Original Research Article

Fatty acid profiles and omega-3 long-chain polyunsaturated fatty acids (LC-PUFA) biosynthesis capacity of three dual purpose chicken breeds

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ABSTRACT

Westernized societies ingest an unhealthy high dietary omega-6/omega-3 fatty acid ratio of 20:1 or even higher. Seafood is the primary source of omega-3 long chain polyunsaturated fatty acids (LC-PUFA) for humans, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are involved in a plethora of physiological and health-related processes. However, the production of marine organisms with aquafeed formulations based on marine ingredients leads to serious environmental impacts on global pelagic fish resources, resulting in an un-sustainable activity. The present study aims to gain further insight into the metabolism of fatty acids in chicken as a potential supply for omega-3 LC-PUFA. To this purpose, lipid classes and fatty acid profiles of thighs and hepatocytes, and the modification of [1-^{14}C]18:3n-3 by hepatocytes isolated from three dual-purpose chicken breeds adapted to free-range culture systems were determined. Arachidonic acid highly accumulated in thighs meat (7.16–8.79 %) despite being barely supplied in the diet, with DHA (1.22–1.71 %) and n-3 docosapentaenoic acid (DPA, 22:5n-3; 1.02–1.14 %) being also relevant. Our experimental design with radiolabeled fatty acids was validated for the first time in terrestrial vertebrates. Chicken hepatocytes incubated with [1-^{14}C]18:3n-3 pro-duced a wide variety of C18-C24 intermediates demonstrating that the set of fatty acyl desaturases and elongases enzymes necessary to metabolize dietary C18 precursors are active for the production of LC-PUFA, including EPA, n-3 DPA and DHA.

1. Introduction

It is projected that by the year 2050, the world population will in-crease up to 9.7 billion people (FAO, 2018). This growth, coupled with socio-economic development and social awareness about human health is expected to boost the demand for richer diets, increasing the con-sumption of animal products. Agriculture, livestock farming and aqua-culture have been intensified in the last decades to meet the dietary necessities of world population (Troell et al., 2014; Føre et al., 2018; Longo et al., 2019). However, the intensification of aquaculture has heightened the environmental impact of the activity (Tacon et al., 2009). Within this context, one current major concern is that feeding of marine carnivorous species still relies on meals and oils derived from small pelagic fish as critical ingredients for aquafeeds (Konar et al.,

2019), contributing to the loss of fish species diversity (Kok et al., 2020). Consequently, there is a need to look for strategies to improve animal production and reduce its environmental impact while generating healthy and safe food (FAO, 2018).

Seafood is the major source of omega-3 long chain polyunsaturated fatty acids (LC-PUFA) including eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Tacon and Metian, 2013; Hicks et al., 2019) which consumption has been associated to a wide range of benefits for human health and well-being (Riediger et al., 2009; Zárate et al., 2017; Calder, 2018) whereas eggs and meat from terrestrial animals are richer in omega-6 LC-PUFA including arachidonic acid (ARA, 20:4n-6) (Calder, 2007). However, most of teleost fish lost the fatty acyl desaturase 1 (FADs1) pool, where Δ5 was found, making it difficult to obtain LC-PUFA from their C18 precursors, linolenic acid

(LNA, 18:3n-3) and linoleic acid (LA, 18:2n-6) (Tocher, 2015). The ability of each species to biosynthesize these physiologically essential fatty acids (FAs) also partially depends on their dietary availability and environmental modulation (Tocher, 2003, 2015; Castro et al., 2016). Thus, a strict carnivorous fish that gets sufficient amounts of EPA, DHA and ARA generally shows reduced capacity to elongate and desaturate C18 FAs (Geay et al., 2015). All these conditioning factors hinder the access to omega-3 LC-PUFA of marine origin in a sustainable way.

Both fish and chicken have significant productive advantages in terms of high conversion rates and nutritional quality. Chicken is characterized by a low-calorie white meat (234 kcal per 200 g serving), with an average protein content of 20 % and a good supply of sodium, iron and zinc (Moreiras et al., 2010; Valero et al., 2010). However, terrestrial herbivores do not have direct access to EPA, DHA and ARA in their diets but to their C18 FA plant-derived precursors, LNA and especially LA and, as a result, these species present higher proportions of C18 PUFAs in tissues compared to the greater presence of omega-3 LC-PUFA in aquaculture products. Nevertheless, poultry can be considered as a potential and sustainable source of LC-PUFA as they possess the enzymatic machinery involved in their biosynthesis (Gregory et al., 2013). However, the profile of PUFAs in their meat differs substantially due to factors such as diet, environmental conditions, or the genetic characteristics and expression of the enzymes involved in the metabolism of these FAs, which has been particularly little studied in poultry (Gregory et al., 2013). Then, studies focusing on the evaluation of the presence/activity of elongases of very long chain fatty acid (Elovl) and Fads enzymes necessary to synthesize EPA, DHA and ARA are currently of great interest in chicken breeds (Castro et al., 2012).

The use of dual-purpose chicken breeds is currently receiving growing attention in alternative systems due to their good productive performance in eggs and superior meat quality, adapting well to different environments and dietary regimes (Torres et al., 2019). Moreover, it is important to emphasize that farmers need to be able to advertise specific process-related and product-related (nutritional and sensorial) qualities in order to be able to fetch a price premium for their product (Muth et al., 2018). For all above, the general objectives of the present work were to evaluate the nutritional value of the meat of three dual purpose chicken breeds, through their capacity to incorporate dietary FAs, and to determine their capacity to biosynthesize LC-PUFA. To these purposes, the lipid profile (total fat content, lipid classes and FAs) of thighs meat and hepatocytes isolated from the three breeds, and the incorporation pattern of [1-^{14}C]18:3n-3 into hepatocytes and their ability to elongate and desaturate this C18 precursor to produce omega-3 LC-PUFAs were determined.

2. Materials and methods

2.1. Experimental design and sampling

Animal procedures were conducted in strict accordance with national ethics of research involving animals, the current European Animal Welfare Legislation (ART13TFEU) and institutional review board-approved protocols.

A total of 20 male chickens from each genotype (Canaria, Les Bleues and Dominant Red Barred D 459) were raised under free-range conditions until 15 wk at the experimental farm of the Instituto Canario de Investigaciones Agrarias (ICIA, Tenerife, Spain). The experimental individuals were housed in three 15 m^2 interior pens, having access to about 25 m^2 outdoors pens.

All chickens were fed with a starter feed (1–28 D), a growth standard feed (29–60 D), and a finisher feed (61–105 D), whose main ingredients were soybean meal, corn, wheat, soybean oil, barley, and calcium carbonate (Graneros de Tenerife SL, Tenerife, Spain). Feed and water were supplied *ad libitum*.

The animals were slaughtered at the slaughterhouse of the SADA P.a. Canarias SA Group (Tenerife, Spain) according to European regulations

(Torres et al., 2017). Thighs from 5 individuals of each breed were taken and immediately frozen until their biochemical analysis. At the same time, livers were removed, washed with cold Ringer's physiological solution and soaked in cold buffered HBSS physiological glucose solution prior to be transferred to the laboratory of the Department of Animal Biology, Soil Science and Geology at the University of La Laguna.

2.2. Isolation and incubation of hepatocytes with [1-^{14}C]18:3n-3

Hepatocytes were isolated as described by Rodríguez et al. (2002). Briefly, approximately 2 g of a central portion of each liver clean of fat and other tissues were finely minced and soaked into 10 mL of cold HBSS solution with collagenase (10 mg mL^{-1}) and incubated in agitation (53 u), for 30 min at 20 °C. The resultant cell suspensions were filtered through a 100 μm nylon mesh with HBSS including 1% (w/v) fatty acid free bovine serum albumin (FAF-BSA). Cells were collected by centrifugation (Beckman Coulter Allegra 25R, Indianapolis, USA) at 716 *g* for 10 min, washed with HBSS and re-centrifuged for 7 min. The isolated cells were then re-suspended in cold M199 medium and a sample was taken to assess the viability of cells (over 90 % in all cases) by the trypan blue exclusion test. A 100 μL-aliquot of each cell suspension was used to determine the protein content (Lowry et al., 1951), using FAF-BSA as standard.

Cells capability to incorporate and metabolize unsaturated FAs was studied by *in vitro* radio tracing of [1-^{14}C]18:3n-3 (specific activity 53.7 μCi mmol^{-1}; PerkinElmer Inc., Waltham, Massachusetts, USA) (Rodríguez et al., 2002). Immediately after isolation, 6 mL of cell preparation were incubated in sterile plastic flasks (TC-flask T25, Cell+; Sarstedt AG & Co., Nümbrecht, Germany) for 3 h with 40 μL (0.20 μCi) of radiolabeled [1-^{14}C]18:3n-3. After incubation, the cells were centrifuged and washed twice to remove the remaining substrate. A control group of 2 mL of cells without the addition of the radiolabeled substrate was also incubated under the same experimental conditions for further determination of hepatocytes FA profiles. Samples were stored at −80 °C until biochemical analysis.

2.3. Lipid analysis

The total lipid (TL) content of samples (diets, thigh meat and radiolabeled and non-radiolabeled isolated cells) was extracted according to Folch et al. (1957) with small modifications (Christie and Han, 2010). Briefly, cell preparations were dissolved in 2 mL of 0.88 % KCl (w/v) and 8 mL of chloroform/methanol (2:1, v/v) containing 0.01 % (w/v) butylated hydroxytoluene (BHT) as antioxidant. For the lipid extraction of diets, pellets (~100 mg) were grinded to a fine powder, 0.5 mL of distilled water added for hydration and after half an hour in the fridge, 5 mL of chloroform-methanol (2:1, v/v) added to the solution, homogenized using a Virtis rotor homogenizer (Virtishear, Virtis, Gardiner, New York, USA) and kept overnight under a N$_2$ atmosphere to prevent oxidation. The day after, a further 5 mL of chloroform/methanol (2:1, v/v) were added and re-homogenized previously to the addition of 2.0 mL of KCl (0.88 %, w/v). For the lipid extraction of chicken thighs, the tissue was directly homogenized in 10 mL chloroform/methanol (2:1, v/v). For all samples, the mixture was vigorously shaken, centrifuged at 716 *g* for 5 min, and the organic solvent collected, filtered, and evaporated under a stream of nitrogen. The whole process was developed under an ice-cold environment to prevent sample degradation. The lipid content was determined gravimetrically, the extracts re-suspended in chloroform/methanol (2:1, v/v) with 0.01 % (w/v) BHT and stored at -20 °C under an inert atmosphere of nitrogen until further analysis.

Lipid extracts of thigh meat and control cells were analyzed for lipid class (LC) and FA composition. Lipid fractions were separated by high-performance thin-layer chromatography (HPTLC, Merck, Darmstadt, Germany) in a single-dimensional double-development, and quantified by calibrated densitometry, using a dual-wavelength flying spot scanner CAMAG TLC Visualizer (Camag, Muttenz, Switzerland) as described by

Reis et al. (2019).

Up to 1 mg of lipid extract were subjected to acid-catalyzed trans-methylation to yield fatty acid methyl esters (FAME). During trans-methylation, FAME are formed simultaneously with dimethylacetals (DMA), which originate from the 1-alkenyl chain of plasmalogenic lipids and must be considered to avoid misidentifying and overestimations in intramuscular fat analysis. Resultant FAME and DMA were purified by thin layer chromatography (TLC, Macherey-Nagel GmbH & Co. KG, Düren, Germany), separated and quantified using a TRACE-GC Ultra gas chromatograph (Thermo Scientific, Milan, Italy) equipped with an on-column injection, a flame ionization detector (FID) and a fused silica capillary column Supelcowax® 10 (30 m ×0.32 mm ID, df 0.25 µm) (Supelco Inc., Bellefonte, PA, USA). Nonadecanoic acid (19:0) was used as an internal standard. Helium was used as carrier gas at a flow rate of 1.5 mL min^{-1}, and oven temperature programming was from 50 to 150 °C at a rate of 40 °C min^{-1}, then from 150 °C to 200 °C at 2 °C min^{-1}, to 214 °C at 1 °C min^{-1} and, finally, to 230 °C at 40 °C min^{-1}, which was maintained for 3 min. Individual FAME were identified by reference to a mixture of authentic standards (Mix C4-C24 and PUFA No. 3 from menhaden oil, Supelco Inc.), and to a well characterized cod roe oil. When necessary, identity of FAME was confirmed by GC–MS (DSQ II, Thermo Scientific).

2.4. Metabolism of [1-^{14}C]18:3n-3 by hepatocytes. Incorporation of radioactivity into total lipids and bioconversion of radiolabeled fatty acids

A 100-µg aliquot of TL extract from hepatocytes incubated with [1-^{14}C]18:3n-3 was transferred to scintillation vials to determine the radioactivity incorporated into isolated cells by means of a LKB Wallac 1214 RackBeta liquid scintillation β-counter (PerkinElmer Inc., Waltham, Massachusetts, USA). Results obtained in dpm were transformed to pmol mg prot^{-1} h^{-1} taking into account the specific activity of the radiolabeled substrate and the cell TL content.

To determine the esterification pattern of [1-^{14}C]18:3n-3 into LC and the capacity of hepatocytes to remodel the uptaken radiolabeled FA, a 0.1-mg aliquot of TL extract was applied to HPTLC plates and separated as detailed in Reis et al. (2019). The developed HPTLC plates were placed in closed exposure cassettes (Exposure Cassette-K, BioRad, Madrid, Spain) in contact with a radioactive-sensitive phosphorus screen (Image Screen-K, BioRad) for two weeks. The screens were then scanned with an image acquisition system (Molecular Imager FX, Bio-Rad) and the bands quantified to obtain the percentage distribution of the radioactivity incorporated into each LC using Quantity One 4.5.2 (BioRad) image-analysis software.

Finally, transformation of the incubated [1-^{14}C]FA by desaturation/ elongation processes was determined using pre-coated TLC plates G-25 (Macherey-Nagel GmbH & Co. KG) pre-impregnated with a solution of 2 g silver nitrate in 20 mL of acetonitrile. The plates were developed in toluene/acetonitrile (95:5, v/v), separating radioactive FAs according to their chain length and degree of unsaturation. Developed TLC plates were placed in closed exposure cassettes, treated and quantified as described above. The identification of labelled bands was confirmed by radiolabeled standards formulated with a blend of commercially available ^{14}C-FA substrates (including 18:1n-9, 18:2n-6, 18:3n-3, 18:4n-3, 20:4n-3, 20:5n-3 and 22:6n-3; Fig. S1) at a concentration of 1 µCi mL^{-1} hexane each, simultaneously run on the same plate (Galindo et al., 2020). Radioactivity of the standard mixture was previously validated by means of a scintillation β-counter (PerkinElmer Inc., Waltham).

2.5. Statistical analysis

Results are presented as mean ± standard deviation (SD). Statistical differences in TL content, lipid classes and FAME profiles, incorporation of the radioactive substrate into TL, esterification into lipid classes and the pattern of elongation and desaturation between breeds were determined by a one-way ANOVA followed by a Tukey multiple comparison

post-hoc test. Prior to analysis, normal distribution of the data and homogeneity of variances were verified by the Kolmogorov-Smirnoff and the Levenne test, respectively. When homocedasticity or normality was not achieved, the arcsine square root, inverse or logarithmic transformations were applied. If transformation did not succeed, the Welch test was performed to check the effect of the factor and, subsequently, multiple posterior comparisons were made using the Games Howell test. A principal component analysis (PCA) was performed to reduce the dimensionality of the data set, specifically for the study of FAs from thigh meat and from control and radioactive hepatocytes. Inspection of the correlation matrix showed that there are significant and sufficiently high correlations; the general Kaiser-Meyer-Olkin (KMO) measure of sampling was greater than 0.5 units, and the Bartlett sphericity test was statistically significant ($p <$ 0.001). All this leading to the verification of the assumptions that corroborate the feasibility of the technique. All differences were considered significant for *p* values of less than 0.05. The statistical study was carried out with the IBM©SPSS Statistics 25.0 software package.

3. Results

3.1. Lipid composition of diets and chicken thigh meat

The diets used to feed the chicken contained 4.70–5.86 % of total lipid (TL) in dry weight basis. The main fatty acids (FA) determined were 18:2n-6 (31.18–50.23 % of total FA), 18:1n-9 (20.47–29.50 %) and 16:0 (14.36–19.19 %). By contrast, 18:3n-3 only represented 2.29–4.59 % of total FA, 22:6n-3 was < 0.40 %, 20:4n-6 < 0.20 %, and 20:5n-3 was not detected (Table 1).

The Canarian genotype tended to have the lowest thigh meat fat content (1.09 ± 0.27 % fresh weight) although the lipid class distribution remained unchanged among the three breeds (Table 2). Thus, phosphatidylcholine (PC, 11.52–14.61 %) and phosphatidylethanolamine (PE, 13.40–14.21 %) were the most abundant lipid fractions within phospholipids whereas triacylglycerols (TAG, 29.33–36.26 %) and cholesterol (CHO, 11.82–12.68 %) were dominant within neutral lipids (Table 2).

Saturated fatty acids (SFA, 31.00–33.07 %) and n-6 PUFA (29.17–30.93 %) were the most relevant families of FAs in thighs (Table 3). Within the n-6 series, the n-6 LC-PUFA proportions ranged between 10.04 and 12.24 % while n-3 LC-PUFA only represented a 2.47–2.97 % of total FAs. Individually, 18:1n-9 (19.97–22.46 %), 16:0 (18.67–19.34 %) and 18:2n-6 (18.52–18.96 %) were the most extensive FAs. Among thighs LC-PUFAs, it must be highlighted the high amount of ARA (7.16–8.79 %) despite being barely supplied in the diet (<0.20 %), and that of DHA (1.22–1.71 %) and n-3 docosapentaenoic acid (DPA, 22:5n-3; 1.02–1.14 %) that were very scarce or absent in the diet, respectively (Table 1). PCA with Varimax orthogonal rotation of meat FA composition retained two components which explained 47.11 and 23.60 % of the variance, respectively (Table S1), with no significant differences between strains for PC1 scores (*p* > 0.05) (Fig. S2).

3.2. Lipid composition of control hepatocytes

No significant differences in the TL content and LC composition of hepatocytes existed between breeds although there was a subtle tendency to reduce TAG in Dominant Red Barred specimens (Table 4). All species presented similar proportions of total polar and total neutral lipids, with PC and PE being the major components of the polar lipid fraction, while TAG and CHO were the most abundant neutral lipids, as also described for thighs.

Regardless of genotype, SFA, mainly C16 (~19 %) and C18 (20.93–23.01 %); n-6 PUFA including 18:2n-6 (15.32–17.59 %) and ARA (12.04–13.11 %), and monounsaturated fatty acids (MUFA) with 18:1n-9 as the main representative (10.45–16.51 %), were the most abundant FAs of hepatocytes. C18 n-3 PUFA exclusively represented

Table 1

Total lipid content (% fresh and dry weight) and main fatty acid composition (% of total fatty acids) of the three diets used to feed the chicken breeds.

Fatty acids	Starter			Growth			Finisher		
16:0	14.36	±	0.05	16.79	±	0.52	19.19	±	0.35
18:0	3.50	±	0.01	5.03	±	0.17	7.17	±	0.16
22:0	0.41	±	0.01	0.27	±	0.01	0.16	±	0.00
Total SFA[1]	19.40	±	0.07	23.43	±	0.78	28.16	±	0.55
16:1n-7	0.25	±	0.00	0.84	±	0.02	1.52	±	0.02
18:1n-9	20.47	±	0.24	26.20	±	1.05	29.50	±	0.66
18:1n-7	1.60	±	0.03	2.31	±	0.27	2.78	±	0.40
Total MUFA[2]	23.19	±	0.20	30.84	±	0.47	35.54	±	0.02
18:2n-6	50.23	±	0.02	39.64	±	0.38	31.18	±	0.07
20:2n-6	0.09	±	0.02	0.24	±	0.03	0.38	±	0.00
20:4n-6	nd			0.10	±	0.01	0.19	±	0.01
22:4n-6	nd			nd			0.09	±	0.01
Total n-6 PUFA	50.31	±	0.04	39.98	±	0.40	31.84	±	0.06
18:3n-3	4.59	±	0.03	3.50	±	0.20	2.29	±	0.13
18:4n-3	nd			0.12	±	0.06	0.10	±	0.04
20:3n-3	nd			nd			0.04	±	0.05
20:5n-3	nd			nd			nd		
22:6n-3	0.37	±	0.02	0.37	±	0.01	0.30	±	0.04
Total n-3 PUFA	4.97	±	0.01	3.99	±	0.27	2.73	±	0.08
Total PUFA	55.28	±	0.03	43.97	±	0.67	34.57	±	0.14
Total n-3 LC-PUFA	0.37	±	0.02	0.37	±	0.01	0.34	±	0.09
Total n-6 LC-PUFA	nd			0.10	±	0.01	0.28	±	0.01
n-6/n-3	10.13	±	0.03	10.04	±	0.59	11.65	±	0.31
Total lipid (% fresh weight)	4.64	±	0.08	4.13	±	0.04	5.21	±	0.01
Total lipid (% dry weight)	5.23	±	0.10	4.70	±	0.03	5.86	±	0.00

Results are expressed as mean ± SD (n = 5). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids ($\geq$C20 and $\geq$3 double bonds). nd, not detected. Other minor components not shown [1] 14:0, 15:0, 17:0, 20:0 and 24:0; [2] 16:1n-9, 17:1n-7, 18:1n-5, 20:1n-9 and 22:1n-9.

Table 2

Total lipid content (% fresh weight) and lipid class composition (% of total lipid) of thigh meat from the three chicken breeds.

Lipid classes	Canaria			Les Bleues			DRB D459		
SM	5.49	±	5.23	6.72	±	5.39	5.55	±	3.97
PC	14.61	±	5.01	11.52	±	6.25	12.59	±	7.78
PS	4.31	±	1.55	3.19	±	1.86	3.84	±	0.85
PI	2.91	±	1.39	2.21	±	0.90	2.47	±	1.22
PG	3.84	±	1.28	3.99	±	0.36	4.21	±	0.85
PE	14.21	±	1.67	13.40	±	2.57	13.56	±	2.21
TPL	45.37	±	7.40	41.03	±	6.72	42.23	±	6.91
MAG	2.16	±	0.81	1.97	±	0.34	2.65	±	1.96
DAG	1.83	±	0.86	1.44	±	0.77	1.76	±	1.56
CHO	12.68	±	1.57	11.82	±	3.33	13.22	±	2.93
FFA	5.69	±	0.95	4.13	±	1.18	5.33	±	1.86
TAG	29.33	±	8.46	36.26	±	11.07	31.77	±	7.91
CE	2.94	±	0.92	3.33	±	0.76	3.04	±	0.51
TNL	54.63	±	7.40	58.97	±	6.72	57.77	±	6.91
Total lipid	1.09	±	0.27	1.48	±	0.54	1.29	±	0.27

Results are expressed as mean ± SD (n = 5). SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; TPL, total polar lipids; MAG, monoacylglycerols; DAG, diacylglycerols; CHO, cholesterol; FFA, free fatty acids; TAG, triacylglycerols; CE, cholesterol esters; TNL, total neutral lipids.

0.29−0.43 % of total FAs whereas interestingly, DHA is the most relevant component (2.11–2.60 %) within the n-3 series (Table 5).

Table 5 and Fig. 1 also show that hepatocytes FA composition from Les Bleues and Dominant Red Barred were similar to each other but not with the Canarian breed. Thus, Canarian chickens contained the lowest proportions of total SFA (40.74 ± 1.63 %) and n-6 PUFA (31.40 ± 2.73 %), and the highest levels of C18 and C20 MUFA of the three genotypes. Moreover, LC-PUFAs, tended to be lower in the Canarian genotype although only significantly for docosatetraenoic acid (DTA, 22:4n-6) and n-3 DPA.

For the PCA of hepatocytes FAs, two components accumulated 60.04 % of the variance. PC1 (44.57 % of variance) was highly negatively correlated with MUFAs and positively related to 18:0, 18:2n-6, the n-6 LC-PUFA, ARA, 22:4n-6 and 22:5n-6, and the n-3 LC-PUFA, n-3 DPA and DHA. PC2 (15.47 % of variance) was positively correlated with 18:3n-3 and 20:3n-3, and negatively and highly correlated with 20:3n-6

(Table S2). PC1 scores significantly differed between Campera and Les Bleues and Red Barred ($p < 0.05$) (Fig. 1).

3.3. Incorporation and bioconversion of [1-^{14}C]18:3n-3 in hepatocytes

[1-^{14}C]18:3n-3 was incorporated to a similar extent into hepatocytes TL of the three chicken breeds (Table 6). Most of the incorporated radioactivity was esterified into neutral lipids, mainly into TAG (46.78–54.73 %) and monoacylglycerols (MAG, 7.01–8.51 %), and also into PC (6.07–8.82 %) and PE (3.12–4.31 %) within phospholipids. Approximately a 15–17 % of total radioactivity remained unesterified as free fatty acids (FFA). The Canarian genotype presented the lowest esterification rates of [1-^{14}C]18:3n-3 into PC and PS and, as a consequence, into the total polar lipid fraction.

PCA with Varimax orthogonal rotation of radioactive LC revealed two components accounting for near 70 % of total variance. PC1 (42.27

Table 3

Total fatty acid content (mg fatty acid 100 g tissue^{-1}) and main fatty acid composition (% of total fatty acids) of thigh meat from the three chicken breeds.

Fatty acids	Canaria			Les Bleues			DRB D459		
14:0	0.39	±	0.09	0.42	±	0.08	0.50	±	0.09
16:0	18.67	±	1.36	19.27	±	1.52	19.34	±	0.61
18:0	13.28	±	1.78	10.95	±	1.16	12.33	±	0.90
Total SFA[1]	33.07	±	1.11[a]	31.00	±	0.77[b]	32.64	±	0.20[a]
16:0 DMA	4.15	±	0.55	2.90	±	1.35	3.67	±	1.06
18:0 DMA	1.37	±	0.38	1.04	±	0.33	1.23	±	0.34
18:1n-9 DMA	0.42	±	0.03	0.38	±	0.16	0.36	±	0.11
Total DMA	5.95	±	0.79	4.32	±	1.83	5.26	±	1.50
16:1n-9	0.51	±	0.09	0.51	±	0.08	0.48	±	0.12
16:1n-7	1.83	±	0.85	2.53	±	0.91	1.98	±	0.70
18:1n-9	19.97	±	5.15	22.46	±	4.34	20.07	±	3.39
18:1n-7	3.43	±	0.17[a]	3.62	±	0.41[a]	2.72	±	0.23[b]
Total MUFA[2]	26.22	±	6.26	29.89	±	5.08	25.51	±	4.31
18:2n-6	18.57	±	0.63	18.96	±	0.92	18.52	±	1.26
18:3n-6	0.13	±	0.02	0.17	±	0.03	0.12	±	0.03
20:2n-6	0.33	±	0.08	0.41	±	0.04	0.41	±	0.04
20:3n-6	0.49	±	0.10	0.46	±	0.14	0.44	±	0.13
20:4n-6	8.59	±	2.03	7.16	±	2.30	8.79	±	1.94
22:4n-6	1.71	±	0.54	1.58	±	0.42	1.95	±	0.64
22:5n-6	0.49	±	0.11	0.44	±	0.10	0.65	±	0.28
Total n-6 PUFA[3]	30.34	±	3.30	29.17	±	2.80	30.93	±	1.98
18:3n-3	0.61	±	0.19	0.82	±	0.18	0.74	±	0.18
20:3n-3	0.13	±	0.05	0.12	±	0.08	0.07	±	0.01
20:5n-3	0.08	±	0.02	0.12	±	0.06	0.09	±	0.03
22:5n-3	1.05	±	0.33	1.02	±	0.40	1.14	±	0.36
22:6n-3	1.71	±	0.37	1.22	±	0.37	1.53	±	0.49
Total n-3 PUFA	3.57	±	0.61	3.29	±	0.71	3.58	±	0.71
Total PUFA	33.92	±	3.81	32.47	±	3.50	34.51	±	2.64
Total n-3 LC-PUFA	2.97	±	0.68	2.47	±	0.83	2.84	±	0.87
Total n-6 LC-PUFA	11.61	±	2.73	10.04	±	2.92	12.24	±	2.99
n-6/n-3	8.51	±	0.93	8.87	±	1.14	8.64	±	1.13
EPA/DHA	0.05	±	0.02[a]	0.09	±	0.02[b]	0.06	±	0.01[a]
EPA/ARA	0.01	±	0.00[a]	0.02	±	0.00[b]	0.01	±	0.00[a]
Total FA content	728.44	±	250.72	1021.02	±	426.72	795.93	±	187.88

Results are expressed as mean ± SD (n = 5). SFA, saturated fatty acids; DMA, dimethyl acetals; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids ($\geq$C20 and $\geq$3 double bonds); EPA, eicosapentaenoic acid (20:5n-3); DHA, docosahexaenoic acid (22:6n-3); ARA, arachidonic acid (20:4n-6). Other minor components not shown [1] 15:0, 17:0, 20:0, 23:0 and 24:0; [2] 17:1n-7, 18:1n-5, 20:1n-9 and 24:1n-9; [3] 22:2n-6. Different letters within a row denote significant differences between species (p < 0.05).

Table 4

Total lipid content (mg lipid mg protein^{-1}) and lipid class composition (% of total lipid) of control hepatocytes from the three chicken breeds.

Lipid classes	Canaria			Les Bleues			DRB D459		
SM	1.80	±	0.73	1.58	±	0.33	2.14	±	0.39
PC	17.16	±	1.51	18.61	±	3.54	19.35	±	3.40
PS	4.82	±	1.72	4.92	±	1.87	5.29	±	1.43
PI	2.21	±	1.34	2.52	±	1.37	2.09	±	0.97
PG/PA	5.19	±	0.97	4.60	±	0.56	4.19	±	0.75
PE	12.89	±	0.87	13.63	±	0.84	14.06	±	0.44
TPL	46.13	±	2.84	48.16	±	4.20	49.30	±	4.85
MAG	5.41	±	1.10	5.33	±	0.23	5.86	±	1.44
DAG	3.62	±	1.41	3.55	±	0.73	3.58	±	1.14
CHO	14.64	±	0.82	15.76	±	1.60	15.57	±	1.12
FFA	8.45	±	1.80	7.28	±	1.51	7.98	±	2.25
TAG	14.53	±	1.69	13.45	±	1.37	11.79	±	1.73
CE	4.62	±	1.08	4.77	±	1.39	4.55	±	1.32
TNL	53.87	±	2.84	51.84	±	4.20	50.70	±	4.85
Total lipid	0.43	±	0.07	0.46	±	0.06	0.41	±	0.04

Results are expressed as mean ± SD (n = 5). SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG/PA, phosphatidylglycerol / phosphatidic acid; PE, phosphatidylethanolamine; TPL, total polar lipids; MAG, monoacylglycerols; DAG.diacylglycerols; CHO, cholesterol; FFA, free fatty acids; TAG, triacylglycerols; CE, cholesterol esters; TNL, total neutral lipids.

% of variance explained) was highly positively related with the incorporation into phospholipids (PE, PI and PS) and negatively related to MAG. PC2 (26.76 % of variance explained) was positively correlated with FFA and negatively with TAG (Table S3). There were significant differences between strains for PC1 scores (p < 0.05) (Fig. S3).

The incubation of hepatocytes with [1-^{14}C]18:3n-3 (Table 7) produced higher amounts of 18:4n-3 in the Canarian breed than in Les Bleues. LC-PUFA including EPA and DHA were also obtained from their [1-^{14}C]C18 precursor. Some bands corresponding to *de novo* biosynthesized FAs were also detected in the TLC plates as a result of beta-oxidation of [1-^{14}C]18:3n-3.

Two components explaining 94.68 % of the variance were obtained from the PCA of radioactive compounds after cellular incubation. PC1 (84.88 % of variance explained), was highly negatively correlated with the radiolabeled substrate and positively correlated with both EPA and DHA, and their metabolic biosynthetic intermediates. PC2 explained 9.80 % of total variance and was highly correlated with 18:4n-3, the intermediary obtained from the desaturation of 18:3n-3 (Table S4). No differences between groups were registered in radioactive FA products obtained with cellular incubation for PC1 scores (p > 0.05) (Fig. S4).

4. Discussion

Genotype-related differences in FA composition have previously been reported among indigenous breeds (Jaturasitha et al., 2008; Zanetti et al., 2010) and commercial chicken lines (Castellini et al., 2006; Sirri et al., 2011). In this regard, Castellini et al. (2008) pointed out that the feeding behavior and the different pasture utilization in free-range systems as the one assayed in our experimental design may be partly responsible for the genotype-related variations. To avoid this possible interference, grass growth was reduced and restricted to limited zones in outdoor areas to which birds had access to in the present trial, ensuring that its nutrient contribution did not give rise to

Table 5

Total lipid content (µg lipid mg protein^{-1}) and main fatty acid composition (% of total fatty acids) of hepatocytes from the three chicken breeds.

Fatty acids	Canaria			Les Bleues			DRB D459		
16:0	18.68	±	0.74	19.62	±	0.39	19.39	±	0.77
18:0	20.93	±	1.33	21.88	±	0.89	23.01	±	1.43
22:0	0.30	±	0.06[b]	0.31	±	0.01[b]	0.44	±	0.06[a]
Total SFA[1]	40.74	±	1.63[b]	42.81	±	0.97[a]	44.20	±	1.11[a]
16:0 DMA	0.50	±	0.18	0.56	±	0.08	0.55	±	0.08
18:0 DMA	0.89	±	0.27	1.02	±	0.16	0.92	±	0.21
Total DMA	1.39	±	0.45	1.58	±	0.24	1.48	±	0.27
16:1n-7	1.19	±	0.26[a]	0.64	±	0.28[b]	0.67	±	0.14[b]
18:1n-9	16.51	±	3.18[a]	11.14	±	0.99[b]	10.45	±	1.67[b]
18:1n-7	2.25	±	0.30[a]	1.76	±	0.31[b]	1.68	±	0.18[b]
Total MUFA[2]	21.41	±	4.01[a]	14.84	±	1.47[b]	13.99	±	1.98[b]
18:2n-6	15.32	±	1.68[b]	17.59	±	0.87[a]	17.07	±	0.97[ab]
18:3n-6	0.23	±	0.06	0.19	±	0.05	0.20	±	0.05
20:2n-6	0.59	±	0.05[b]	0.73	±	0.06[a]	0.67	±	0.05[ab]
20:3n-6	1.37	±	0.21	1.06	±	0.09	1.22	±	0.47
20:4n-6	12.04	±	1.00	12.46	±	0.81	13.11	±	1.09
22:4n-6	1.11	±	0.25[b]	1.57	±	0.08[a]	1.46	±	0.12[a]
22:5n-6	0.73	±	0.15	1.02	±	0.10	1.10	±	0.23
Total n-6 PUFA[3]	31.40	±	2.73[b]	34.61	±	1.02[a]	34.83	±	1.06[a]
18:3n-3	0.29	±	0.06[b]	0.43	±	0.02[a]	0.30	±	0.03[b]
20:3n-3	0.08	±	0.01[a]	0.14	±	0.07[b]	0.07	±	0.01[a]
20:5n-3	0.33	±	0.04	0.29	±	0.05	0.29	±	0.04
22:5n-3	0.59	±	0.16[b]	0.78	±	0.09[a]	0.90	±	0.18[a]
22:6n-3	2.11	±	0.59	2.58	±	0.38	2.60	±	0.44
Total n-3 PUFA	3.39	±	0.62	4.22	±	0.43	4.17	±	0.61
Total PUFA	34.78	±	3.28[b]	38.84	±	1.42[a]	39.00	±	1.38[a]
Total n-3 LC-PUFA	3.10	±	0.65	3.80	±	0.43	3.87	±	0.59
Total n-6 LC-PUFA	15.85	±	1.09	16.84	±	0.93	17.56	±	1.05
n-6/n-3	9.26	±	1.01	8.20	±	0.63	8.35	±	1.09
EPA/DHA	0.16	±	0.04	0.12	±	0.03	0.11	±	0.03
EPA/ARA	0.03	±	0.00	0.02	±	0.00	0.02	±	0.00
TL content	287.50	±	25.26	268.81	±	41.49	229.20	±	19.28

Results are expressed as mean ± SD (n = 5). TL, total lipid. SFA, saturated fatty acids; DMA, dimethyl acetals; MUFA, monounsaturated fatty acids; PUFA, poly-unsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids ($\geq$C20 and $\geq$3 double bonds); EPA, eicosapentaenoic acid (20:5n-3); DHA, docosahexaenoic acid (22:6n-3); ARA, arachidonic acid (20:4n-6). nd, not detected. Other minor components not shown [1] 15:0, 17:0, 20:0, 23:0 and 24:0; [2] 17:1n-7, 18:1n-5, 20:1n-9 and 24:1n-9; [3] 22:2n-6. Different letters within a row denote significant differences between species (p < 0.05).
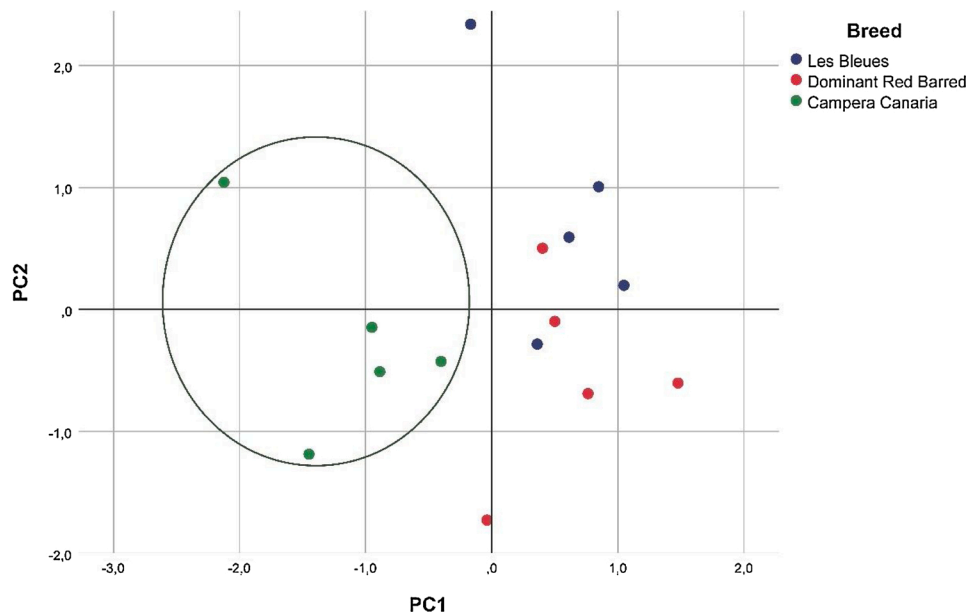

Fig. 1. Factor scores for principal components 1 and 2 of the fatty acid composition of hepatocytes total lipids according to the breeds.

genotype-related differences in the lipid and FA profiles.

Feed has been described as one major factor influencing the resultant lipid and FA composition of edible products from farmed terrestrial and aquatic vertebrates (Pérez et al., 2014; Moallem, 2018; Akif Boz et al., 2019; Paszkiewicz et al., 2020). The lipid and FA profiles of the meat from the three different genotypes studied remained fairly stable suggesting that identical diets and rearing system may override genetic differences. Thus, regardless of the breed, thigh meat presented low lipid deposition (<1.5 % fresh weight) coinciding with the relatively low intramuscular fat (IMF) content previously reported by Jin et al. (2019).

Table 6
Incorporation of radioactivity from [1-^{14}C]18:3n-3 into total lipids (pmol mg protein^{-1} h) and lipid classes (% of total lipid) of hepatocytes of the three chicken breeds.

Lipid classes	Canaria			Les Bleues			DRB D459		
PC	6.07	±	1.53[b]	8.82	±	1.26[a]	8.15	±	1.54[ab]
PS	0.98	±	0.28[b]	1.60	±	0.49[a]	1.77	±	0.24[a]
PI	0.76	±	0.35	1.33	±	0.47	1.22	±	0.13
PE	3.12	±	0.66	4.26	±	1.16	4.31	±	0.46
TPL	10.94	±	2.49[b]	16.01	±	2.90[a]	15.44	±	2.13[a]
MAG	8.51	±	2.71	8.36	±	1.42	7.01	±	1.10
DAG	3.48	±	0.48	3.80	±	0.76	4.03	±	1.05
FFA	17.29	±	7.56	18.76	±	3.16	15.37	±	6.84
UK	5.06	±	0.82	6.31	±	0.70	5.88	±	0.64
TAG	54.73	±	7.69	46.78	±	2.51	52.27	±	9.47
TNL	89.06	±	2.49[a]	83.99	±	2.90[b]	84.56	±	2.13[b]
Total incorporation	60.53	±	11.90	49.25	±	9.30	57.27	±	18.62

Results are expressed as mean ± SD (n = 5). PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; TPL, total polar lipids; MAG, monoacylglycerols; DAG, diacylglycerols; FFA, free fatty acids; UK, unknown lipids; TAG, triacylglycerols; TNL, total neutral lipids. Different letters within a row denote significant differences between species (p < 0.05).

Table 7
Products obtained from the incubation with [1-^{14}C]18:3n-3 of hepatocytes from the three chicken breeds.

Fatty acids	Canaria			Les Bleues			DRB D459		
De novo synthesis	4.80	±	2.39	4.16	±	1.81	4.21	±	2.58
18:3n-3	82.85	±	6.25	85.12	±	5.27	84.68	±	6.70
18:4n-3	2.84	±	0.93[a]	1.24	±	0.60[b]	2.09	±	0.43[ab]
20:3n-3	2.19	±	0.83	2.31	±	0.52	2.12	±	0.83
20:4n-3	0.71	±	0.43	0.49	±	0.48	0.52	±	0.49
20:5n-3	0.78	±	0.36	0.75	±	0.25	0.69	±	0.42
22:3n-3	1.37	±	0.43	1.36	±	0.28	1.28	±	0.58
22:5n-3	0.34	±	0.28	0.28	±	0.27	0.30	±	0.28
22:6n-3	1.31	±	0.52	1.52	±	0.34	1.38	±	0.66
24:5n-3	0.37	±	0.21	0.27	±	0.21	0.27	±	0.23
24:6n-3	0.56	±	0.47	0.48	±	0.43	0.47	±	0.47

Results are expressed as mean ± SD (n = 5). Different letters within a row denote significant differences between species (p < 0.05).

Moreover, meat contained high omega-6/omega-3 ratios (8.59–9.05), although slightly lower than those in the feed (from 10.13 in the starter feed to 11.65 in the finishing diet). It is widely accepted that not only the absolute amounts of omega-6 and omega-3 FAs, but also increased omega-6/omega-3 ratio, promote the pathogenesis of many diseases (Simopoulos, 2002, 2004, 2016; Zárate et al., 2017). The consumption of omega-6 FAs has increased in industrialized societies, rising the omega-6/omega-3 ratio from an optimal 1-4:1 in primitive human diets to an unhealthy 20:1 or even higher in today's Western diets (Simopoulos, 2016). This unbalance is related to a decrease in the intake of marine fish/seafood, which are the primary source of omega-3 LC-PUFAs, and to a higher consumption of vegetable oils, cereals, animal fats, and wholegrain bread particularly rich in 18:2n-6 (Patterson et al., 2012). Hence, the omega-6/omega-3 proportions obtained in the present study are higher than desirable, and its modulation should be targeted to make it healthier for humans.

Regardless of genotype, palmitic (16:0), oleic (18:1n-9) and linoleic (18:2n-6) acids were highly incorporated into thigh meat. In fact, 18:2n-6 and C20 and C22 FAs derived from its progressive desaturation and elongation were more abundant than 18:3n-3 and its C20-C22 derivatives. It is especially relevant the high proportion of ARA in meat (38 to 46-fold higher than in the finisher diet) suggesting that this biosynthetic pathway is highly active in the three strains, and therefore, promotes the excessive production of its derived pro-inflammatory eicosanoids (Simopoulos, 2004; Tallima and El Ridi, 2018; Huang et al., 2020).

LC-PUFA, including ARA, EPA and DHA, and more recently n-3 DPA, have been reported to play a central role in numerous physiological and biochemical events within the cells, and are considered essential to health in humans (Siriwardhana et al., 2012; Hussein, 2013; Zárate et al., 2017; Drouin et al., 2019). Hence, most National Health Bodies and Government agencies recommend consuming between 500 and 1000 mg day^{-1} of n-3 LC-PUFA (EPA + DHA) for all life-stages (2 year+), to maintain health and well-being (International Society for the Study of Fatty Acids and Lipids (ISSFAL, 2004; National Health and Medical Research Council (NHMRC, 2006; FAO, 2010; Sioen et al., 2017). Currently, the production of suitable functional foods with adjusted PUFA content is considered as a suitable option to meet nutritional recommendations (see review by Alagawany et al., 2019). Despite being given a very scarce level of omega-3 FAs, the species studied here provide 21.63–25.22 mg omega-3 LC-PUFA, mainly n-3 DPA and DHA, per 100 g meat and might be considered as moderate sources of n-3 LC-PUFA according to a recent study by Fayet-Moore et al. (2015). These authors concluded that the suggested dietary target (SDT) recommendations for n-3 LC-PUFA consumption could be met with 3–4 serving sizes of red meat/week plus 1 serve of "excellent" fish (*i.e.* 100 g Atlantic salmon), 1 serve of "good" or "moderate" fish (*i.e.* 100 g king prawns) and 3–26 serves of n-3 LC-PUFA enriched foods (*i.e.* eggs, milk). Within this nutritional context our present results indicate that red meat may be substituted by chicken meat to reach SDT for n-3 LC-PUFA in this dietary model.

Interestingly, the biochemical composition of chicken thighs meat suggests the presence of all the enzymatic machinery involved in the biosynthesis of LC-PUFA from their C18 precursors. The unbalanced proportion of 18:2n-6 (LA) present in the diet may also justify a preference of enzymes involved for C18 omega-6 substrates and the high levels of ARA observed in the tissues. Biosynthesis of ARA and EPA from LA and ALA, respectively, follows the same metabolic pathways and involves the same enzymes (Fig. 2). In this respect, increasing proportions of dietary LA reduce the conversion of ALA into EPA, n-3 DPA or DHA whereas adequate levels of ALA might promote the biosynthesis of the three n-3 LC-PUFA in meat and eggs (Ebeid et al., 2008; Yalcin and Unal, 2010; Domenichiello et al., 2015; Konieczka et al., 2017).

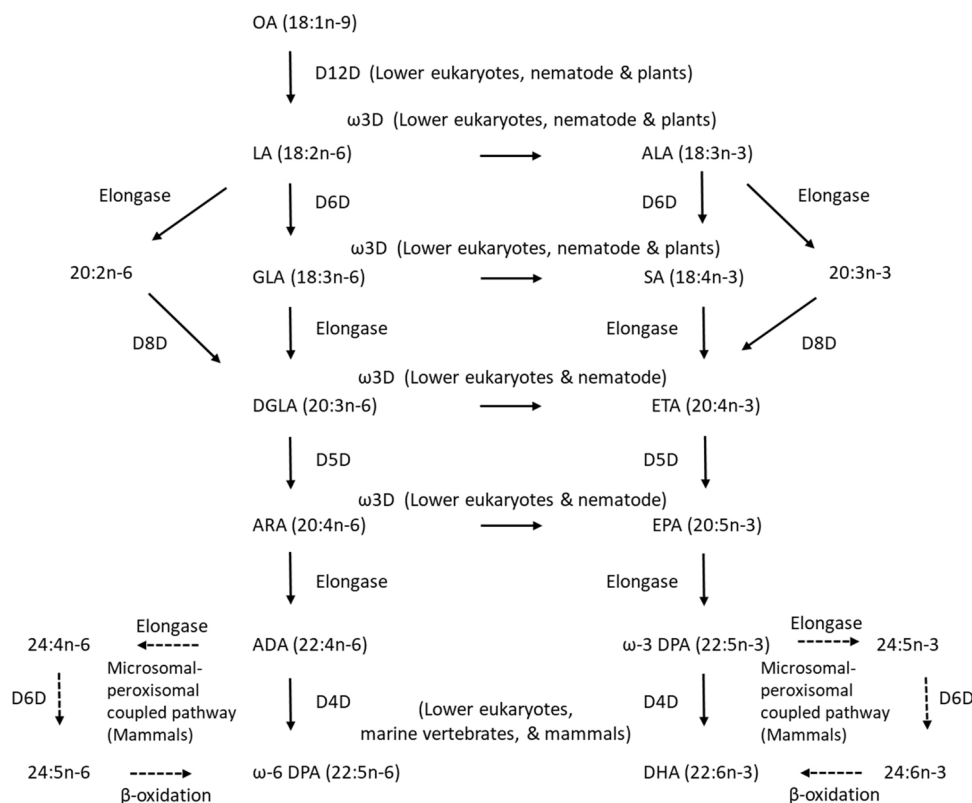In chickens, as in humans, liver rather than adipose tissue is the

Fig. 2. LC-PUFA synthesis pathway from oleic acid (OA, 18:1n-9) in eukaryotes (Lee et al., 2016).

primary site of *de novo* lipogenesis (O'Hea and Leveille, 1968; Leveille et al., 1975; Shrago and Spennetta, 1976). The present *in vitro* experimental design where hepatocytes are incubated with radiolabelled FA has proven a reliable methodology to characterize the ability and metabolic pathways to synthesize LC-PUFA in fish, cephalopods and other marine invertebrates (Rodríguez et al., 2002; Bell and Tocher, 2009; Lee et al., 2016; Reis et al., 2016, 2019; Galindo et al., 2020; Lund et al., 2020). The limited fraction of radioactivity (15.37–18.76 %) recovered unesterified (*i.e.* as free fatty acid, FFA) in isolated cells in our present experimental design demonstrate the validity of this methodology as a suitable tool to study n-3 LC-PUFA biosynthetic capacities and its modulation by dietary and environmental factors for the first time in livestock.

Alternative steps comprising a Δ6-desaturation, followed by a chain elongation and a subsequent Δ5-desaturation of dietary C18 FAs are involved in the biosynthesis of ARA and EPA. The production of DHA from EPA can be mediated *via* the Sprecher pathway (Sprecher, 2000), which requires two consecutive elongation steps, a Δ6 desaturation, and a final peroxisomal β-oxidation, or through an alternative and more direct pathway with the participation of a Δ4 desaturase (Fig. 2). Sprecher's route has been characterized in mammals and fish (Oboh et al., 2017). Elovl proteins involved in the biosynthesis of DHA from 18:3n-3 have been characterized in chicken, duck and turkey (Gregory et al., 2013; Gregory and James, 2014). Specifically, in the three poultry species, Elovl2 had a preference for omega-3 PUFA over omega-6 PUFA substrates whereas Elovl5 showed similar activity with both FA series for the C18 and C20 compounds. By contrast, there was an Elovl5 preference for C22 omega-3 over C22 omega-6 PUFA in chicken, which was not active in duck and turkey (Gregory et al., 2013; Gregory and James, 2014). Our present [1-^{14}C] metabolic approach establishes that the enzymes involved in the LC-PUFA synthesis are similarly functional and active in the three genotypes studied being able to produce EPA and DHA from 18:3n-3, even when the dietary inclusion of 18:2n-6 is high (Table 7; Fig. S4). The incubation of hepatocytes with radiolabeled

18:3n-3 produced an important variety of C18-C24 intermediates to finally produce DHA indicating that, unlike many fish, chickens have undergone a large and unique expansion of the Fads1 lineage with respect to other tetrapod with three genes, thus mainly possessing Δ5 and Δ6 desaturases (Castro et al., 2012). Our results demonstrate that the two possible routes to produce DHA are active, also highlighting the potential presence of a desaturase Δ4 in these species.

5. Conclusions

The present metabolic approach demonstrates that chickens possess the set of enzymes necessary to elongate and desaturate C18 precursors to produce LC-PUFA such as ARA, EPA and DHA and that this enzymatic machinery is highly active. However, future studies should be carried out to clarify the preferences over omega-6 or omega-3 substrates in order to determine a balanced dietary 18:2n-6/18:3n-3 ratio that could induce a greater synthesis of EPA and DHA diminishing the endogenous production of ARA to improve the nutritional value of chicken meat for humans. Finally, the Canarian genotype can be considered as a good nutritional candidate for a novel dual-purpose chicken model.

Author statement

J.A. Pérez: Writing-Review and Editing, **A. Castro:** Writing-original draft preparation, Investigation, **C. Rolo:** Writing-original draft preparation, Investigation, **A. Torres:** Investigation, Resources, Writing and Reviewing, **R. Dorta-Guerra**: Formal analysis, **N.G. Acosta:** Methodology, Supervision, **C. Rodríguez:** Conceptualization, Supervision, Funding acquisition, Review and Editing.

Declaration of Competing Interest

The authors of the present work declare NO affiliations with or involvement in any organization or entity with any financial or non-

financial interest in the subject matter or materials discussed in this manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jfca.2021.104005.

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