© 2019 Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Docosahexaenoic acid enrichment of layer hen tissues and eggs through dietary supplementation with heterotrophically grown *Aurantiochytrium limacinum*

C.A. Moran,[*,1] M. Morlacchini,[†] J.D. Keegan,[‡] F. Rutz,[§] and G. Fusconi[†]

[*]*Regulatory Affairs Department, Alltech SARL, Rue Charles Amand, 14500 Vire, France;* [†]*CERZOO S.r.l., Zootechnical Research Centre, 29122 Piacenza, Italy;* [‡]*Regulatory Affairs Department, Alltech European Bioscience Centre, Co. Meath, Ireland; and* [§]*Zootechnical Department, Faculty of Agronomy, Federal University of Pelotas, Eliseu Maciel Av., University Campus, 96010-900 Capão do Leão, RS, Brazil*

Primary Audience: Layer Hen Nutritionists, Researchers, Feed Formulators, Egg Producers

SUMMARY

Omega-3 fatty acids play an important role in various aspects of human health, but many people do not consume them in sufficient quantities, resulting in deficiencies in some populations. The enrichment of commonly consumed foods with omega-3 fatty acids has been proposed to address this deficit. Feeding omega-3–rich ingredients to animals can enrich their products, increasing the consumption by the population without requiring any major dietary changes. Eggs can be enriched to a high degree, and currently, omega-3–enriched eggs are widely available. Oftentimes, a missed opportunity for the poultry industry is in valorizing the spent hen. In this short-term study, a docosahexaenoic acid (**DHA**)–rich protist was fed to layer hens at three different inclusion levels to determine the degree of enrichment observed in the eggs and concomitantly in the breast, thigh, liver, and kidney. The addition of the protist ingredient had no negative impact on the bird health or performance. Significant increases in egg DHA concentration were observed, with 60, 164, 259, and 410 mg DHA/100g of egg collected from birds supplemented with the DHA-rich protist at a rate of 0, 0.5, 1, and 2.5% of the diet, respectively. This enrichment could increase the value of spent hen meat when used in the production of human and companion animal food as DHA-enriched products can be sold for a premium. Moreover, this enriched spent hen meat could potentially be used as a partial substitute for less-sustainable sources of dietary omega-3 including fish meal and fish oil.

Key words: algae, layer, DHA, EPA, omega-3

2019 J. Appl. Poult. Res. ■:■–■
https://doi.org/10.1016/j.japr.2019.10.002

[1]Corresponding author: cmoran@alltech.com

ARTICLE IN PRESS

DESCRIPTION OF PROBLEM

The consumption of adequate amounts of omega-3 long-chain polyunsaturated fatty acids (**n-3 LC-PUFA**) has been associated with a number of benefits to various aspects of human health [1,2]. In our previous work, we were able to demonstrate that the fatty acid content of eggs was altered by dietary manipulation by feeding a docosahexaenoic acid (**DHA**)–rich *Aurantiochytrium* biomass ingredient, thereby significantly increasing eicosapentaenoic acid and DHA concentrations and reducing the n-6:n-3 ratio of the eggs [3]. *Aurantiochytrium limacinum* is a member of a group of protists called thraustochytrids, which are commonly classified as microalgae but also display fungus-like characteristics. These organisms are primary producers of LC-PUFA in the marine food chain and can be grown heterotrophically on suitable carbon sources. The addition of these LC-PUFA–rich ingredients to the hens' diet involves an extra economic cost; however, these eggs can be sold at a premium, with many consumers willing to pay extra for the healthful benefits of the enriched eggs [4, 5].

The value of the hen is the second highest cost to the layer egg industry behind feed [6]. Spent hen meat is rich in protein, amino acids, and PUFA and low in fat [7–9]. Despite these nutritive qualities, spent hens have low economic value, primarily due to low market weight, low yield of meat, and a high heat-stable collagen content, which results in tougher meat. When recovered, the meat and other by-products of spent hens are used in the production of processed foods such as soups, pies, sausages, chicken nuggets [10, 11], or processed into pet food ingredients, with an estimated 2.6 billion spent hens annually being used for this purpose [11]. However, the majority are simply disposed, frequently because large abattoirs are no longer willing to slaughter spent hens [12], which can incur additional costs or lost earnings for the farmer or integrator [11]. There continues to be a major need to upgrade and valorize spent hens, for business and environmental sustainability [13, 14], as inputs for new products in the bioeconomy, to be transformed into a wide array of valuable human or companion animal food products.

Previously, feeding linseed or fishmeal sources of n-3 LC-PUFA to layer hens, for omega-3–rich egg production, concomitantly enriched their meat tissues and organs and increased their nutritional value, thereby providing the egg industry with an additional valuable commercial product [15]. However, the use of linseed is considered inefficient [16] and use of fishmeal becomes less useful because of sensory off-flavors being formed in the egg [17] and marine conservation concerns [18].

The objectives of the current study were to investigate the effect of inclusion of heterotrophically grown *Aurantiochytrium* as a dietary supplement for laying hens on the DHA enrichment of eggs, breast, thigh, liver, and kidneys at different inclusion levels. The efficiency of DHA transfer from feed to the egg and from feed to the edible tissues was also investigated.

MATERIALS AND METHODS

Animals and Housing

The research protocol and animal care were in accordance with European Union Directive 2010/63/EU pertaining to the protection of animals used for experimental or other scientific purposes and in compliance with G.L.P. guidelines (Directives 2004/9/CE and 2004/10/CE) for the collection and handling of data documentation. The live animal portion of this experiment was conducted at CERZOO S.r.L (Piacenza, Italy), which holds authorization by the Italian Ministry of Health to use animals for experimental or other scientific purposes. Animals and husbandry practices represent current state of the art for a modern commercial layer operation. Feed and water were supplied ad libitum, and the environment within the facility was controlled by a dynamic ventilation system. Temperatures and relative humidity were recorded every 30 min during each day of the study by a computerized system. The lighting scheme was 18:6 h light: dark for the duration of the study.

The study was carried out using thirty-six 300/320-day-old, ISA Brown laying hens which were evenly divided between 12 pens (3 birds per pen) [19]. The birds used in the

Table 1. Ingredients and analytical composition of the experimental layer hen diets supplemented with 0, 0.5, 1.0 and 2.5% unextracted *Aurantiochytrium limacinum* biomass.

Diet composition	Units	0%[3]	0.5%	1.0%	2.5%
Wheat meal	%	46.83	46.80	46.68	46.50
Soybean meal 44%	%	22.47	22.35	22.21	21.92
Barley meal	%	14.64	14.63	14.60	14.54
Calcium gritted	%	5.86	5.85	5.84	5.81
Animal fat (lard)	%	3.42	3.41	3.41	3.39
Soya oil	%	1.40	1.10	0.90	-
Limestone	%	2.95	2.94	2.94	2.93
Monocalcium phosphate	%	1.46	1.46	1.46	1.45
Salt	%	0.29	0.29	0.29	0.29
Sodium bicarbonate	%	0.15	0.15	0.15	0.15
Choline liquid	%	0.10	0.10	0.10	0.10
Fungistatic product	%	0.10	0.10	0.10	0.10
DL-methionine	%	0.13	0.13	0.13	0.13
Vitamins and minerals[1]	%	0.20	0.19	0.19	0.19
AURA[2]	%	-	0.50	1.00	2.50
Analytical characteristics					
DM	%	90.76	90.80	90.82	90.94
CP	%	17.83	17.95	17.72	17.58
Ether extract	%	5.65	5.69	5.39	5.33
Crude fiber	%	3.54	3.32	3.28	3.49
Ash	%	12.06	12.56	12.35	12.43
Starch	%	38.83	38.50	38.59	38.87
Sugar	%	3.60	4.03	3.80	3.75
Lutein	mg/kg	1.00	0.71	0.46	<0.01
Zeaxanthin	mg/kg	1.43	0.92	0.57	<0.01
β-carotene	mg/kg	<0.01	<0.01	0.01	<0.01
ME	kcal/kg	2528	2536	2503	2506
DHA	mg/g	<0.01	0.64	1.47	3.53

Abbreviation: DHA, docosahexaenoic acid.

[1]Provided per kilogram of diet (as per label): vitamin A: 2500 IU; vitamin D3: 600 IU; vitamin E: 15 IU; vitamin K: 1.2 mg; vitamin B1: 400 mg; vitamin B2: 1.6 mg; vitamin B6: 4.9 mg; vitamin B12: 1.2 mg; vitamin PP: 63 mg; pantothenic acid: 17.5 mg; folic acid: 2.1 mg; biotin: 120 mg; ferrous carbonate: 72 mg; copper sulfate pentahydrate: 48 mg; manganese oxide: 108 mg; zinc oxide: 87 mg; potassium iodide: 1.38 mg; and sodium selenite: 0.46 mg.

[2]AURA: unextracted *Aurantiochytrium limacinum* algae containing 17.0 g DHA/100g.

[3]All birds were fed the 0% diet for an acclimatization period of 8 D before the beginning of the trial.

study were taken from a larger group of laying hens on the CERZOO farm and were chosen based on weight to ensure maximum possible homogeneity within each group and to ensure minimum differences between the groups. The birds were allowed to acclimatize to the experimental set up for 8 D during which they were fed a control diet (Table 1). Pens were randomly allocated to one of four treatment groups, with each treatment replicated three times. The diets included one untreated control and three treatment diets supplemented with a heterotrophically grown unextracted *A. limacinum* biomass (**AURA**) [20] at a rate of 0.5, 1.0, and 2.5% (Table 1).

The analytical composition of the AURA used was determined before the start of the study using the following methods: CP (AOAC 990.03), crude fat (AOAC 954.02), fatty acid composition [21], moisture (AOAC 930.15), and ash (AOAC 942.05). The analytical composition of each diet was determined at the Institute of Food Science and Nutrition (Faculty of Agricultural Sciences, Food and Environment, Catholic University of Sacred Heart), Piacenza, Italy, using standard procedures, as follows: dry matter/moisture (ISO 6496), CP (ISO 5983–1), crude fat (ISO 6492), crude fiber (ISO 6865), crude ash (NEN 3329; ISO 5984–2002), starch (ISO 10520), and sugar

(71/250/EEC, L155, 12/07/71). Dietary DHA content was determined by extracting lipids using the Floch method [22], after which they were converted to fatty acid esters and analyzed using the method of Bannon et al. [23]. The ME content of the experimental diets was calculated from feed analysis as per the equation proposed by Legislation (EC 152/2009).

Bird health, mortality, and culling records were maintained. Individual bird weights were recorded on days 0 and 28. ADFI for the treatment period was calculated per pen. This information along with the egg mass output per pen was used to calculate the average feed conversion ratio (**FCR**). A daily count of the number of eggs produced per pen was recorded throughout the experimental period along with a count of dirty, cracked, broken, shell-less, or otherwise-unsalable eggs. The total weight of eggs produced per pen was also recorded daily throughout the experimental period. Egg quality was assessed by analyzing the carotenoids [24], fat (ISO 6492), protein (ISO 5983–1), and energy content of two whole eggs from each pen [25] on days −1, 20, and 27. On days 0, 21, and 28, the DHA content was established [26] for two eggs per pen. On day 29, 6 animals per treatment were slaughtered, and the liver, both thighs, breasts, and kidneys were removed postmortem, with the right side of each tissue/organ sample frozen until analysis for DHA content.

Fatty acid profiles of tissue and organ samples were determined at Chelab S.r.L. [27] utilizing a gas chromatography–flame ionization method (AOAC 996.06), which was recently validated to demonstrate the fitness for purpose in analyzing seven fatty acids in five different chicken tissues, that is, the breast, thigh, skin, kidney, and liver [28]. Samples of chicken tissues (liver, kidney, breast, and thigh) were ground thoroughly using a mortar and pestle and left in a freezer at $< -16°C$ for 12 h before freeze drying. Frozen samples were freeze dried with VirTis Benchtop Pro Freeze Dryer [29]. Fat was extracted from a 0.5-g sample of the homogenized freeze-dried tissue into ether; pyrogallic acid was added to minimize oxidative degradation of fatty acids during analysis, followed by methylation resulting in fatty acid methyl esters (**FAME**) using boron trifluoride ($\mathbf{BF_3}$) in methanol. The internal standard for sample extraction, 1,2,3- triundecanoylglycerol (common name: triundecanoin), the internal standard for calibration curve and quality control, methyl undecanoate, and DHA were purchased from Nu-Chek Prep Inc. [30]. FAME were quantitatively measured on an Agilent 6890 N gas chromatograph [29] equipped with a SP2560 100-cm-long capillary column, a hydrogen flame ionization detector set at 250°C temperature and an Agilent 7683 autosampler [29]. The concentration of DHA (mg/g) in the fatty acid form in each sample type was calculated using the following formula:

$$\frac{R\ DHA\ FAME \times Weight\ of\ IS \times CF\ IS}{(R\ IS\ \times RF\ DHA) \times CF\ DHA} \times \frac{1000}{SW\ (g)}$$

where R DHA FAME is the GC area detection value of the DHA FAME analyte; weight of IS (g) is the weight of the internal standard used; CF IS is the conversion factor of the internal standard; R IS is the GC area detection value of the internal standard; RF DHA is the ratio of the detection area values of DHA to IS; CF DHA is the conversion factor of DHA; and SW is the sample weight.

An estimate of the efficiency of transfer of DHA from the feed to the eggs was established as follows: First, DHA intake per bird was estimated as the mean daily feed intake per bird multiplied by the milligrams of DHA detected per gram of each experimental diet. Second, the mean DHA deposited for all control eggs was subtracted from the mean yolk DHA detected for each pen to provide an estimate of the amount of DHA deposited in the eggs of birds supplemented with AURA. Then, transfer efficiency was calculated as follows:

$$\frac{DHA\ deposited\ (mg\ per\ egg)}{DHA\ intake\ (mg\ per\ bird\ per\ day)} \times 100$$

Statistical Analysis

The raw data were tested for normality with the Shapiro-Wilk test. The performance parameters (live weight, ADFI, FCR, and feed

ARTICLE IN PRESS

conversation efficiency), the egg quality characteristics (egg weight, laying (%), egg protein, egg fat, and egg energy), and organ and tissue DHA content were analyzed using the GLM procedure of Minitab [31] for each sampling day. Tukey's tests were used to compare the means of each group. The level of significance to indicate differences was $P \leq 0.05$. As differences between the groups were observed at day -1 for egg lutein and zeaxanthin concentrations, these values were used as a covariate analysis of day 20 and 27 results. Regression analysis was conducted to investigate the relationship between mean DHA intake/day and DHA (mg/100g) content of the breast, thigh, liver, kidneys, and egg.

RESULTS AND DISCUSSION

Diet Analysis and Zootechnical Performance

The test article, AURA, used in the study primarily consisted of 66.9 g crude fat/100 g biomass, with a significant level of palmitic acid and DHA (50.1 and 31.5 g/100 g fat, respectively). The eicosapentaenoic acid accounted for only 0.4 g/100 g fat. The analytical composition of the experimental diets is shown in Table 1. The hens were considered healthy and husbandry to be generally good, with normal consistency of feces observed during the study. No veterinary drugs were provided to the hens, and no mortality or culling occurred during the entire study period. Supplementation with AURA had no effect on hen weight (day 0 and 28), ADFI, FCR, or feed conversation efficiency (Table 2). Egg weight and laying were also unaffected by treatment (Table 2). In agreement with the findings of this study, previous authors have demonstrated that AURA supplementation had no major impact on the productivity of layer hens [3, 32]. Supplementation of layer diets with fish oils has been shown to reduce egg/yolk weight in some circumstances [17] and have no impact in others [33]. These differing effects of n-3 LC-PUFA supplementation on productivity have been attributed to differences in experimental design, with genetics, age, and feed formulation suggested as possible causes [34].

No differences in egg protein (%) were observed between the treatment groups on days -1 and 20, whereas on day 27, the 0.5% AURA group had eggs with more protein than the control, with no differences observed between the control group and those supplemented at a rate of 1 or 2.5% (Table 2). For both the egg fat and egg energy content, no differences were observed between treatments on days -1 or 27, whereas on day 20, a significant effect of treatment was observed. However, the post hoc Tukey's test failed to find significant differences in the pairwise comparisons of the treatment group means. Considering the differences observed were between the control and 0.5% AURA group, with no dose-dependent effects observed when adding additional AURA, it is unlikely that these differences in egg quality are a result of supplementation. In addition, no differences in the egg carotenoid lutein or zeaxanthin content were observed between treatments (Table 3), whereas β-carotene was undetected. The closely related thraustochytrid *Schizochytrium* sp. has been shown to contain the carotenoids, β-carotene, and canthaxanthin and, when fed to layer hens, can influence the color of the yolk [35]. Ao et al. [32] also fed AURA as a dietary supplement and noted no differences in yolk color at a rate of 1% of the diet; however, color changes were observed when AURA was fed at a rate of 2 and 3%. In contradiction, when analyzing the carotenoid concentrations of the experimental diets used in the current study, no β-carotene was detected in any of the diets and the content of lutein and zeaxanthin inversely correlated with increasing inclusion of AURA, in agreement with the carotenoid content of the AURA test substance. The literature indicates that carotenoid production in the Thraustochytriaceae family (e.g. *Schizochytrium* sp., *Aurantiochytrium* sp) is associated with autotrophic growth and not heterotrophic growth on a carbon source in deep liquid culture which agrees with our finding [36].

Enrichment of Eggs, Tissues, and Organs With DHA

The mean DHA content detected in the eggs on days 0, 21, and 28 is shown in Table 4. No differences were observed between the treatment groups at the beginning of the trial. By day 21 and on day 28, supplementation significantly increased egg DHA content, with each increase

Table 2. Effect of dietary supplementation with 0, 0.5, 1.0 and 2.5% unextracted *Aurantiochytrium limacinum* biomass on various layer hen and egg performance characteristics.

		AURA[1]%					
Parameter	Units	0.0	0.5	1.0	2.5	SE	*P*-value
Initial weight, day 0	g	1949	2043	1966	2007	42.8	0.448
Final weight, day 28	g	2002	2096	1987	2063	35.2	0.176
ADFI	g/D	112.5	126.3	115.7	121.3	6.62	0.503
Feed conversion ratio		1.92	2.10	1.97	2.12	0.107	0.503
Feed conversion efficiency		0.53	0.48	0.51	0.47	0.025	0.478
Egg weight	g	59.9	62.2	60.8	59.9	0.91	0.300
Laying	%	97.6	96.8	96.8	95.6	0.79	0.790
Egg protein, day -1	%	12.74	12.77	12.84	13.02	0.161	0.628
Egg protein, day 20	%	13.78	14.12	13.85	14.06	0.262	0.760
Egg protein, day 27	%	12.37[b]	13.13[a]	13.06[a,b]	12.85[a,b]	0.188	0.040
Egg fat, day -1	%	8.36	8.56	8.97	8.30	0.302	0.407
Egg fat, day 20	%	8.98	9.10	7.95	7.86	0.352	0.035
Egg fat (%), day 27	%	8.32	8.47	9.17	8.47	0.320	0.269
Egg energy, day -1	Kcal/100g	126.14	128.10	132.08	126.73	2.49	0.384
Egg energy, day 20	Kcal/100g	135.97	138.40	126.92	127.01	3.29	0.040
Egg energy, day 27	Kcal/100g	124.40	128.73	134.74	127.61	2.611	0.122

Means in rows that do not share a superscript differ significantly.

[1]AURA: unextracted *Aurantiochytrium limacinum* algae containing 17.1 g docosahexaenoic acid/100g.

in the inclusion level of AURA corresponding to a significant increase in DHA content ($P < 0.001$). These results are in agreement with those of other authors who have observed significant increases in egg DHA content after dietary supplementation of hens with DHA-rich protists [3, 32, 34, 37]. The level of enrichment observed in these studies is typically similar to when fish oils are used as a supplement [34]; however, higher levels of egg enrichment can be achieved with marine protists without impacting the sensory characteristics of the eggs [35].

A similar trend of increasing levels of DHA enrichment was observed for the sampled tissues and organs (Table 4). Supplementation with 1 and 2.5% AURA resulted in a significantly higher breast DHA content than the control, whereas the content for 0.5% AURA was numerically higher but not statistically different. For the thigh, the DHA content increased numerically with each increase in the AURA inclusion level; however, only the 2.5% AURA had significantly more DHA than the control. In the current study, supplementation at 0.5 or 2.5% of the diet resulted in

Table 3. Main effects of dietary supplementation with 0.0, 0.5, 1.0 and 2.5 % unextracted *Aurantiochytrium limacinum* biomass (AURA) on egg Lutein, Zeaxanthin or β carotene content over a 28-day period.

	AURA[1]%					
mg/kg	0.0	0.5	1.0	2.5	SE	*P*-value
Lutein, day -1	0.57[b]	0.46[a,b]	0.37[a]	0.43[a,b]	0.046	0.037
Lutein, day 20	0.59	0.64	0.61	0.55	0.102	0.808
Lutein, day 27	0.53	0.59	0.50	0.60	0.085	0.414
Zeaxanthin, day -1	0.81[y]	0.64[x,y]	0.46[x]	0.55[x,y]	0.088	0.091
Zeaxanthin, day 20	0.72	0.87	0.78	0.71	0.118	0.624
Zeaxanthin, day 27	0.68	0.97	0.76	1.03	0.205	0.561
β–Carotene, day -1	<0.01	<0.01	<0.01	<0.01	<0.01	-
β-Carotene, day 20	<0.01	<0.01	<0.01	<0.01	<0.01	-
β-Carotene, day 27	<0.01	<0.01	<0.01	<0.01	<0.01	-

[a,b]Means that do not share a superscript differ significantly ($P < 0.05$). [x,y]Means that do not share a superscript differ ($0.05 < P < 0.1$).

[1]AURA: unextracted *Aurantiochytrium limacinum* algae containing 17.1 g docosahexaenoic acid/100g.

Table 4. Mean docosahexaenoic-acid (DHA) content (mg/100 g) of eggs, breast, thigh, liver and kidney, following dietary supplementation of layer hens for 28 D with 0, 0.5, 1.0 or 2.5% unextracted *Aurantiochytrium limacinum* biomass.

	AURA[1]%					
mg DHA/100g	0.0	0.5	1.0	2.5	SE	*P*-value
Egg, day 0	63.90	65.60	65.90	68.30	2.54	0.677
Egg, day 21	56.30^{d}	166.40^{c}	243.20^{b}	365.70^{a}	9.56	<0.001
Egg, day 28	60.30^{d}	163.80^{c}	258.90^{b}	410.10^{a}	21.9	<0.001
Breast	6.68^{c}	15.47^{b,c}	19.62^{b}	41.72^{a}	2.79	<0.001
Thigh	4.03^{b}	14.27^{a,b}	18.87^{a,b}	32.87^{a}	5.64	0.014
Liver	62.63^{d}	184.27^{c}	259.77^{b}	430.72^{a}	13.02	<0.001
Kidney	28.55^{c}	59.70^{b,c}	86.80^{b}	159.83^{a}	9.21	<0.001

^{a,b,c}Means that do not share a superscript differ significantly ($P < 0.05$).

[1]AURA: unextracted *Aurantiochytrium limacinum* algae containing 17.04 g DHA/100g.

breast tissues of layers being enriched with 16 and 42 mg DHA/100g of layer breast tissue, respectively. This is considerably lower than the levels achieved when supplementing broilers with similar levels of AURA, with 0.5 and 2.5% treatments resulting in 35 and 89 mg of DHA/100g of broiler breast tissue, respectively [38]. A similar trend was observed for the thigh tissues, with 14 vs 43 mg of DHA/100g detected in the layer vs broiler thigh samples when supplemented at 0.5% of the diet and 33 vs 115 mg DHA detected in the 2.5% group. Huang et al. [39] investigated the enrichment of layer hen eggs and thigh meat using menhaden oil and found significant increases in egg DHA content with every 1% increase in oil inclusion (1, 2, and 3%), whereas only the 3% oil supplement resulted in a significantly higher thigh DHA content than the control, 1, and 2% treatments. The authors suggested that the maturity of layer hens would result in the majority of dietary lipids being used for the production of eggs [39], which would account for the lower breast and thigh DHA content of the layers in the current study, when compared with broilers in other studies fed similar amounts of DHA-rich ingredients [36].

The DHA content of the kidney from the 1.0 and 2.5% AURA treatments was significantly higher than that of the control, whereas in the liver, each increase in AURA supplementation resulted in a significantly higher DHA content. This high DHA content is expected because of the key role that the liver plays in lipid metabolism [40]. As mean DHA intake/day increased, the DHA content of the layer breast, thigh, and kidney increased linearly, whereas the DHA content of the liver and eggs was best fit by a quadratic model (Figure 1). Similar trends of DHA accumulation in the liver and egg have been reported by Neijat et al [41, 42] when feeding increasing concentrations of DHA-rich protists to layer hens.

The efficiency of transfer of DHA from the feed to the egg differed significantly between treatments on day 21 but not 28 (Table 5). A similar trend was observed on both days, with transfer efficiency decreasing numerically with increasing AURA inclusion level; however, only the 2.5% AURA treatment on day 21 had a significantly lower transfer efficiency than the 0.5 and 1.0% AURA treatments. The lack of significance on day 28 is likely due to the sample size, with only three replicates for each treatment available for the transfer efficiency calculation. Using the same DHA-rich protist supplement, Ao et al. [32] reported a similar trend, with estimated transfer efficiencies of 45, 31, and 21% achieved when feeding AURA at a rate of 1, 2, and 3% of the diet, respectively. Other authors have also reported that increases in DHA intake are not always directly proportional to the level deposited in the eggs [37, 43]. A similar reduction in the efficiency of transfer from feed to the egg has been reported when using fish oil as a supplement [17, 44]. This drop in transfer efficiency has been attributed to a possible limit to the amount of lipid-soluble substances that can be deposited in eggs [41, 45]. In addition, the bioaccessibility of the n-3 LC-PUFA may also play a role. In one study, fish oil was shown to result in a slightly higher transfer efficiency than algae, with the authors suggesting the cell wall affected the bioavailability of n-3 LC-PUFA from this source [46].

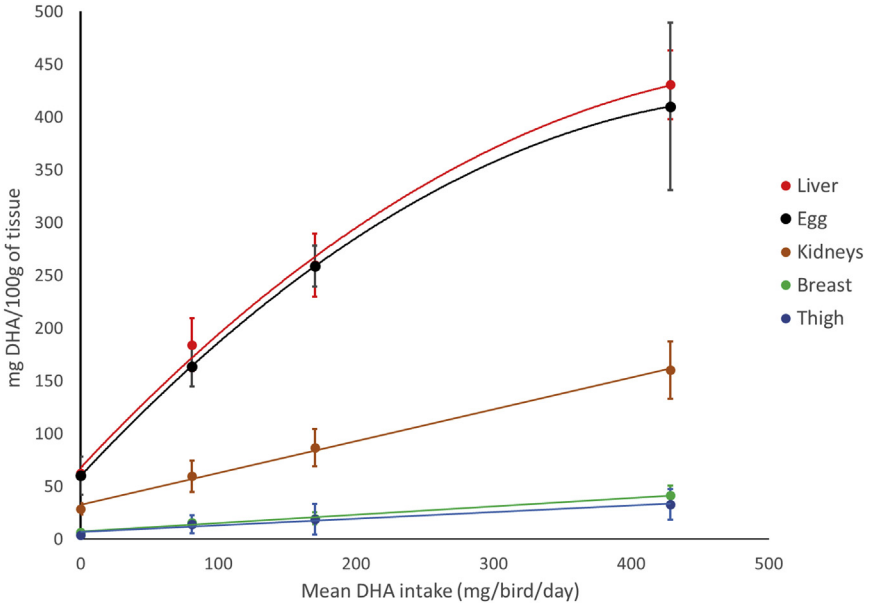

Figure 1. The mean DHA content (±95% C.I.) detected in the liver, eggs, kidneys, breast, and thigh in relation to the mean DHA intake/bird/day after supplementation for 28 D with an unextracted *Aurantiochytrium limacinum* biomass. The regression analysis was calculated based on the raw data for each sample type and the lines on the graph are described by the following regression equations. Breast = linear, r^2 = 77.4, $P < 0.001$, Y = 7.614 + 0.07806 X. Thigh = linear, r^2 = 42.16, $P < 0.001$, Y = 6.370 + 0.06558 X. Liver = quadratic, r^2 = 94.25, $P < 0.001$, Y = 70.89 + 1.329 X - 0.001131 X^2. Kidney = linear, r^2 = 83.40, $P < 0.001$, Y = 33.09 + 0.2986 X. Egg = quadratic, r^2 = 85.28, $P < 0.001$, Y = 58.31 + 1.453 X - 0.001488 X^2.

With millions of spent hens going unused as food every year, [12] the poultry industry has emphasized the need to increase the value of spent hen meat [6, 13, 47], enhancing the economic value for the producer and reducing the waste of a significant amount of nutritious food. Food waste prevention is an important aspect of the United Nations Sustainable Development goals, with an aim to half the level of food waste per capita by 2030. The choice of optimum downstream valorization of spent hen products, whether to human or companion animal food, would depend on the local demands for particular products in any specific geographic or socioeconomic region. For example, various methods of processing spent hen meats to

Table 5. Estimated efficiency of docosahexaenoic-acid (DHA) transfer from the feed to the eggs of laying hens fed diets supplemented for 28 D with 0, 0.5, 1.0 or 2.5% an unextracted *Aurantiochytrium limacinum* biomass.

	AURA[1]%					
	0.0	0.5	1.0	2.5	SE	*P*-value
[2]Mean DHA intake (mg/bird/d)	0	80.9	170.1	428.4		
[3]Mean DHA deposited, day 21	n/a	19.9	35.5	54.7		
[3]Mean DHA deposited, day 28	n/a	19.8	35.1	63.5		
[4]Transfer efficiency (%), day 21	n/a	24.7[a]	20.9[a,b]	12.8[b]	1.79	0.015
[4]Transfer efficiency (%), day 28	n/a	24.8	20.7	15.0	2.66	0.140

Means in rows that do not share a superscript differ significantly.

[1]AURA: unextracted *Aurantiochytrium limacinum* algae containing 17.1 g DHA/100g.

[2]DHA intake was calculated per pen by multiplying the feed intake recorded by the DHA content of each diet.

[3]DHA deposited was calculated by subtracting the mean DHA content of the control group eggs from the mean egg DHA content recorded for each pen.

[4]Transfer efficiency was calculated by dividing DHA deposited by DHA intake and multiplying by 100.

ARTICLE IN PRESS

improve consumer acceptability have been suggested including their use in sausages with a "healthy appeal" [47]. The use of omega-3–enriched spent hen meat could further increase the value and desirability of these processed products, especially considering consumers are already willing to pay more for omega-3–enriched products such as eggs [4, 5]. In addition, the current primary sources of LC-PUFA, fish oil and fish meal, are insufficient to meet the requirements of the world's population [48]. Processed spent hens, enriched with sustainably produced, DHA-rich protists, could offer an alternative to fish oil or fishmeal, increasing our ability to meet the LC-PUFA nutrient requirements of the population.

CONCLUSIONS AND APPLICATIONS

1. Dietary supplementation with AURA significantly increased the DHA concentration in hen eggs and tissues, with the liver and eggs enriched to the highest degree.
2. From a business sustainability perspective, producers of LC-PUFA/DHA-enriched eggs have not capitalized on the concomitant enrichment of hen tissues, which could be potentially marketed as premium ingredients in the human and companion animal markets.
3. Further work needs to be undertaken to assess the nutritive value, full fatty acid profile, oxidative stability, and economics of the production of a DHA-enriched spent hen meal ingredient for the companion animal market or the spent hen meat for human consumption.

REFERENCES AND NOTES

1. Gogus, U., and C. Smith. 2010. N-3 omega fatty acids: a review of current knowledge. Int. J. Food Sci. Technol. 45:417–436.

2. Sun, G. Y., A. Simonyi, K. L. Fritsche, D. Y. Chuang, M. Hannink, Z. Gu, C. M. Greenlief, J. K. Yao, J. C. Lee, and D. Q. Beversdorf. 2018. Docosahexaenoic acid (DHA): an essential nutrient and a nutraceutical for brain health and diseases. Prostaglandins, Leukot. Essent. Fat. Acids 136:3–13.

3. Moran, C. A., M. Morlacchini, J. D. Keegan, and G. Fusconi. 2019. Increasing the omega-3 content of hen's eggs through dietary supplementation with Aurantiochytrium limacinum microalgae: effect of inclusion rate on the temporal pattern of docosahexaenoic acid enrichment, efficiency of transfer, and egg characteristics. J. Appl. Poult. Res. 28:329–338.

4. Marshall, A. C., K. S. Kubena, K. R. Hinton, P. S. Hargis, and M. E. Van Elswyk. 1994. n-3 fatty acid enriched table eggs: a Survey of consumer acceptability. Poult. Sci. 73:1334–1340.

5. Surai, P. F., and N. H. C. Sparks. 2001. Designer eggs: from improvement of egg composition to functional food. Trends Food Sci. Technol. 12:7–16.

6. Trindade, M. A., P. E. Felício, and C. J. Contreras Castillo. 2004. Mechanically separated meat of broiler breeder and white layer spent hens. Sci. Agric. 61:234–239.

7. Cheng, C. H., T. F. Shen, W. L. Chen, and S. T. Ding. 2004. Effects of dietary algal docosahexaenoic acid oil supplementation on fatty acid deposition and gene expression in laying Leghorn hens. J. Agric. Sci. 142:683–690.

8. Mello, J. L. M., R. A. Souza, G. C. Paschoalin, F. B. Ferrari, M. P. Berton, A. Giampietro-Ganeco, P. A. Souza, and H. Borba. 2017. Physical and chemical characteristics of spent hen breast meat aged for 7 days. Anim. Prod. Sci. 57:2133.

9. Semwogerere, F., J. Neethling, V. Muchenje, and L. C. Hoffman. 2018. Effects of production systems on the carcass and meat quality characteristics of spent laying hens. Poult. Sci. 97:1990–1997.

10. Hollender, R., J. H. Mac Neil, and M. G. Mast. 1987. Effect of fragmentation method and formulation on the quality of patties made from restructured spent layer meat. J. Food Sci. 52:290–293.

11. Kumar, R., S. Biswas, V. Singh, and M. Ram. 2015. Quality and Shelf Life Evaluation of nuggets. *Explor.* Anim. Med. Res. 5:176–182.

12. Mueller, S., M. Kreuzer, R. E. Messikommer, and I. D. M. Gangnat. 2016. Carcass and meat quality of spent hens from dual purpose Vs layer Genotypes when fed a by-product diet. Int. Congr. Meat Sci. Technol. Bangkok 12–15.

13. Kondaiah, N., and B. Panda. 1992. Processing and Utilization of spent hens. Worlds Poult. Sci. J. 48:255–268.

14. Lyons, J. J., and J. M. Vandepopuliere. 1997. Alternate procedures used to process spent Leghorn hens. J. Appl. Poult. Res. 6:74–80.

15. Ajuyah, A. O., R. T. Hardin, K. Cheung, and J. S. Sim. 1992. Yield, lipid, cholesterol and fatty acid composition of spent hens fed full-fat oil seeds and fish meal diets. J. Food Sci. 57:338–341.

16. Ribeiro, T., M. M. Lordelo, S. P. Alves, R. J. B. Bessa, P. Costa, J. P. C. Lemos, L. M. A. Ferreira, C. M. G. A. Fontes, and J. A. M. Prates. 2013. Direct supplementation of diet is the most efficient way of enriching broiler meat with n-3 long-chain polyunsaturated fatty acids. Br. Poult. Sci. 54:753–765.

17. Gonzalez-Esquerra, R., and S. Leeson. 2000. Effect of feeding hens regular or deodorized menhaden oil on production parameters, yolk fatty acid profile, and sensory quality of eggs. Poult. Sci. 79:1597–1602.

18. Salem, N., and M. Eggersdorfer. 2015. Is the world supply of omega-3 fatty acids adequate for optimal human nutrition? Curr. Opin. Clin. Nutr. Metab. Care 18:147–154.

19. The pens measured 160 cm x 140 cm giving a stocking density was of 0.24 m^2 per animal.

20. ALL-G-RICH; CCAP 4087/2; Alltech Inc., Nicholasville, KY.

ARTICLE IN PRESS

21. Dillon, G. P., A. Yiannikouris, W. Brandl, C. Cardinall, W. Yuan, and C. A. Moran. 2019. Analytical method assessment for the determination of DHA and fatty acids present in unextracted Aurantiochytrium limacinum biomass. Food Nutr. Sci. 10:469–483.

22. Floch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226:497–509.

23. Bannon, C. D., J. D. Craske, and A. E. Hilliker. 1985. Analysis of fatty acid methyl esters with high accuracy and reliability. IV. Fats with fatty acids containing four or more carbon atoms. J. Am. Oil Chem. Soc. 62:1501–1507.

24. Panfili, G., A. Fratianni, and M. Irano. 2004. Improved normal-phase high-performance liquid chromatography procedure for the determination of carotenoids in cereals. J. Agric. Food Chem. 52:6373–6377.

25. Martillotti, F. 1987. Metodi di analisi per la valutazione degli alimenti d'impiego zootecnico. CNR. IPRA, Rome, Italy.

26. Dillon, G. P., A. Yiannikouris, W. Brandl, C. Cardinall, W. Yuan, and C. A. Moran. 2019. Matrix extension with fitness for purpose and stability assessment of DHA and additional fatty acids in individual whole chicken eggs. Food Nutr. Sci. 10:517–532.

27. Mérieux NutriSciences, Resana, Italy.

28. Dillon, G. P., A. Yiannikouris, W. Brandl, C. Cardinall, W. Yuan, and C. A. Moran. 2019. Fitness for purpose and stability assessment of long-chain polyunsaturated fatty acids in chicken tissues. Regul. Toxicol. Pharmacol. 103:93–99.

29. SP Scientific, Warminster, PA.

30. Nu-Chek Prep, Inc., Elysian, MN.

31. Minitab Ltd., Coventry, United Kingdom.

32. Ao, T., L. M. Macalintal, M. A. Paul, A. J. Pescatore, A. H. Cantor, M. J. Ford, B. Timmons, and K. A. Dawson. 2015. Effects of supplementing microalgae in laying hen diets on productive performance, fatty-acid profile, and oxidative stability of eggs. J. Appl. Poult. Res. 24:394–400.

33. Cachaldora, P., P. García-Rebollar, C. Alvarez, J. C. De Blas, and J. Méndez. 2008. Effect of type and level of basal fat and level of fish oil supplementation on yolk fat composition and n-3 fatty acids deposition efficiency in laying hens. Anim. Feed Sci. Technol. 141:104–114.

34. Fraeye, I., C. Bruneel, C. Lemahieu, J. Buyse, K. Muylaert, and I. Foubert. 2012. Dietary enrichment of eggs with omega-3 fatty acids: a review. Food Res. Int. 48:961–969.

35. Herber-Mcneill, S. M. M., and M. E. E. Van Elswyk. 1998. Dietary marine algae Maintains egg consumer acceptability while enhancing yolk color. Poult. Sci. 77:493–496.

36. Yokoyama, R., and D. Honda. 2007. Taxonomic rearrangement of the genus Schizochytrium sensu lato based on morphology, chemotaxonomic characteristics, and 18S rRNA gene phylogeny (Thraustochytriaceae, Labyrinthulomycetes): emendation for Schizochytrium and erection of Aurantiochytrium and Oblongichytrium gen. nov. Mycoscience 48:199–211.

37. Herber, S. M., and M. E. Van Elswyk. 1996. Dietary marine algae promotes efficient deposition of n-3 fatty acids for the production of enriched shell eggs. Poult. Sci. 75:1501–1507.

38. Moran, C. A., D. Currie, J. Keegan, and A. Knox. 2018. Tolerance of broilers to dietary supplementation with high levels of the DHA-rich Microalga, Aurantiochytrium limacinum: effects on health and productivity. Animals 8:180.

39. Huang, Z., H. Leibovitz, C. M. Lee, and R. Millar. 1990. Effect of dietary fish oil on omega-3 fatty acid levels in chicken eggs and thigh flesh. J. Agric. Food Chem. 38:743–747.

40. Nguyen, P., V. Leray, M. Diez, S. Serisier, J. Le Bloc'h, B. Siliart, and H. Dumon. 2008. Liver lipid metabolism. J. Anim. Physiol. Anim. Nutr. 92:272–283.

41. Neijat, M., O. Ojekudo, and J. D. House. 2016. Effect of flaxseed oil and microalgae DHA on the production performance, fatty acids and total lipids of egg yolk and plasma in laying hens. Prostaglandins Leukot. Essent. Fat. Acids 115:77–88.

42. Neijat, M., P. Eck, and J. D. House. 2017. Impact of dietary precursor ALA versus preformed DHA on fatty acid profiles of eggs, liver and adipose tissue and expression of genes associated with hepatic lipid metabolism in laying hens. Prostaglandins Leukot. Essent. Fat. Acids 119:1–17.

43. Bruneel, C., C. Lemahieu, I. Fraeye, E. Ryckebosch, K. Muylaert, J. Buyse, and I. Foubert. 2013. Impact of microalgal feed supplementation on omega-3 fatty acid enrichment of hen eggs. J. Funct. Foods 5:897–904.

44. Lemahieu, C., C. Bruneel, R. Termote-Verhalle, K. Muylaert, J. Buyse, and I. Foubert. 2013. Impact of feed supplementation with different omega-3 rich microalgae species on enrichment of eggs of laying hens. Food Chem. 141:4051–4059.

45. Leeson, S., and L. Caston. 2004. Enrichment of eggs with lutein. Poult. Sci. 83:1709–1712.

46. Lemahieu, C., C. Bruneel, E. Ryckebosch, K. Muylaert, J. Buyse, and I. Foubert. 2015. Impact of different omega-3 polyunsaturated fatty acid (n-3 PUFA) sources (flaxseed, Isochrysis galbana, fish oil and DHA Gold) on n-3 LC-PUFA enrichment (efficiency) in the egg yolk. J. Funct. Foods 19:821–827.

47. Souza, K. M. R., R. B. Araujo, A. L. Santos, C. E. C. Rodrigues, D. E. Faria, and M. A. Trindade. 2011. Adding value to the meat of spent laying hens manufacturing sausages with a healthy appeal. Braz. J. Poult. Sci. 13:57–63.

48. Tocher, D., M. Betancor, M. Sprague, R. Olsen, and J. Napier. 2019. Omega-3 long-chain polyunsaturated fatty acids, EPA and DHA: Bridging the Gap between supply and demand. Nutrients 11:89.

Acknowledgments

The authors would like to express their gratitude to the following people: Rebecca Timmons (Alltech Inc., USA) for her technical input on AURA and Tuoying Ao (Alltech Inc., USA) for his knowledge of the application of AURA in layer hen nutrition. Funding for this work was provided by Alltech SARL (France).