Feeding laying hens docosa hexaenoic acid-rich microalgae oil at 40 g/kg diet causes hypotriglyceridemia, depresses egg production, and attenuates expression of key genes affecting hepatic triglyceride synthesis and secretion, but is rescued by dietary co-supplementation of high-oleic sunflower oil

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ABSTRACT The primary goal of this study was to investigate the effect of feeding White Leghorn hens graded levels of a docosahexaenoic acid (**DHA**)-rich microalgae oil (**MAO**) on productive performance and enrichment of eggs with very long-chain (**VLC**) omega-3 (**n-3**) polyunsaturated fatty acids (**PUFA**). Forty-nine-week-old hens (8 per diet) were fed the following diets for 28 d: 1) A corn-soybean meal-based diet with no supplemental oil (**CON**); 2) CON + 10 g/kg MAO; 3) CON + 20 g/kg MAO; 4) CON + 30 g/kg MAO; 5) CON + 40 g/kg MAO; 6) CON + 40 g/kg MAO + 20 g/kg high-oleic sunflower oil (**HOSO**); and 7) CON + 40 g/kg MAO + 40 g/kg HOSO. Diets 6 and 7 were included because we previously reported that co-feeding high-oleic acid oils with n-3 PUFA-containing oils attenuated egg yolk n-3 PUFA contents vs. feeding hens the n-3 oils alone. All data were collected on an individual hen basis. Egg VLC n-3 PUFA enrichment plateaued, in terms of statistical significance, at the 30 g/kg MAO level (266 mg/yolk). Hens fed 40 g/kg MAO had greatly attenuated measures of hen performance, marked liver enlargement, an altered ovarian follicle hierarchy, greatly lowered circulating triglyceride levels, and depressed hepatic expression of key genes involved in triglyceride synthesis and secretion. As compared to hens fed 40 g/kg MAO alone, feeding hens 40 g/kg MAO co-supplemented with HOSO (Diets 6 and 7) restored egg production, ovarian morphology, and all other measures of hen productive performance to CON levels, elevated plasma triglyceride levels, prevented liver enlargement, and increased the hepatic expression of key genes involved in triglyceride synthesis and secretion. In conclusion, MAO can greatly enrich hens' eggs with VLC n-3 PUFA, but its recommended dietary inclusion should not exceed 20 g/kg. This would allow for near-maximal yolk VLC n-3 PUFA enrichment without impairing hen productive performance, altering the ovarian follicle hierarchy or, based on the work of others, presumably imparting off-flavors in the egg.

Key words: docosahexaenoic acid, high-oleic sunflower oil, hypotriglyceridemia, laying hen, microalgae oil

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INTRODUCTION

The many human health-promoting effects of consuming very long-chain (**VLC**) omega-3 (**n-3**) polyunsaturated fatty acids (**PUFA**), specifically eicosapentaenoic acid (**EPA**; 20:5 n-3), and docosahexaenoic acid (**DHA**; 22:6 n-3) are well documented and include improved outcomes in the areas of cardiovascular disease, cancer risk, eye health, and cognitive function in older healthy adults (Backes et al., 2016; Shahidi and Ambigaipalan, 2018). Dietary sources of VLC n-3 PUFA include cold water fatty (oily) marine fish as well as nonfish sources, such as microalgae oils (**MAO**; Ryckebosch et al., 2014), and enriched foods such as eggs (Hargis and Van Elswyk, 1993; Fraeye et al., 2012) and poultry meat (Leskanich and Noble, 1997; Betti et al., 2009). Concerns regarding fish sources include the health risks of methylmercury, which is found in certain species of cold-water marine fish, the sustainability of the world's fish stocks, and the environmental effects of aquaculture (Nesheim and Nestle, 2014).

With regard to the VLC n-3 PUFA enrichment of poultry meat and eggs, many researchers have fed chickens fish oil or fish meal, as well as flaxseed meal or flaxseed oil, with initial reports dating back 50 yr (Navarro

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et al., 1972). Fish oil is rich in EPA and DHA, but feeding laying hens or broilers fish meal or fish oil can impart a "fishy taint" in eggs and meat; in order to avoid this problem, it is recommended that supplemental dietary levels of fish oil should be at or below 15 g/kg (Hargis and Van Elswyk, 1993; Leskanich and Noble, 1997). Flaxseed meal and flaxseed oil are rich sources of α-linolenic acid (**ALA**; 18:3 n-3). However, in order to be converted to VLC n-3 PUFA, ALA must undergo a series of elongation and desaturation steps in the liver (Saini and Keum, 2018). Like humans, chickens have a limited ability to synthesize VLC n-3 PUFA from ALA or stearidonic acid (**SDA**: 18:4 n-3), the desaturation product of ALA (Fraeye et al., 2012; Elkin et al., 2015, 2016; El-Zenary et al., 2021) and egg and tissue contents of DHA can be more readily enriched by direct dietary supplementation of DHA-rich ingredients (Leskanich and Noble, 1997; Cachaldora et al., 2008; Fraeye et al., 2012, Ribeiro et al., 2013, Elkin et al., 2015; Neijat et al., 2017). It is also probable that, as in the rat (Tu et al., 2010), VLC n-3 PUFA synthesis in chickens is regulated more by substrate levels than gene expression (Elkin et al., 2018).

In addition to fish meal and fish oil, algal biomass (Rymer et al., 2010; Fraeye et al., 2012; Lemahieu et al., 2013; Neijat et al., 2016; Manor et al., 2019; Moran et al., 2019, 2020; Tolba et al., 2019; Wu et al., 2019; Liu et al., 2020; Kalia and Lei, 2022) and MAO (Feng et al., 2020; Khan et al., 2021) are the other main commercially available ingredients containing preformed EPA and DHA that have been fed to poultry. Although oilseed crops, such as camelina (*Camelina sativa*) and canola (*Brassica napus*), which naturally are devoid of EPA and DHA, have recently been genetically engineered to contain VLC n-3 PUFA (Tejera et al., 2016; Walsh et al., 2016), to the authors' knowledge, they are not yet commercially available in the United States as animal feedstuffs. Moreover, *Camelina* is a member of the Brassicaceae family and contains glucosinolates, which may cause digestive tract irritation (Burrows and Tyrl, 2001); thus, any oil obtained from the engineered crop will need to be demonstrated to be free of this antinutrient.

In human medicine, oral prescription formulations of VLC n-3 PUFA are used effectively for the treatment of hypertriglyceridemia, which is defined as >150 mg/100 mL (Backes et al., 2016; Wolska et al., 2020). In contrast, White Leghorn hens *normally* have very high levels of circulating triglycerides, which can range from approximately 1,200 mg/100 mL to 3,800 mg/100 mL (Elkin et al., 2003, 2006) depending upon the strain and age of the hens. The triglycerides are primarily contained within hepatically synthesized very low-density lipoprotein particles (**VLDL**), which are secreted into the bloodstream and taken up by growing oocytes (future egg yolks) by receptor-mediated endocytosis (Schneider, 2009). VLDL is the main egg yolk precursor macromolecule and typically constitutes approximately 60% of egg yolk mass (Burley et al., 1993). Therefore, depending upon the dietary level, it is possible that feeding hens preformed VLC n-3 PUFA may attenuate circulating triglyceride levels and affect yolk formation and egg production.

The primary goal of the present study was to evaluate the effect of feeding laying hens graded levels of a DHA-rich MAO on the enrichment of eggs and tissues with VLC n-3 PUFA. A second objective was to observe the effect of the dietary DHA-rich MAO on hen performance and the expression of key genes related to VLDL and triglyceride synthesis and secretion. Lastly, based on prior studies in our laboratory which showed that co-feeding hens a high-oleic acid (**OLA**; 18:1 n-9) oil along with either a high-ALA flaxseed oil or a high-SDA soybean oil, attenuated egg yolk and tissue n-3 PUFA contents compared to feeding the ALA-rich or SDA-rich oils alone (Elkin et al., 2018, 2021), 2 additional diets were included in which high-OLA sunflower oil (**HOSO**) was co-fed with the high-DHA MAO.

MATERIALS AND METHODS

Animals, Diets, and Management

One-hundred, 48-wk-old Hy-Line W-36 White Leghorn hens were obtained from the University flock, housed in individual 30 cm (w) × 43 cm (d) × 36 cm (h) cages with sloping plastic-coated wire floors in an environmentally controlled room located in the Penn State Poultry Education and Research Center layer facility, and provided with 16 h of light daily. The hens had been acquired as 1-d-old chicks from the Hy-Line Hatchery (Elizabethtown, PA) and subjected to management and feeding protocols in general accordance with the breeder recommendations (Hy-Line W-36 Commercial Management Guide, Hy-Line International, West Des Moines, IA; www.hyline.com; accessed May 8, 2019). When the hens were 49-wk-old (d 0 of the experiment), the 56 best-performing hens were selected based on 5-day pre-experimental egg weights and egg production and were allocated to 1 of 7 dietary treatment groups ($n = 8$). Each group averaged approximately 95% hen-day egg production and had average egg weights of approximately 59.5 g for the 5 d.

The following diets were fed to the hens for 28 d: 1) A de-germinated corn-ground yellow corn-soybean meal-based diet with no supplemental oil (control diet; **CON**); 2) CON + 10 g/kg DHA-rich MAO (DHA Natur oil, refined and bleached, Archer Daniels Midland Company, Clinton, IA); 3) CON + 20 g/kg MAO; 4) CON + 30 g/kg MAO; 5) CON + 40 g/kg MAO; 6) CON + 40 g/kg MAO + 20 g/kg HOSO; and 7) CON + 40 g/kg MAO + 40 g/kg HOSO (Table 1). The diets were formulated to meet or exceed the hens' nutrient requirements as listed in the Hy-Line W-36 Commercial Management Guide. The 7 diets were isonitrogenous and isocaloric (167 g/kg crude protein and approximately 2,839 kcal ME/kg) using corn starch and cellulose to balance caloric content and volume, respectively. Degerminated corn meal was substituted for half of the dietary ground yellow corn because it has comparatively

Table 1. Diet compositions and nutrient contents (g/kg, as-is basis).

	Diet						
Ingredient	1	2	3	4	5	6	7
DHA-rich microalgae oil[1]	0.0	10.0	20.0	30.0	40.0	40.0	40.0
High-oleic sunflower oil[2]	0.0	0.0	0.0	0.0	0.0	20.0	40.0
Corn starch[3]	180.0	157.5	135.0	112.5	90.0	45.0	0.0
Cellulose[4]	0.0	12.5	25.0	37.5	50.0	75.0	100.0
Basal mixture[5]	820.0	820.0	820.0	820.0	820.0	820.0	820.0
Analyzed composition[6]							
Total fatty acids[7]	16.50	24.59	35.73	47.83	59.41	73.24	95.91
Calculated composition							
Crude protein	166.6	166.6	166.6	166.6	166.6	166.6	166.6
Metabolizable energy (kcal/kg)	2839	2839	2839	2839	2839	2839	2839
Arginine, digestible	10.42	10.42	10.42	10.42	10.42	10.42	10.42
Isoleucine, digestible	6.57	6.57	6.57	6.57	6.57	6.57	6.57
Lysine, digestible	8.47	8.47	8.47	8.47	8.47	8.47	8.47
Methionine, digestible	4.25	4.25	4.25	4.25	4.25	4.25	4.25
Methionine + Cyst(e)ine, digestible	6.60	6.60	6.60	6.60	6.60	6.60	6.60
Threonine, digestible	5.60	5.60	5.60	5.60	5.60	5.60	5.60
Tryptophan, digestible	1.86	1.86	1.86	1.86	1.86	1.86	1.86
Valine, digestible	7.05	7.05	7.05	7.05	7.05	7.05	7.05
Calcium	45.11	45.11	45.11	45.11	45.11	45.11	45.11
Available phosphorus	5.03	5.03	5.03	5.03	5.03	5.03	5.03

[1]DHA-Natur oil, refined and bleached, Archer Daniels Midland, Clinton, IA.
[2]Jedwards International, Inc., Braintree, MA.
[3]Argo corn starch, ACH Food Companies, Inc., Memphis, TN.
[4]Solka-Floc 200 FCC, Solvaira Specialties, North Tonawanda, NY.
[5]The proprietary basal mixture (Wenger Feeds, Rheems, PA) consisted primarily of soybean meal (282 g/kg), ground corn (183 g/kg), de-germinated course yellow corn meal (183 g/kg), ground wheat (36 g/kg), alfalfa meal (12 g/kg), calcium chips (63 g/kg), limestone (42 g/kg), monocalcium phosphate (12.9 g/kg), sodium chloride (4.2 g/kg), DL-methionine (2.05 g/kg), L-threonine (0.05 g/kg), choline, 70% (0.3 g/kg), supplemental vitamins (including 25 IU/kg of vitamin E), trace minerals, and enzymes.
[6]Mean of 2 analyses per diet.
[7]See Table 2 for individual fatty acid contents.

lower levels of linoleic acid (**LA**; 18:2 n-6). LA has been shown to compete with ALA for access to elongase and desaturase enzymes of the VLC n-3 PUFA biosynthetic pathway in the liver (Fraeye et al., 2012; Kartikasari et al., 2012; Jing et al., 2013). Therefore, by keeping dietary LA levels low, any absorbed dietary ALA will have greater access to the desaturases and elongases and will contribute (albeit to a minor extent) to the DHA pool available for transfer from the liver to the egg yolk and tissues. Analysis of the diets and oils verified both the incremental increases in DHA from MAO in Diets 1 to 5, and the addition of OLA from HOSO in Diets 6 and 7 (Table 2). The MAO contained 47.45% DHA and the HOSO contained 82.40% OLA by analysis. Feed and water were provided for ad libitum consumption.

All data were collected on an individual basis and feed allocations and egg weights were recorded daily. With some exceptions, 1 egg per hen was collected on d 0 and d 28, or on d 27 if an egg was not laid on 28 (or on d 26 if an egg was not laid on d 27) for the determination of component (shell, albumen, and yolk) weights and percentages by standard procedures described in Elkin et al. (1999). The exceptions were that 1 hen from Diet 2 (CON + 10 g/kg MAO) died of unknown causes on d 25, and 1 hen from Diet 6 (CON + 40 g/kg MAO + 20 g/kg HOSO) that went out of production on d 19 and subsequently molted was removed from the study. Five hens from Diet 5 (CON + 40 g/kg MAO) ceased egg production entirely on d 12, 22, 23 (2 hens), and 27. Thus, 1 egg was obtained for analysis from each of the 8 hens fed Diets 1, 3, 4, and 7; 1 egg was obtained for analysis from each of the 7 hens fed Diets 2 and 6; and 1 egg each was obtained for analysis from 4 hens fed Diet 5 (an egg laid on d 26 from the hen that ceased laying on d 27 was included).

Egg yolks obtained were frozen (−20°C) and subsequently lyophilized. Hen body weights were recorded on d 0 and d 28. All 55 hens (1 hen died during the study) were euthanized at the end of the experiment (d 28) by electric stunning followed by exsanguination via severing of the jugular vein. Blood was collected in 9 mL Greiner K3 EDTA vacutainer tubes (VWR, Radnor, PA; catalog # 95057-237). The tubes were placed on ice until subsequent centrifugation at 1,300 × *g* for 15 min at 4°C. The plasma was decanted and frozen at −80°C until analyzed for fatty acids, triglycerides, and total estrogens content. Whole livers, muscle (boneless and skinless half breast), and abdominal fat pads were excised and weighed, and samples were snap frozen in liquid nitrogen, and stored at −80°C until subsequent analysis for fatty acid content (all tissues) and expression of key genes regulating triglyceride, VLDL, and fatty acid synthesis and metabolism (liver). All of the vitellogenic follicles (i.e., those that had been recruited into the yolk-filled hierarchy; Etches and Petite, 1990) were individually excised from the ovaries of each hen, transferred to a plastic petri dish, and weighed. All animal protocols were approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University (#PROTO201900793).

Table 2. Fatty acid content of the diets (g/kg, as-is basis) and oils (% of fatty acids)[1].

		Diet							Oil	
		1	2	3	4	5	6	7		
	DHA-rich microalgae oil (MAO)	0	10	20	30	40	40	40		
Fatty acid	High-oleic sunflower oil (HOSO)	0	0	0	0	0	20	40	MAO	HOSO
14:0		0.04	2.96	5.59	9.04	11.77	10.98	11.12	26.42	0.06
14:1 n-5		0.01	0.04	0.06	0.10	0.13	0.12	0.12	0.29	0
15:0		0.01	0.03	0.03	0.03	0.03	0.03	0.04	0.05	0.02
16:0		2.53	3.34	4.63	5.90	7.20	7.35	8.52	10.83	4.61
16:1 n-7		0.05	0.06	0.07	0.09	0.12	0.15	0.18	0.18	0.18
17:0		0.04	0.03	0.03	0.03	0.03	0.03	0.05	0.01	0.03
18:0		0.49	0.49	0.58	0.61	0.66	1.24	1.93	0.39	3.11
18:1 n-9		3.43	2.95	3.33	3.18	3.54	19.46	36.29	0.62	82.40
18:1 n-7		0.20	0.21	0.22	0.24	0.24	0.57	1.17	0.19	0
18:1 t12		0	0.03	0.05	0.08	0.10	0.09	0.09	0.21	0
18:2 n-6		8.67	7.46	8.53	8.06	8.82	8.65	10.84	1.39	7.26
18:3 n-6		0.06	0.05	0.07	0.07	0.08	0.14	0.20	0.07	0.29
18:3 n-3		0.80	0.72	0.80	0.85	0.89	0.80	0.85	0.33	0.06
18:4 n-3		0	0.03	0.06	0.09	0.12	0.11	0.12	0.26	0.02
20:0		0	0.01	0.02	0.03	0.04	0.04	0.04	0.09	0
20:1 n-9		0.05	0.05	0.05	0.05	0.06	0.11	0.17	0.01	0.26
20:3 n-6		0.05	0.05	0.04	0.04	0.05	0.05	0.05	0.18	0.98
20:3 n-3		0	0.03	0.06	0.11	0.14	0.13	0.13	0.33	0
20:4 n-6		0	0.01	0.04	0.03	0.04	0.04	0.05	0.08	0.01
20:4 n-3		0	0.05	0.09	0.15	0.19	0.18	0.18	0.46	0.04
20:5 n-3		0	0.03	0.07	0.10	0.14	0.13	0.13	0.34	0
22:5 n-6		0.02	0.02	0.05	0.05	0.07	0.07	0.07	0.14	0.02
22:5 n-3		0	0.01	0.02	0.01	0.02	0.02	0.01	0.05	0
22:6 n-3		0.02	4.98	9.40	15.80	20.83	18.82	19.24	47.45	0.02
24:0		0.03	0.02	0.05	0.04	0.05	0.11	0.19	0.03	0.35
24:1 n-9		0	0	0	0	0	0	0	0	0.01
Others		0	0.93	1.79	3.05	4.05	3.82	4.13	9.60	0.27
Total		16.50	24.59	35.73	47.83	59.41	73.24	95.91	100.00	100.00

[1]Values are means of 2 analyses per diet or oil sample.

Diet, Egg, and Tissue Analyses

Diets, oils, egg, and tissue fatty acid contents were subjected to a dual methylation procedure using 0.5 M sodium methoxide in methanol followed by 5% methanolic HCl (Jenkins, 2010). Individual fatty acids (**FA**) were separated and quantified by gas-liquid chromatography using an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a fused-silica capillary column (SP2560; 100 m × 25 mm i.d. with 0.2-μm film thickness; Supelco, Inc., Bellefonte, PA) and a flame ionization detector (Elkin et al., 2016). Total FA concentration and methylation efficiencies were determined using 3 external standards (13:0 and 19:0 free FA and 17:1 methyl ester; Nu-Chek Prep, Inc., Elysian, MN). Peaks were identified based on purified standards (GLC780, GLC461, GLC 566, and pure 22:1 n-9 [Nu-Chek Prep]; and pure 20:4 n-3 [Caymen Chemical Company, Ann Arbor, MI]), with the exception of SDA, which was identified based on the analysis of a high-SDA soybean oil (Elkin et al., 2015). An equal weight standard (GLC461; Nu-Chek Prep) was used for calculation of recovery factors.

The efficiency of conversion (g/g) of dietary DHA to egg yolk DHA, or the conversion of dietary DHA to egg yolk VLC n-3 PUFA, took into account average daily egg production but did not take into account the very small amount of dietary ALA (Table 2) that may have been elongated and desaturated, and was calculated as follows: [DHA or VLC n-3 PUFA (mg/yolk) × (hen-day egg production %/100)]/[Daily feed intake (g) × dietary DHA content (mg/g)]. For example: (130.6 × 0.9184)/(101.2 × 4.98) = 0.2380 or 23.80% conversion.

For the hepatic gene expression analyses, total RNA extraction, RNA concentration and quality assessment, cDNA synthesis, and quantitative real-time RT-PCR using SYBR green were determined as previously described (Elkin et al., 2015). Expression of key genes or transcription factors related to triglyceride synthesis (diacylglycerol acetyltransferase 2 [**DGAT2**]), VLDL synthesis and secretion (apolipoproteinVLDL-II [**apoVLDL-II**]; apolipoproteinB [**apoB**]; microsomal triglyceride transfer protein [**MTTP**]; and lysophosphatidylcholine acetyltransferase 3 [**Lpcat3**]), and fatty acid synthesis (sterol regulatory element binding protein-1 [**SREBP1**] and fatty acid synthase [**FASN**]) were determined in quadruplicate using validated primers (Table 3) and gene expression was quantified as previously described (Elkin et al., 2018). The geometric mean of the housekeeping genes (Vandesompele et al., 2002) was used as a covariant in the statistical analysis and data are reported relative to the CON treatment (Diet 1 of Table 1) which was set to 100.

Plasma Analyses

Plasma FA were determined following lipid extraction in hexane-isopropanol (3:2, vol:vol) as described by Hara and Radin (1978), and methylation and analysis

Table 3. Primers used for quantitative PCR.

Gene[1]	Sequence[2]	Primers 5' to 3'	Amplicon size (bp)	Gene accession number
ApoB	F	5′-GCTCAGCAGTGGTGTTCTCA-3′	142	NM_001044633
	R	5′-TGTTCCCGTGATCCCACTTG-3′		
ApoVLDL-II	F	5′-GCCTGGGAGAGAGAAAGCAG-3′	142	NM_205483
	R	5′-CTTTGAGTGCACTTCAGGGAC-3′		
DGAT2	F	5′-CACGTTCCTCATCATGGGTATT-3′	142	XM_040661934
	R	5′-CTGGGATCTTCTTCCACCTTTC-3′		
FASN	F	5′-CTTGAGTTGGCACAGTGG-3′	120	NM_205155
	R	5′-GATTCCCAGCGCCTTCCA-3′		
Lpcat3	F	5′-TCGTTACGGAAGGTGTCTGC-3′	168	XM_046908955
	R	5′-CCCAAGCGTTGGTGTTGATG-3′		
MTTP	F	5′-GGGCAGTCCAGCATGATTGT-3′	94	NM_001109784
	R	5′-AGCTTGGTCCAGTAGTGTGC-3′		
SREBP1	F	5′-ACCGCTCATCCATCAACGAC-3′	87	XM_046927256
	R	5′-TCAGGATCGCCGACTTGTTG-3′		

[1]ApoB, apolipoprotein B; ApoVLDL-II, apolipoprotein VLDL-II; DGAT2, diacylglycerol acetyltransferase 2; FASN, fatty acid synthase; Lpcat3, lysophosphatidylcholine acetyltransferase 3; MTTP, microsomal triglyceride transfer protein; and SREBP1, sterol regulatory element binding protein 1.
[2]F, forward; R, reverse.

by gas-liquid chromatography as described above for the diets, oils, and tissues. Plasma triglycerides were measured using a glycerol-blanked kit (L-Type Triglyceride M; FujiFilm Healthcare Solutions, Valhalla, NY). Briefly, plasma was diluted 3-fold in double distilled water. Two standard curves were used including 105 to 630 mg/100 mL by increasing amounts of Wako Multi-Calibrator Lipids (Cat. No. 464-01601) and by dilution of a high lipid standard available from the manufacturer through special request.

Plasma total estrogens concentration was determined by a commercial kit (ImmuChem Double Antibody Total Estrogens ^{125}I RIA Kit; Cat. No. 07-140202; MP Biomedicals, LLC, Solon, OH). The kit detects the combined concentrations of unconjugated estradiol-17β (**E2**) and estrone (**E1**). It does not detect estrogen sulfates or glucuronides. Therefore, the total estrogens measurements are an approximation of plasma estrogenicity, since E2 is biologically more active than E1. For the analysis of plasma total estrogens, an extraction step was necessary prior to running the assay according to the manufacturer's recommendations. Briefly, 6 mL of ethyl acetate: hexane (3:2, vol:vol) was added to 500 μL of plasma. Tubes were vortexed vigorously for 60 s and phases were allowed to separate. Next, 5 mL of the organic phase (top phase) was withdrawn and evaporated under nitrogen. The sample residue was then reconstituted with 2.5 mL of steroid diluent provided by the manufacturer and incubated at room temperature for 1 h while being gently mixed on a plate shaker. Extracted samples were stored at −20°C. The intraassay CV was less than 5%, the interassay CV was less than 4%, and the sensitivity of this assay was 2.5 to 100 pg/mL.

Statistical Analyses

Data were analyzed as a one-way ANOVA using JMP Pro 14 and 16 (SAS Institute, Cary, NC). Data were log transformed when necessary and back transformed data are reported. Data points outside of ±3 Studentized residuals were considered outliers and were removed from the analysis. This rarely occurred for more than 1 value per variable. When significant ($P \leq 0.05$) treatment effects were observed, means were separated by a Protected LSD.

RESULTS

Diets, Animal Performance, and Tissue Weights

The dietary FA contents reflected the FA profiles of the oils (Table 2) and differed mainly in the contents of myristic acid (14:0), palmitic acid (16:0), OLA, and DHA. The total FA contents of the diets reflected the varying amounts of supplemental oils.

Dietary MAO appeared to have little effect on hen performance, with the exception of hens fed Diet 5 (40 g/kg MAO), which exhibited significantly depressed average body weights, feed intakes, hen-day egg production, as well as poorer feed conversion efficiencies compared to the other dietary treatments (Table 4). Average egg weights over the entire study were also lower (but $P > 0.05$; Table 4). With regard to hen-day egg production, 5 of the 8 hens fed the 40 g/kg MAO diet (Diet 5) completely ceased laying eggs prior to the end of the study (specifically, on d 12, 21, 23, 23, and 27; data not shown) and by d 28, 3 of the hens had completely regressed ovaries (Figure 1). Individual 28-day feed intakes of hens fed 40 g/kg MAO averaged 82.5. 83.0, 91.9, and 96.7 g/hen/d for the 4 hens that remained in production until at least d 27 of the study; conversely, 28-d feed intakes were markedly lower for the 4 hens that ceased egg production between d 12 and d 23, and were 53.5, 56.9, 61.3, and 64.7 g/hen/d (data not shown). However, feed intakes for all 8 hens fed Diet 5 were initially similar to the other treatment groups for the first week of the 28-d study, but began to decrease after average egg weights for the treatment group began to decrease on d 8 (data not shown). This suggested that changes in feed intake were driven by whether the hen was producing eggs.

Simultaneous supplementation of the 40 g/kg MAO diet with either 20 g/kg or 40 g/kg HOSO (Diets 6 and

Table 4. Performance of hens fed diets containing DHA-rich microalgae oil (MAO) with or without high-oleic sunflower oil (HOSO) for 28 d[1,2].

	Diet								
Oil (g/kg)	1	2	3	4	5	6	7		
MAO	0	10	20	30	40	40	40		
HOSO	0	0	0	0	0	20	40	SEM	*P*-value
Body weight change (g)[3]	32.9[a]	55.0[a]	39.5[a]	20.2[a]	-88.0[b]	40.2[a]	54.1[a]	26.1	<0.01
Daily feed consumption (g)	103.8[a]	101.2[a]	105.4[a]	99.2[a]	73.8[b]	96.7[a]	98.9[a]	3.31	<0.0001
Hen-day egg production (%)[4]	90.18[a]	91.84[a]	95.54[a]	91.07[a]	62.50[b]	95.41[a]	92.86[a]	4.50	<0.0001
Average egg weight (g)[5]	59.49	59.22	60.02	59.30	57.69	58.14	60.48	0.93	0.30
Feed conversion efficiency (g feed/g egg mass)	1.96[a,b]	1.85[b]	1.84[b]	1.84[b]	2.20[a]	1.79[b]	1.76[b]	0.09	0.02

[1]Values are means of 8 hens each for each diet except for Diets 2 and 6 ($n = 7$).
[2]Each dietary group averaged 95.00% hen-day egg production in the 5 d immediately prior to the initiation of the experiment and had egg weights (± SD) of 59.43 ± 3.08 g, 59.46 ± 2.85 g, 59.61 ± 2.53 g, 59.58 ± 2.26 g, 59.51 ± 2.20 g, 59.52 ± 2.03 g, and 59.31 ± 1.95 g for birds assigned to diets 1 to 7, respectively.
[3]The initial body weights (±SD) of hens fed diets 1 to 7 were: 1,563.3 ± 110.8 g, 1,418.4 ± 138.7 g, 1,527.7 ± 144.9 g, 1,548.0 ± 140.5 g, 1,521.4 ± 82.8 g, 1,485.4 ± 95.9 g, and 1,462.4 ± 115.4 g, respectively.
[4]Hen-day egg production was calculated as (100 × number of eggs laid)/(number of hens × d).
[5]Values are the means of 202, 180, 214, 204, 140, 187, and 208 eggs from hens fed Diets 1 to 7, respectively.
[a-b]Within a row, values with no common superscript differ significantly.

7, respectively; Table 1) appeared to completely rescue egg production, egg weights, yolk weights, and other measures of hen performance (Tables 4 and 5). Due to the smaller (weight-wise) yolks, eggs from hens fed 40 g/kg MAO (Diet 5) had greater relative amounts of albumen, which was normalized by co-feeding hens either 20 g/kg HOSO or 40 g/kg HOSO (Diets 6 and 7, respectively), while the absolute and relative amounts of shell did not vary greatly among the treatment groups (Table 5).

Absolute and relative liver weights were markedly increased ($P \leq 0.05$) in hens fed 40 g/kg MAO (Diet 5), while co-supplementation with either 20 g/kg HOSO or 40 g/kg HOSO (Diets 6 and 7, respectively) restored liver weights to CON levels (Table 6). In contrast, abdominal fat pad weights were decreased ($P \leq 0.05$) in hens fed 40 g/kg MAO (Diet 5) and restored to CON levels by co-supplementation with either 20 g/kg HOSO or 40g/kg HOSO (Diets 6 and 7, respectively; Table 6). Markedly lower weights (Table 6) and numbers (Figure 1) of vitellogenic (growing, yolk-accumulating) ovarian follicles were observed in hens fed 40 g/kg MAO (Diet 5), with 5 of the 8 hens out of production and 3 with completely regressed ovaries by d 28. Co-feeding hens with either 20 g/kg HOSO (Diet 6) or 40 g/kg HOSO (Diet 7) completely restored the ovarian follicular hierarchy (Figure 1) and function, in terms of egg production and egg weights (Table 4) and egg components (Table 5).

Egg Yolk and Tissue Fatty Acid Compositions

Dietary supplementation of MAO greatly enriched egg yolk contents of DHA and VLC n-3 PUFA, which

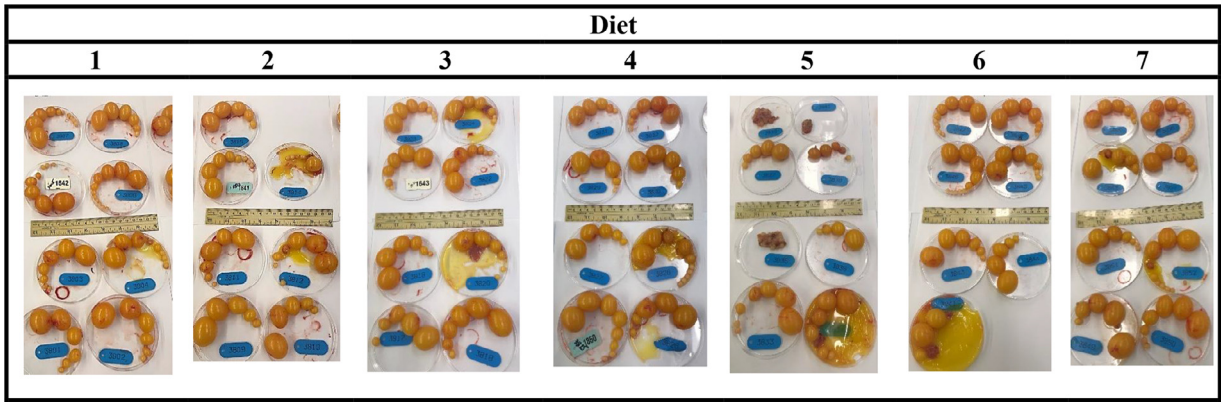

Figure 1. Influence of dietary MAO with or without co-supplemental HOSO on ovarian morphology. Ovarian follicles excised on d 28 from 8 hens each fed 0 (Diet 1), 20 (Diet 3), 30 (Diet 4), or 40 g/kg MAO (Diet 5) or 40 g/kg MAO + 40 g/kg HOSO (Diet 7), or 7 hens each fed 10 g/kg MAO (Diet 2) or 40 g/kg MAO + 20 g/kg HOSO (Diet 6) for 28 d. One hen from Diet 2 died of unknown causes on d 25 and 1 hen from Diet 6 went out of production on d 19 and subsequently molted. All of the follicles were excised intact but some broke after being placed in the petri dish, most likely due to weak vitelline membranes. Three of the 8 hens fed 40 g/kg MAO (Diet 5) had completely regressed ovaries. Note the restoration of the ovarian follicle hierarchy with co-supplementation of 20 or 40 g/kg HOSO to the 40 g/kg MAO diet (Diets 6 and 7, respectively).

Table 5. Weights (g) of eggs and egg components from hens fed diets containing DHA-rich microalgae oil (MAO) with or without high-oleic sunflower oil (HOSO) for 28 d[1].

	Diet								
Oil (g/kg)	1	2	3	4	5	6	7		
MAO	0	10	20	30	40	40	40		
HOSO	0	0	0	0	0	20	40	SEM	*P*-value
Egg weight (g)	60.1	58.8	60.4	57.8	55.5	58.0	59.8	1.55	0.13
Yolk									
Weight (g)	16.27[a]	14.99[bc]	15.63[ab]	15.12[b]	13.70[c]	15.16[ab]	15.39[ab]	0.53	0.02
% of Egg weight	26.80[a]	25.68[ab]	26.13[a]	26.07[a]	24.32[b]	26.20[a]	25.76[a]	0.56	0.05
Albumen									
Weight (g)	38.40	38.32	39.30	37.48	36.77	38.04	39.15	1.00	0.31
% of Egg weight	63.71[c]	65.24[ab]	65.12[b]	64.68[bc]	66.44[a]	65.13[b]	65.50[ab]	0.50	<0.01
Shell									
Weight (g)	5.76[a]	5.30[b]	5.24[b]	5.33[b]	5.17[b]	5.03[b]	5.24[b]	0.17	<0.01
% of Egg weight	9.55[a]	9.03[ab]	8.72[b]	9.29[a]	9.24[ab]	8.70[b]	8.71[b]	0.26	<0.01

[1]Values are means of 8, 7, 8, 8, 4, 7, and 8 eggs collected on d 27 or 28 from all hens in production from Diets 1 to 7, respectively. One hen from Diet 2 died of unknown causes on d 25 and 1 hen from Diet 6 went out of production on d 19 and subsequently molted. Five hens from Diet 5 ceased egg production entirely on d 12, 22, 23 (2 hens), and 26; the egg laid by the latter hen on d 26 was included in the study.

[a-c]Within a row, values with no common superscript differ significantly.

appeared to plateau, in terms of statistical significance, at the 30 g/kg MAO treatment (Diet 4; Table 7). Moreover, there appeared to be a linear relationship between dietary DHA content and egg yolk DHA or VLC n-3 PUFA levels over the first 2 supplemental MAO levels, with an additional ~100 mg of DHA or VLC n-3 PUFA being deposited into egg yolks for every additional 10 g/kg diet of MAO consumed.

The efficiency of conversion (g/g) of dietary DHA to egg yolk DHA decreased with increasing dietary DHA intake as follows for Diets 2 to 5 that contained 10, 20, 30, or 40 g/kg MAO: 0.24, 0.21, 0.14, and 0.10, respectively (data not shown). The efficiency of conversion of dietary DHA to egg yolk VLC n-3 PUFA was slightly higher for the 4 diets: 0.25, 0.23, 0.15, and 0.11, respectively (data not shown). Therefore, for the 4 diets that contained MAO, the efficiency of conversion of dietary DHA to yolk DHA or yolk VLC n-3 PUFA ranged from 24 to 10% or from 25 to 11%, respectively, and decreased with each additional 10 g/kg of dietary MAO.

The greatest amounts of both DHA and VLC n-3 were observed in the egg yolks of hens fed Diet 6 (40 g/kg MAO + 20 g/kg HOSO; 266 and 282 mg/yolk, respectively), although those values did not differ ($P > 0.05$) from those of hens fed Diets 4 and 5 (30 or 40 g/kg MAO, respectively). Moreover, VLC n-3 accounted for 65.4, 87.8, 91.6, 93.3, 94.2, 94.0, and 94.6% of total egg yolk n-3 PUFA in Diets 1 to 7, respectively (Table 7), with DHA accounting for most of the VLC n-3 PUFA. Thus, 1 or 2 of the eggs from hens fed 30 g/kg MAO would exceed a number of health organizations' recommended daily intake of VLC n-3 PUFA for healthy adults (e.g., 250 or 500 mg combined EPA and DHA), while eggs from hens fed 20 g/kg MAO would approach those values. Furthermore, in contrast to results of previous work (Elkin et al., 2018, 2021), dietary supplemen-

Table 6. Absolute and relative weights of tissues from hens fed diets containing DHA-rich microalgae oil (MAO) with or without high-oleic sunflower oil (HOSO) for 28d[1].

	Diet								
Oil (g/kg)	1	2	3	4	5	6	7		
MAO	0	10	20	30	40	40	40		
HOSO	0	0	0	0	0	20	40	SEM	*P*-value
Liver[2]									
Weight (g)	35.2[bc]	32.5[c]	34.5[bc]	44.6[b]	61.9[a]	33.2[bc]	32.9[c]	2.70	<0.0001
% of Body weight	2.21[b]	2.20[b]	2.21[b]	2.84[b]	4.30[a]	2.19[b]	2.18[b]	0.17	<0.0001
Abdominal fat pad[2]									
Weight (g)	43.6[a]	29.5[bc]	41.0[ab]	38.5[abc]	27.6[c]	40.6[abc]	42.0[ab]	4.84	0.10
% of Body weight	2.71[ab]	1.97[ab]	2.60[ab]	2.44[ab]	1.93[b]	2.68[ab]	2.75[a]	0.30	0.20
Vitellogenic follicles[3]									
Weight	30.3[a]	29.7[a]	29.4[a]	32.4[a]	23.3[b]	32.9[a]	30.7[a]	2.88	0.02
% of Body weight	1.91[a]	1.98[a]	1.90[a]	2.06[a]	1.61[b]	2.17[a]	2.04[a]	0.18	0.01

[1]Mean 28-day body weights ± SD (g) of the hens fed Diets 1 to 7 were: 1596.3 ± 126.7, 1483.3 ± 112.5, 1567.2 ± 128.5, 1568.3 ± 89.0, 1433.4 ± 121.5, 1514.8 ± 50.9, and 1516.5 ± 114.8, respectively.

[2]Values are the d 28 least square means of 8 hens per diet except for Diets 2 and 6 ($n = 7$).

[3]Values are the d 28 least square means of hens from Diets 1, 3, 4, and 7 ($n = 8$); Diets 2 and 6 ($n = 7$); and Diet 5 ($n = 5$). One hen from Diet 2 died of unknown causes on d 25 and 1 hen from Diet 6 went out of production on d 19 and subsequently molted. Five hens from Diet 5 ceased egg production entirely on d 12, 22, 23 (2 hens), and 26, but vitellogenic follicles were present in 2 of the 5 hens that had ceased laying eggs.

[a-c]Within a row, values with no common superscript differ significantly.

Table 7. Fatty acid content (mg/yolk) of eggs from hens fed diets containing DHA-rich microalgae oil (MAO) with or without high-oleic sunflower oil (HOSO) for 28 d[1].

	Diet								
Oil (g/kg)	1	2	3	4	5	6	7		
MAO	0	10	20	30	40	40	40		
HOSO	0	0	0	0	0	20	40	SEM	*P*-value
Fatty acid									
14:0	20.0^{e}	33.1^{d}	55.5^{c}	61.7^{bc}	62.5^{bc}	79.4^{a}	69.0^{b}	3.90	<0.0001
14:1 n-5	6.01^{c}	9.62^{b}	14.1^{a}	13.8^{a}	10.7^{ab}	13.7^{a}	10.5^{b}	1.39	<0.0001
15:0	2.07^{c}	2.08^{c}	2.56^{b}	2.89^{ab}	3.20^{a}	2.73^{b}	2.62^{b}	0.17	<0.0001
16:0	1307^{a}	1162^{bc}	1263^{ab}	1211^{abc}	972^{d}	1192^{bc}	1119^{c}	54.6	<0.001
16:1 n-7	216^{a}	160^{b}	136^{b}	107^{c}	73^{d}	90.2^{cd}	65.7^{d}	12.3	<0.0001
17:0	5.40^{c}	5.12^{c}	6.04^{bc}	7.14^{ab}	8.53^{a}	6.15^{bc}	6.26^{bc}	0.68	<0.001
18:0	389^{a}	333^{bc}	373^{ab}	378^{ab}	353^{abc}	325^{c}	316^{c}	21.7	<0.01
18:1 n-7	154^{a}	115^{b}	93.5^{c}	86.3^{c}	68.5^{d}	70.8^{d}	64.9^{d}	4.75	<0.0001
18:1 n-9	1853^{ab}	1659^{c}	1649^{c}	1580^{c}	1340^{d}	1681^{bc}	1874^{a}	63.4	<0.0001
18:2 n-6	402	349	389	357	322	380	362	27.7	0.14
18:3 n-6	4.96^{a}	2.69^{b}	2.28^{bc}	2.05^{cd}	2.03^{cd}	1.74^{d}	1.58^{d}	0.24	<0.0001
18:3 n-3	15.6^{bc}	16.6^{b}	20.8^{a}	17.8^{b}	15.5^{bc}	16.8^{b}	13.5^{c}	1.22	<0.0001
18:4 n-3	2.29^{a}	1.61^{b}	1.31^{c}	1.17^{cd}	1.02^{d}	1.08^{d}	1.05^{d}	0.07	<0.0001
20:0	1.14^{ab}	1.00^{bc}	1.27^{a}	1.24^{a}	1.20^{a}	1.00^{bc}	0.96^{c}	0.07	<0.001
20:1 n-9	11.5^{a}	8.93^{bc}	8.18^{cd}	7.19^{de}	6.32^{e}	8.13^{cd}	9.82^{b}	0.52	<0.0001
20:2 n-6	4.28^{a}	3.33^{b}	3.21^{b}	2.71^{b}	2.99^{b}	2.74^{b}	3.01^{b}	0.32	<0.001
20:3 n-3	0.29	0.29	0.27	0.21	0.21	0.22	0.20	0.04	0.16
20:3 n-6	9.40^{a}	6.24^{b}	5.10^{c}	4.58^{cd}	4.55^{cd}	4.00^{d}	3.98^{d}	0.43	<0.0001
20:4 n-6	74.3^{a}	40.7^{b}	32.8^{c}	28.4^{d}	27.2^{d}	27.6^{d}	30.5^{cd}	2.09	<0.0001
20:5 n-3	0.80^{e}	2.76^{d}	8.16^{bc}	9.69^{b}	12.16^{a}	8.98^{bc}	7.43^{c}	0.77	<0.0001
22:0	0.08^{c}	0.11^{c}	0.19^{b}	0.28^{a}	0.25^{ab}	0.27^{a}	0.33^{a}	0.04	<0.0001
22:4 n-6	5.18^{a}	2.73^{b}	2.63^{b}	2.57^{b}	2.52^{bc}	1.84^{cd}	1.49^{d}	0.28	<0.0001
22:5 n-3	3.36^{e}	3.66^{e}	7.37^{bc}	8.23^{ab}	9.11^{a}	6.68^{c}	5.11^{d}	0.66	<0.0001
22:5 n-6	18.3^{b}	10.6^{c}	18.5^{b}	20.4^{ab}	21.2^{ab}	22.9^{a}	20.7^{ab}	1.56	<0.0001
22:6 n-3	29.5^{d}	131^{c}	223^{b}	248^{ab}	253^{ab}	266^{a}	234^{b}	15.1	<0.0001
24:0	0.22	0.13	0.20	0.20	0.16	0.13	0.15	0.05	0.31
24:1 n-9	0.15^{ab}	0.20^{a}	0.20^{a}	0.13^{abc}	0.12^{abc}	0.06^{c}	0.10^{bc}	0.04	0.03
Others	71.3^{a}	52.7^{b}	47.2^{b}	49.7^{b}	50.7^{b}	46.5^{b}	46.4^{b}	4.38	<0.0001
Total	4604^{a}	4113^{bc}	4364^{ab}	4159^{b}	3678^{c}	4260^{ab}	4271^{ab}	193	0.02
Total n-3	51.8^{d}	156^{c}	261^{b}	285^{ab}	291^{ab}	300^{a}	261^{b}	16.8	<0.0001
Total VLC n-3	33.9^{d}	137^{c}	239^{b}	266^{ab}	274^{ab}	282^{a}	247^{b}	16.0	<0.0001

[1]Values are the d 28 least square means of 1 egg each from 8 hens fed Diets 1, 3, 4, and 7, 1 egg each from 7 hens fed Diets 2 and 6, or 1 egg each from 4 hens fed Diet 5. One hen from Diet 2 died of unknown causes on d 25 and 1 hen from Diet 6 went out of production on d 19 and subsequently molted. Five hens from Diet 5 ceased egg production entirely on d 12, 22, 23 (2 hens), and 26; the egg laid by the latter hen on d 26 was included in the study.

[a-e]Within a row, values with no common superscript differ significantly.

tal HOSO did not appear to attenuate egg yolk DHA or VLC n-3 PUFA contents, but it did enrich egg yolks with OLA as compared to the diet with 40 g/kg MAO alone (Diets 6 and 7 vs. Diet 5; Table 7). Egg yolk OLA contents also appeared to be inversely related to dietary MAO levels, a trend which was reversed by feeding the hens supplemental dietary HOSO.

Increasing dietary MAO incrementally increased liver DHA and VLC n-3 PUFA contents by between ~100 and 140 mg/100 g fresh tissue for every 10 g/kg increase in MAO, with a plateau at the 30 g/kg level (Diet 4; Table 8). Similar to that observed in egg yolks (Table 7), co-feeding hens either 20 or 40 g/kg HOSO along with 40 g/kg MAO resulted in further ($P \leq 0.05$) DHA and VLC n-3 PUFA liver enrichment, with the greatest contents observed in hens fed Diet 6 (40 g/kg MAO + 20 g/kg HOSO). Moreover, VLC n-3 PUFA accounted for >94% of liver n-3 PUFA (Table 8). Dietary supplemental HOSO increased ($P > 0.05$) liver contents of OLA as compared to those of hens fed the 40 g/MAO (Diets 6 and 7 vs. Diet 5) but the levels were much less than those of the CON birds. Although the absolute FA contents varied greatly between the tissues, a similar pattern of DHA and VLC n-3 PUFA enrichment was observed in adipose tissue (abdominal fat pads; Table 9), boneless skinless breast (Table 10), and plasma (Table 11). There was no significant effect of either supplemental dietary MAO or HOSO on breast OLA content (Table 10).

Plasma Triglycerides and Total Estrogens Concentrations

Circulating levels of plasma triglycerides were approximately 2,950 mg/100 mL in hens fed either the CON diet (Diet 1) or the diet containing 10 mg/kg MAO (Diet 2), significantly ($P \leq 0.05$) less and about 1,560 mg/100 mL each in hens fed the next 2 successive dietary levels of MAO, and lowest (663 mg/100 mL) in hens fed 40 g/kg MAO (Diet 5; Figure 2). Co-supplementation with dietary HOSO significantly increased plasma triglycerides concentrations to levels similar to or slightly higher ($P > 0.05$) than hens fed 20 mg/kg MAO (Diet 3) or 30 mg/kg MAO (Diet 4). The pattern of plasma total estrogens (Figure 2) was somewhat opposite that of plasma triglycerides, with the lowest levels (195−218 pg/mL) observed in hens fed Diets 1 to

Table 8. Fatty acid content (mg/100 g fresh tissue) of livers from hens fed diets containing DHA-rich microalgae oil (MAO) with or without high-oleic sunflower oil (HOSO) for 28 d[1].

	Diet								
Oil (g/kg)	1	2	3	4	5	6	7		
MAO	0	10	20	30	40	40	40		
HOSO	0	0	0	0	0	20	40	SEM	*P*-value
Fatty acid									
14:0	22.6^{d}	29.3^{d}	63.0^{c}	108^{a}	95.0^{ab}	85.0^{b}	57.7^{c}	7.90	<0.0001
14:1 n-5	6.25^{a}	6.46^{a}	9.69^{a}	8.52^{a}	2.46^{b}	7.67^{a}	2.53^{b}	1.31	<0.001
15:0	1.97^{c}	1.71^{c}	2.39^{c}	3.53^{b}	4.33^{a}	2.15^{c}	1.85^{c}	0.26	<0.0001
16:0	1055^{a}	757^{bc}	952^{ab}	947^{ab}	710^{c}	712^{c}	596^{c}	78.8	<0.001
16:1 n-7	204^{a}	115^{b}	112^{b}	78.8^{bc}	31.6^{d}	47.2^{cd}	21.4^{c}	17.4	<0.0001
17:0	4.64^{c}	3.62^{c}	5.48^{c}	8.97^{b}	12.6^{a}	4.72^{c}	4.50^{c}	0.89	<0.0001
18:0	428^{ab}	312^{c}	381^{bc}	443^{ab}	470^{a}	307^{c}	319^{c}	31.8	<0.001
18:1 n-6	2.67^{a}	2.50^{a}	3.33^{a}	3.34^{a}	1.06^{b}	1.51^{b}	0.98^{b}	0.31	<0.0001
18:1 n-7	98.7^{a}	57.4^{b}	57.2^{b}	54.4^{b}	34.1^{c}	31.5^{c}	26.7^{c}	6.73	<0.0001
18:1 n-9	1503^{a}	967^{bc}	1185^{ab}	1125^{b}	665^{c}	871^{bc}	852^{bc}	141	<0.01
18:2 n-6	333	258	301	309	266	251	225	29.9	0.12
18:3 n-6	4.49^{a}	2.65^{b}	2.50^{b}	1.85^{bc}	1.42^{cd}	1.32^{cd}	0.46^{d}	0.37	<0.0001
18:3 n-3	11.2^{a}	9.18^{ab}	11.4^{a}	11.2^{a}	6.30^{bc}	9.07^{ab}	5.60^{c}	1.22	0.001
20:0	1.40^{cd}	0.96^{d}	1.40^{cd}	2.21^{b}	2.69^{a}	1.41^{cd}	1.51^{c}	0.18	<0.0001
20:1 n-9	6.84^{a}	5.14^{bc}	5.33^{bc}	5.54^{bc}	4.86^{c}	5.19^{bc}	6.26^{ab}	0.43	0.01
20:2 n-6	3.94	3.77	3.69	3.76	3.91	2.99	3.85	0.33	0.45
20:3 n-6	19.1^{a}	16.1^{ab}	14.9^{b}	13.8^{bc}	15.3^{b}	10.9^{c}	13.1^{bc}	1.25	0.001
20:4 n-6	157^{a}	114^{b}	91.5^{c}	77.5^{cd}	82.4^{cd}	67.4^{d}	80.2^{cd}	8.12	<0.0001
20:5 n-3	0.30^{d}	2.38^{d}	5.84^{c}	8.76^{bc}	19.8^{a}	10.1^{b}	11.0^{b}	1.22	<0.0001
22:4 n-6	7.14^{a}	4.76^{b}	3.98^{b}	3.81^{bc}	4.99^{b}	2.56^{c}	2.51^{c}	0.51	<0.0001
22:5 n-3	3.04^{de}	2.72^{e}	4.94^{cd}	7.10^{b}	10.3^{a}	6.39^{bc}	5.50^{bc}	0.76	<0.0001
22:5 n-6	25.8^{c}	15.5^{d}	23.0^{cd}	40.4^{b}	39.8^{b}	57.8^{a}	52.3^{a}	3.34	<0.0001
22:6 n-3	42.0^{f}	141^{e}	256^{d}	395^{bc}	338^{c}	475^{a}	424^{ab}	22.1	<0.0001
24:0	0.07	0.00	0.00	0.00	0.00	0.03	0.00	0.03	0.52
24:1 n-9	0.15^{bc}	0.12^{bc}	0.83^{ab}	1.11^{a}	0.17^{bc}	0.78^{ab}	0.00^{c}	0.28	0.02
Others	88.6^{a}	60.5^{bc}	60.8^{bc}	68.3^{b}	75.5^{ab}	44.1^{c}	38.8^{d}	7.37	0.0001
Total	4043^{a}	2889^{bc}	3557^{abc}	3730^{ab}	2898^{c}	3017^{bc}	2753^{c}	308	0.02
Total n-3	56.5^{f}	155^{e}	278^{d}	422^{bc}	374^{c}	501^{a}	446^{ab}	23.0	<0.0001
Total VLC n-3	45.3^{f}	146^{e}	267^{d}	411^{bc}	368^{c}	492^{a}	441^{ab}	22.3	<0.0001

[1]Values are the d 28 least square means of livers from 8 hens each fed Diets 1, 3, 4, 5, and 7, or 7 hens each fed Diets 2 and 6. One hen from Diet 2 died of unknown causes on d 25 and 1 hen from Diet 6 went out of production on d 19 and subsequently molted.

[a-f]Within a row, values with no common superscript differ significantly.

3, significantly ($P \leq 0.05$) higher levels vs. Diet 3 in hens fed Diets 4 to 6 (286−339 pg/mL), and then a decrease to approximately 243 pg/mL in hens fed Diet 7 (40 g/kg MAO + 40 g/kg HOSO).

Hepatic Gene Expression

The hepatic mRNA abundance of apoB, apoVLDL-II, MTTP, and DGAT, which are all important genes with regard to the synthesis of triglycerides or VLDL, all followed a somewhat similar pattern (Figure 3), although the differences were nonsignificant ($P > 0.05$) with regard to the latter 2. For apoB and apoVLDL-II, the highest levels were observed in hens fed Diets 1 to 3 (0−20 mg/kg MAO, respectively), followed by a precipitous significant ($P \leq 0.05$) stepwise decrease in hens fed Diet 4 (30 mg/kg MAO) and Diet 5 (40 mg/kg MAO), which was the nadir, and then significant ($P \leq 0.05$) increases in hens fed the 2 HOSO-supplemented diets (Diets 6 and 7). Both Lpcat3 and FASN exhibited a somewhat similar pattern of mRNA expression for the 5 diets not supplemented with HOSO, with Diets 1 to 3 being significantly greater than Diets 4 to 7 (Figure 3). Relative hepatic expression of SREBP1 decreased with increasing dietary MAO and reached a nadir in the 2 co-supplemented HOSO treatments (1.00, 0.42, 0.33, 0.18, 0.16, 0.07, and 0.02 for Diets 1−7, respectively). Diets 2 to 7 all differed ($P \leq 0.05$) vs. the CON diet and Diet 2 differed ($P \leq 0.05$) from Diets 6 and 7 (data not shown).

DISCUSSION

The results of the present study revealed 3 important novel findings: 1) Egg VLC n-3 PUFA enrichment plateaued, in terms of statistical significance, at the 30 g/kg MAO level (266 mg/yolk); 2) Compared to the CON group, hens fed the highest level of MAO (40 g/kg diet) had greatly attenuated measures of productive performance, with 5 of the 8 hens ceasing egg production entirely by d 27 or earlier, extremely enlarged livers, markedly lowered circulating triglyceride levels, and depressed expression of key hepatic genes involved in VLDL synthesis and secretion; and 3) As compared to hens fed the 40 g/kg MAO diet, feeding hens the 40 g/kg MAO diet co-supplemented with either 20 g/kg or 40 g/kg of an OLA-rich HOSO restored egg production and all other measures of hen productive performance, as well as the ovarian follicle hierarchy, to normal (CON) levels or morphology, elevated plasma triglyceride levels, prevented liver enlargement, and increased

Table 9. Fatty acid content (mg/100 g fresh tissue) of abdominal fat pads from hens fed diets containing DHA-rich microalgae oil (MAO) with or without high-oleic sunflower oil (HOSO) for 28 d[1].

	Diet								
Oil (g/kg)	1	2	3	4	5	6	7		
MAO	0	10	20	30	40	40	40		
HOSO	0	0	0	0	0	20	40	SEM	*P*-value
Fatty acid									
14:0	537[e]	1000[de]	1593[cd]	2192[b]	1826[bc]	3193[a]	3353[a]	223	<0.0001
14:1 n-5	80.9[c]	120[bc]	161[ab]	189[a]	122[bc]	160[ab]	126[b]	16.2	<0.001
15:0	87.8	92.7	101	102	90.8	92.8	97.2	4.93	0.30
16:0	15582	15194	16599	15595	14338	15059	14539	595	0.11
16:1 n-7	2619[ab]	2643[ab]	2715[a]	2813[a]	2103[bc]	2113[bc]	1857[c]	210	0.01
17:0	146	139	149	138	132	129	134	5.80	0.15
18:0	4131	3924	4276	4016	4454	4014	4026	227	0.61
18:1 n-7	1698[a]	1546[ab]	1455[bc]	1446[bc]	1311[c]	1328[c]	1419[bc]	70.6	<0.01
18:1 n-9	27626[b]	26359[b]	28242[b]	27926[b]	26221[b]	28349[b]	33924[a]	962	<0.0001
18:2 n-6	18933	17472	18933	18223	16649	17376	17777	729	0.20
18:3 n-6	75.0	81.5	80.2	85.9	101	89.1	97.7	11.1	0.55
18:3 n-3	608	619	686	664	533	649	654	36.9	0.07
20:0	165	131	120	148	124	115	96.5	18.2	0.15
20:1 n-9	224	221	225	231	274	229	246	17.3	0.27
20:2 n-6	37.4	37.3	39.9	39.1	45.8	36.4	36.5	3.08	0.27
20:3 n-6	36.5	32.6	27.8	30.5	31.2	27.6	24.3	2.92	0.08
20:4 n-6	30.9[d]	36.6[cd]	49.1[bcd]	54.6[abc]	67.9[ab]	71.4[a]	62.3[ab]	7.15	<0.001
20:5 n-3	6.07	14.1	13.0	12.8	13.7	11.9	14.5	2.28	0.16
22:2 n-9	0.00[c]	6.46[c]	38.5[b]	51.3[ab]	53.2[ab]	59.2[a]	57.1[ab]	7.41	<0.0001
22:4 n-6	7.49	6.90	6.68	4.69	8.49	5.62	3.97	1.62	0.38
22:5 n-3	1.01[e]	4.78[de]	7.97[cde]	12.6[bc]	21.6[a]	17.0[ab]	10.0[bcd]	2.88	<0.0001
22:5 n-6	3.33[c]	13.0[c]	52.9[c]	139[b]	123[b]	289[a]	260[a]	25.5	<0.0001
22:6 n-3	5.14[c]	70.3[c]	257[c]	641[b]	776[ab]	1083[a]	1079[a]	122	<0.0001
24:1 n-9	0.31[b]	1.86[b]	5.27[a]	5.21[a]	5.75[a]	5.03[a]	4.76[a]	0.87	0.0001
Others	1031	1081	1033	1027	893	888	1027	54.5	0.06
Total	73890[b]	71009[b]	77082[ab]	75990[ab]	70988[b]	75815[ab]	81070[a]	2302	0.02
Total n-3	620[c]	714[c]	964[bc]	1331[b]	1344[b]	1741[a]	1757[a]	144	<0.0001
Total VLC n-3	12.2[c]	95.4[c]	278[c]	667[b]	812[ab]	1109[a]	1103[a]	124	<0.0001

[1]Values are the d 28 least square means of abdominal fat pads from 8 hens each fed Diets 1, 3, 4, 5, and 7, or 7 hens each fed Diets 2 and 6. One hen from Diet 2 died of unknown causes on d 25 and 1 hen from Diet 6 went out of production on d 19 and subsequently molted.

[a-e]Within a row, values with no common superscript differ significantly.

the hepatic expression of key genes involved in triglyceride and VLDL synthesis and secretion.

The decreased efficiency of conversion of dietary DHA to egg yolk DHA or VLC n-3 PUFA associated with increased dietary DHA levels in hens fed MAO is reminiscent of that reported by others in laying hens fed fish oil (reviewed by Fraeye et al., 2012). Moreover, Van Elswyk et al. (1994) were among the first researchers to report impaired production, specifically decreased egg weights and yolk weights, as well as reduced circulating triglyceride levels, in laying hens fed 30 g/kg menhaden fish oil, albeit all of these negative effects were of a much lesser degree than observed in hens fed 40 g/kg MAO (Diet 5) in the present study. Van Elswyk et al. (1994) postulated that VLC n-3 PUFA consumption likely impaired the availability of lipids for yolk formation. However, they observed no effects of feeding hens 30 g/kg of fish oil on egg production or yolk weights as a relative percentage of egg weights and, in a second experiment, hens fed 20 g/kg of fish oil had normal liver, ovary, and oviduct weights. Other researchers have reported that fish oil feeding impaired hen productive performance (reviewed by Fraeye et al., 2012; Gao et al., 2021) but none reported a complete cessation of egg production, a reduction in circulating triglycerides to the degree observed in the present study, or effects on the hepatic expression of key genes involved in triglyceride and VLDL synthesis or secretion (discussed below).

In the second experiment of Van Elswyk et al. (1994) referred to above, some of the hens fed 20 g/kg of fish oil were also individually injected subcutaneously with 100 μg of 17β-estradiol, which caused enlarged livers with greater lipid content. Whitehead et al. (1993), who also reported reduced egg production, egg weights, and yolk weights in hens fed 60 g/kg fish oil for 5 wk, concluded that egg weight is mediated by an effect of fats on estrogen metabolism and reported that plasma estradiol concentrations were highly (positively) correlated with changes in egg weights. However, in the present study, as compared to the other dietary groups, hens fed 40 g/kg MAO (Diet 5) had greatly enlarged livers (Table 6) and the highest level of circulating total estrogens (Figure 2), but laid the *smallest* (weight-wise) eggs (Table 4). Therefore, the present findings do not align with the conclusions of Whitehead et al. (1993).

Alternately, the markedly elevated plasma total estrogen levels in hens fed 40 mg/kg MAO may represent an attempt by follicular thecal cells to produce more estrogen in order to stimulate more yolk precursor macromolecule synthesis via a feedback response to the reduced rate of ovulation. Although we present no evidence, this hypothesis is based on the fact that apoB, the main

Table 10. Fatty acid content (mg/100 g fresh tissue) of boneless skinless breasts from hens fed diets containing DHA-rich microalgae oil (MAO) with or without high-oleic sunflower oil (HOSO) for 28 d[1].

	Diet								
Oil (g/kg)	1	2	3	4	5	6	7		
MAO	0	10	20	30	40	40	40		
HOSO	0	0	0	0	0	20	40	SEM	*P*-value
Fatty acid									
14:0	4.60[b]	8.42[ab]	8.80[a]	10.2[a]	10.3[a]	11.6[a]	11.7[a]	1.42	0.01
14:1 n-5	0.06	0.41	0.14	0.26	0.10	0.06	0.05	0.12	0.24
15:0	0.81	1.05	0.86	0.88	0.87	0.72	0.76	0.10	0.27
16:0	171	205	173	173	168	153	150	18.0	0.30
16:1 n-7	12.6[ab]	17.3[a]	10.4[b]	11.4[b]	8.65[b]	7.40[b]	7.25[b]	2.29	0.02
17:0	1.44	1.56	1.33	1.25	1.39	1.09	1.12	0.16	0.25
18:0	81.9	87.1	80.1	80.0	83.5	75.6	72.1	6.36	0.58
18:1 n-7	17.5	19.7	16.0	16.3	15.6	14.1	13.2	1.78	0.12
18:1 n-9	185	245	191	188	194	160	179	28.1	0.45
18:2 n-6	149	189	145	132	138	108	110	20.1	0.06
18:3 n-6	1.45	1.66	1.09	1.12	1.13	0.81	0.88	0.24	0.11
18:3 n-3	3.05	4.74	3.67	3.22	3.05	2.53	2.83	0.67	0.25
20:0	0.55	0.69	0.58	0.58	0.67	0.57	0.54	0.07	0.61
20:1 n-9	1.23	1.71	1.49	1.26	1.56	1.10	1.18	0.25	0.46
20:2 n-6	2.21[a]	2.12[a]	1.70[b]	1.44[bc]	1.44[bc]	1.26[c]	1.21[c]	0.16	<0.0001
20:3 n-6	5.11[a]	4.72[ab]	3.89[bc]	3.41[cd]	2.96[de]	2.76[de]	2.31[e]	0.32	<0.0001
20:4 n-6	71.8[a]	65.1[a]	65.1[ab]	62.3[bc]	62.5[c]	59.7[c]	58.9[c]	2.07	<0.001
20:5 n-3	0.00[c]	0.71[c]	1.84[b]	2.61[a]	3.21[a]	3.23[a]	3.23[a]	0.28	<0.0001
22:0	0.73[a]	0.60[a]	0.50[ab]	0.25[bc]	0.03[c]	0.08[c]	0.12[c]	0.10	<0.0001
22:1 n-9	0.20	0.29	0.23	0.20	0.28	0.20	0.19	0.04	0.20
22:5 n-3	2.29[a]	2.39[a]	2.32[a]	1.82[a]	1.15[b]	0.99[b]	1.13[b]	0.24	<0.0001
22:4 n-6	9.39[a]	5.85[b]	3.45[c]	2.56[d]	2.24[d]	2.15[d]	1.98[d]	0.33	<0.0001
22:5 n-6	6.60[bc]	5.95[bc]	5.69[c]	6.66[bc]	6.82[b]	8.72[a]	8.93[a]	0.39	<0.0001
22:6 n-3	8.40[e]	28.3[d]	45.0[c]	55.6[b]	59.5[b]	70.0[a]	65.8[a]	2.51	<0.0001
Others	22.8	22.8	23.3	22.5	22.7	20.5	21.1	1.42	0.77
Total	759	923	778	779	789	706	709	81.0	0.47
Total n-3	13.7[f]	36.6[e]	53.5[d]	63.2[c]	66.9[bc]	76.8[a]	73.3[ab]	2.84	<0.0001
Total VLC n-3	10.7[f]	31.8[e]	49.6[d]	60.0[c]	63.8[bc]	74.3[a]	70.4[ab]	2.67	<0.0001

[1]Values are the d 28 least square means of half-breasts from 8 hens each fed diets 1, 3, 4, 5, and 7, or 7 hens each fed diets 2 and 6. One hen from Diet 2 died of unknown causes on d 25 and 1 hen from Diet 6 went out of production on d 19 and subsequently molted.

[a-f]Within a row, values with no common superscript differ significantly.

protein component essential for the synthesis of VLDL and the sole apoprotein through which VLDL binds to the chicken oocyte receptor (Nimpf et al., 1988), apoVLDL-II, a second protein component of VLDL that is an inhibitor of lipoprotein lipase (Schneider et al., 1990), and vitellogenin I, II, and III, the other main yolk precursor macromolecules (Burley et al, 1993), are all hepatically synthesized following induction by estrogen. This is at least due in part to the presence of estrogen-responsive elements in the 5' upstream regions of the respective genes (Herrmann et al., 1997). Estrogen also shifts hepatic lipoprotein production from "generic" VLDL to smaller (by ~50%) yolk-targeted VLDL particles (Walzem et al., 1999). Together, VLDL and vitellogenins account for approximately 84% of egg yolk mass (Burley et al., 1993).

Chicken plasma VLDL particles, which are comprised of a core of neutral triglycerides and cholesteryl esters, surrounded by polar phospholipids, free cholesterol, and apolipoproteins (Hermann et al., 1997), contain approximately 69% triglycerides, 7% cholesteryl esters, 18% phospholipids, and 6% free cholesterol (Kouba et al., 1995; Sato et al., 2009). Egg yolk consists of approximately 65% triglycerides, 28% phospholipids, 5% free cholesterol, and 1% cholesteryl esters by weight (Kuksis, 1992). Thus, the similarity of plasma VLDL and yolk triglyceride contents reflects the lack of appreciable hydrolysis of triglycerides as the VLDL particles are transported via the bloodstream from the liver to growing oocytes. This is attributed to the presence of apoVLDL-II on the surface of the VLDL particles (Schneider et al., 1990).

Microalgae biomass and oils have emerged as viable alternatives to fish oil for the enrichment of eggs and poultry meat with VLC n-3 PUFA (Cheng et al., 2004; Cachaldora et al., 2005, 2008; Lemahieu et al., 2013; Neijat et al., 2016; Wu et al., 2019; Moran et al., 2019, 2020; Feng et al., 2020; Liu et al., 2020; Świątkiewicz et al., 2020). However, in contrast to the present findings, all of the above researchers, as well as Gao et al. (2021) who recently reviewed this topic, reported that feeding hens DHA-rich MAO or algal biomass did not negatively affect egg weight, yolk weight, or egg production. However, the inclusion of DHA-rich MAO in the diets of laying hens has been reported to produce an off-flavor in eggs often described as "fishy" (Swiatkiewicz et al., 2020) which can lead to reduced consumer acceptance. Based on the reports of Fraeye et al. (2012) and Swiatkiewicz et al. (2020), Gao et al. (2021) concluded that the dietary inclusion of MAO at more than 25 g/kg will impart negative sensory characteristics in eggs. Feng et al. (2020) suggested that, in order to obtain eggs with acceptable sensory properties, dietary DHA supplementation from MAO should not exceed 6.64 mg/g (equal to

Table 11. Fatty acid content (mg/100 mL) of plasma from hens fed diets containing DHA-rich microalgae oil (MAO) with or without high-oleic sunflower oil (HOSO) for 28 d[1].

	Diet								
Oil (g/kg)	1	2	3	4	5	6	7		
MAO	0	10	20	30	40	40	40		
HOSO	0	0	0	0	0	20	40	SEM	*P*-value
Fatty acid									
14:0	14.9^{d}	30.2^{c}	40.1^{bc}	44.3^{b}	30.1^{c}	59.2^{a}	45.4^{b}	4.38	<0.0001
14:1 n-5	3.67^{c}	6.50^{ab}	7.31^{ab}	6.85^{ab}	3.41^{c}	8.61^{a}	5.78^{bc}	0.98	0.003
15:0	1.60	1.89	1.83	1.98	1.98	1.91	1.74	0.16	0.63
16:0	965^{a}	935^{ab}	868^{ab}	769^{bc}	477^{d}	757^{bc}	638^{cd}	64.9	<0.0001
16:1 n-7	138^{a}	125^{a}	93.9^{b}	64.8^{c}	31.7^{d}	55.6^{cd}	39.1^{d}	9.15	<0.0001
17:0	4.30	4.36	4.38	4.76	5.09	4.11	3.75	0.46	0.49
18:0	307^{a}	276^{a}	258^{ab}	252^{ab}	164^{c}	220^{bc}	181c	21.9	<0.0001
18:1 n-7	105^{a}	89.0^{a}	68.1^{b}	56.1^{bc}	33.0^{d}	48.4^{cd}	43.7^{cd}	6.40	<0.0001
18:1 n-9	1466^{a}	1352^{ab}	1226^{ab}	1140^{b}	681^{c}	1102^{b}	1095^{b}	100	<0.0001
18:2 n-6	292^{ab}	304^{a}	269^{ab}	244^{bc}	161^{d}	245^{bc}	213^{cd}	22.3	0.0004
18:3 n-6	0.92	1.01	0.93	0.92	0.67	0.95	0.85	0.09	0.20
18:3 n-3	9.58^{a}	7.54^{b}	5.81^{c}	5.07^{c}	3.21^{d}	5.16^{c}	5.58^{c}	0.68	<0.0001
20:0	3.36^{a}	2.81^{b}	1.97^{c}	1.46^{cd}	1.24^{d}	1.31^{d}	0.98^{d}	0.19	<0.0001
20:1 n-9	9.46^{abc}	12.5^{a}	12.1^{a}	10.6^{ab}	7.16^{c}	11.0^{ab}	8.15^{bc}	1.13	0.0122
20:3 n-6	6.47^{a}	5.59^{a}	3.86^{b}	3.22^{bc}	3.04^{bcd}	2.51^{cd}	2.22^{d}	0.40	<0.0001
20:4 n-3	0.14^{d}	0.22^{cd}	0.32^{bc}	0.44^{b}	0.37^{bc}	0.64^{a}	0.42^{b}	0.05	<0.0001
20:4 n-6	64.1^{a}	43.2^{b}	25.8^{c}	21.0^{cd}	17.7^{d}	19.3^{cd}	18.1^{d}	3.08	<0.0001
20:5 n-3	0.53^{e}	1.51^{e}	3.50^{d}	5.72^{c}	8.17^{ab}	8.56^{a}	6.33^{bc}	0.77	<0.0001
22:5 n-3	2.29^{d}	2.28^{d}	3.13^{cd}	3.87^{bc}	5.64^{a}	4.57^{ab}	2.82^{cd}	0.46	<0.0001
22:5 n-6	15.5^{bcd}	9.93^{d}	11.4^{cd}	16.5^{bc}	15.4^{bcd}	26.5^{a}	21.0^{ab}	2.06	<0.0001
22:6 n-3	25.4^{e}	94.3^{d}	136^{cd}	179^{bc}	159^{bc}	243^{a}	191^{b}	16.5	<0.0001
24:0	0.14^{c}	0.13^{c}	0.18^{bc}	0.22^{bc}	0.19^{bc}	0.27^{ab}	0.38^{a}	0.05	0.003
24:1 n-9	0.65^{ab}	0.66^{a}	0.52^{b}	0.37^{c}	0.25^{c}	0.33^{c}	0.29^{c}	0.05	<0.0001
Total	3506^{a}	3365^{a}	3090^{ab}	2879^{ab}	1903^{c}	2863^{ab}	2563^{bc}	240	0.0005
Total n-3	37.9^{e}	106de	149^{cd}	194^{bc}	176^{bc}	262^{a}	206^{b}	24.0	<0.0001
Total VLC n-3	28.3^{e}	98.5^{d}	143^{cd}	189^{b}	173^{bc}	257^{a}	201^{b}	19.0	<0.0001

[1]Values are the d 28 least square means of plasma samples from 8 hens each fed diets 1, 3, 4, 5, and 7, or 7 hens each fed diets 2 and 6. One hen from Diet 2 died of unknown causes on d 25 and 1 hen from Diet 6 went out of production on d 19 and subsequently molted.

[a-e]Within a row, values with no common superscript differ significantly.

13.22 g/kg dietary MAO oil, since the MAO oil used in their study contained 502 mg DHA/g oil), and could result in production of eggs containing as much as 261 mg of DHA/yolk.

The negative sensory characteristics of eggs from hens fed DHA-rich MAO reported by others is not unanticipated. It has been known for almost a century, since the initial report by Carrick and Hague (1926), that feeding broilers or laying hens VLC n-3 PUFA-rich fish oil or fish meal can impart a fishy flavor or aroma in poultry meat and eggs (reviewed by Hargis and Van Elswyk, 1993; Leskanich and Noble, 1997; Feng et al., 2020). Leskanich and Noble (1997) concluded that the amount of fish oil or fishmeal in the diet should be limited to about 10 g/kg or 120 g/kg, respectively, in order to prevent the development of fishy taint, although Nash et al. (1996) detected undesirable off-flavors in enriched eggs when hens were fed >80 g/kg fishmeal.

Although the exact mechanisms of how n-3 PUFA lower plasma triglycerides is still not completely

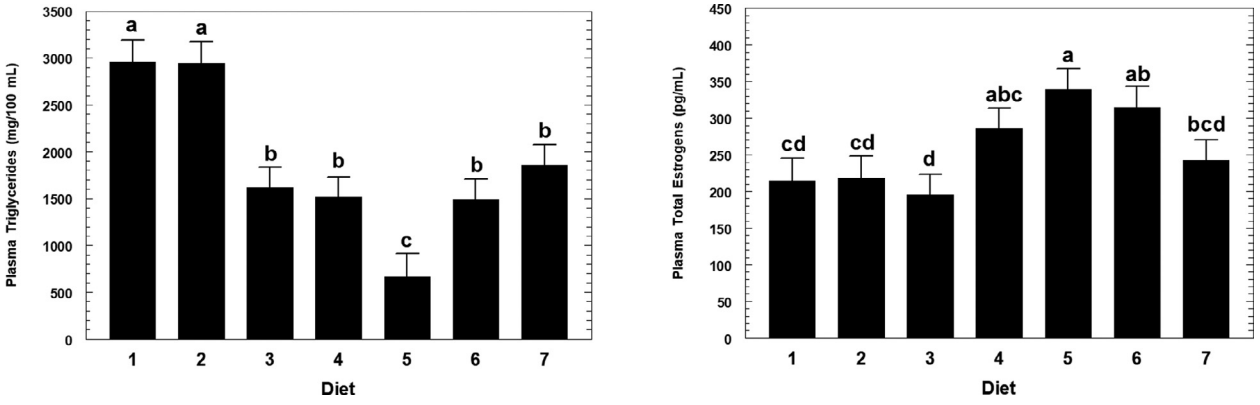

Figure 2. Influence of dietary MAO with or without co-supplemental HOSO on plasma triglycerides and plasma total estrogens. Plasma was prepared from blood samples obtained on d 28 from 8 hens each fed 0 (Diet 1), 20 (Diet 3), 30 (Diet 4), or 40 g/kg MAO (Diet 5) or 40 g/kg MAO + 40 g/kg HOSO (Diet 7), or 7 hens each fed 10 g/kg MAO (Diet 2) or 40 g/kg MAO + 20 g/kg HOSO (Diet 6) for 28 d. One hen from Diet 2 died of unknown causes on d 25 and 1 hen from Diet 6 went out of production on d 19 and subsequently molted. Bars with different letters are significantly different ($P \leq 0.05$).

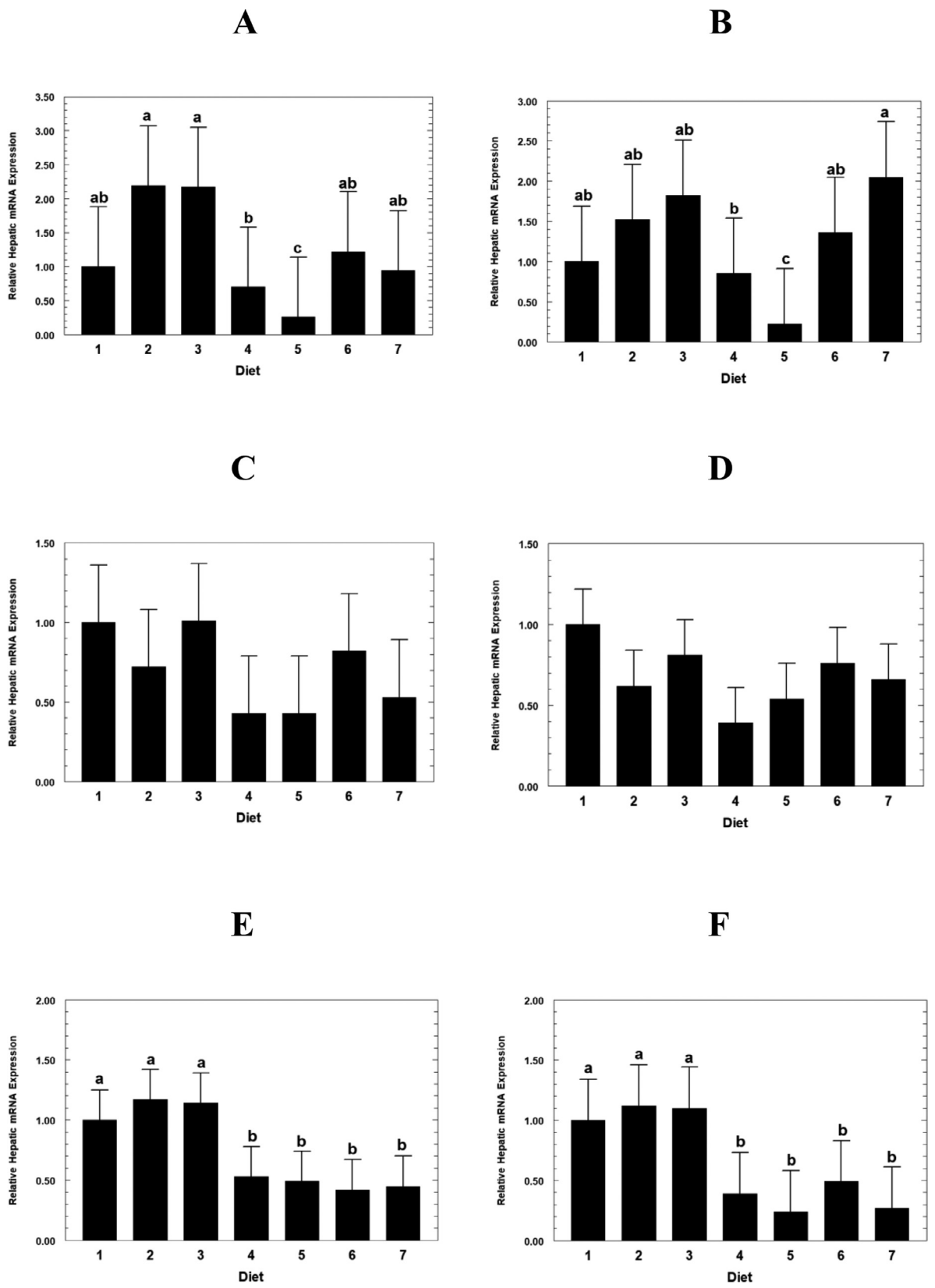

Figure 3. Influence of dietary MAO with or without co-supplemental HOSO on hepatic expression of key genes involved in triglyceride, very low-density lipoprotein, or fatty acid synthesis or secretion. Liver samples were excised, weighed, and snap-frozen in liquid nitrogen on d 28 until subsequent analysis for expression of apolipoprotein B (Panel A), apolipoproteinVLDL-II (Panel B), microsomal triglyceride transport protein (Panel C), diacylglycerol acyltransferase 2 (Panel D), lysophosphatidylcholine acyltransferase 3 (Panel E), and fatty acid synthase (Panel F). Each bar represents the least squares mean of quadruplicate analyses of livers from 8 hens each fed 0 (Diet 1), 20 (Diet 3), 30 (Diet 4), or 40 g/kg MAO (Diet 5) or 40 g/kg MAO + 40 g/kg HOSO (Diet 7), or 7 hens each fed 10 g/kg MAO (Diet 2) or 40 g/kg MAO + 20 g/kg HOSO (Diet 6) for 28 d. One hen from Diet 2 died of unknown causes on d 25 and 1 hen from Diet 6 went out of production on d 19 and subsequently molted. Values are scaled to 0 g/kg MAO (Diet 1) equal to 1.00. Bars with different letters are significantly different ($P \leq 0.05$).

understood, based on mammalian studies, it has been proposed that n-3 PUFA inhibit hepatic DGAT2, increase plasma lipoprotein lipase activity and apoB degradation, decrease hepatic lipogenesis through down-regulating SREBP1 transcription factor, and increase hepatic FA β-oxidation (Backes et al., 2016; Mason, 2019; Wolska et al., 2020). With one exception, mechanistic studies in birds are virtually nonexistent. Daggy et al. (1987) investigated the influence of dietary fish oil on hepatic VLDL production rates in roosters injected (via the brachial vein) hourly for 4 h with chicken adipose lipoprotein lipase antiserum raised in a goat and fed them a diet containing 100 g/kg of either corn oil or fish oil. The antiserum was shown to block removal of VLDL from the serum. Hepatic secretion rates of VLDL-cholesterol and VLDL-triglycerides were 39% and 49% lower in roosters fed the fish oil diet compared to the corn oil diet. In a second experiment, the researchers added 5 g/kg cholesterol in addition to the oils and observed VLDL-cholesterol and VLDL-triglycerides rates that were lower by 39 and 38%, respectively, in the fish oil-fed group vs. the corn oil-fed group.

Several observations in the present study might help to elucidate mechanistically how dietary DHA-rich MAO causes the attenuation of egg production, egg weights, and yolk weights. First, hens fed 40 g/kg MAO (Diet 5) had circulating triglyceride levels that were only 22% of CON bird values (663 mg/100 mL vs. 2,960 mg/100 mL; Figure 2), which suggested a marked impairment of hepatic VLDL secretion, since chicken plasma VLDL particles contain approximately 65 to 69% triglycerides (Kouba et al., 1995; Sato et al., 2009). Second, the relative hepatic mRNA levels of both apoB and apoVLDL-II, the 2 key apolipoproteins of VLDL particles (Hermann et al., 1997), were also only 26 and 22% of the levels in CON birds (Figure 3), which paralleled the degree of reduction in circulating triglycerides. Although the differences were not significant ($P > 0.05$), somewhat similar patterns of hepatic expression were observed for MTTP, which is another key gene involved in assembly of VLDL (Shelness and Ledford, 2005; Eresheim et al., 2014) and DGAT2, which catalyzes the de novo synthesis of triglycerides (Zammit, 2013), both of which were depressed in hens fed 40 g/kg MAO (43 and 54% of CON values, respectively; Figure 3). In addition, the hepatic expression of Lpcat3, a critical determinant of triglyceride secretion (Rong et al., 2015), and FASN, which catalyzes FA synthesis, were significantly lowered to ~50 and 30% of CON values in hens fed 30 or 40 mg/kg MAO (Figure 3). Expression of SREBP1, which regulates lipid biosynthesis and adipogenesis by controlling the expression of several enzymes required for FA, triglyceride, and phospholipid synthesis (Bertolio et al., 2019), was also severely depressed by dietary MAO, either alone or when co-fed with HOSO.

One of the most striking findings of the current experiment is that co-feeding hens either 20 mg/kg or 40 mg/kg HOSO along with 40 g/kg MAO rescued hen productive performance, the ovarian follicle hierarchy, and other variables that were negatively affected by the highest dietary level of MAO (Tables 4–6 and Figure 1). This was somewhat reminiscent of the effect of co-supplementing hens' diets with OLA-rich olive oil to prevent the adverse effects of conjugated linoleic acid on chick embryo mortality and egg quality (Aydin et al., 2001). Previous work in our laboratory has shown that co-feeding an OLA-rich plant oil with either ALA-rich flaxseed oil (Elkin et al., 2018) or SDA-rich soybean oil (Elkin et al., 2021) attenuated egg n-3 PUFA contents compared to feeding the ALA-rich or SDA-rich oils alone. It was hypothesized that dietary OLA may have "outcompeted" ALA and SDA for uptake from the intestine, that competition existed between OLA and ALA or SDA for esterification within intestinal and/or liver cells, or that OLA promoted the β-oxidation of ALA. An analogous result (i.e., lower DHA transfer to the egg) was anticipated in the present study with the 2 co-supplemented HOSO diets vs. the 40 g/kg MAO diet, but instead hen productive performance was rescued and a normal ovarian follicle hierarchy was restored. However, it is possible that co-supplementation of the 40 g/kg MAO diet with HOSO did in fact prevent the intestinal absorption of *some* of the dietary DHA to the extent that the amount which was absorbed and reached the liver was below the threshold level that impaired hepatic triglyceride and VLDL synthesis and secretion, thereby allowing normal hepatic yolk precursor production without impairing egg production or attenuating yolk weight.

To the authors' knowledge, the complete cessation of egg production resulting from laying hens consuming a high level of MAO (e.g., providing ~1.50–1.75 g DHA/hen/d) has not been previously reported. However, it is not a completely unexpected finding, since anything that would interfere significantly with the production or secretion of VLDL from the laying hens' liver would be expected to impair yolk formation and egg production. In conclusion, MAO can greatly enrich hens' eggs with VLC n-3 PUFA, but its recommended dietary inclusion should not exceed 20 g/kg. This would allow for near-maximal yolk VLC n-3 PUFA enrichment (~240 mg per egg) without impairing hen productive performance, altering the ovarian follicle hierarchy or, based on the work of others, presumably imparting off-flavors in the egg. However, whether any egg sensory issues would arise awaits further investigation.

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DISCLOSURES

The authors declare no conflicts of interest.

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