levels of ammonia and nitrite below 0.1 mg L^{-1}. Six isoproteic and isolipidic experimental diets, varying only in the dietary lipid source, were formulated to contain 15% lipid and 47% protein. Three different lipid sources, sunflower oil, linseed oil, and beef tallow, were utilized to formulate the five experimental diets with various ratios of ALA/LA, maintaining a constant level of total C_{18} PUFA, whereas fish oil was used for the sixth/control diet (CD) (**Table 1**). The five experimental diets were named T1−T5 and were formulated to achieve a graded ALA/LA ratio, with a constant total C_{18} PUFA (ALA+LA) content. Diets were prepared and stored as previously reported in detail (*13*).

At the commencement of the experiment, a sample of 15 fish was taken and euthanized in excess anesthetic (Aqui-S; 0.5 mL/L) for chemical analysis. The feeding trial consisted of 840 individually weighed juvenile Murray cod (16.0 ± 0.42 g) that were randomly distributed into the 24 tanks (35 fish per tank) and randomly assigned to one of the six experimental treatments (four replicate tanks for each treatment; $N = 4$). Fish were fed twice daily at 8:00 a.m. and 4:00 p.m. to apparent satiation for a period of 74 days. Fecal samples were collected during the last week of the feeding experiment, freeze-dried, and stored at −20 °C until analyzed. At the termination of the experiment, all fish were anaesthetized and individually weighed, and a random sample of 12 fish from each treatment (3 per tank) was culled and stored at −20 °C for chemical analyses.

Chemical Analysis. Nutrient composition of the experimental diets and fish whole bodies was evaluated via proximate composition analysis according to standard procedures previously described (*11*,*14*). Fatty acid analysis was performed on duplicate subsamples of each of the experimental diets and on three whole-body samples from each of the replicates. Following the lipid extraction (*15*), fatty acids were esterified into methyl esters using the acid-catalyzed methylation method (*16*) and analyzed by gas chromatography (*11*, *14*). Fatty acid digestibility was measured using the inert marker chromium oxide and calculated using standard formulas (*17*).

Whole-Body Fatty Acid Balance Calculations. The in vivo assessment of fish fatty acid metabolism was performed using the whole-body fatty acid balance method, as described previously in detail by Turchini et al. (*18*) and formerly implemented on Murray cod (*10*−*12*) and rainbow trout (*Oncorhynchus mykiss*) (*19*), among other animals.

Statistical Analysis. All data were reported as mean ± standard error (SEM) ($N = 4$). Comparisons were made among treatments by one-way analysis of variance (ANOVA) at a significance level of 0.05 following confirmation of normality and homogeneity of variance. When significant differences were detected by ANOVA, data were subjected to a Student−Newman−Keuls post hoc test for individuating homogeneous subsets. Statistical analyses were computed using SPSS v17.0 (SPSS Inc., Chicago, IL). Data were analyzed further by linear or nonlinear regression when appropriate and computed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA). After analysis of the residual plots, the simplest model able to efficiently describe the trend was selected and reported. In all instances, the full equation and the 95% confidence band of the regression line are reported. For linear regression analyses, the R^2 value and P value describing the slope of deviation from zero were computed. Only linear regressions with a significant slope of deviation from zero ($P < 0.05$) were reported. For curvilinear regression analyses, R^2 values and the P values relative to the D'Agostino−Pearson test of normality of residuals were computed. Only curvilinear regression analyses with normally distributed residuals ($P > 0.1$) were reported.

RESULTS

The six experimental diets were isoproteic and isolipidic; the main differences between them were relative to their fatty acid composition (**Table 1**). Fatty acid analysis of different experimental diets (T1−T5) clearly showed that the formulated composition was achieved with specific ALA/LA ratios of 0.3, 0.6, 1.0, 1.6, and 2.9 in diets T1−T5, respectively, and a constant total amount of C_{18} PUFA (ALA+LA) at ∼51% (**Table 1**). The levels of SFA, MUFA, PUFA, and LC-PUFA were close to identical across all five experimental diets (T1−T5). In these diets, the amounts of EPA ranged from 20 to 21 mg g $lipid^{-1}$ (2.2−2.4% of total fatty acids) and DHA from 37 to 40 mg g $lipid^{-1}$ (4.2−4.4% of total fatty acids). This LC-PUFA was derived from the lipid content of the fishmeal included in the formulation. The control diet (CD), which was formulated with the inclusion of fish oil, was characterized by a high content of n-3 LC-PUFA, consisting mainly of EPA (106 mg g $lipid^{-1}$, 13.8%) and DHA (90 mg g $lipid^{-1}$, 11.7%). The CD also contained a notably higher concentration of SFA and lower concentrations of PUFA in comparison to treatments T1−T5.

During the 74 day experimental period, the overall mortality was low and not influenced by the dietary treatment. All experimental diets were well accepted by all treatment groups, and subsequently, no significant differences ($P > 0.05$) were noted in feed intake, which ranged from 91.5 to 105.0 g of diet per fish. There was a 4-fold increase in fish weights across the six dietary treatments with no significant differences ($P > 0.05$) recorded for growth performance or feed efficiency parameters. Briefly,

Table 1. Formulation and Proximate and Fatty Acid Compositions of the Experimental Diets

	dietary treatments[a]					
	CD	T1	T2	T3	T4	T5
ingredients (mg g^{-1})						
fish meal[b]	525	525	525	525	525	525
defatted soybean meal[b]	280	280	280	230	230	230
CMC[c]	20	20	20	20	20	20
wheat flour[d]	63	63	63	63	63	63
vitamin + mineral[b]	3	3	3	3	3	3
choline bitartrate[e]	2	2	2	2	2	2
Cr_2O_3[e]	2	2	2	2	2	2
fish oil[b] (FO)	105					
beef tallow[b] (BT)		2.6	6.6	10.5	14.4	18.4
linseed oil[f] (LO)		28.2	43	57.8	72.2	86.6
sunflower oil[f] (SO)		74.2	55.5	36.8	18.4	-
proximate composition (mg g^{-1})						
moisture	67.7	78.9	60.1	57.1	55.3	54.5
crude protein	465.9	467.5	473.3	485.1	474.7	462.4
crude lipid	152	151.3	151.7	153.3	150	149.7
ash	103.5	100.7	105.2	103	103.9	102.3
NFE[g]	210.9	201.6	209.8	201.6	216.2	231.1
fatty acids (mg g $lipid^{-1}$, % in parentheses)						
14:0	61.8 (8.0)	10.1 (1.2)	11.8 (1.3)	11.5 (1.3)	13.0 (1.5)	13.4 (1.6)
16:0	171.8 (22.4)	92.4 (10.7)	103.7 (11.2)	101.7 (11.6)	107.5 (12.1)	107.7 (12.5)
18:0	38.0 (4.9)	37.1 (4.3)	43.3 (4.7)	43.9 (5.0)	48.3 (5.4)	49.3 (5.7)
20:0	2.1 (0.3)	2.2 (0.3)	2.0 (0.2)	1.7 (0.2)	1.7 (0.2)	1.5 (0.2)
22:0	1.4 (0.2)	3.7 (0.4)	3.3 (0.4)	2.5 (0.3)	1.9 (0.2)	1.3 (0.1)
24:0	0.6 (0.1)	1.0 (0.1)	1.7 (0.2)	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)
16:1n-7	72.8 (9.5)	12.7 (1.5)	14.1 (1.5)	13.7 (1.6)	14.5 (1.6)	14.6 (1.7)
18:1n-9	82.3 (10.7)	164.5 (19.1)	169.4 (18.3)	153.2 (17.5)	146.4 (16.5)	133.9 (15.6)
18:1n-7	24.1 (3.1)	12.0 (1.4)	13.5 (1.5)	13.1 (1.5)	13.9 (1.6)	14.3 (1.7)
20:1n-11	1.2 (0.2)	0.7 (0.1)	0.8 (0.1)	0.7 (0.1)	1.01.0 (0.1)	1.1 (0.1)
20:1n-9	8.4 (1.1)	3.8 (0.4)	4.1 (0.4)	3.9 (0.4)	3.9 (0.4)	3.7 (0.4)
22:1n-11	4.8 (0.6)	1.5 (0.2)	1.5 (0.2)	1.4 (0.2)	1.4 (0.2)	1.4 (0.2)
22:1n-9	1.6 (0.2)	0.8 (0.1)	0.8 (0.1)	0.8 (0.1)	0.7 (0.1)	0.7 (0.1)
24:1n-9	0.2 (0.0)	1.0 (0.1)	1.3 (0.1)	1.7 (0.2)	1.9 (0.2)	2.2 (0.3)
18:2n-6	33.0 (4.3)	338.3 (39.3)	303.5 (32.7)	230.4 (26.3)	175.8 (19.8)	113.6 (13.2)
18:3n-6	3.2 (0.4)	0.7 (0.1)	0.8 (0.1)	0.8 (0.1)	1.0 (0.1)	1.0 (0.1)
20:2n-6	1.4 (0.2)	0.7 (0.1)	0.6 (0.1)	0.8 (0.1)	0.7 (0.1)	0.8 (0.1)
20:3n-6	1.5 (0.2)	0.4 (0.0)	0.4 (0.0)	0.4 (0.0)	0.3 (0.0)	0.4 (0.0)
20:4n-6	11.5 (1.5)	4.4 (0.5)	4.7 (0.5)	4.5 (0.5)	4.7 (0.5)	4.1 (0.5)
22:2n-6	nd	nd	0.2 (0.0)	nd	0.3 (0.0)	0.3 (0.0)
22:4n-6	1.7 (0.2)	0.6 (0.1)	0.6 (0.1)	0.6 (0.1)	0.6 (0.1)	0.7 (0.1)
18:3n-3	9.4 (1.2)	106.5 (12.4)	174.6 (18.8)	222.4 (25.4)	281.4 (31.6)	325.7 (37.8)
18:4n-3	14.6 (1.9)	2.4 (0.3)	3.0 (0.3)	2.8 (0.3)	2.9 (0.3)	3.0 (0.3)
20:3n-3	0.9 (0.1)	0.4 (0.0)	0.5 (0.1)	0.6 (0.1)	0.6 (0.1)	0.5 (0.1)
20:4n-3	6.5 (0.9)	1.2 (0.1)	1.2 (0.1)	1.1 (0.1)	1.2 (0.1)	1.1 (0.1)
20:5n-3	106.1 (13.8)	19.9 (2.3)	21.2 (2.3)	19.6 (2.2)	20.7 (2.3)	20.9 (2.4)
22:5n-3	17.9 (2.3)	4.5 (0.5)	4.7 (0.5)	4.5 (0.5)	4.6 (0.5)	4.6 (0.5)
22:6n-3	89.5 (11.7)	37.5 (4.4)	40.3 (4.3)	37.2 (4.2)	38.7 (4.4)	39.0 (4.5)
SFA	275.7 (35.9)	146.5 (17.0)	165.8 (17.9)	161.5 (18.4)	172.5 (19.4)	173.5 (20.2)
MUFA	195.5 (25.4)	196.9 (22.9)	205.5 (22.2)	188.6 (21.5)	183.6 (20.6)	171.7 (19.9)
PUFA	297.1 (38.7)	517.4 (60.1)	556.3 (60.0)	525.6 (60.0)	533.5 (60.0)	515.7 (59.9)
n-6 PUFA	52.2 (6.8)	345.1 (40.1)	310.9 (33.5)	237.5 (27.1)	183.3 (20.6)	121.0 (14.0)
n-6 LCPUFA	16.0 (2.1)	6.0 (0.7)	6.4 (0.7)	6.2 (0.7)	6.3 (0.7)	6.0 (0.7)
n-3 PUFA	244.9 (31.9)	172.3 (20.0)	245.4 (26.5)	288.1 (32.9)	350.1 (39.4)	394.7 (45.8)
n-3 LCPUFA	220.9 (28.8)	63.4 (7.4)	67.9 (7.3)	62.9 (7.2)	65.8 (7.4)	66.1 (7.7)
ALA+LA[h]	42.4 (5.5)	444.8 (51.7)	478.1 (51.5)	452.8 (51.7)	457.2 (51.4)	439.3 (51.0)
n3/n6[i]	4.7	0.5	0.8	1.2	1.9	3.3
ALA/LA[j]	0.3	0.3	0.6	1.0	1.6	2.9

[a] Diet abbreviations: CD, control diet; 100% FO; T1, (0.3 ALA/LA ratio in 100% alternative oils (AO) diet with SO, LO, and BT); T2, (0.6 ALA/LA ratio in 100% AO diet with SO, LO, and BT); T3, 1.0 ALA/LA ratio in 100% AO diet with SO, LO, and BT); T4, 1.6 ALA/LA ratio in 100% AO diet with SO, LO, and BT); T5, (2.9 ALA/LA ratio in 100% AO diet with SO and LO). [b] Ridley Agriproducts, Narangba, QLD, Australia. [c] Carboxymethyl cellulose, BDH Laboratory Supplies, Poole, U.K. [d] Black and Gold, Tooronga, VIC, Australia. [e] Sigma-Aldrich, Inc. St. Louis, MO. [f] Sceney Chemical Pty., Ltd. Sunshine, VIC, Australia. [g] Nitrogen-free extract: calculated by difference. [h] Sum of ALA (α-linolenic acid, 18:3n-3) and LA (linoleic acid, 18:2n-6). [i] Ratio of total omega 3 fatty acids to omega 6 fatty acids. [j] Ratio of ALA (α-linolenic acid, 18:3n-3) to LA (linoleic acid, 18:2n-6).

Murray cod achieved a specific growth rate (SGR) ranging from 2.2 to 2.5 (% day^{-1}), with feed conversion ratio (FCR) and protein efficiency ratio (PER) values ranging from 1.1 to 1.3 and from 1.6 to 1.8, respectively. Detailed information on growth results, feed efficiency, and fillet chemical and fatty acid modification have been reported in a previous paper (*6*).

Table 2. Fatty Acid Composition (Milligrams per Gram of Lipid) of Murray Cod Whole Bodies at the Commencement and End of the Feeding Trial[a]

		dietary treatments[b]					
fatty acid	initial	CD	T1	T2	T3	T4	T5
14:0	47.2	59.2 ± 1.2 b	17.4 ± 0.6 a	18.0 ± 1.4 a	17.3 ± 0.2 a	18.1 ± 0.2 a	18.5 ± 0.5 a
16:0	171.2	181.5 ± 1.4 b	122.5 ± 2.1 a	128.8 ± 2.4 a	121.8 ± 1.8 a	126.1 ± 3.2 a	124.0 ± 3.3 a
18:0	38.6	37.8 ± 0.6 a	39.8 ± 0.6 ab	42.2 ± 0.9 b	41.1 ± 0.6 b	42.3 ± 1.0 b	42.1 ± 0.9 b
20:0	1.3	1.4 ± 0.0 b	1.3 ± 0.0 b	1.3 ± 0.1 b	1.1 ± 0.0 a	1.1 ± 0.1 a	1.0 ± 0.0 a
22:0	0.9	1.0 ± 0.0 b	1.8 ± 0.0 e	1.5 ± 0.0 d	1.2 ± 0.1 c	1.0 ± 0.0 b	0.8 ± 0.0 a
24:0	0.5	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.4 ± 0.2	0.2 ± 0.1
16:1n-7	64.4	78.8 ± 1.3 b	23.3 ± 0.7 a	24.3 ± 0.8 a	23.0 ± 0.6 a	24.3 ± 0.6 a	24.5 ± 0.5 a
18:1n-9	187.8	123.7 ± 4.9 a	195.5 ± 2.7 d	191.0 ± 1.2 d	179.7 ± 3.4 c	175.8 ± 1.6 c	164.9 ± 0.4 b
18:1n-7	32.5	31.5 ± 0.2 b	18.3 ± 0.3 a	18.9 ± 0.2 a	18.3 ± 0.2 a	19.1 ± 0.1 a	19.3 ± 0.3 a
24:1n-9	0.1	0.1 ± 0.0 a	0.2 ± 0.1 ab	0.4 ± 0.1 bc	0.7 ± 0.1 cd	0.7 ± 0.1 cd	0.9 ± 0.1 d
20:1n-11	1.6	1.1 ± 0.1 b	0.7 ± 0.1 a	0.7 ± 0.1 a	0.7 ± 0.1 a	0.8 ± 0.1 a	0.8 ± 0.1 a
20:1n-9	15.3	9.3 ± 0.5 b	6.6 ± 0.2 a	6.6 ± 0.4 a	6.1 ± 0.2 a	6.0 ± 0.2 a	6.0 ± 0.4 a
22:1n-11	5.1	3.1 ± 0.1 b	1.4 ± 0.1 a	1.4 ± 0.2 a	1.3 ± 0.1 a	1.3 ± 0.1 a	1.4 ± 0.1 a
22:1n-9	1.9	1.3 ± 0.1 b	0.9 ± 0.0 a	1.0 ± 0.1 a	0.8 ± 0.0 a	0.8 ± 0.0 a	0.9 ± 0.1 a
18:2n-6	55.7	50.1 ± 1.4 a	294.7 ± 4.3 f	246.6 ± 3.4 e	203.2 ± 4.4 d	161.1 ± 2.9 c	109.9 ± 2.1 b
18:3n-6	3.9	3.2 ± 0.3 a	10.0 ± 0.3 e	8.3 ± 0.3 d	7.0 ± 0.2 c	5.5 ± 0.1 b	3.6 ± 0.1 a
20:2n-6	1.4	1.8 ± 0.16 a	4.2 ± 0.1 e	3.4 ± 0.0 d	2.8 ± 0.1 c	2.3 ± 0.1 b	1.9 ± 0.0 a
20:3n-6	1.5	2.0 ± 0.1 c	3.3 ± 0.1 e	2.6 ± 0.1 d	2.0 ± 0.0 c	1.6 ± 0.0 b	1.2 ± 0.0 a
20:4n-6	11.1	13.2 ± 0.3 b	5.7 ± 0.2 a	5.6 ± 0.3 a	5.7 ± 0.1 a	5.9 ± 0.2 a	6.1 ± 0.3 a
22:2n-6	0.1	0.0 ± 0.0 a	0.4 ± 0.0 d	0.4 ± 0.0 cd	0.3 ± 0.0 bc	0.3 ± 0.1 b	0.3 ± 0.0 b
22:4n-6	1.8	2.5 ± 0.1 b	1.2 ± 0.1 a	1.1 ± 0.1 a	1.1 ± 0.0 a	1.1 ± 0.0 a	1.1 ± 0.0 a
18:3n-3	8.6	10.3 ± 0.2 a	76.7 ± 1.4 b	114.1 ± 2.9 c	152.2 ± 2.0 d	188.9 ± 2.8 e	220.8 ± 12.1 f
18:4n-3	12.6	13.3 ± 0.2 c	8.4 ± 0.3 a	10.5 ± 0.3 b	12.7 ± 0.1 c	15.7 ± 0.4 d	15.8 ± 0.2 d
20:3n-3	0.8	1.1 ± 0.0 a	4.1 ± 0.1 b	5.4 ± 0.1 c	6.8 ± 0.2 d	7.9 ± 0.1 e	9.0 ± 0.5 f
20:4n-3	7.3	9.0 ± 0.2 d	5.8 ± 0.2 a	7.1 ± 0.2 b	8.2 ± 0.1 c	9.6 ± 0.0 d	9.8 ± 0.2d
20:5n-3	81.8	71.0 ± 1.5 b	18.5 ± 0.7 a	18.7 ± 2.5 a	18.8 ± 0.5 a	19.9 ± 1.3 a	21.4 ± 2.1 a
22:5n-3	41.5	46.7 ± 1.3 b	15.8 ± 0.6 a	15.7 ± 1.2 a	15.6 ± 0.4 a	16.2 ± 0.6 a	17.1 ± 1.1 a
22:6n-3	85.0	109.1 ± 2.3 b	49.7 ± 0.6 a	50.6 ± 2.0 a	51.1 ± 1.0 a	52.1 ± 1.4 a	54.1 ± 2.1 a
SFA	259.6	281.0 ± 2.1 b	182.9 ± 2.9 a	192.0 ± 2.0 a	182.7 ± 2.7 a	188.9 ± 3.8 a	186.6 ± 4.0 a
MUFA	308.7	248.9 ± 4.9 c	247.0 ± 3.6 c	244.4 ± 0.9 c	230.7 ± 4.4 b	228.8 ± 2.1 b	218.6 ± 1.0 a
PUFA	313.0	333.4 ± 6.7 a	498.6 ± 5.5 b	490.3 ± 2.2 b	487.5 ± 8.1 b	487.9 ± 3.1 b	472.0 ± 9.7 b
n-6 PUFA	75.5	72.7 ± 1.6 a	319.6 ± 4.5 f	268.1 ± 3.2 e	222.1 ± 4.8 d	177.8 ± 2.8 c	124.0 ± 1.8 b
n-6 LC-PUFA	15.9	19.4 ± 0.4 e	14.9 ± 0.5 d	13.2 ± 0.3 c	11.9 ± 0.2 b	11.2 ± 0.2 ab	10.5 ± 0.4 a
n-3 PUFA	237.5	260.6 ± 5.4 c	179.0 ± 1.7 a	222.2 ± 3.4 b	265.4 ± 3.5 c	310.1 ± 0.8 d	348.0 ± 8.0 e
n-3 LC-PUFA	216.3	237.0 ± 5.1 c	93.9 ± 2.0 a	97.5 ± 5.7 ab	100.5 ± 1.9 ab	105.5 ± 3.1 ab	111.4 ± 4.9 b
ALA+LA[c]	64.3	60.4 ± 1.5 a	371.4 ± 5.7 c	360.7 ± 6.4 c	355.3 ± 5.8 bc	350.0 ± 3.2 bc	330.6 ± 14.1 b
n3/n6[d]	3.1	3.6 ± 0.1 f	0.6 ± 0.0 a	0.8 ± 0.0 b	1.2 ± 0.0 c	1.7 ± 0.0 d	2.8 ± 0.0 e
ALA/LA[e]	0.2	0.2 ± 0.0 a	0.3 ± 0.0 a	0.5 ± 0.0 b	0.7 ± 0.0 c	1.2 ± 0.0 d	2.0 ± 0.1 e

[a] Data are reported as the mean ± SEM ($N = 4$). [b] Values in the same row with different letters are significantly different ($P < 0.05$; ANOVA and Student−Newman−Keuls post hoc test, $n = 4$); initial sample was not included in the statistical analysis. See **Table 1** for diet abbreviations. [c] Sum of ALA (α-linolenic acid, 18:3n-3) and LA (linoleic acid, 18:2n-6). [d] Ratio of total omega 3 fatty acids to omega 6 fatty acids. [e] Ratio of ALA (α-linolenic acid, 18:3n-3) to LA (linoleic acid, 18:2n-6).

The total lipid content of fish whole bodies showed no significant differences among treatments, whereas the dietary fatty acid makeup was clearly mirrored in the fish body (**Table 2**). In general, and as expected, fish fed the CD diet had the highest levels of LC-PUFA in comparison to the five experimental groups. The overall fatty acid classes of whole bodies of fish fed the five experimental diets (T1−T5) were close in terms of SFA (~185 mg g lipid^{-1}) and PUFA (~485 mg g lipid^{-1}) concentrations. Within the PUFA class, the contents of ALA and LA clearly reflected the composition of the experimental diets and the ALA/LA ratios of whole bodies tended to increase, recording 0.3, 0.5, 0.7, 1.2, and 2.0 in T1−T5, respectively. Comparatively lower ALA/LA ratios in fish tissue in contrast to that of the experimental diets were recorded, with this trend tending to amplify as the treatments progressed from T1 through T5. Irrespective of their identical dietary levels, clear trends manifested in the whole-body content of n-3 and n-6 LC-PUFA among the treatment groups (T1−T5). In particular, significantly higher levels ($P < 0.05$) of all fatty acids along the n-3 desaturation and elongation pathway (namely, 18:4n-3, 20:3n-3, and 20:4n-3) were found in fish receiving diets with higher ALA/LA ratios (**Table 2**). However, no statistically significant differences were noted in the concentrations of EPA, docosapentaenoic acid (22:5n-3, DPA), and DHA, even though the same numerical trend was present. Despite the corresponding dietary levels, the whole bodies contained higher levels of DHA compared to EPA in all dietary groups.

The results of the first step of the whole body balance method, reporting the individual fatty acid appearance/disappearance (mg of fatty acid per fish) during the experimental period are reported in **Table 3**. The highest disappearance of fatty acids in the CD group was recorded for EPA followed by 16:0, 16:1n-7, and 14:0, whereas in the five experimental treatments the highest disappearances were recorded for LA (T1 and T2 groups) and ALA (T3−T5 groups). Fish receiving T1 recorded a greater appearance of fatty acids along the n-6 desaturation and elongation pathway, whereas fish receiving the T5 diet had the highest appearance of fatty acids along the n-3 desaturation and elongation pathway. In the CD group, a significant appearance of DPA was noted ($P < 0.05$).

The apparent in vivo fatty acid β-oxidation (μmol g^{-1} day^{-1}) of major fatty acid classes is shown in **Figure 1**. Fish fed a higher ratio of ALA/LA exhibited a numerically higher (although not significant, $P > 0.05$) oxidation of SFA and MUFA (**Figure 1A**).

Table 3. Individual Fatty Acid Appearance/Disappearance (Milligrams per Fish)[a]

	dietary treatments[b]					
fatty acid	CD	T1	T2	T3	T4	T5
14:0	−329.5 ± 44.3 a	−35.6 ± 10.1 b	−37.2 ± 10.3 b	−55.6 ± 11.6 b	−67.1 ± 12.4 b	−83.9 ± 21.5 b
16:0	−715.6 ± 123.0	−235.9 ± 104.3	−202.9 ± 131.2	−401.8 ± 81.7	−424.1 ± 125.7	−552.6 ± 170.9
18:0	−164.2 ± 20.9 ab	−110.7 ± 32.6 b	−128.1 ± 43.0 b	−204.2 ± 28.5 ab	−249.5 ± 31.7 ab	−298.1 ± 62.5 a
20:0	−16.0 ± 1.2	−14.5 ± 1.5	−11.8 ± 1.5	−12.5 ± 1.0	−13.1 ± 0.8	−12.3 ± 1.6
22:0	−10.3 ± 0.7 d	−26.4 ± 2.3 a	−24.5 ± 1.9 ab	−20.1 ± 1.3b c	−16.4 ± 0.8 c	−10.2 ± 1.3 d
24:0	−7.2 ± 0.6 c	−9.8 ± 0.4 b	−18.3 ± 0.9 a	−1.9 ± 0.4 d	0.2 ± 1.2 d	−1.6 ± 0.5 d
16:1n-7	−330.2 ± 59.0 a	−36.2 ± 17.4 b	−22.9 ± 16.3 b	−52.7 ± 15.8 b	−50.5 ± 23.7 b	−72.9 ± 25.7 b
18:1n-9	−149.5 ± 53.5	−574.8 ± 142.8	−523.2 ± 163.8	−635.9 ± 121.2	−568.3 ± 134.1	−623.3 ± 205.6
18:1n-7	−56.1 ± 21.3	−30.4 ± 13.7	−28.8 ± 15.2	−50.5 ± 11.6	−53.2 ± 15.1	−74.0 ± 21.8
20:1n-11	−8.3 ± 0.7 a	−4.5 ± 0.7 b	−5.7 ± 0.2 ab	−6.9 ± 1.1 ab	−7.8 ± 0.5 ab	−8.9 ± 1.5 a
20:1n-9	−45.1 ± 4.2 a	−10.6 ± 3.8 b	−7.9 ± 3.7 b	−16.6 ± 2.8 b	−17.8 ± 2.4 b	−21.7 ± 5.1 b
22:1n-11	−44.6 ± 2.6 a	−12.3 ± 1.4 b	−12.5 ± 1.5 b	−13.5 ± 0.9 b	−14.4 ± 0.6 b	−13.6 ± 1.6 b
22:1n-9	−9.9 ± 0.7 a	−3.5 ± 0.6b	−2.5 ± 1.2 b	−4.0 ± 0.5 b	−3.9 ± 0.6 b	−3.5 ± 0.8 b
24:1n-9	−1.8 ± 0.3 e	−10.2 ± 1.0 d	−12.3 ± 1.0 cd	−15.3 ± 1.3 bc	−17.7 ± 0.9 ab	−20.0 ± 2.3 a
18:2n-6	−30.0 ± 23.2 d	−1705.5 ± 257.4 a	−1520.3 ± 238.0 ab	−1266.9 ± 159.9 ab	−918.2 ± 102.9 bc	−620.8 ± 153.1 c
18:3n-6	−18.3 ± 3.4 a	76.7 ± 2.8 d	70.4 ± 10.4 d	51.3 ± 4.4 c	36.5 ± 7.6 c	14.1 ± 3.2 b
20:2n-6	−3.1 ± 1.1 a	27.0 ± 1.2 c	24.8 ± 3.7 cd	14.6 ± 2.3 b	11.5 ± 2.3 b	3.4 ± 2.0 a
20:3n-6	−1.4 ± 1.5 a	23.6 ± 1.1 c	20.3 ± 3.4 c	12.7 ± 1.5 b	9.7 ± 1.7 b	3.9 ± 1.1 a
20:4n-6	−44.9 ± 8.3 a	−16.7 ± 2.5 b	−17.0 ± 3.1 b	−18.9 ± 3.6 b	−19.5 ± 1.8 b	−15.7 ± 5.6 b
22:2n-6	−0.1 ± 0.0 b	3.9 ± 0.2 c	0.8 ± 0.6 b	3.0 ± 0.3 c	−1.8 ± 0.6 a	−2.2 ± 0.4 a
22:4n-6	−1.6 ± 2.0	0.9 ± 0.5	1.0 ± 1.0	0.4 ± 0.8	−0.4 ± 0.9	−1.5 ± 1.2
18:3n-3	−40.7 ± 6.1 e	−599.9 ± 77.0 d	−1022.4 ± 121.9 cd	−1471.7 ± 130.4 bc	−1898.0 ± 116.7 ab	−2273.8 ± 389.6 a
18:4n-3	−99.3 ± 9.3 a	26.4 ± 4.0 b	49.5 ± 8.3 bc	65.9 ± 8.0b cd	94.7 ± 16.6 d	81.6 ± 14.6 cd
20:3n-3	−2.7 ± 0.8 a	30.1 ± 1.4 b	47.8 ± 6.5 bc	56.8 ± 5.6 c	67.7 ± 10.3 c	73.1 ± 11.2c
20:4n-3	−11.3 ± 6.8 a	27.3 ± 1.8 b	46.0 ± 5.9 bc	52.9 ± 5.4 bc	67.6 ± 11.5 c	61.8 ± 9.0c
20:5n-3	−993.2 ± 62.8 a	−206.8 ± 17.7 b	−214.1 ± 18.5 b	−208.0 ± 13.8 b	−217.0 ± 11.4 b	−220.3 ± 19.7 b
22:5n-3	179.3 ± 40.6 b	26.0 ± 6.8 a	37.0 ± 7.1 a	29.8 ± 10.3 a	34.0 ± 10.6 a	27.2 ± 10.2 a
22:6n-3	−281.9 ± 74.7	−132.2 ± 32.5	−118.8 ± 36.2	−124.0 ± 34.2	−136.7 ± 21.2	−161.6 ± 49.8

[a] Data are reported as the mean ± SEM ($N = 4$). Values in the same row with different letters are significantly different ($P < 0.05$; ANOVA and Student−Newman−Keuls post hoc test, $N = 4$). [b] See **Table 1** for diet abbreviations.

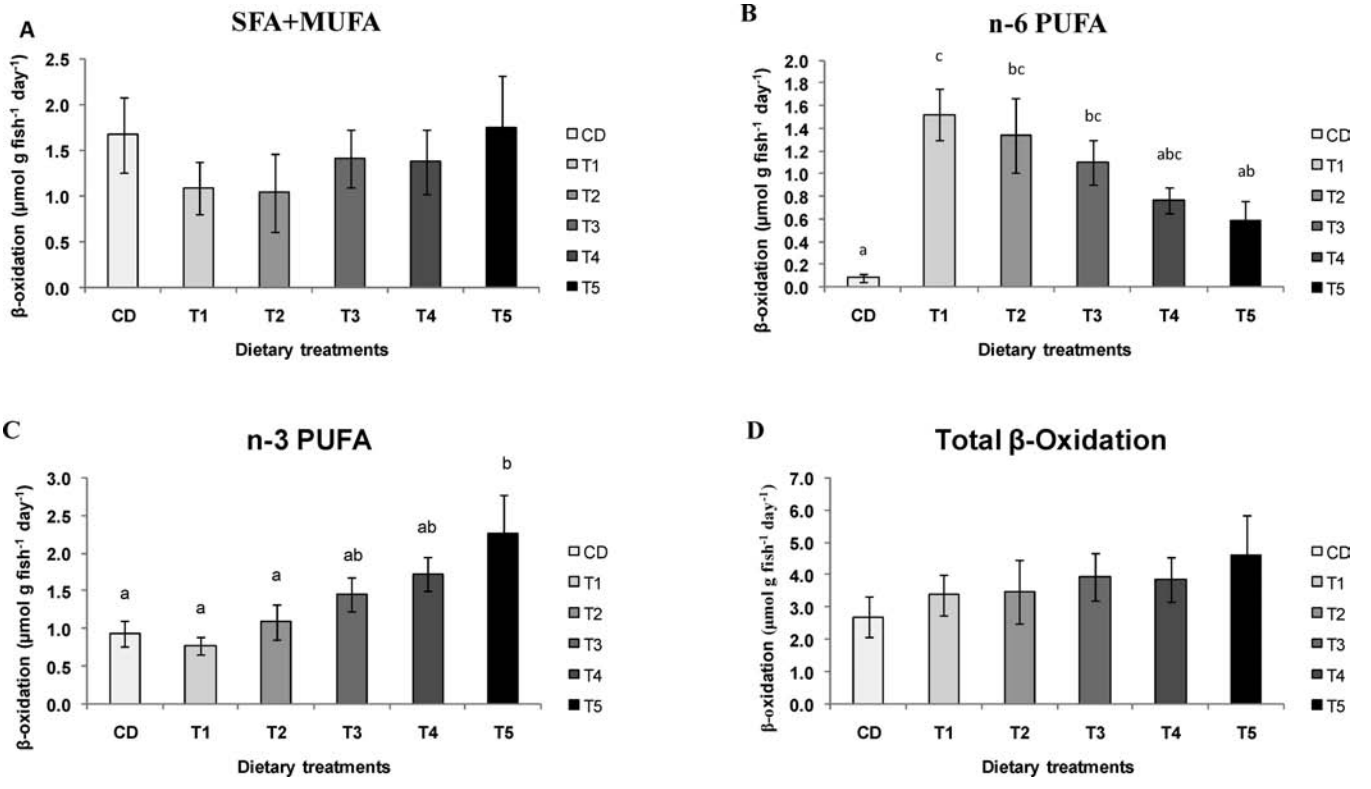

Figure 1. Apparent in vivo fatty acid β-oxidation (μmol g^{-1} day^{-1}) in Murray cod: (**A**) total SFA and MUFA; (**B**) n-6 PUFA; (**C**) n-3 PUFA; (**D**) total fatty acids.

n-6 PUFA were oxidized at significantly higher level ($P < 0.05$) in treatments containing higher concentrations of LA (**Figure 1B**), and higher n-3 PUFA oxidation was observed in treatments containing higher ALA (**Figure 1C**). Total fatty acid oxidation, although not

Table 4. Individual Apparent in Vivo Fatty Acid β-Oxidation (Expressed as Percent of Net Intake) of Selected Fatty Acids (Mean ± SEM; $N = 4$)[a]

	dietary treatments[b]					
	CD	T1	T2	T3	T4	T5
14:0	36.5 ± 5.99	26.7 ± 9.06	21.2 ± 8.84	36.2 ± 7.27	39.6 ± 8.17	46.4 ± 10.88
16:0	29.6 ± 6.47	22.5 ± 7.55	17.2 ± 9.95	30.3 ± 6.05	30.9 ± 9.78	38.2 ± 11.42
18:0	31.9 ± 5.17	25.8 ± 7.46	25.7 ± 8.68	37.4 ± 5.02	41.8 ± 6.69	47.4 ± 8.80
18:1n-9	13.0 ± 5.38	27.1 ± 6.41	24.1 ± 7.56	30.3 ± 5.72	29.1 ± 7.19	33.3 ± 10.58
18:2n-6	6.9 ± 5.53 a	36.2 ± 5.17 b	35.6 ± 6.37 b	37.6 ± 5.08 b	36.2 ± 5.10 b	38.4 ± 9.44 b
20:4n-6	25.8 ± 5.92	30.3 ± 4.74	27.3 ± 5.80	31.5 ± 6.67	32.4 ± 5.21	28.3 ± 9.92
18:3n-3	29.3 ± 5.64	40.0 ± 5.45	40.7 ± 6.38	44.4 ± 4.44	45.4 ± 4.95	47.8 ± 8.60
20:5n-3	50.6 ± 5.24	71.8 ± 7.09	64.2 ± 5.54	67.4 ± 8.06	65.6 ± 5.12	68.7 ± 6.93
22:6n-3	21.2 ± 6.46	28.3 ± 6.61	23.4 ± 6.76	25.1 ± 6.79	27.2 ± 5.18	30.7 ± 8.88

[a] Data are reported as the mean ± SEM ($N = 4$). Values in the same row with different letters are significantly different ($P < 0.05$; ANOVA and Student−Newman−Keuls post hoc test, $N = 4$). [b] See **Table 1** for diet abbreviations.

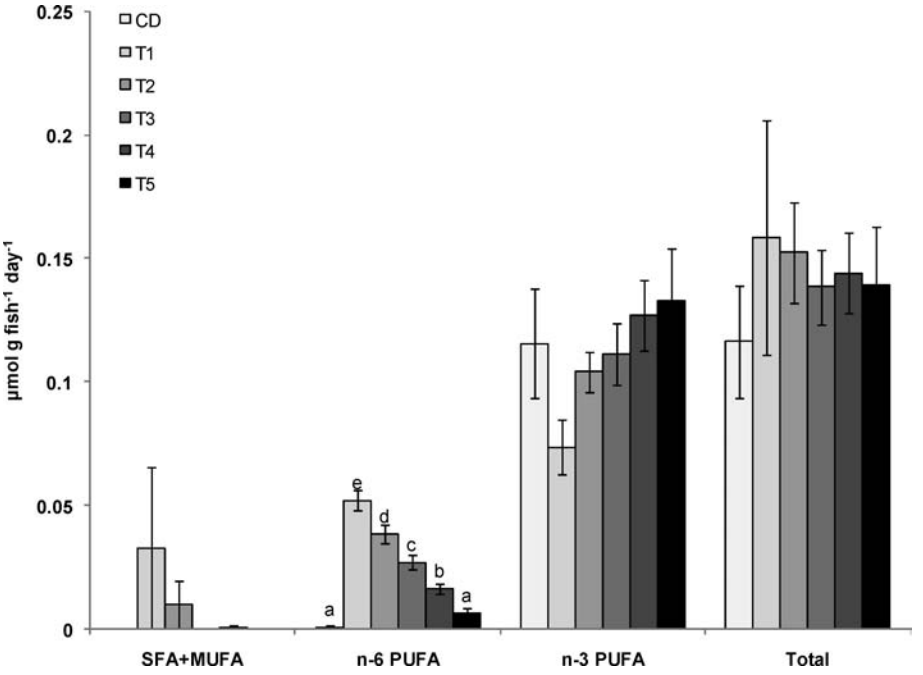

Figure 2. Apparent in vivo total elongase activity (μmol g fish^{-1} day^{-1}).

significantly different ($P > 0.05$), was higher in groups fed the five experimental diets in comparison to fish receiving the CD treatment, and, among the five experimental treatments, an increasing trend was evident with increasing ALA/LA ratio (**Figure 1D**).

The apparent in vivo β-oxidation of individual major fatty acids, reported as a percentage of net intake, is reported in **Table 4**. The only statistically significant difference was a lower level of LA β-oxidation in the CD treatment in comparison to all other treatments. Among the five experimental treatments, LA was oxidized at ~36%, with no clear trend among treatments, whereas ALA was oxidized in the range of 40−48%, with a noticeable increasing trend toward the higher dietary inclusion levels of ALA. In general, the β-oxidation of oleic acid (OA, 18:1n-9) (as % of net intake) was lower than that of LA and ALA and followed the order ALA > LA > OA. Moreover, although no statistically significant differences were noted, a trend toward increased SFA and MUFA oxidation in higher ALA diets was observed, with, for example, 16:0 varying from 22.5 to 38.2% and OA from 27.1 to 33.3% in T1 and T5, respectively. Among LC-PUFA, EPA was highly oxidized (> 65%), whereas the oxidation of DHA was significantly lower (~27%).

Total neogenesis of fatty acids was minimal and detected only in T1 and T2 (0.028 ± 0.028 and 0.004 ± 0.004 μmol g^{-1} fish day^{-1}, respectively). The apparent in vivo elongase activity is reported in **Figure 2**. In general, the large variability of data resulted in a lack of statistically significant differences, even if clear trends were evident. Independent of the dietary treatments, higher elongase activity was recorded on n-3 PUFA, followed by n-6 PUFA, with minimal elongase activity recorded on SFA and MUFA. The fatty acid elongase activity on n-6 PUFA and n-3 PUFA decreased and increased, respectively, with the elevated dietary ALA/LA ratios, resulting from the provision of more or less substrate. High in vivo apparent elongase activity acting on n-3 PUFA (mainly ascribable to EPA being elongated to DPA) was observed in the CD treatment, whereas the activity acting on n-6 fatty acids was negligible.

No apparent in vivo desaturase (Δ-9, Δ-6, or Δ-5) activity was detected in fish fed the CD treatment, and no Δ-5 desaturase activity was observed in any of the experimental treatments (**Figure 3**). On the other hand, Δ-6 desaturase activity on n-6 and n-3 fatty acids was apparent and tended to decrease and increase, respectively, from T1 to T5, in synchrony with the increasing dietary ALA/LA ratio. Interestingly, the highest apparent Δ-6 desaturase activity acting on ALA (0.1320 μmol g^{-1} day^{-1}) was recorded not in fish receiving the highest ALA supply (T5), but in fish receiving T4, whereas the highest activity

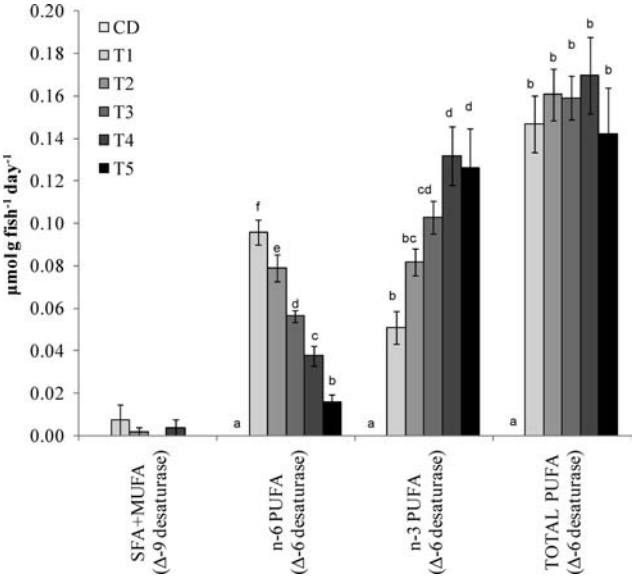

Figure 3. Apparent in vivo total desaturase (Δ-9 and Δ-6) activity (μmol g fish^{-1} day^{-1}).

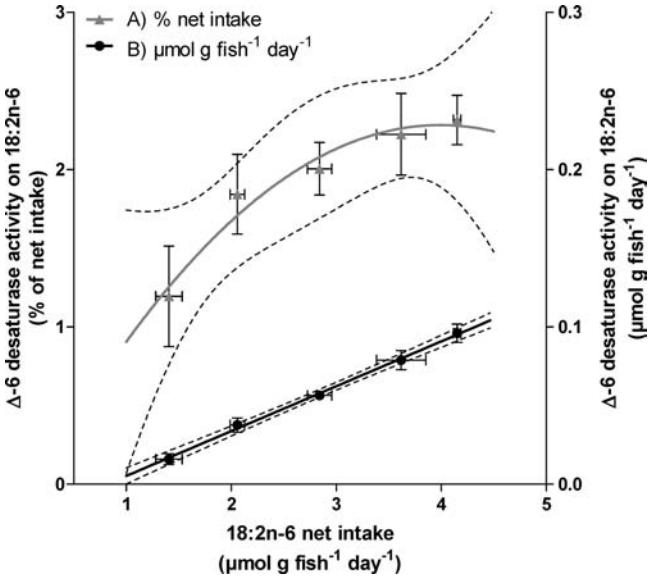

Figure 4. Apparent in vivo Δ-6 desaturase activity on 18:2n-6 expressed as (**A**) % of net intake and as (**B**) μmol g of fish^{-1} day^{-1}, in relationship to the18:2n-6 net intake (μmol g of fish^{-1} day^{-1}). Error bars represent SEM, and dotted lines represent 95% confidence band of the regression lines. Regression equations: (**A**) $Y = -0.1542X^2 + 1.230X - 0.1705$; $R^2 = 0.96$; test of normality of residuals D'Agostino−Pearson $P = 0.97$; (**B**) $Y = 0.02851X - 0.02327$; $R^2 = 0.99$; slope deviation from zero $P < 0.0001$.

acting on LA (0.0960 μmol g^{-1} day^{-1}) was detected in fish receiving the highest concentration of this fatty acid (T1).

The relationships between in vivo apparent Δ-6 desaturase activity acting on LA and ALA against their respective net dietary inclusion, expressed as μmol g^{-1} day^{-1} or percentage of net intake, are shown in **Figures 4** and **5**, respectively. In the case of LA, a positive linear regression described the relationship between dietary content (substrate availability) and enzyme activity when expressed as μmol g^{-1} day^{-1}. However, when expressed as a percentage of net intake, the trend was better described by a second-order polynomial equation (**Figure 4**). Similarly, the relationship between apparent Δ-6 desaturase activity acting on ALA (as μmol g^{-1} day^{-1}) and its net intake level could also be

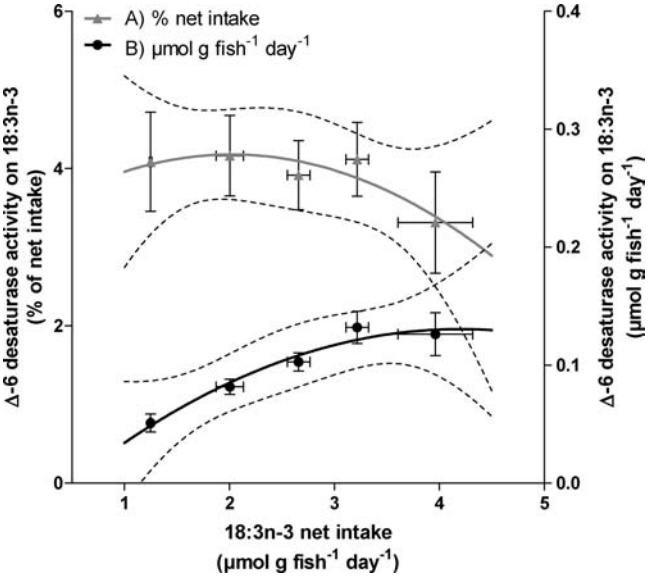

Figure 5. Apparent in vivo Δ-6 desaturase activity on 18:3n-3 expressed as (**A**) % of net intake and as (**B**) μmol g of fish^{-1} day^{-1}, in relationship to the18:3n-3 net intake (μmol g of fish^{-1} day^{-1}). Error bars represent SEM, and dotted lines represent 95% confidence band of the regression lines. Regression equations: (**A**) $Y = -0.2097X^2 + 0.8483X + 3.321$; $R^2 = 0.81$; test of normality of residuals D'Agostino−Pearson $P = 0.77$; (**B**) $Y = -0.009417X^2 + 0.07916X + 0.03560$; $R^2 = 0.96$; test of normality of residuals D'Agostino−Pearson $P = 0.10$.

described by a second-order polynomial equation, which showed that the rate of increase was proportionally reduced with the increase of substrate availability following a peak at the net intake of 3.2186 μmol g^{-1} day^{-1} (**Figure 5**). The relative apparent Δ-6 activity acting on ALA (as a percentage net intake), described by a negative second-order polynomial equation, showed that the actual efficiency of the enzyme was reduced by an increase in substrate availability. The total apparent in vivo Δ-6 desaturase activity (on ALA and LA) expressed as both percentage of net intake and μmol g fish^{-1} day^{-1}, in relation to the dietary ALA/LA ratio, is shown in **Figure 6**. Both relationships were described by second-order polynomial equations, with the activity tending to increase with the increasing dietary ALA/LA ratio. This activity peaked at a ratio of 1.60 and 1.47 for percentage of net intake and μmol g fish^{-1} day^{-1}, respectively, and then tended to decrease as the dietary ALA/LA ratio elevated. The maximum activity was detected in T4, therefore indicating that an ALA/LA ratio of 1.54 (the mean value of the two peaks computed on the two regression lines) is the most efficient ratio for maximal fatty acid Δ-6 desaturase activity in juvenile Murray cod.

The relationship between the percentage of total apparent in vivo Δ-6 desaturase activity acting on ALA or LA against the in vivo available substrate ratio (dietary ALA/LA ratio) is reported in **Figure 7**. Essentially, the greater the amount of ALA, the greater the rate of desaturation and vice versa for the amount of dietary LA. Additionally, by plotting these equations, it is noted that the theoretical maximum percentage activity on ALA was 91.89% and the minimum percentage activity acting on LA was 8.11%, independent of the ALA/LA ratio in the diets.

Figure 8 describes the relationship between the dietary ALA/LA ratio and the ratio of Δ-6 desaturase activity on the two substrates (activity on ALA/activity on LA ratio). A linear regression equation (with a slope of 3.2) best described this relationship. Thus, it was clearly shown that the Δ-6 desaturase activity acting on ALA, across either experimental treatment

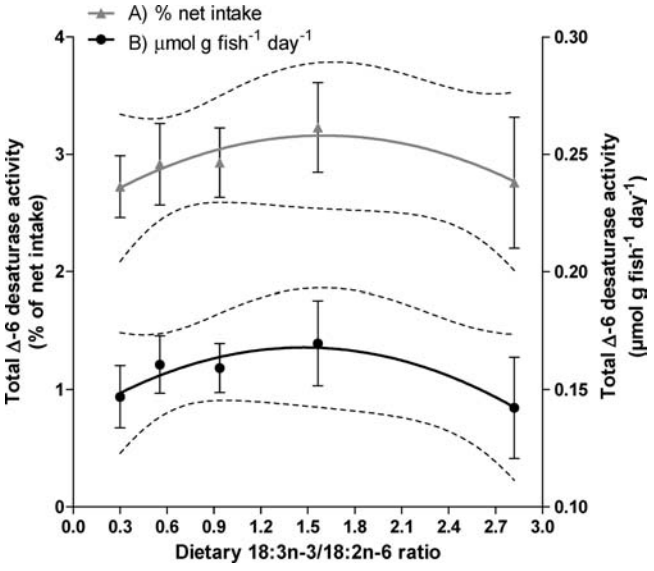

Figure 6. Total apparent in vivo Δ-6 desaturase activity (on 18:3n-3 and 18:2n-6) expressed as (**A**) % of net intake and as (B) μmol g of fish^{-1} day^{-1}, in relationship to the dietary18:3n-3/18:2n-6 ratio. Error bars represent SEM, and dotted lines represent 95% confidence band of the regression lines. Regression equations: (**A**) $Y = -0.2636X^2 + 0.8440X + 2.484$; $R^2 = 0.87$; test of normality of residuals D'Agostino−Pearson $P = 0.87$; (**B**) $Y = -0.0141X^2 + 0.04157X + 0.1373$; $R^2 = 0.90$; test of normality of residuals D'Agostino−Pearson $P = 0.84$.

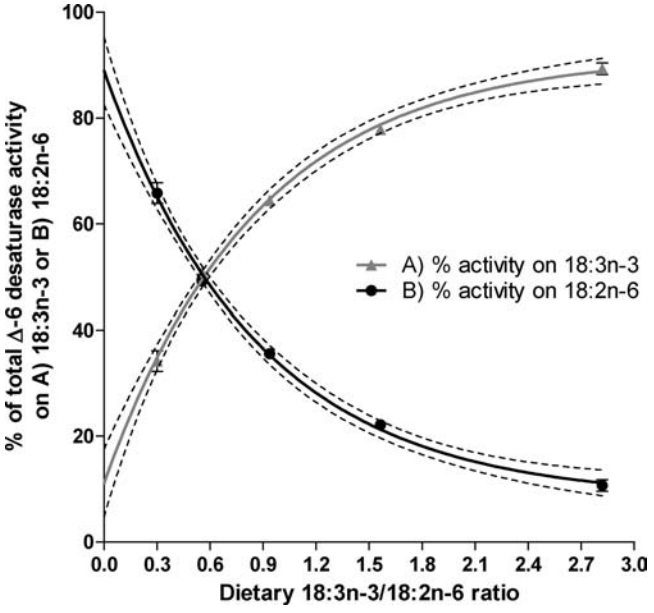

Figure 7. Percentage of total apparent in vivo Δ-6 desaturase activity acting on (**A**) 18:3n-3 and (**B**) 18:2n-6 plotted against the in vivo available substrate ratio (dietary 18:3n-3/18:2n-6 ratio). Error bars represent SEM, and dotted lines represent 95% confidence band of the regression lines. Regression equations: (**A**) $Y = -80.78\, e^{-1.160X} + 91.89$; $R^2 = 0.99$; test of normality of residuals D'Agostino−Pearson $P = 0.81$; (**B**) $Y = 80.78\, e^{-1.160X} + 8.107$; $R^2 = 0.99$; test of normality of residuals D'Agostino−Pearson $P = 0.81$.

(T1−T5), was higher than the activity on LA (evident if compared to the line of equity), clearly indicating that the affinity of the Δ-6 desaturase was 3.2-fold higher for ALA in comparison to LA.

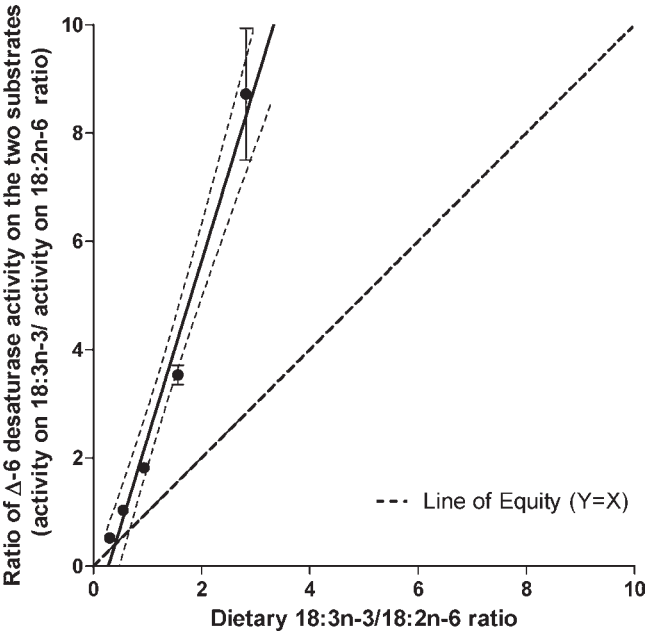

Figure 8. Relationship between the dietary 18:3n-3/18:2n-6 ratio and the ratio of Δ-6 desaturase activity on the two substrates (activity on 18:3n-3/ activity on 18:2n-6 ratio). Error bars represent SEM, and dotted lines represent 95% confidence band of the regression line. Regression equation: $Y = 3.275X - 0.09183$; $R^2 = 0.88$; slope deviation from zero $P < 0.0001$. The line of equity ($Y = X$) has also been reported for visual reference.

DISCUSSION

The major focus of the present study was to evaluate the in vivo fatty acid metabolism of juvenile Murray cod fed with various ALA/LA ratios at a constant level of total C_{18} PUFA. For specific details on growth performance, feed utilization, and fillet fatty acid composition the reader is referred to Senadheera et al. (*6*).

As expected, across each of the six treatments, there was a clear reflection of the dietary fatty acid composition in the whole bodies of Murray cod, an observation consistent with previous studies on the same species (*10*−*12*, *20*) and many other species (*1*, *21*, *22*). The levels of LC-PUFA, particularly EPA and DHA, were severely affected by diets devoid of fish oil, as previously reported in numerous studies (*4*, *22*−*26*). However, irrespective of the identical levels of total C_{18} PUFA (ALA+LA) in the five experimental diets, it was interesting to note that the corresponding levels in fish whole bodies decreased significantly with the increasing dietary ALA/LA ratio, indicating a pronounced higher rate of deposition of total C_{18} PUFA (LA + ALA) in treatments with high-LA diets. In addition, the relatively lower ALA/LA ratios detected in whole bodies in comparison to those found in corresponding diets further support the observation of apparent preferential utilization observed between ALA and LA, where ALA tended to be utilized more readily (β-oxidized or bioconverted), whereas LA appeared to be more efficiently and directly deposited in the fish tissues (*1*, *12*, *27*). Despite the major and direct influences caused by dietary fatty acid composition, other factors such as digestibility (*28*, *29*), transport and uptake, desaturation and elongation processes (*23*, *30*), and β-oxidation of fatty acids (*29*, *31*) are also known to have impacts on the overall membrane and depot fatty acid compositions.

Numerous studies suggest that dietary fatty acids can remarkably affect overall lipid metabolism (*4*, *10*, *19*, *22*). However, little information is available on the direct effects of the dietary

ALA/LA ratio on overall fatty acid metabolism. The results of this study indicate that the majority of consumed LA, ALA, and OA was accumulated in the fish body, whereas significantly lower proportions were β-oxidized for energy production, or bioconverted. Specifically, Murray cod displayed a preferential order of accumulation (as a percentage of net intake) of OA, followed by LA and ALA, and an inverse order of preference in β-oxidizing ALA over LA and OA. These results are consistent with the observations of previous studies on Murray cod using both fish oil-based experimental diets (*32*) and vegetable oil-based diets (*10*, *11*). However, this observation is in contrast with previous results for other fish species, where n-6 and n-9 fatty acids were reported to be more actively β-oxidized in comparison with n-3 fatty acids (*33*). This further validates the hypothesis that, when compared to other cultured finfish, n-6 fatty acids play a different role in the general fatty acid metabolism of Murray cod, as previously suggested (*11*, *12*, *32*).

Generally, it is recognized that β-oxidation of specific fatty acids is proportional to their dietary availability. In the present study, n-6 PUFA and n-3 PUFA were β-oxidized to a greater extent in treatments with high LA and high ALA, respectively. However, observing the results of β-oxidation, expressed as a percentage of net intake (**Table 4**), it was interesting to note that LA was β-oxidized at a constant level independently from dietary availability (~36% of net intake), whereas ALA was proportionally oxidized at a greater rate, if provided in surplus, as would normally be expected (*34*). Additionally, ALA was found to be β-oxidized to a greater extent than LA irrespective of the dietary level, as previously recorded for Murray cod (*10*, *11*).

Currently available information on fatty acid β-oxidation in fish suggests the existence of a substrate preference for SFA and MUFA over PUFA (*35*,*36*). A previous study on Atlantic salmon (*Salmo salar*) fed a diet with low n-3 LC-PUFA showed that these fatty acids were selectively retained in fish tissues. Specifically, the fish fed a fish oil-based diet stored approximately 20% EPA and 30% DHA, whereas vegetable oil-fed fish stored 70% EPA and 80% DHA during the fast growth period in seawater (*34*), suggesting a switch in fatty acid substrate used for β-oxidation when dietary levels are low. On the contrary, the present study observed a higher level of β-oxidation and lower retention levels of EPA and DHA in fish fed the five experimental diets containing low n-3 LC-PUFA, compared to those fed the fish oil-based diet. This may be ascribable to differences in the overall fatty acid metabolism between the two species. This result is not surprising, especially in consideration that Atlantic salmon are cold-water anadromous species, whereas Murray cod inhabit warm water and are a strictly freshwater species. In general, in the present study, EPA was highly oxidized, whereas DHA seemed to be spared this fate. This finding is in agreement with previous observations (*19*) and likely relative to the fact that DHA is less easily oxidized as it requires an additional step to remove the Δ-4 double bond prior to β-oxidization for energy production (*2*).

In agreement with similar studies in which fish oil was replaced with alternative sources (*33*, *34*, *37*), only marginal effects on β-oxidation were recorded in the present study. Nonetheless, during the parr−smolt transformation, vegetable oil-fed Atlantic salmon had significantly decreased β-oxidation capacity in comparison to fish fed fish oil-based diets (*34*). Additionally, in vitro studies using Atlantic salmon hepatocytes have indicated that n-3 LC-PUFA stimulated total β-oxidation activity, but through increased uptake of fatty acids into the cells, rather than by stimulating the actual β-oxidation system (*38*).

Freshwater fish fed diets containing alternative lipid sources lacking in n-3 LC-PUFA generally have increased rates of enzyme activity and elevated transcription rates in comparison to those fed n-3 LC-PUFA-rich fish oil-based diets (*1*−*4*, *33*, *39*−*43*). However, this metabolic effort is reported to be inadequate to compensate for the decreased n-3 LC-PUFA intake, resulting in a significant reduction in the n-3 LC-PUFA content in fish tissue and adversely affecting the nutritional quality of farmed fish (*1*, *26*). This was also the case in the present study, in which a significant increase in desaturase and elongase activity was evident in the five experimental treatments in comparison to the control group. Despite an identical intake of LC-PUFA in the five experimental diets, the levels of LC-PUFA in Murray cod whole bodies were significantly affected by the dietary ALA/LA ratio, manifestly showing modified fatty acid bioconversion. Accordingly, similar results were observed in yellow catfish (*Pelteobagrus fulvidraco*) fed diets containing different ALA/LA ratios (*44*).

In general, the elongase activity recorded in the present study was manifestly substrate dependent, as previously reported for the same species (*12*). The relatively high elongase activity acting on n-3 PUFA recorded in fish receiving the CD treatment was attributable to an elongation of EPA to DPA and then DHA. These results are in agreement with similar observations made for rainbow trout fed a fish oil-based experimental diet (*19*).

Murray cod have been shown to exhibit Δ-5 desaturase activity (*11*). However, in the present study, no apparent Δ-5 activity was recorded in any of the treatments. As previously reported (*12*), an absence of apparent Δ-5 desaturase activity does not indicate a lack of Δ-5 desaturase capability, but may be attributable to a combination of minimal activity of this enzyme and a masking effect resulting from the presence of the enzyme product in the experimental diets and initial fish tissues. Similarly, a previous study investigating differing dietary ALA/LA ratios with partial fish oil substitution observed a lack of Δ-5 desaturase activity compared to Δ-6 desaturase activity in Eurasian perch (*Perca fluviatilis*) along the n-6 bioconversion pathway (*45*).

As per the elongase activity, the in vivo apparent Δ-6 desaturase activity acting on n-3 and n-6 PUFA both increased and decreased with the elevation of the dietary ALA/LA ratio, clearly indicating that this enzymatic activity is substrate dependent. However, the specific kinetics of Δ-6 desaturase activity acting on the two possible substrates (LA or ALA) were quite different. The apparent in vivo Δ-6 desaturase activity acting on LA (μmol g of fish^{-1} day^{-1}) showed an increasing trend in relation to the substrate availability (LA net intake), as previously reported (*12*). However, when this activity was expressed as a percentage of net intake, thus indicating the efficiency of this enzyme, the Δ-6 desaturase activity acting on LA initially increased, then peaked at the substrate level of 4.1512 (μmol g of fish^{-1} day^{-1}), and subsequently remained constant, indicating that any further increase of substrate availability is inefficient in terms of n-6 LC PUFA production. On the other hand, the apparent in vivo Δ-6 desaturase activity on ALA was observed to increase with the increased substrate availability (ALA net intake), but reached its maximum at an average substrate level. The trend of Δ-6 desaturase activity (as a percentage of net intake) on ALA appears similar to previous findings (*12*), suggesting that the amount of ALA desaturated is proportionally reduced by an increasing dietary substrate level. Interestingly, the maximum Δ-6 desaturase activity acting on ALA peaked at the substrate level of 3.2186 (μmol g fish^{-1} day^{-1}) (T4), suggesting that any additional amount of ALA is not only wasteful, but counterproductive, in terms of n-3 LC-PUFA bioconversion. A similar trend was noted for both absolute (μmol per g fish day^{-1}) and relative (as a percentage of net intake) in vivo total apparent desaturase activity in relation to the dietary ALA/LA ratio, peaking at an ALA/LA ratio of 1.54, suggesting that this could be considered the optimal dietary ALA/LA ratio for Murray cod. In a previous study on

Eurasian perch (*45*), it was concluded that fish fed a linseed-oil based diet, with an ALA/LA ratio of 3, was the optimal for maximal n-3 LC-PUFA biosynthesis, which is a ratio comparatively higher than that observed in Murray cod during the present investigation. However, in this study the actual total substrate availability (ALA+LA) was not balanced, and it is likely that the increased n-3 LC-PUFA biosynthesis recorded in fish fed the linseed oil-based diet was primarily derived from the total higher substrate availability. In human hepatocytes, the optimal ALA/LA ratio for maximal DHA production has been suggested to be 1:1, but higher EPA production was recorded in cells fed ALA alone (ALA/LA ratio 1:0) (*46*). The LC-PUFA biosynthesis pathway has most commonly been assayed in vitro by investigating, for example, the conversion of radiolabeled [1-^{14}C]ALA in isolated cell preparations, and mainly in salmonids (*39*, *47*). The desaturation of radiolabeled [1-^{14}C]ALA and [1-^{14}C]LA in hepatocytes of Atlantic salmon fed vegetable oil, compared to fish fed fish oil-based, diets was significantly increased in salmon parr (*22*) and smolts (*48*). These studies observed that the overall level of LC-PUFA synthesis from LA was less than half of that from ALA, irrespective of the diet. Accordingly, the present study detected a comparatively higher overall bioconversion of ALA compared to that of LA regardless of the dietary inclusion level. Specifically, a 3.2-fold greater Δ-6 desaturase affinity toward ALA over LA was recorded in Murray cod.

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