

Effect of *Escherichia coli* lipopolysaccharide challenge on eggshell, tibia, and keel bone attributes in ISA brown hens exposed to dietary n-3 fatty acids prior to onset of lay

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ABSTRACT The impact of *Escherichia coli* lipopolysaccharide (LPS) challenge on eggshell, tibia, and keel bone characteristics in ISA brown hens derived from breeders and pullets fed omega-3 polyunsaturated fatty acids (n-3 PUFA) was examined. The breeders were fed the following diets: 1) Control (CON); 2) CON + 1% microalgae as the source of docosahexaenoic acid (DHA); and 3) CON + 2.6% of a co-extruded mixture of full-fat flaxseed and pulses as a source of α -linolenic acid (ALA). During the pullet phase, offspring from breeders fed CON were fed CON or supplemented diets, and offspring from supplemented diets either continued with respective n-3 PUFA diets or CON. At 18 weeks of age (WOA), pullets were fed a common layer diet to 42 WOA. A total of 5 birds were selected based on the average body weight (BW) of each treatment and moved to an individual cage at 41 WOA. Three

days before the end of 42 WOA, all the birds were weighed and subcutaneously injected with either saline or 4 mg LPS/kg BW. Eggs were recorded, labeled, and kept for egg quality analyses. At 42 WOA, birds were necropsied for tibia and keel bone samples. Administration of LPS reduced eggshell breaking strength, eggshell weight, tibia, and keel bone ash content ($P < 0.05$). Specifically, LPS challenged hens had 14.9, 11.1, 9.2, and 11.6% lower eggshell breaking strength, eggshell weight, keel, and tibia ash content, respectively relative to unchallenged hens. Hens from breeders and pullets fed n-3 PUFA had similar ($P > 0.05$) eggshell, tibia, and keel bone attributes to control hens. In conclusion, the provision of ALA and DHA to breeders and their offspring did not alleviate the negative effects of LPS on eggshell, tibia, and keel bone characteristics in laying hens.

Key words: α -linolenic acid, docosahexaenoic acid, inflammatory response, eggshell, tibia, keel bone

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INTRODUCTION

Poor eggshell quality is a global challenge in the egg industry. The eggshell quality is influenced by the strain, hen age, general stress, heat stress, disease, production system, and nutritional factors such as minerals, vitamins, water quality, nonstarch polysaccharides, enzymes, contamination of feed (Roberts, 2004; Xie et al., 2019; Abo Ghanima et al., 2020; Reda et al., 2020). In addition, eggshell quality is inextricably linked to skeletal integrity (Whitehead, 2004). After the surge of estrogen during puberty, bone formation switches from forming structural parts such as cortical to creating medullary bone, giving only around 18 wk for building a foundation for the lay cycle (Korver, 2020). Several

approaches have been used to optimize bone mineral content and strength during the rearing phase with subsequent effect on bone and eggshell quality in hens. For example, dietary calcium level, limestone particle size (Khanal et al., 2019; Khanal et al., 2020), and housing style (Casey-Trott et al., 2017a,b).

The omega-3 (n-3) and omega-6 (n-6) PUFA are important components of cell membranes and improve the performance, antioxidant system and immunity in animals (Alagawany et al., 2019, 2021a). Linoleic acid (LA) and α -linolenic acid (ALA), the 2 essential polyunsaturated fatty acids (PUFA), are the precursors of other PUFA, such as docosahexaenoic acid (DHA) and arachidonic acid (Brenner, 1971). n-3 and n-6 PUFA are believed to possess various physiological effects and are crucial for many biological processes (Boeyens et al., 2014). In recent decades, there has been increased interest in nutritional factors that influence skeletal development in pre-hatch and their impact on leg problems in birds during the post-hatch period (Torres and Korver, 2018, Akbari Moghaddam Kakhki, 2021). Liu et al. (2003)

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reported an increase in tibia ash percentage in day-old quail from breeders fed 50 g/kg fish oil compared with those fed soybean oil group. Previously, it has been reported that the inclusion of n-3 PUFA sources in Shaver white breeders increased tibia breaking strength and cortical ash content in their offspring at sexual maturity (Akbari Moghaddam Kakhki et al., 2020c).

The ability of n-3 PUFA to modulate bone metabolism is associated with the production of inflammatory mediators by reducing the expression of locally produced prostaglandins (Mazzucco et al., 2005) and inflammatory cytokines (Moon et al., 2012). Inflammatory mediators increase the osteoclastogenesis and activity of osteoclasts, leading to an increase in bone resorption (Nakanishi et al., 2013). Therefore, the effect of n-3 PUFA on bone metabolism might be more evident throughout a provoked immune response by offsetting the side-effect of elevated inflammatory mediators (Trebble et al., 2005). For instance, Mireles et al. (2005) studied the lipopolysaccharides (LPS) model to provoke an acute inflammatory response and its effect on bone attributes in broilers. It has been reported by Mireles et al. (2005) that bone homeostasis can be affected by provoked inflammatory response via LPS in broiler chickens. Thus, it was hypothesized that early-life exposure to n-3 PUFA could offset the adverse impact of inducing systemic stress on eggshell and bone quality in laying hens. This study aimed to evaluate the effect of embryonic and pullet exposure to n-3 PUFA on eggshell quality and bone parameters in 42 WOA ISA brown hens challenged with LPS.

MATERIALS AND METHODS

All birds in this trial were raised under the approved experimental protocol (#3675) by the University of Guelph Animal Care Committee based on the Canadian Council on Animal Care guidelines (CCAC, 2009).

Breeder and Rearing Periods

A total of 240 female and 30 male ISA brown breeders were housed in 3 large groups based on body weight (BW) and fed one of three dietary treatments from 26 week of age (WOA). Dietary treatments were included 1) Control (CON), a corn, soybean meal, wheat, and corn gluten diet, 2) DMA: CON plus 1% of dried microalgae (*Aurantiochytrium limacinum*) supplement as a rich source of DHA contained 17.9% DHA (Alltech, Nicholasville, KY), and 3) FFF: CON plus 2.6% of a 1:1 (wt/wt) co-extruded full-fat flaxseed and pulse mixture containing 10.5% ALA (linPRO, O & T Farms Ltd, Regina, SK, Canada). The inclusion level of full-fat flaxseed was chosen based on the given concentration of the total n-3 and n-6 in dried microalgae at 1% inclusion level, which was reported to double the enrichment of n-3 PUFA in eggs. All the diets were formulated to meet the nutrient specifications (Parent Stock Management Guide: ISA Brown, 2018: Parent Stock

Management Guide: Shaver White, 2018). The experimental diets were offered for 30 d, enough time to reach the plateau of the deposition of n-3 PUFA in eggs. Confirmation of deposition in hatching eggs was previously reported (Akbari Moghaddam Kakhki et al., 2020b). Eggs were labeled, incubated at 37.5°C with 55% humidity for 19 d, and hatched at 36.9°C with 66% humidity in a commercial-grade incubator and hatcher (Nature Form, Jacksonville, FL) at the Arkell Poultry Research Station (University of Guelph, Guelph, ON, Canada).

Female hatchlings were weighed and distributed into post-hatch treatments with five replications as described in Akbari Moghaddam Kakhki et al. (2020c). Hatchlings from the CON breeder group were divided into 3 post-hatch treatments: CON, DMA, and FFF. Hatchlings from the DMA breeder group were divided into 2 post-hatch treatments: CON and DMA. Hatchlings from the FFF breeder group were divided into 2 post-hatch treatments: CON and FFF. Breeder and pullet diets containing DMA and FFF had similar total n-3 FA and n-6: n-3 FA and met nutrient specifications (Shaver white, 2018, ISA Brown, 2018).

Laying Hen Management and LPS Challenge

Birds were transitioned to conventional cages at the onset of lay (18 WOA), retained on breeder-rearing treatment combinations, and fed a common commercial laying diet without a supplemental source of n-3 FA containing 39.16% LA, 2.79% ALA (of total fat), and 4.55% total fat throughout the laying phase as described by Akbari Moghaddam Kakhki et al. (2020a). Further details on the housing system, temperature, and lighting regimens post-hatch to 42 WOA are available in Akbari Moghaddam Kakhki et al. (2020a) and Akbari Moghaddam Kakhki et al. (2020c). All birds were weighed, and 5 birds were selected based on average BW \pm 150 g of each treatment and moved to an individual cage at 41 WOA. Four days post-placement, all the birds were weighed and subcutaneously injected with 4 mg/kg of BW LPS from *Escherichia coli* (L4130, Sigma-Aldrich, St. Louis, MO) as described in Mireles et al. (2005). Five additional birds from CON-CON were selected based on an average BW and injected with saline solution (Sham). Housing the additional birds from other treatments injected with saline solution was not feasible due to space limitation. All produced eggs during 72-h postchallenge were recorded, labeled and kept for egg quality analysis. At 42 WOA, birds were weighed and euthanized before the light turned on. Birds were sampled for tibia, and keel bones.

Sampling and Analyses

Eggs were weighed and eggshell breaking strength was measured according to Mwaniki et al. (2018) by Force Reader (ORKA Food Technology Ltd. Ramat HaSharon, Israel). Eggs then were cracked open, and the eggshells were washed by water, dried for 24 h at

105°C and weighed as described by Akbari Moghaddam Kakhki et al. (2019a). Dry weight, ash content, and ash concentration of keel bone and subparts of the left tibia were measured according to Akbari Moghaddam Kakhki et al. (2019a). Right tibia samples were used for measuring breaking strength via an Instron material tester (Instron corp, Canton, MA) with the crosshead speed of 2 mm/s, according to Khanal et al. (2019).

Calculations and Statistical Analyses

Data were tested for normality using the Univariate Plot normal procedure of SAS 9.4. Dry weight, ash content of tibia, and keel bone were normalized based on BW (Akbari Moghaddam Kakhki et al., 2019b). The data were subjected to a nested design with fixed effects of breeder diet and offspring diet using the GLIMMIX procedure of SAS 9.4. To measure the time-effect, the data were subjected to ANOVA with the fixed effect of time postchallenge. Significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Body Weight Change

The initial BW was not different among breeder diets, offspring diet, and challenge treatments (Table 1, $P >$

Table 1. Effects of feeding sources of docosahexaenoic and α -linolenic acids to ISA brown breeders and offspring pullets to 18 weeks of age on body weight change in 42-wk-old laying hens upon challenge with lipopolysaccharide (LPS)^{1,2}.

Items	Initial body weight, g	%
Challenge		
LPS	2,177.2	0.93
Sham	2,216.2	0.03
SEM	54.213	0.785
Breeder diets		
CON	2,269.2	0.93 ^a
DMA	2,262.6	-3.65 ^b
FFF	2,186.2	-10.02 ^c
SEM	78.915	1.297
Offspring diets		
CON-CON	2,177.2	0.93 ^a
CON-DMA	2,338.0	-16.90 ^b
CON-FFF	2,292.4	-2.20 ^a
DMA-CON	2,190.4	-3.65 ^b
DMA-DMA	2,334.8	-3.71 ^a
FFF-CON	2,293.2	-10.02 ^c
FFF-FFF	2,079.2	-4.26 ^a
SEM	111.60	1.834
Probabilities (P -value)		
Challenge	0.771	0.399
Breeder diets	0.692	0.006
Offspring diets	0.453	<0.001

^{a-c}Values with uncommon superscripts within each column are significantly different ($P < 0.05$).

CON, control; DMA, microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid; and FFF, co-extruded full-fat flaxseed and pulses of field peas mixture (50/50, wt/wt), as a source of α -linolenic acid.

¹n = 5.

²The day-old female pullets from breeders fed CON, DMA, and FFF were divided into 3 (CON, DMA and FFF), 2 (CON and DMA), and 2 (CON, FFF) post-hatch treatments, respectively.

0.05). Administration of LPS did not affect BW change in 42 WOA ISA brown hens ($P > 0.05$). Administration of LPS has been reported to reduce feed intake and BW in poultry (Mireles et al., 2005; Ahiwe et al., 2019; Bai et al., 2019) due to the changes in nutrients metabolism to combat and eliminate the presence of the foreign or exogenous threat (Korver, 2006; Ahiwe et al., 2019). Body weight loss was more severe in offspring from breeders fed with FFF compared with DMA ($P = 0.010$). The BW loss was more severe in offspring from CON-DMA and FFF-CON compared with the rest of the treatments ($P < 0.001$). Breeder feeding and pullet feeding with DMA and FFF have been previously reported not to influence BW in 42 WOA ISA brown hens (Akbari Moghaddam Kakhki et al., 2020a). Dietary supplementation of conjugated LA has been shown to reduce BW in 48 and 58 WOA layer hens (Shang et al., 2004; Yin et al., 2008). However, there is no report on the effect of an early or continues n-3 PUFA feeding in immune-challenged egg-type birds. Maroufyan et al. (2012) observed that increasing the inclusion level of n-3 PUFA from 11.5 to 16.5% of total fat reduced the BW gain with no change in feed intake in 4 WOA broiler chickens challenged with the infectious bursal disease.

Egg Weight and Eggshell Quality

The administration of LPS did not influence the egg weight or eggshell breaking strength ($P > 0.05$), while it reduced the eggshell weight and eggshell percentage (Table 2, $P < 0.05$). Daily eggshell formation equates to removing 2 to 3 g of calcium from the skeletal system (Gilbert, 1983). Jing et al. (2014) observed a reduction in serum calcium and phosphorus levels in laying hens challenged with 8 mg LPS/kg of BW, suggesting that LPS-induced stress may adversely affect mineral metabolism. In addition, calcium homeostasis is created by balancing intestinal absorption, renal excretion, and bone mineral metabolism to meet the bird's requirements (Elaroussi et al., 1994). The administration of 1.5 mg LPS/kg BW for nine consecutive days has been reported to adversely affect intestinal morphology, intestinal mucosal cell growth, and the digestion and absorption of nutrients in laying hens (Nie et al., 2018). In response to administering 1.5 mg LPS/kg BW in laying hens, Nie et al. (2018) observed an increase in the eggshell thickness and strength, caused by a lower laying rate and longer eggshell formation time.

Egg weight, absolute eggshell weight, and eggshell percentage were not influenced by breeder and offspring treatments ($P > 0.05$). Eggshell breaking strength was not affected by the breeder diet, but pullets from CON-DMA had a lower value of eggshell breaking strength than the CON-CON group ($P = 0.006$). The reason for the observed difference and how n-3 PUFA might adversely influence eggshell breaking strength compared with n-6 PUFA, CON, in challenged birds is unknown. Neither breeder feeding nor pullet feeding with DMA and FFF has been reported to affect egg production, egg

Table 2. Effects of feeding sources of docosahexaenoic and α -linolenic acids to ISA brown breeders and offspring pullets to 18 weeks of age on egg weight and eggshell quality in 42-wk-old laying hens upon challenge with lipopolysaccharide (LPS)^{1,2}.

Items	Egg weight, g	Eggshell breaking strength, kgf	Eggshell, g	Eggshell, %
Challenge				
LPS	61.73	4.49	6.01 ^b	9.43 ^b
Sham	65.81	4.33	6.72 ^a	10.24 ^a
SEM	1.682	0.282	0.199	0.283
Breeder diets				
CON	64.09	3.44	5.74	8.96
DMA	65.71	3.70	5.77	8.84
FFF	63.68	2.92	5.41	8.45
SEM	1.312	0.299	0.237	0.377
Offspring diets				
CON-CON	64.09	3.44 ^a	5.74	8.96
CON-DMA	64.88	2.49 ^b	5.47	8.41
CON-FFF	65.67	3.35 ^a	5.76	8.91
DMA-CON	65.71	3.70 ^a	5.77	8.84
DMA-DMA	68.19	3.48 ^a	5.57	8.63
FFF-CON	63.68	2.92 ^{ab}	5.41	8.45
FFF-FFF	64.69	3.10 ^a	5.77	8.91
SEM	1.624	0.309	0.262	0.456
Probabilities (<i>P</i> -value)				
Challenge	0.105	0.685	0.008	0.010
Breeder diets	0.387	0.130	0.453	0.539
Offspring diets	0.054	0.006	0.499	0.144

^{ab}Values with uncommon superscripts within each column are significantly different ($P < 0.05$).

CON, control; DMA, microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid; and FFF, co-extruded full-fat flaxseed and pulses of field peas mixture (50/50, wt/wt), as a source of α -linolenic acid.

¹The day-old female pullets from breeders fed CON, DMA, and FFF were divided into 3 (CON, DMA, and FFF), 3 (CON and DMA), and 2 (CON, FFF) post-hatch treatments, respectively.

²Unchallenged CON-CON produced 13 eggs during 72-h postchallenge. Challenged birds from CON-CON, CON-DMA, CON-FFF, DMA-CON, DMA-DMA, FFF-CON, and FFF-FFF produced 9, 8, 10, 9, 12, 5, and 9, respectively. All the produced eggs were weighed and used for eggshell-quality analysis.

weight, and eggshell quality in unchallenged 42 WOA ISA brown hens (Akbari Moghaddam Kakhki et al., 2020a). The results of eggshell quality analysis in eggs produced by all challenged birds at different times are shown in Table 3. Egg weight was not differed at different postchallenge times by LPS ($P = 0.852$). Eggshell breaking strength, eggshell weight, and eggshell percentage were decreased after 48-h postchallenge by LPS ($P < 0.05$). Therefore, the administration of 4 mg LPS/kg of BW can be considered a potential stressor for eggshell quality.

Bone Quality

Polyunsaturated fatty acids play essential roles in the improvement of egg quality and nutritional value of eggs, mineral metabolism including bone formation, growth, and development (Alagawany et al., 2021). The reservoir of minerals amassed in the hen's skeletal system before puberty is crucial for eggshell formation, especially considering the unprecedented calcium output associated with the record number of eggs per bird in a lay cycle of modern hens (Anderson et al., 2013). The ash percentage of keel bone was reduced by 3.81% post-LPS challenge (Table 4, $P = 0.008$). In addition, tibia dry weight, ash content, ash percentage, and breaking strength were reduced by the LPS challenge (Table 5, $P < 0.05$). Administration of LPS has been reported to decrease serum calcium and phosphorous levels and deteriorate intestine morphology in laying hens (Jing et al., 2014; Nie et al., 2018), demonstrating LPS-

Table 3. Effects of a lipopolysaccharide challenge on egg weight and eggshell quality at 24, 48, and 72-h postchallenge¹.

Items	Egg weight, g	Eggshell breaking strength, kgf	Eggshell, g	Eggshell, %
0-h	65.00	4.15 ^a	6.39 ^a	9.84 ^a
24-h	65.44	3.57 ^{ab}	6.06 ^{ab}	9.30 ^{ab}
48-h	65.09	3.67 ^b	5.97 ^b	9.16 ^b
72-h	64.40	3.53 ^b	5.68 ^b	8.83 ^b
SEM	0.901	0.208	0.155	0.241
<i>P</i> -value	0.852	0.043	0.003	0.008

^{ab}Values with uncommon superscripts within each column are significantly different ($P < 0.05$).

¹All the produced eggs in each day were weighed and used for eggshell quality analysis.

induced stress may adversely influence mineral absorption and metabolism (Jing et al., 2014). An acute inflammatory response via LPS in broiler chickens has been reported to increase bone catabolism, which was demonstrated by reducing tibia relative weight, calcium content, and breaking strength (Mireles et al., 2005).

Under an inflammatory state, various skeletal cells, such as synovial tissue, produce different cytokines, including interleukin-1, interleukin-6, interleukin-17, and tumor necrosis factor- α (Kajarabille et al., 2013). These cytokines increase osteoclastogenesis and, thereby, bone resorption (Kruger et al., 2010). One example is the abnormal generation and activity of osteoclasts, which is a significant factor in inhibited chondrocyte proliferation and induced cartilage degradation attributed to rheumatoid arthritis, osteoarthritis (Kajarabille et al., 2013) and tibial dyschondroplasia in

Table 4. Effects of feeding sources of docosahexaenoic and α -linolenic acids to ISA brown breeders and offspring pullets to 18 weeks of age on keel bone attributes in 42-wk-old laying hens challenged with lipopolysaccharide (LPS)^{1,2}.

Items	Wt ³ , g/kg BW	Ash, g/kg BW	Ash, %
Challenge			
LPS	2.69	1.01	37.67 ^b
Sham	2.23	0.96	41.48 ^a
SEM	0.223	0.080	0.777
Breeder diets			
CON	2.50	0.99	39.83 ^{ab}
DMA	2.49	1.06	42.30 ^a
FFF	2.82	1.05	37.45 ^b
SEM	0.110	0.046	1.111
Offspring diets ²			
CON-CON	2.50	0.99	39.83 ^{ab}
CON-DMA	2.55	1.02	38.73 ^{ab}
CON-FFF	2.92	0.93	43.10 ^a
DMA-CON	2.49	1.16	42.30 ^a
DMA-DMA	2.44	1.01	41.34 ^a
FFF-CON	2.82	1.05	37.45 ^b
FFF-FFF	2.73	1.12	40.95 ^{ab}
SEM	0.165	0.065	1.572
Probabilities (<i>P</i> -value)			
Challenge	0.202	0.431	0.008
Breeder diets	0.059	0.411	0.017
Offspring diets	0.099	0.357	0.007

^{ab}Values with uncommon superscripts within each column are significantly different ($P < 0.05$).

CON, control; DMA, microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid; and FFF, co-extruded full-fat flaxseed and pulses of field peas mixture (50/50, wt/wt), as a source of α -linolenic acid.

¹ $n = 5$.

²The day-old female pullets from breeders fed CON, DMA, and FFF were divided into 3 (CON, DMA, and FFF), 2 (CON and DMA), and 2 (CON, FFF) post-hatch treatments, respectively.

³Weight.

poultry. Lipopolysaccharide challenge has been reported to downregulate osteoprotegerin, an inhibitory factor for osteoclastogenesis, subsequently inducing bone resorption (De Boever et al., 2009; Kruger et al., 2010). Bone mineral loss can lead to a higher chance of bone fractures and osteoporosis in laying hens.

Physiological stress increases protein catabolism and hydroxyapatite turnover (Trey and Kushner, 1995; Roux and Orsel, 2000), leading to bone mass loss in the extracellular matrix (Mireles et al., 2005), and reducing tibia breaking strength. In addition, tibia breaking strength was dose-dependent on the level of LPS (Mireles et al., 2005). However, the observations on the effect of the administration of LPS on bone metabolism have not been consistent. Nie et al. (2018) did not observe any impact of administering 1.5 mg LPS/kg BW for 9 consecutive days in laying hens on tibia calcium and phosphorous content. However, the egg production was dropped by 25.5% caused by LPS induced stress, meaning fewer minerals were shunted out of the skeletal system for bone formation (Nie et al., 2018). Moreover, the dietary nutrient composition has been reported to influence the inflammatory response in poultry. For instance, the expression of liver inflammatory cytokines were shown to be increased in LPS challenged birds fed diets with 3,100 kcal/kg metabolizable energy (ME) compared with challenged birds fed diets with 2,800, 2,900 kcal/kg ME (Bai et al., 2019). Therefore, various factors such as dosage of LPS (Mireles et al., 2005), type of birds (Mireles et al., 2005; Nie et al., 2018), egg production rate (Nie et al., 2018), and dietary

Table 5. Effects of feeding sources of docosahexaenoic and α -linolenic acids to ISA brown breeders and offspring pullets to 18 weeks of age on whole tibia attributes in 42-wk-old layer hens challenged with lipopolysaccharide (LPS)^{1,2}.

Items	Wt ³ , g/kg BW	Ash, g/kg BW	Ash, %	Breaking strength, N/kg BW
Challenge				
LPS	3.43 ^b	1.34 ^b	38.97 ^b	87.67 ^b
Sham	3.84 ^a	1.69 ^a	44.06 ^a	117.50 ^a
SEM	0.114	0.073	1.445	1.99
Breeder diets				
CON	3.40	1.27	37.49	93.50
DMA	3.45	1.26	36.64	89.69
FFF	3.58	1.38	38.58	96.35
SEM	0.087	0.049	1.115	4.040
Offspring diets ²				
CON-CON	3.40	1.27	37.49	93.50
CON-DMA	3.64	1.24	34.23	96.05
CON-FFF	3.13	1.23	39.26	96.77
DMA-CON	3.45	1.26	36.64	89.69
DMA-DMA	3.40	1.23	36.21	90.59
FFF-CON	3.58	1.38	38.58	96.35
FFF-FFF	3.50	1.40	39.89	93.19
SEM	0.123	0.069	1.534	5.374
Probabilities (<i>P</i> -value)				
Challenge	0.036	<0.001	0.038	<0.001
Breeder diets	0.295	0.172	0.476	0.512
Offspring diets	0.071	0.726	0.124	0.695

^{ab}Values with uncommon superscripts within each column are significantly different ($P < 0.05$).

CON, control; DMA, microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid; and FFF, co-extruded full-fat flaxseed and pulses of field peas mixture (50/50, wt/wt), as a source of α -linolenic acid.

¹ $n = 5$.

²The day-old female pullets from breeders fed CON, DMA and FFF were divided into 3 (CON, DMA and FFF), 2 (CON and DMA), and 2 (CON, FFF) post-hatch treatments, respectively.

³Weight.

Table 6. Effects of feeding sources of docosahexaenoic and α -linolenic acids to ISA brown breeders and offspring pullets to 18 weeks of age on tibia subparts attributes in 42-wk-old layer hens challenged with lipopolysaccharide (LPS)^{1,2}.

Items	Epiphysis			Medullary			Cortical		
	Wt ³ , g/kg BW	Ash, g/kg BW	Ash, %	Wt, g/kg BW	Ash, g/kg BW	Ash, %	Wt, g/kg BW	Ash, g/kg BW	Ash, %
Challenge									
LPS	1.96	0.65	32.85 ^b	0.38	0.05	14.44	1.09 ^b	0.64 ^b	58.65
Sham	2.10	0.81	38.28 ^a	0.43	0.07	16.16	1.31 ^a	0.82 ^a	62.54
SEM	0.114	0.050	1.130	0.025	0.001	2.605	0.051	0.040	1.804
Breeder diets									
CON	1.91	0.60	31.67	0.43	0.05	12.23	1.07	0.62	58.00
DMA	1.95	0.61	31.25	0.42	0.05	11.30	1.07	0.61	56.52
FFF	2.02	0.66	32.70	0.43	0.04	8.52	1.13	0.68	60.28
SEM	0.059	0.028	1.192	0.028	0.007	1.659	0.033	0.025	1.455
Offspring diets									
CON-CON	1.91	0.60	31.67	0.43 ^{ab}	0.05	12.23	1.07	0.62	58.00
CON-DMA	2.01	0.60	30.26	0.54 ^a	0.04	7.57	1.09	0.60	55.24
CON-FFF	1.74	0.56	31.90	0.36 ^b	0.05	14.67	1.04	0.62	60.11
DMA-CON	1.95	0.61	31.25	0.42 ^{ab}	0.05	11.30	1.07	0.61	56.52
DMA-DMA	1.88	0.58	30.69	0.45 ^{ab}	0.05	11.20	1.06	0.60	56.62
FFF-CON	2.02	0.66	32.70	0.43 ^{ab}	0.04	8.52	1.13	0.68	60.28
FFF-FFF	2.00	0.67	33.59	0.41 ^{ab}	0.04	10.22	1.10	0.68	61.99
SEM	0.093	0.042	1.610	0.038	0.010	2.37	0.049	0.036	2.011
Probabilities (<i>P</i> -value)									
Challenge	0.402	0.052	0.009	0.225	0.325	0.655	0.017	0.015	0.165
Breeder diets	0.364	0.294	0.674	0.983	0.638	0.233	0.332	0.079	0.201
Offspring diets	0.156	0.415	0.740	0.018	0.593	0.170	0.715	0.959	0.383

^{ab}Values with uncommon superscripts within each column are significantly different ($P < 0.05$).

CON, control; DMA, microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid; and FFF, co-extruded full-fat flaxseed and pulses of field peas mixture (50/50, wt/wt), as a source of α -linolenic acid.

¹ $n = 5$.

²The day-old female pullets from breeders fed CON, DMA, and FFF were divided into 3 (CON, DMA and FFF), 2 (CON and DMA), and 2 (CON, FFF) post-hatch treatments, respectively.

³Weight.

nutrients can influence the impact of LPS administration on the skeletal system (Bai et al., 2019).

Breeder and offspring diets did not affect dry weight and ash content of keel bone (Table 4; $P > 0.05$). Ash percentage of keel bone was higher in birds from breeders fed DMA compared with FFF ($P = 0.017$). The offspring treatment of CON-FFF, DMA-CON, and DMA-DMA maintained a higher ash percentage in keel bone compared with FFF-CON ($P = 0.007$). The exposure to n-3 PUFA during embryonic and pullet-phase have been reported not to modify the keel bone characteristics in 42 WOA ISA brown and Shaver white hens without any induced stress (Akbari Moghaddam Kakhki et al., 2020a). Breeder and offspring diets did not affect tibia dry weight, ash content, ash percentage, and breaking strength (Table 5, $P > 0.05$).

Administration of LPS reduced the tibia epiphysis ash percentage, cortical dry weight, and ash content (Table 6, $P < 0.05$). The majority of tibia subparts attributes were not influenced by breeder and offspring diet (Table 6, $P > 0.05$). Only dry weight of the tibia medullary was higher in the CON-DMA group than CON-FFF ($P = 0.018$). Similarly, there was no effect on tibia epiphysis and cortical attributes in 42 WOA ISA brown and Shaver white hens without any induced stress (Akbari Moghaddam Kakhki et al., 2020a). The potential of n-3 PUFA in offsetting the adverse impact of LPS administration on bone attributes was not observed in any dietary n-3 PUFA treatments compared with CON group. Higher ash percentage in keel bone

and tibia medullary dry weight in birds fed with DMA compared with FFF can show the higher potential of DHA than ALA in offsetting the adverse effect of LPS-induced challenge. Dietary inclusion of highly unsaturated fatty acids, such as eicosapentaenoic acid and DHA, has been reported to increase the eicosapentaenoic acid and DHA content in the cell membranes (Lee et al., 2018). The increase in eicosapentaenoic acid and DHA content occurs at the expense of arachidonic acid in cell membranes associated with inflammation pathways, resulting in decreased availability of the substrate of eicosanoids and inflammatory mediators (Calder, 2017). In addition, dietary inclusion of DMA has been reported to increase the synthesis of different pro-resolving mediators, reducing the expression of inflammatory mediators, expedite the return to homeostasis, and protect from host defence and collateral tissue damage that can occur during excessive inflammation (Calder, 2017). The limitations of this study should be noted since they might contribute to the results. Administration of LPS at an earlier age, when the pullets were exposed to the dietary n-3 PUFA, could increase the possibility of observing effect of an early n-3 PUFA feeding in offsetting the impact of LPS stress on bone attributes. Also, assessing other responses to LPS challenge such as daily feed intake and body temperature could demonstrate the gradual physiological changes in birds.

In conclusion, administration of 4 mg LPS/kg of BW reduced the eggshell quality, keel bone, and tibia attributes at 72 h postchallenge in 42 WOA ISA brown hens,

showing a disruption in mineral metabolism. The eggshell quality started to react to LPS-induced stress after 48 h postchallenge. Using the breeder-feeding technique to expose embryos to n-3 PUFA and direct feeding sources of n-3 PUFA to pullets did not influence their eggshell quality, tibia, and keel bones attribute compared with CON group after 24 wk of removing dietary n-3 PUFA sources. The provision of DHA could offset the effect of LPS-induced stress in tibia medullary and keel bones compared with ALA. The lack of effect of an early n-3 PUFA feeding on skeletal and eggshell quality might be associated with the cessation of n-3 PUFA inclusion in the diet after the onset of lay for 24 wk. Future studies are needed to investigate the potential of continuous feeding with n-3 PUFA for alleviating the impact of immune-system challenge on the skeletal system in laying hens.

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DISCLOSURES

The authors did not declare any conflict of interest.

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