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EFFECT OF CONJUGATED LINOLEIC ACID FEEDING PERIODS ON FATTY ACID PROFILE AND NUTRITIONAL QUALITY OF *Astyanax altiparanae**

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ABSTRACT

The effects of feeding diets with conjugated linoleic acid (CLA) to *Astyanax altiparanae* for different periods were investigated on carcasses' fatty acid profile and nutritional quality. The trial was laid out in a complete randomized design with eight feeding periods (0, 7, 14, 21, 28, 35, 42, and 49 days) and four replicates. A total of 240 fish (3.4 ± 0.3 g) were distributed into four aquariums and fed with the diet containing 32% crude protein, 15 MJ kg^{-1} gross energy, and 2.5% of CLA. Feeding CLA diet for 49 days resulted in the higher deposition of CLA, CLA isomers, and docosahexaenoic acid (DHA). The isomer *c*9,*t*11 was highly deposited than the *t*10,*c*12. Polyunsaturated fatty acids (PUFA), n-3, and n-6 increased with feeding period, whereas saturated (SFA), monounsaturated, and medium-chain fatty acids decreased. PUFA/SFA ratio, DHA/EPA, EPA+DHA, and thrombogenicity index increased linearly. Whereas atherogenicity index reduced, hypocholesterolemic/hypercholesterolemic ratio increased in quadratic effects over feeding time. No difference was observed for the n-6/n-3 ratio. Feeding *A. altiparanae* with 2.5% of CLA for a minimum of 35 days improves the fatty acid profile and provides a commercial product with good nutritional quality and functional benefits.

Keywords: CLA; deposition rate; fatty acids; healthy traits; nutrition.

EFEITO DO TEMPO DE ALIMENTAÇÃO COM ÁCIDO LINOLEICO CONJUGADO NO PERFIL DE ÁCIDOS GRAXOS E QUALIDADE NUTRICIONAL DE *Astyanax altiparanae*

RESUMO

Os efeitos da alimentação com dietas contendo ácido linoleico conjugado (CLA) por diferentes períodos foram investigados no perfil de ácidos graxos e na qualidade nutricional das carcaças de *Astyanax altiparanae*. O experimento foi conduzido em delineamento inteiramente causalizado com oito períodos de alimentação (0, 7, 14, 21, 28, 35, 42 e 49 dias) e quatro repetições. Um total de 240 peixes (3,4 ± 0,3 g) foram distribuídos em quatro aquários e alimentados com dieta contendo 32% de proteína bruta, 15 MJ kg^{-1} de energia bruta e 2.5% de CLA. A alimentação com CLA por 49 dias resultou em maior deposição de CLA, isômeros e ácido docosahexaenóico (DHA). O isômero *c9,t11* foi mais depositado do que o *t10,c12*. Os ácidos graxos n-3 e n-6 aumentaram com o período de alimentação, enquanto os ácidos graxos saturados (SFA), monoinsaturados e de cadeia média diminuíram. A razão PUFA/SFA, DHA/EPA, EPA + DHA e índice de trombogenicidade aumentaram linearmente. Enquanto o índice de aterogenicidade reduziu, a razão hipocolesterolêmica/hipercolesterolêmica aumentou em efeito quadrático com o aumento do tempo de alimentação com CLA. Nenhuma diferença foi observada para a razão n-6/n-3. Assim, a alimentação de *A. altiparanae* com 2.5% de CLA por um mínimo de 35 dias melhora o perfil de ácidos graxos e fornece um produto comercial com boa qualidade nutricional e benefícios funcionais.

Palavras-chave: CLA; taxa de deposição; ácidos graxos; produtos saudáveis; nutrição.

INTRODUCTION

Conjugated linoleic acid (CLA) refers to a group of geometric and positional isomers of linoleic acid (18:2n-6) with two conjugated double bonds. The physiological effects and health benefits of CLA for humans, which were thoroughly reviewed by Chen and Park (2019), are primarily linked to the 18:2 *cis*-9, *trans*-11 (*c9,t11*), and 18:2 *trans*-10, *cis*-12 (*t10,c12*) isomers. Although daily CLA intake can vary between countries and

individuals (Schmid et al., 2006), the average intake is well below the recommended range of 0.6-3.2 g day^{-1} (Siurana and Calsamiglia, 2016). Although it benefits, there are some concerns regarding the safety of CLA supplementation in humans (Dilzer and Park, 2012) like hepatic steatosis (Ramos et al., 2009), milk fat depression impacting infants nutrition (Masters et al., 2002), and increase on insulin resistance, the negative effects of CLA are still inconsistent (Dilzer and Park, 2012). Because animals are not able to synthesize CLA, enriching foods with CLA is one way to increase human consumption of this fatty acid, besides the enrichment with CLA in foods.

Enhancing CLA levels in animals through the diet can improve the nutritional quality of their carcass. It is known that CLA levels are related to the expression of transcription factors and other genes as well as to the activity of enzymes involved in lipid metabolism (Minghetti et al., 2011; Mondragón, 2016; Zou et al., 2018) in a lowering-lipid effect. It has been indicated that CLA induces the expression of PPARγ and PPARα (Dong et al., 2014), important transcription factors for lipid metabolism regulation involved in the reduction of lipogenesis and increase on lipolysis, through β-oxidation (Minghetti et al., 2011). Also, CLA reduced adipose cell mass by inhibiting the activity of lipoprotein lipase, stearoyl-CoA desaturase, and fatty acid-binding protein (Park et al., 1999), reducing fatty acids uptake and transport (Royan and Navidshad, 2015). As a result of CLA and its effects on PPARs, activation of Δ5 and Δ6 desaturases, enzymes responsible for desaturation of polyunsaturated fatty acids into highly unsaturated fatty acids have been reported (Kennedy et al., 2006; Pereira et al., 2003; Zuo et al., 2013). Thus, enrichment with CLA in fish diets will not only result in increased CLA in fish tissues, but it can also reduce total lipid deposition (Mersmann, 2002) and improve its nutritional quality (Kennedy et al., 2007).

Though not able to synthesize CLA as well as other animals, when fed dietary CLA, some fish species can incorporate this fatty acid into the muscle, e.g. *Salmon salar* (Kennedy et al., 2005), *Oncorhynchus mykiss* (Valente et al., 2007), and *Dicentrarchus labrax* (Makol et al., 2013). Others incorporate it into the whole body, such as *Pelteobagrus fulvidraco* (Tan et al., 2010); whole body and filet, such as *Oreochromis niloticus* (Dos Santos et al., 2011); or in the carcass, as seen in *Astyanax altiparanae* (Campelo et al., 2015).

Astyanax altiparanae is an omnivorous species with a high carcass yield (70-85%) (Ferreira et al., 2014; Campelo et al., 2015; Salaro et al., 2015). The *A. altiparanae* market consists of two slaughter sizes, 4-5 g and 10 g, and the fish has great relevance as bait, in sport fishing, in snacks (Porto-Foresti et al., 2005), and as a canned product (Dutra et al., 2012). *Astyanax altiparanae* has also been highlighted as a useful experimental research model (Pontes et al., 2019) due to its small size, prolificacy, and short production cycle. The ability of *A. altiparanae* to incorporate CLA by dietary intake was verified by Campelo et al. (2015). After 90 days of trial, the authors concluded that the highest incorporation of CLA in *A. altiparanae* carcass was archived by fish fed the diet with the highest CLA inclusion, 2.5% of total fatty acids diet. In general, the studies that evaluate the ability of fish to incorporate CLA through dietary intake focused on increasing levels of CLA into the diets (Kennedy et al., 2005; Valente et al., 2007; Tan et al., 2010; Dos Santos et al., 2011; Makol et al., 2013). However, the ability of fish to modify its fatty acid reflects not only the species or dietary composition but also the time of feed administration (Twibell et al., 2001; Justi et al., 2003). Few studies have evaluated the effect of feeding time on the deposition of CLA in fish regarding the quality of the final product for the consumers (Dos Santos et al., 2011; Ramos et al., 2008) with the ideal feeding time varying between 30 to 56 days for Nile tilapia (Dos Santos et al., 2011) and rainbow trout (Ramos et al., 2008), respectively. Considering the differences between species and the costs involved in the inclusion of commercial CLA into the diets, become essential to determinates the minimum feeding period of dietary CLA that allows high and acceptable deposition of CLA into fish tissues.

Therefore, this study was carried out to evaluate the effects of feeding a diet with CLA to *A. altiparanae* for different periods on CLA incorporation by the animal, fatty acid profile, and carcass nutritional quality index.

MATERIAL AND METHODS

Research on animals was conducted according to the institutional committee on animal use and was approved by the Ethical Principles for Animal Research (approval. nº 01/2014) established by the National Council of Animal Experimentation Control (CONCEA) on February 12, 2014.

The formulated test diet contained 32% of crude protein, 15 MJ kg^{-1} gross energy and 2.5% of CLA (Table 1). The CLA inclusion level was based on Campelo et al. (2015). Commercial CLA (LUTA-CLA® 60-BASF, São Paulo, SP, Brazil) was used, containing 60% CLA methyl esters as a 50:50 mixture of *c9,t11* and *t10,c12* isomers.

The experimental diet was prepared by grinding all dry macro ingredients in a hammer mill (TRF-400 Trapp, Jaraguá do Sul, SC, Brazil) to a powder (0.5-mm sieve). Micro and macro ingredients were manually mixed. Conjugated linoleic acid was first mixed with soybean oil in the amount of 2.5%, which was then added to the dry mixture along with 500 mL kg^{-1} of water (50°C). The diet was pelleted in an electric meat grinder (Tecnal, Piracicaba, SP, Brazil) and then dried in a forced-recirculation oven at 50°C for 24 h. Pellets were ground in a hammer mill (TRF-400 Trapp), passed through a 2.0-2.5 mm sieve (Tecnal), and then stored at –20°C until use.

The trial was set up using a completely randomized design with eight treatment durations (0, 7, 14, 21, 28, 35, 42, and 49 days in which a diet supplemented with CLA was provided), with four replicates each. To compound the control group (time zero), fish were sampled at the beginning of the trial, without any contact with the diet containing CLA. *Astyanax altiparanae* juveniles (3.4 ± 0.3 g) were stocked at a density of 0.3 fish L^{-1} in circular polyethylene aquariums (200 L of water), with each experimental unit consisting of an aquarium with 60 fish. The aquariums were equipped with mechanical and biological filters, constant aeration systems, and continuous water flow systems.

Table 1. Formulation, chemical composition, and fatty acid profile[1] of the experimental diet (dry matter basis) with 2.5% of CLA inclusion.

Ingredient[2]	(%, as-fed)
Soybean meal	40.0
Cottonseed meal	10.0
Corn gluten meal	12.73
Cornmeal	8.05
Wheat bran	10.0
Rice meal	9.0
Soybean oil	0.5
CLA[3]	4.5
L-lysine	0.4
DL-methionine	0.2
Dicalcium phosphate	3.5
Mineral and vitamin mix[4]	0.5
Vitamin C	0.1
Salt	0.5
Butylated hydroxytoluene[5]	0.02
Total	100.0
Chemical composition	**(%)[d]**
Dry matter (%, as-fed)	93.5
Crude protein	31.9
Crude lipid	9.4
Ash	10.2
Gross energy (MJ kg^{-1})[6]	15.9
Fatty acid profile	**Results expressed as a percentage (%) of the total fatty acids**
14:00	0.015
16:00	1.10
18:00	0.32
18:1 n-9	3.09
18:2 n-6	2.45
18:3 n-6	0.007
18:3 n-3	0.16
18:2 (*c9,t11*)	1.18
18:2 (*t10,c12*)	1.67
SFA	1.44
MUFA	3.09
PUFA	5.48
PUFA/SFA	0.38
n-6	2.46
n-3	0.16
n-6/n-3	15.38
Total CLA	2.86

CLA - conjugated linoleic acid; SFA - saturated fatty acids; MUFA - monounsaturated fatty acids); PUFA - polyunsaturated fatty acids. [1]The fatty acids were identified and determined at the Cromalimentos Laboratory (State University of Maringa, PR, Brazil). [2]Manufacturers: Soybean meal, corn meal, corn gluten meal (Cargill Inc., Brazil); cottonseed meal (Maeda S.A., Brazil), wheat bran (Vilma Alimentos, Brazil); rice meal (Rozcampo, Brazil); dicalcium phosphate (Serrana S.A., Brazil); salt (National Refinery S.A., Brazil); soybean oil (ADM Ltd., Brazil); DL-methionine (Evonik Ind., Brazil); L-lysine (Ajinomoto Ind. and Com. Ltd., Brazil); vitamin C (Saint Charbel Farm., Brazil). [3]LUTA-CLA 60®, BASF, São Paulo, SP, Brazil, containing 60% of CLA. [4] Provides per kilogram of product: vit. A - 1,200,000 IU; vit. D3 - 200,000 IU; vit. E - 12,000 mg; vit. K3 - 2,400 mg; vit. B1 - 4,800 mg; vit. B2 - 4,800 mg; vit. B6 - 4,000 mg; vit. B12 - 4,800 mg; folic acid - 1,200 mg; calcium pantothenate - 12,000 mg; vit. C - 48,000 mg; biotin - 48 mg; choline - 65,000 mg; niacin - 24,000 mg; Fe - 10,000 mg; Cu - 6,000 mg; Mg - 4,000 mg; Zn - 6,000 mg; I - 20 mg; Co - 2 mg; Se - 20 mg (Guabi Nutrição Animal, Brazil). [5] ISOFAR Ind., Brazil. [6]Values determined at the Laboratory of Animal Science of Federal University of Viçosa, MG, Brazil. Crude protein was analyzed according to Silva and Queiroz (2002). Total lipids, dry matter, and ash were determined based on the methods of the Association of Official Analytical Chemists (AOAC, 2000) and gross energy was determined using a bomb calorimeter.

The system allowed a total water exchange of five times a day in each aquarium. Water temperature was maintained at 28 ± 1 °C, dissolved oxygen levels were 6.5 ± 1 mg L^{-1}, total ammonia was below 0.02 mg L^{-1}, and pH was 6.5 ± 0.3. Water parameters were measured weekly using a multiparameter meter (HI 9828, Hanna Instruments, Barueri, SP, Brazil). The water temperature was monitored daily using a standard alcohol thermometer. The photoperiod was adjusted to 12 h using fluorescent lamps (60 W) with a timer control.

Fish were fed the experimental diet four times daily (8h00, 11h00, 14h00, and 17h00) to satiation, for a total of 49 days. On the first day and every seven days thereafter, seven fish were sampled per replicate. They were then slaughtered with a super dosage of clove oil (400 mL L^{-1}). The fish carcasses were washed with distilled water to remove possible residues of clove oil, according to Kildea et al. (2004). Carcass was considered fish without scales and viscera.

For the total lipid determination, the fish from each replicate were crushed and homogenized. Then 15-g samples were extracted with a mixture of chloroform, methanol, and water (2:2:1, 8 v/v), according to the Bligh and Dyer (1959) method.

Fatty acid methyl esters (FAME) were prepared according to Hartman and Lago (1973). The methyl esters were separated using a CP-3380 gas chromatograph (Varian, USA) equipped with a flame ionization detector and a fused silica capillary column (100 m × 0.25 mm id × 0.25 µm cyanopropyl film thickness) (Varian FAME Select CP-7420). The gas (White Martins) flow rates were 1.2 mL min^{-1} for the carrier gas (H_2), 30 mL min^{-1} for the make-up gas (N_2), 30 mL min^{-1} for the flame gas (H_2), and 300 mL min^{-1} for synthetic air. The sample split ratio was 1/100. The chromatographic conditions for the FAME separations were optimized from the previous study, it was used injection point and detector temperatures of 220 and 240°C, respectively. The oven conditions of the method used an initial column temperature programmed to 165°C for 12 min, increased from 165 to 235°C at 5°C min^{-1}, and kept at 235°C for 9 min (Campelo et al., 2015). The FAME were identified by comparison to the retention times of a mixture of FAME and methyl esters containing the *c9, t11* and *t10, cis12* geometric isomers of CLA (189-19 and O5632 Sigma, Saint Louis, MO, USA) and by spiking samples with the standard. All samples (2 µL) were injected in duplicate. Peak areas were determined using Star 5.0 software (Varian, Palo Alto, CA, USA), and data were expressed as percentages of the normalized area of fatty acids.

To investigate the nutritional lipid quality of fish as well as the modifications along the time of feed administration, important indicators of nutritional quality index (NQI) were used as suggested by Ulbricht and Southgate (1991), Santos-Silva et al. (2002), and Rodrigues et al. (2017). Polyunsaturated/saturated fatty acid (PUFA/SFA) ratio, n-6/n-3 ratio, docosahexaenoic/eicosapentaenoic acid (DHA/EPA) ratio, EPA+DHA, atherogenicity index (AI), thrombogenicity index (TI), and hypocholesterolemic/hypercholesterolemic ratio (h/H) were determined as follows:

$$"AI = (4 \times C14:0 + C16:0) / (\sum MUFA + \sum n\text{-}6 + \sum n\text{-}3)"$$

"TI = (C14:0 + C16:0 + C18:0)/(0.5 × ∑MUFA + 0.5 × ∑n-6 + 3 × ∑n-3 + 100 × ∑n-3/∑n-6)"

"h/H = (C18:1n9 + C18:2n-6 + C20:4n-6 + C18:3n-3 + C20:5n-3 + C22:6n-3) / (C14:0 + C16:0)"

The statistical model was as follows:

$$yij = \mu + ti + eij\ ;$$

in which y: response variable; μ: parametric mean; ti: effect of treatment level i; and eij: experimental error.

Data were expressed as mean ± pooled standard error of the mean (SEM). Normality and homogeneity of variances were tested using the Shapiro-Wilk and Levene tests, respectively. All statistical procedures were performed using SPSS software package for Windows (IBM® SPSS® Statistics, New York, USA). ANOVA of the regression was applied, with a decomposition of the treatment effect in orthogonal polynomial contrasts for linear and quadratic effects of days of CLA feeding. Principal component analysis (PCA) was conducted on the specific and total fatty acids and nutritional quality indexes to identify clustering. Pearson's correlation coefficient test was selected to perform correlation analyses.

RESULTS

Feeding duration significantly modified the incorporation in % of total fatty acids of CLA and its isomers and the fatty acid profile of the fish carcasses (Table 2). The incorporation of CLA in the fish carcasses increased quadratically throughout the feeding trial. The CLA isomers showed distinct incorporation patterns, which included a linear effect for *t10,c12* and a quadratic effect for *c9,t11* (Figure 1).

Table 2. Fatty acid profile (% of total fatty acids) and nutritional quality indexes in the carcass of *A. altiparanae* fed a diet with 2.5% of CLA for different periods[1].

Fatty acid	Days of feeding								SEM	Contrast[2]	
	0	7	14	21	28	35	42	49		Linear	Quadratic
16:00	22.87	22.99	21.78	20.94	21.71	21.53	21.13	21.25	0.2	0.003	0.002
18:00	7.45	7.66	7.34	7.31	7.42	7.27	7.29	7.36	<0.1	0.040	0.366
16:1n5	4.01	3.61	3.52	3.43	3.34	3.21	3.03	3.07	0.1	<0.001	<0.001
18:1n9	43.23	41.29	42.00	43.07	41.54	40.68	39.81	39.63	0.3	<0.001	0.020
18:2n6	11.29	12.31	12.32	12.53	12.50	13.18	13.43	13.58	0.1	<0.001	0.149
18:2c9t11	0.00	0.21	0.31	0.36	0.75	1.10	1.37	1.29	0.1	<0.001	0.003
18:2t10c12	0.00	0.22	0.26	0.32	0.57	0.80	0.96	0.96	0.1	<0.001	0.094
18:3n3	0.74	0.77	0.77	0.83	0.83	0.76	0.83	0.79	<0.1	0.038	0.038
20:4n6	0.05	0.05	0.06	0.07	0.06	0.05	0.06	0.05	<0.1	0.456	0.006
20:5n3	0.16	0.17	0.18	0.21	0.18	0.16	0.17	0.18	<0.1	0.618	0.014
22:6n3	1.14	1.25	1.52	1.26	1.22	1.36	1.54	1.61	<0.1	<0.001	0.147
Total CLA	0.00	0.44	0.57	0.68	1.32	1.90	2.33	2.25	0.2	<0.001	0.003
SCFA	nd	nd	nd	nd	nd	nd	nd	nd			
MCFA	0.07	0.05	0.05	0.05	0.06	0.04	0.04	0.05	<0.1	<0.001	0.029
LCFA	95.31	94.74	94.12	94.55	94.72	94.40	93.94	93.82	0.1	<0.001	0.618
SFA	32.09	32.29	30.78	29.90	30.85	30.40	30.01	30.21	0.2	0.004	0.008
MUFA	57.07	51.03	52.16	52.90	51.43	50.55	49.92	49.62	0.3	<0.001	0.002
PUFA	14.84	16.57	17.06	17.21	17.72	19.06	20.07	20.16	0.4	<0.001	0.580
n-6	12.08	13.14	13.16	13.40	13.35	14.03	14.31	14.45	0.2	<0.001	0.144
n-3	2.76	3.00	3.33	3.13	3.04	3.13	3.43	3.47	0.1	<0.001	0.786
c10,t12/c9,t11	0.00	1.02	0.86	0.87	0.76	0.73	0.70	0.75	<0.1	<0.001	<0.001
PUFA/SFA	0.46	0.51	0.55	0.58	0.57	0.63	0.67	0.67	<0.1	<0.001	0.213
n6/n3	4.37	4.39	3.96	4.28	4.39	4.49	4.17	4.17	<0.1	0.425	0.767
DHA/EPA	7.12	7.14	8.62	6.15	6.90	8.31	9.15	9.09	0.2	0.001	0.001
EPA+DHA	1.30	1.42	1.69	1.47	1.39	1.52	1.70	1.78	<0.1	<0.001	0.255
AI	0.44	0.46	0.43	0.40	0.43	0.43	0.42	0.42	<0.1	0.032	0.002
TI	0.16	0.17	0.19	0.16	0.16	0.16	0.18	0.18	<0.1	0.031	0.442
h/H	2.38	2.34	2.52	2.67	2.50	2.52	2.56	2.54	<0.1	0.013	0.002

CLA (Conjugated Linoleic Acid); SFA (Saturated fatty acid); MUFA (Monounsaturated fatty acid); PUFA (Polyunsaturated fatty acid); MCFA (Medium-chain fatty acids); LCFA (Long-chain fatty acids); EPA (Eicosapentaenoic acid); DHA (Docosahexaenoic acid); AI (Atherogenicity Index); TI (Thrombogenicity Index); h_H (Hypocholesterolemic/hypercholesterolemic ratio). [1]Values presented as means (n = 12) and pooled standard error of the mean (SEM).

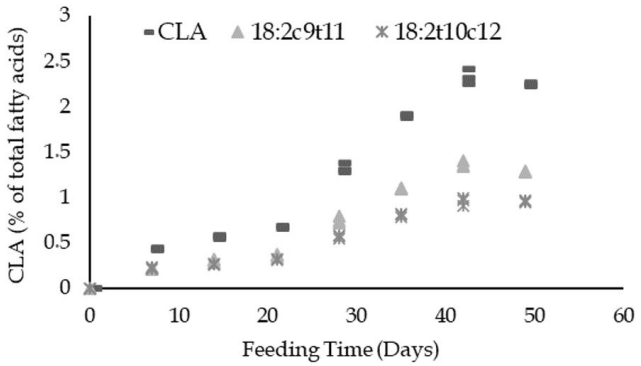

Figure 1. CLA incorporation in the carcass of *A. altiparanae* fed a diet with 2.5% of CLA for different periods. CLA - conjugated linoleic acid ($y = 0.0001x^2 + 0.0443x - 0.0124$, $r^2 = 0.9511$); 18:2c9t11 ($y = 1E\text{-}04x^2 + 0.025x - 0.0232$, $r^2 = 0.9436$); 18:2t10c12 ($y = 0.0209x - 0.0006$, $r^2 = 0.9578$).

A linear increase for linoleic acid (18:2n-6) and a quadratic effect for linolenic acid (18:3n-3) was observed as the feeding period increased. Docosahexaenoic acid (22:6n-3) increased linearly after 49 days of feeding, while EPA (20:5n-3) and arachidonic acid (ARA, 20:4n-6) presented quadratic effects.

Quadratic effects were observed for saturated (SFA) and monounsaturated fatty acid (MUFA) incorporation with feeding time. After 49 days of feeding, the content of SFA reduced from 32 to 30% of total fatty acids, whereas MUFA decreased from 57 to 50%. Polyunsaturated fatty acids and n-3 and n-6 fatty acids increased linearly with the feeding period. Every seven days throughout the trial, a 1.03% increase in PUFA was observed in the fish carcasses. There were not detected short-chain fatty acids in fish carcasses. The sum of medium-chain (MCFA) and long-chain fatty acids (LCFA) also decreased linearly with feeding time. Additionally, the *t10,c12/c9,t11* ratio in the carcasses reduced quadratically with the CLA feeding period, with ratios close to 1.0 observed in the fish fed the CLA diet for seven days and 0.75 in those which received it for 49 days.

As the CLA feeding period increased, PUFA/SFA, EPA+DHA, and TI in the fish carcasses also increased (Table 2). A quadratic effect was observed for DHA/EPA and AI. No difference was observed for the n-6/n-3 ratio.

Principal component analysis was used to visualize data groupings, associations, and correlations between feeding period, specific fatty acids, total fatty acids (SFA, MUFA, PUFA, MCFA, LCFA, n-3, and n-6), and NQI (TI, AI, h/H, n-6/n-3, PUFA/SFA, DHA/EPA, and EPA+DHA). Strong associations were found as evaluated by Pearson's correlation coefficient between specific fatty acids, total fatty acids, and NQI for the fish fed CLA for increasing periods (Table 3). Similarly, CLA isomers and total CLA were positively correlated (r >0.90) with PUFA, n-6 fatty acid, PUFA/SFA ratio, and feeding period. A negative correlation was observed for CLA (-0.67) and its isomers *c9t11* and *t10c12* (-0.68 and -0.67, respectively) for the sum of MCFA. There was no strong correlation between feeding period or CLA and the h/H, AI, and TI indexes (r<0.50); however, SFA were negatively correlated with h/H (r = -0.96) and positively with the AI index (r = 0.93). The TI index was negatively correlated with the n-6/n-3 ratio (r = -0.89) and positively with DHA (0.86), n-3 fatty acids (0.84), and EPA+DHA (0.87). The DHA and n-3 fatty acid contents were positively correlated with EPA + DHA (r ≥0.97). Between the n-6 and n-3 fatty acids, n-6 was mainly responsible for the increases in PUFA (r = 0.99) and PUFA/SFA (r = 0.98) in fish carcasses.

Principal component analysis explained 75.40% of the observed variance (Figure 2); principal component 1 contributed most to the variance (59.39%), followed by component 2 (16.02%). Principal component 1 divided the data into two groups. The first, which consisted of 35, 42, and 49 days of feeding with CLA, was positively correlated with most healthy fatty acids, including CLA, PUFA, n-3, and NQI (h/H, PUFA/SFA, DHA/EPA, and EPA+DHA). The second group, consisting of 0, 7, 14, 21, and 28 days of feeding with CLA, was associated with parameters correlated with the excess of lipid deposition and consequently health disturbs (SFA, MUFA, MCFA, and respective specific fatty acids) and NQI (AI and n-6/n-3).

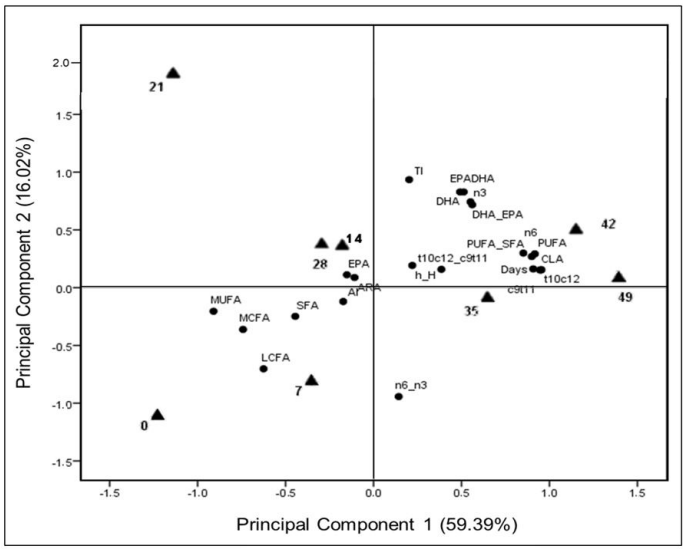

Figure 2. Specific fatty acids (FA), total FAs, nutritional quality index (NQI) and days of the different feeding time in the plane defined by two principal components. CLA (Conjugated Linoleic Acid); SFA (Saturated fatty acid); MUFA (Monounsaturated fatty acid); PUFA (Polyunsaturated fatty acid); MCFA (Medium-chain fatty acids); LCFA (Long-chain fatty acids); EPA (Eicosapentaenoic acid); DHA (Docosahexaenoic acid); DHA_EPA (DHA/EPA ratio); PUFA_SFA (PUFA/SFA ratio); EPADHA (EPA+DHA); AI (Atherogenicity Index); TI (Thrombogenicity Index); h_H (Hypocholesterolemic/hypercholesterolemic ratio).

Table 3. Results of Pearson's correlation between days of feeding, specific fatty acids, total fatty acids, and nutrient quality index of fish fed a diet with 2.5% CLA.

	Days	**Total CLA**	**SFA**	**MUFA**	**PUFA**	**MCFA**	**LCFA**	***n-6***	***n-3***	***n6/n3***	**PUFA/ SFA**	***c10.t12/ c9.t11***	**DHA/ EPA**	**EPA+ DHA**	**AI**	**TI**	**h/H**
C20:4n6	.094	-.007	-.327	.174	.064	.053	-.195	.078	.247	-.341	.123	.382*	-.272	.101	-.377*	.255	.432*
C20:5n3	.051	-.109	-.358*	.218	.051	.070	-.247	.150	.285	-.325	.137	.472**	-.349*	.205	-.415*	.191	.530**
C22:6n3	.658***	.645***	-.606**	-.569**	.764***	-.660***	-.963***	.750***	.966***	-.741***	.772***	.329	.876***	.997***	-.378*	.862***	.464*
C18:2*c9t11*	.970***	.999***	-.611**	-.844***	.966***	-.669***	-.720***	.917***	.681***	-.055	.931***	.229	.665***	.621**	-.337	.342	.386*
C18:2*t10c12*	.979***	.999***	-.622**	-.850***	.977***	-.683***	-.741***	.938***	.698***	-.060	.943***	.288	.654***	.636***	-.352*	.348*	.403*
Days	1.000	.975***	-.684***	-.785***	.965***	-.619**	-.756***	.931***	.722***	-.109	.950***	.299	.591**	.653***	-.433*	.363*	.494**
Total CLA		1.000	-.614**	-.848***	.971***	-.674***	-.729***	.926***	.688***	-.057	.936***	.254	.661***	.627**	-.342	.345*	.392*
SFA			1.000	.164	-.701***	.381	.694***	-.719***	-.717***	.350*	-.815***	-.256	-.384*	-.623**	.932***	-.324	-.960***
MUFA				1.000	-.819***	.709***	.621**	-.783***	-.554**	.008	-.704***	-.352*	-.650***	-.544**	-.152	-.392*	.077
PUFA					1.000	-.734***	-.853***	.985***	.819***	-.210	.984***	.404*	.694***	.757***	-.433*	.473*	.503**
MCFA						1.000	.730***	-.751***	-.647***	.199	-.683***	-.583**	-.666***	-.642***	.162	-.464***	-.215
LCFA							1.000	-.859***	-.986***	.648***	-.865***	-.507**	-.779***	-.969***	.467*	-.800*	-.558**
n-6								1.000	.819***	-.192	.978***	.513**	.633***	.750***	-.467*	.446***	.545**
n-3									1.000	-.720***	.842***	.457**	.760***	.975***	-.503**	.835***	.600**
n6/n3										1.000	-.257	-.170	-.523**	-.759***	.286	-.893*	-.362*
PUFA/SFA											1.000	.391*	.656***	.770***	-.582**	.460	.647***
c10.t12/c9.t11												1.000	.056	.360*	-.186	.323	.259
DHA/EPA													1.000	.837***	-.135	.701***	.166
EPA+DHA														1.000	-.402*	.868***	.496**
AI															1.000	-.148	-.976***
TI																1.000	.242
h/H																	1.000

CLA (Conjugated Linoleic Acid); SFA (Saturated fatty acid); MUFA (Monounsaturated fatty acid); PUFA (Polyunsaturated fatty acid); MCFA (Medium-chain fatty acids); LCFA (Long-chain fatty acids); EPA (Eicosapentaenoic acid); DHA (Docosahexaenoic acid); AI (Atherogenicity Index); TI (Thrombogenicity Index); h_H (Hypocholesterolemic/hypercholesterolemic ratio). *p <0.05; **p <0.01; ***p <0.001.

DISCUSSION

The observed incorporation of CLA in *A. altiparanae* carcasses was higher than the levels naturally found in beef (0.12 - 1.0%), sheep meat (0.43 - 1.9%), dairy products (0.03 - 0.55%) (Schmid et al., 2006) which are considered to be the main natural sources of CLA in the human diet, and so for some fish species (0.01 - 0.09%) (Fritsche and Steinhart, 1998). The incorporation of CLA in *A. altiparanae* carcasses obtained in the present study highlighted the ability of this species to incorporate CLA, compared to other fish species also fed diets that included CLA.

For instance, feeding 2.5% of CLA to juvenile *A. altiparanae* for 49 days resulted in 2.25% of CLA in the fish carcass, while feeding 10% of CLA to juvenile *D. labrax* for 140 days resulted in CLA incorporation of 1.45% in the carcass (Makol et al., 2013). Similarly, *O. mykiss* fed 1% of CLA for 140 days showed a CLA content of only 1.3% in the carcass (Ramos et al., 2008). *Oreochromis niloticus* fed 1% of CLA for 60 days (Dos Santos et al., 2011) and *Piaractus mesopotamicus* fed 1% of CLA for 49 days (Barilli et al., 2014) had similar CLA incorporation in the filet when compared with *A. altiparanae* (3.3 and 2.0% of CLA, respectively). In addition to species, other factors such as diet supplementation, feeding time, ingredient composition, and the proportion of CLA isomers in the diet may also affect CLA incorporation in animal tissues (Azain, 2003).

Greater incorporation of *c9,t11* relative to *t10,c12* (1.37 and 0.96%, respectively) may be associated with differences in the metabolism of isomers by this fish species. According to Martin et al. (2000), the *t10,c12* isomer could promote lipid oxidation as a result of the position of its double bonds. Higher incorporation of *c9,t11* relative to *t10,c12* was also observed for *O. mykiss* (Bandarra et al., 2006), *P. fulvidraco* (Tan et al., 2010), *O. niloticus* (Dos Santos et al., 2011), and *Synechogobius hasta* (Tan et al., 2014) fed increasing levels of CLA. Considering the observed reductions in the *t10,c12/c9,t11* ratio as the duration of feeding increased, it can be inferred that it was caused by the difference between the incorporation rates of CLA isomers. These results suggest that CLA isomers could be included in the diet in certain proportions, to maintain equimolar proportions of isomers incorporated into animal tissues, with a higher dietary intake of *t10,c12* than *c9,t11*.

The duration of feeding a diet with 25 g CLA kg^{-1} has a significant impact on the fatty acid profile of fish carcass. Increased incorporation of n-6, n-3 in the fish carcass, in addition to reductions in the levels of SFA and MUFA until a certain feeding period, may be associated with changes in lipid metabolism caused by CLA, such as induction of β-oxidation and changes in the gene expression of transcription factors and enzymes, increase on the expression of PPARγ and PPARα (Dong et al., 2014) and downregulation of enzymes involved on fatty acids uptake and *de novo* fatty acids synthesis (Royan and Navidshad, 2015). In the group of fatty acids, SFA and MUFA are preferable substrates for β-oxidation when compared with PUFA, and n-6 fatty acids are more suitable for oxidation than n-3 (Henderson, 1996). Because one of the effects of CLA on lipid metabolism is to induce β-oxidation, due to increases in activity and expression of carnitine palmitoyltransferase type I (Nagao et al., 2005), this effect may have caused reductions of SFA and MUFA in *A. altiparanae* carcasses. Moreover, several studies have suggested that CLA can alter the expression of genes related to lipid metabolism (Yessoufou et al., 2009; Minghetti et al., 2011; Mondragón, 2016; Zou et al., 2018), especially the peroxisome proliferator activated receptors (PPARs), which act as transcription factors in various tissues such as liver, muscle, and adipose tissue (Yessoufou et al., 2009). Peroxisome proliferator-activated receptor alpha (PPARα) and gamma (PPARγ) are of great importance to the regulation of lipid metabolism (Minghetti et al., 2011), with increases in their activity being correlated with reductions in lipogenesis and increases in lipolysis through β-oxidation.

For instance, diets with increasing levels of CLA (up to 3% of) fed to *Ctenopharyngodon idella* altered the activation of transcription factors (PPARα and PPARγ), lipogenic enzymes (fatty acid synthetase and acetyl-CoA carboxylase), lipolytic enzymes (hormone-sensitive lipase), and lipoprotein transporters (lipoprotein lipase) (Dong et al., 2014). In *Salmo salar L.* fed 10 and 20 g CLA kg^{-1}, a positive relationship was found between the PPARα gene expression levels and the activation of the Δ5 and Δ6 desaturase enzymes (Kennedy et al., 2006), front-end desaturases responsible for catalyzing the introduction of double bonds into linoleic acid (18:2n-6) and linolenic acid (18:3n-3) chains, that after the action of elongases, convert them to ARA (20:4n-6), EPA (20:5n-3), and DHA (22:6n-3), respectively (Pereira et al., 2003). Zuo et al. (2013) suggested that the increase in highly unsaturated fatty acids (HUFA) in the muscle of *Larimichthys crocea* fed diets with increasing levels of CLA (0, 0.42, 0.83, and 1.7% of) may be due to an increase in the expression of the Δ5 and Δ6 desaturases.

Throughout the duration of feeding with CLA, fatty acids probably underwent the elongation and desaturation processes, ultimately being converted to HUFA, mainly through the n-3 series, since increases in DHA in fish carcasses were much more apparent than its precursor. The decreases in EPA observed in the *A. altiparanae* carcass, after 21 days of feeding with CLA, may be related to preference for DHA synthesis in this species, as DHA is synthetized from EPA (Sprecher, 2000). Additionally, Murru et al. (2018) proposed that a ratio of CLA to α-linolenic acid (ALA) around 3:1 may enhance DHA production from ALA. In this respect, our data demonstrate that the inclusion of CLA in fish feed is clearly a good strategy to improve the DHA content in fish by-products. It is even more efficient than dietary ALA intake, since the CLA/ALA ratio after 35, 42, and 49 days of feeding ranged between 2.5 to 2.8, which is close to ratios recommended by Murru et al. (2018).

The opposite was observed for ARA and linoleic acid, where only a slight level of incorporation of ARA was observed. It is known that this fatty acid and CLA compete for biding sites (Whigham et al., 2002; Park and Pariza, 2007; Stachowska et al., 2009), which could be a reason for the observed reductions in ARA in fish carcasses after 21 days of feeding. Another reason could be due to a potential preference for the elongation and desaturation of the n-3-series fatty acids (Tocher and Sargent, 1990) over the n-6.

Considering the biological effects and functions of fatty acids on human health, calculating the nutritional quality indexes of fatty acid profiles is highly important for indicating the quality of products as well as for recommendations to be made about food intake for humans. To be considered a healthy product for human consumption, the PUFA/SFA ratio must be at least 0.45, and the greater the PUFA concentration relative to SFA, the healthier the product (England Department of Health, 1994). Therefore, considering the PUFA/SFA ratios observed in the fish carcasses, *A. altiparanae* fed 2.5% of CLA for 49 days can be considered a healthy product for human consumption. Another notable highlight from this study is that the longer the duration of feeding with CLA the higher PUFA/SFA ratio. Even the increase in PUFA (in % of total fatty acids) might be due to the reduction on SFA as a dilution effect, the increase in PUFA/SFA ratio reflects the benefits of CLA incorporation into the final product.

The recommended n-6/n-3 ratio to which health benefits for humans have been attributed is around 1:1 to 5:1 (England Department of Health, 1994; Gómez-Candela et al., 2011). The duration of feeding with a CLA-rich diet did not promote changes in n-6/n-3 ratios in the tested fish carcasses, which remained close to 4:1 and within the optimal range recommended for human health. However, the differences observed with respect to the EPA + DHA content in the fish carcasses highlight the importance of the duration of feeding with CLA in improving the nutritional value of this species for human consumption. Fish fed the CLA diet for 49 days had 1.79 g of EPA + DHA in 100 g^{-1} of Total Lipids, which corresponds to 603.8 mg per 100 g of tissue. According to the European Commission Regulation, 2010 (EU) 116/2010 products with an EPA + DHA content of over 40 mg 100 g^{-1} can be considered good sources of omega 3 fatty acids for human consumption. Thus, *A. altiparanae* fed a commercial diet is considered an omega 3 source for humans. However, when fed 2.5% of CLA for 49 days, its nutritional value in terms of EPA + DHA content improved by 40%. Therefore, it is important to highlight *A. altiparanae* as a good source of PUFA n-3 for humans, which is even more true for fish fed diets that would maximize this characteristic.

The hypocholesterolemic/hypercholesterolemic ratio is used to express the effects of fatty acids on cholesterol metabolism, and high values are correlated with health benefits (Santos-Silva et al., 2002). On the other hand, low atherogenicity and thrombogenicity indexes are preferable and have been related to reduced risk of coronary disease through reductions in plaque aggregation, circulating cholesterol, and clot formation in circulating blood (Ulbricht and Southgate, 1991). Even though slight increases in were observed in TI in the present study, those values remained well below the maximum recommended level of 1.27 (Ulbricht and Southgate, 1991).

Principal component analysis was performed to identify the relationships between treatment groups (duration of feeding a CLA-containing diet to fish) and improvements observed concerning the fatty acid profile and nutritional quality of fish. Based on the results, the data were clustered in two different groups, from which 35, 42, and 49 days of feeding were positively associated with healthier NQI indexes. This behavior can be associated with the biological effects of CLA on lipid metabolism, leading to positive changes in the fatty acid composition of the fish carcass. This results in a product of improved nutritional quality for human consumption (Kennedy et al., 2007).

Thus, it can be inferred that apart from the intrinsic characteristics of the species, the ability of *A. altiparanae* to incorporate CLA within a short duration of feeding is very relevant. Therefore, feeding a diet containing CLA to *A. altiparane* as a feeding strategy aimed to improve the CLA and DHA content of fish is an alternative approach to enhance daily levels of CLA and DHA consumed by humans.

CONCLUSIONS

Astyanax altiparanae has great potential, as it can incorporate high levels of conjugated linoleic acid within a short time. Feeding diets with 2.5% of conjugated linoleic acid to *A. altiparanae* for 35 days improves the fish fatty acid profile, providing a commercial product with good nutritional quality and functional benefits.

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