Investigating the role of n-3 fatty acids on embryonic and rearing skeletal development and the subsequent impact on egg production, bone and eggshell quality in ISA brown and Shaver white hens

by

Reza Akbari Moghaddam Kakhki, MSc.

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ABSTRACT

INVESTIGATING THE ROLE OF N-3 FATTY ACIDS ON EMBRYONIC AND REARING SKELETAL DEVELOPMENT AND THE SUBSEQUENT IMPACT ON EGG PRODUCTION, BONE AND EGGSHELL QUALITY IN ISA BROWN AND SHAVER WHITE HENS

Reza Akbari Moghaddam Kakhki — Advisor:

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Optimal skeletal development prior to sexual maturity is pivotal in mitigating progressive structural bone and eggshell quality deterioration associated with egg production. Herein are investigations on the impact of omega-3 polyunsaturated fatty acids (**n-3 PUFA**) on skeletal development during embryonic and rearing phases and subsequent effects on egg production, bone and eggshell quality in ISA brown and Shaver white hens. Hatching eggs were procured from breeders fed control or diets supplemented with n-3 PUFA sources: docosahexaenoic acid **(DHA**) or α-linolenic acid (**ALA**). During rearing, pullets from breeders fed control diet were fed control or supplemented diets, and pullets from supplemented diets either continued with respective n-3 PUFA diets or control diet. Pullets were transitioned to a common layer diet at 18 weeks of age (**WOA**) and monitored to 42 WOA. The use of n-3 PUFA in breeder feed increased embryonic utilization of DHA. The effects of n-3 PUFA on skeletal development were strain-dependent. Although feeding ALA did not affect bone quality, DHA fed to breeders supported tibia and femur structural (cortical) development in Shaver white hens. However, egg production, bone, and eggshell quality were not influenced by pre-lay exposure to n-3 PUFA. Although n-3 PUFA supported skeletal development pre-lay, there were no residual effects evident in 42 WOA ISA brown and Shaver white hens. Further studies should focus on the impact of various doses of n-3 PUFA, mainly DHA, and the subsequent effect on bone and eggshell quality over the entire lay cycle.

DEDICATION

To the soul of my grandfather,

Zabih Sharifi Kakhki

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PUBLISHED CONTENT

1) **R. Akbari Moghaddam Kakhki**, K. R. Price, J. Moats, N. A. Karrow, and E. G. Kiarie (2020). Impact of feeding n-3 fatty acids to breeders and their offspring on concentration of antibody titers against infectious bronchitis, and Newcastle diseases and plasma fatty acids in the offspring. British Poultry Science. In Press. https://doi.org/10.1080/00071668.2020.1847254

2) **R. Akbari Moghaddam Kakhki**, and E. G. Kiarie (2020). Effects of feeding ISA brown and Shaver white layers breeder sources of n-3 fatty acids on hatching eggs profile, apparent embryonic utilization of egg components and body composition of day-old chicks. Canadian Journal of Animal Science. In Press. https://doi.org/10.1139/CJAS-2020-0026

3) **R. Akbari Moghaddam Kakhki**, K. R. Price, J. Moats, V. Shouldice, and E. G. Kiarie (2020). n-3 fatty acids fed to ISA brown and Shaver white breeders and their female progeny during rearing: Impact on egg production, eggshell and select bone attributes from 18 to 42 weeks of age. Poultry Science. 99: 3959-3970. https://doi.org/10.1016/j.psj.2020.03.061

4) **R. Akbari Moghaddam Kakhki**, K. R. Price, J. Moats, G. Bédécarrats, N. A. Karrow, and E. G. Kiarie (2020). Impact of feeding sources of n-3 fatty acids to ISA brown and Shaver white breeders and progeny on pullet skeletal attributes at hatch through to 18 weeks of age. Poultry Science. 99: 2087-2099. https://doi.org/10.1016/j.psj.2019.12.016

5) **R. Akbari Moghaddam Kakhki,** D.W.L. Ma, K. R. Price, J. Moats, N. A. Karrow, and E. G. Kiarie (2020). Enriching ISA brown and Shaver white breeder diets with sources of n-3 polyunsaturated fatty acids increased embryonic utilization of docosahexaenoic acid. Poultry Science. 99: 1038-1051. https://doi.org/10.1016/j.psj.2019.09.002

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ABBREVIATION

AA	Arachidonic acid
ALA	α-linolenic acid
AME	Apparent metabolizable energy
BMC	Bone mineral content
BW	Body weight
Ca	Calcium
CI	Change Index
CoA	Coenzyme A
CON	Control diet
CP	Crude Protein
DHA	Docosahexaenoic acid
DM	Dry matter
DMA	Control diet + 1 % of microalgae
DOH	Day of Hatch
EPA	Eicosapentaenoic acid
FA	Fatty acids
FABP	Fatty acid binding protein
FFF	Control diet + 2.60 % of co-extruded full-fat flaxseed and pulse mixture
GPR 120	G-protein coupled receptor 120
IL	Interleukin
LA	Linoleic acid
M-CSF	Macrophage colony-stimulating factor
MDA	Malondialdehyde

NADPH	Nicotinamide adenine dinucleotide phosphate
NF-KB	Nuclear Factor kappa-light-chain-enhancer of activated B cells.
OM	Organic matter
OSB	Osteoblast
OSC	Osteoclast
P	Phosphorous
PG	Prostaglandin
PUFA	Polyunsaturated fatty acids
RANK	Receptors for activation of nuclear factor-KB
RANKL	Receptors for activation of nuclear factor-KB ligand
ROS	Reactive oxygen species
RUNX2	Runt-related transcription factor 2
RY	Residual yolk
TBS	Tibia breaking strength
TNF-α	Tumor necrosis factor-α
TRAP	Tartrate-resistant acid phosphatase
WOA	Week of age

Chapter 1. Literature review

1.1. The lingering issues in egg production

There will likely be two billion more human to feed by the mid-21st century (FAO 2009). The demand for egg products has increased seven-fold, driving a considerable expansion of large-scale animal operations (Foley 2019). The increase in egg production comes with environmental challenges; however, egg production in Canada today is more efficient and more effectively uses many resources compared to farming in the 1960s (Pelletier et al. 2018). In addition to the needed increase in flocks to produce eggs, there are other advances in farming to give more "crop per drop" from the water and nutrients used (Powers and Angel 2008; Leinonen et al. 2012).

Egg is one of the most affordable animal proteins, which may explain the rapid increase in the number of hens in developing countries. Hybrids have been selected and bred for a variety of traits associated with egg production. There are commercial, economic, and environmental inspirations for continued genetic improvements for production indices such as eggs per hen housed and feed efficiency (Preisinger 2018). However, selection merely on production traits is no longer sufficient to meet all market demands, and a wider range of traits associated with the sustainability of egg production, including economics, environment, welfare, and social justice, should be taken into consideration (Fernyhough et al. 2020).

The biological limitation of an egg a day at peak lay is a reality in genetic improvement. Therefore, genetic companies are now targeting extended laying cycles to deliver continued commercial and economic benefits. In developed countries, increasing egg production by breeding for lay persistency and stability in egg quality are the main goals and priorities (Bain et al. 2016). One main characteristic presently being further developed is the lay persistency or the concept of the "long-life" layer. The egg production was increased from 313 eggs per hen in 2010 (Preisinger

2018) to 400 eggs per hen in 2016 (Bain et al. 2016). Breeding companies have been pursuing developing the "long-life" layer capable of producing more eggs in a longer production cycle (Bain et al. 2016; Hy-line International, 2014). The current target is a long-life layer capable of producing 500 eggs in 100 weeks without molting (Bain et al. 2016). Such an increase in egg production will allow the agriculture industries to benefit from more efficient utilization of diminishing resources, including land, water, raw materials for feed, as well as a reduction in waste, and an overall reduced carbon footprint (Bain et al. 2016). Making hens lay for longer appears to be the most logical approach for the efficient utilization of resources, both financial and environmental (Bain et al. 2016). It has been estimated that the number of breeding hens could be reduced by 2.5 million hens per year in the United Kingdom by sustaining 25 more eggs in a hen's lifespan (Bain et al. 2016), resulting in a drastic change in the overall shape of the breeding pyramid. From an environmental perspective, by expanding the lifespan of laying hens for ten weeks, around 1 g of nitrogen could be saved per dozen eggs (Fernyhough et al. 2020). However, the persistency in lay requires consideration of how to maintain birds' health and welfare in longer laying cycles. Therefore, genetic companies should not only focus on extending laying cycle, but also on improving bird resilience and coping with longer productive life (Fernyhough et al. 2020).

Deterioration in eggshell quality and a decline in egg number are the main reasons for replacing laying flocks. Poor eggshell quality due to aging does not imply all birds produce eggs with poor eggshell quality, rather that there is variability in eggshell quality. Therefore, long-term maintenance of organs and tissues associated with egg production is a requirement for extending the laying cycle of commercial flocks (Dunn, 2013). However, despite the body of research in this area spanning over 50 years, there is still more to learn of all the processes and mechanisms governing the complexity of egg formation (Bain et al. 2016). Daily eggshell formation requires

the removal of 2-3 grams of calcium (**Ca**), equivalent to 10% of the hen's body Ca reserve (Gilbert 1983). Calcium homeostasis is created through a balance between intestinal absorption, renal excretion, and bone mineral metabolism to meet the bird's requirements (Elaroussi et al. 1994). In modern layers, this daily bone remodeling for eggshell formation will occur more than 500 times during the laying cycle, which represents 1,500 grams of Ca being shunted out of the hen's body in a lifetime. Therefore, the reservoir of minerals amassed in the hen's skeletal system before puberty is crucial especially considering the Ca output associated with the number of eggs per bird in a lay cycle (Anderson et al. 2013) such that Ca required for eggshell formation is greater than the Ca the medullary bone can supply. Consequently, the structural bone becomes utilized for eggshell production, and subsequently, bones become osteoporotic, resulting in bone fragility (Rufener et al. 2019). This skeletal fragility is mainly due to the loss of structural bone mass throughout the life of the hen, leading to osteoporotic fractures and osteoporosis with implications on hen welfare and health (Kim et al. 2007). Although the transition from conventional cages to other housing systems has been reported to improve bone material properties due to the increased bone formation and remodeling, it has not entirely prevented skeletal disorders (Riber et al. 2018). New cage systems may allow hens to have the freedom to exercise and improve bone strength but can increase the risk of keel bone damage (Fleming et al. 2006). Although alternative housing systems might be beneficial for improving bone strength, the effect of alternative housing systems on eggshell quality and microbial population on eggshell should be taken into consideration as well (Vlčková et al. 2018). Once a skeletal fracture occurs, egg production appears to fall, suggesting there is a tradeoff or re-partitioning of resources (Rufener et al. 2019). Therefore, a healthy skeletal system is required for achieving the potential of 500 eggs per hen and is important as part of the wider sustainability plan of egg production.

1.2. Avian skeletal biology

Bone is a dynamic tissue that is continually adapting not only to external mechanical stimuli but also to internal metabolic Ca demands. Bone mass is continuously maintained by bone-resorbing osteoclasts (**OSC**), which digest the collagenous bone matrix and release Ca, after which bone-depositing osteoblasts (**OSB**) form unmineralized collagen matrices, which eventually become mineralized (Nakanishi and Tsukamoto 2015). Bone growth and mineralization begin during early embryo development. Embryonic bone development occurs in two ways. Mesenchymal cells can directly differentiate into bone or cartilage through intramembranous ossification and endochondral ossification, respectively (Pechak et al. 1986). Endochondral ossification starts in the first week of incubation with the differentiation of mesenchymal cells into chondrocytes and OSB in the diaphysis. This process leads to the formation of the appendicular and axial skeleton of chickens (Pechak et al. 1986). Longitudinal growth in long bones of the appendicular skeleton occurs through endochondral ossification and expansion of both the proximal and distal epiphyses, as opposed to the radial growth of the bone diaphysis. In the chicken embryos, as the bone elongates, expansion of the proliferative zone occurs more quickly than bone resorption at the distal area of the growth plate, leading to an increase in the thickness of the growth plate. As a result, the bones develop fast, and tibia has been reported to elongate by about 2.3-fold between day 15 of incubation and hatching (Torres and Korver 2018). Proliferative chondrocytes form columns of flattish cells, which contain a high content of type II collagen closely packed within an extracellular matrix (Whitehead 2004). These chondrocytes become more separated within their columns, enlarged, rounded, and start to secret collagen type X, proteoglycans, and growth factors (Whitehead 2004). Chondroclasts then resorb the matrix and enlarged chondrocytes secret alkaline phosphatase to initiate the formation of crystals of hydroxyapatite followed by their

apoptosis. Then collagen type I is secreted by OSB, which becomes mineralized by hydroxyapatite (Whitehead 2004). Osteoclasts perform the bone resorption by dissolving bone mineral through creating a sealed highly acidic zone at the bone surface and decomposing the collagenous bone matrix (Kerschnitzki et al. 2014). This collaboration leads to the development of a network of trabecular bone, a woven bone based on an irregular structure of collagen fibrils.

Bone width growth requires a process involving intramembranous ossification. Osteoblasts progress in the perichondrium and produce spicules of bone that merge to produce a network of bone with cavities. The secretion of concentric layers of lamellar cortical bone by OSB fills up the cavities (Whitehead 2004). The medullary cavity increases in volume as resorption of existing bone from the endosteum, and bones get thicker by synthesis and deposition of new mineral by OSB on the periosteum (Torres and Korver 2018). These patterns of bone growth continue throughout the rearing period in pullets. During the early stages of the rearing phase, the ring (cross-section of bone) expands so that cavities do not become entirely filled with the bone before endosteal resorption begins, but as growth slows the degree of infilling increases (Whitehead 2004). At the end of the rearing phase, secondary osteons are formed in which OSC cut a tunnel in the bone followed by OSB forming new concentric layers of lamellar bone, embedding within the bone, and differentiating to osteocytes (Whitehead 2004).

At the onset of sexual maturity, a dramatic change takes place in bone biology due to the surge of estrogen, which has a direct effect on bone and an indirect effect through the intestine, kidney, and immune cells. The increased demand for Ca for eggshell formation over the formation of the eggshell in the shell gland mainly occurs during the night, when the supply of Ca from the digestive tract is low. Therefore, a high proportion of eggshell Ca is shunted out from medullary bone and transported to the oviduct for eggshell formation (Whitehead 2004; Kerschnitzki et al.

2014). This skeletal transferable Ca accounts for 20-40% of eggshell Ca (Kerschnitzki et al. 2014). To meet this demand, the OSB function switches from forming lamellar cortical bone to producing a woven bone called medullary bone (Whitehead 2004). Medullary bone forms a labile Ca reservoir for the eggshell formation and is laid down on the inner surfaces of structural bone and in spicules within the medullary cavities, especially in the leg bones (Kerschnitzki et al. 2014). The high rate of medullary bone formation has been reported during the early stages of lay and can continue to accumulate slowly over the remainder of the laying period (Whitehead 2004). During this time, OSC continue to resorb the structural bone leading to a decline in structural bone content of the hen (Whitehead 2004). An increase in ionic Ca in blood has been reported six hours after oviposition during the early stages of the laying cycle, which was concomitant with the dramatic increase in mineralization of medullary bone (Kerschnitzki et al. 2014). After the initiation of the eggshell calcification process, blood ionic Ca and mineralization of medullary bone are decreased along with an increase in blood P level (Luck and Scanes 1979; Kerschnitzki et al. 2014).

Since the OSC are not specific to the medullary bone, resorption can also occur at exposed structural bone surfaces and the continuous loss of bone mass in structural bone results in weakening of the skeleton and increase the risk of osteoporotic fracture. However, the accumulation of bone mass in medullary bone remains constant or increases over the course of the laying period (Kerschnitzki et al. 2014). Although medullary bone plays a vital role in eggshell formation, there is not a direct relationship between medullary bone content and eggshell quality (Whitehead 2004). The lack of relationship between eggshell quality and medullary bone content is illustrated by 1) good eggshell quality at an early stage of lay when little medullary bone has been formed, and 2) the decline in eggshell quality at a late stage of lay when medullary bone mass

is high (Whitehead 2004; Kerschnitzki et al. 2014). When the hen goes out of lay, the formation of structural bone begins while the medullary bone gradually disappears. The mechanism behind these changes is driven by the reduction in the secretion of estrogen, which stimulates OSB function to lay down structural bone and decreases the OSC function (Whitehead 2004). These changes allow birds to improve structural bones, quality, and mass. Molting has been used as a practical technique to improve eggshell quality at the end of a long laying cycle; however, this procedure has raised concerns over animal welfare (Akbari Moghaddam Kakhki et al. 2018).

1.3. Current strategies for managing skeletal issues in layers

Nutritional and management practices during rearing focus on a good quality pullet in terms of body conformation at sexual maturity. The fastest rate of growth for the skeleton occurs between 6 and 12 weeks of age (**WOA**), when pullets gain an average of 90 to 110 g of body weight per week and the skeleton is 95% developed at 12 WOA (Whitehead 2004). Once the bone growth plates close at sexual maturity, no more bone length can be added. Any delay in growth will affect the size of the mature bird and delay the onset of production. Once birds have reached the proper level of development as determined by body weight (**BW**) and BW uniformity, the flock will be receptive to light stimulation to start egg production (Bain et al. 2016; Whitehead 2004). Laying hens continue to add muscle, fat, and bone mass (medullary) until around 32 WOA when the full mature BW is achieved. Therefore, it is vital that sexually mature pullets meet the recommended target weight, have correct body uniform and composition among the flock, and an adequately developed skeletal system to sustain their egg production (Bain et al. 2016; Whitehead 2004). There are various strategies used to improve skeletal quality in pullets and subsequently hens.

1.3.1. Genetic selection

Genetic selection for optimizing production performance and eggshell quality of laying hens may create birds with poor structural bone content, resulting in an increase in bone fragility (Budgell and Silversides 2004). Hocking et al. (2003) compared production performance and bone quality among 25 commercial and traditional breeds. They showed that commercial breeds had lower tibia breaking strength (**TBS**) compared with traditional breeds, but there was relatively little genetic variation in terms of eggshell strength, which suggested that eggshell quality is maintained in genetically selected lines at the expense of bone strength (Hocking et al. 2003; Fleming et al. 2006).

Skeletal disorders, especially osteoporosis, have a strong genetic component (Fleming et al. 2006), in which bone density has been shown to be a heritable trait, and genetic manipulation has been effective in alleviating the severity of skeletal disorders (Budgell and Silversides 2004). Genetic selection after seven generations led to an increase in TBS among different breeds of hens with no difference in egg production (Bishop et al. 2000). However, those breeds with a higher TBS showed poor eggshell quality (Bishop et al. 2000). The differences among breeds in bone strength have been reported to be attributed to a different rate of bone formation during rearing and lower bone resorption due to lower numbers and activity of OSC (Bishop et al. 2000; Whitehead 2004). Sparke et al. (2002) reported the difference in TBS between breeds was associated with quantities of crosslinking in the collagen matrix, particularly pyrrolic crosslinking. Other bones, such as the keel bone, have been reported to be influenced by genetic selection mainly pertaining to a higher susceptibility for deformity and fracture (Fleming et al. 2006).

The bone strength and quality of white and brown strain hens have been compared in different studies (Riczu et al. 2004; Silversides et al. 2012; Regmi et al. 2016). For instance, Riczu et al. (2004) compared bone strength and production performance in brown (Shaver 579) and white

layers (Shaver 2000). These results showed that egg production of the white strain was higher than the brown strain, but the brown strain exhibited heavier egg weight and better eggshell quality with more frequent pauses in egg production (Riczu et al. 2004). The brown strains had greater femur and humerus breaking strength and mineral density than in the white strain hens, suggesting that the brown strains were more resistant to bone fragility (Riczu et al. 2004). The pauses in egg production in brown strains may have provided more time for medullary reserves to be replenished and subsequently were less reliant on structural bone mass for eggshell formation (Riczu et al. 2004). So, there is an interplay of egg production and bone quality in laying hens. In addition, bone strength has been reported to be positively correlated with BW (Fleming et al. 2006; Cruz et al. 2012). Similar results of the heavier bones (absolute value) and greater cortical bone density in brown strains compared to white strains have been reported by Silversides et al. (2012) and Khanal et al. (2019). Around 40% of the variation in bone quality in laying hens has been reported to be related to genetic selection (Bain et al. 2016). Therefore, genetic selection to improve bone quality can be one approach. However, due to the complex etiology of bone disorders, such as osteoporosis, other factors besides genetics, such as housing and nutrition, have also been implicated (Bain et al. 2016).

1.3.2. Environment

Beginning in the 1950s, the egg industry moved away from floor pens or range to a cage system to improve management, improve egg cleanliness, and reduce disease. However, the impact of this change on welfare and behavior remains a controversial topic (Silversides et al. 2012), leading to the planned or current ban of conventional cage systems with various phase-in periods for movement to alternative systems. Although the restriction of natural behavior

expression is a major concern in conventionally caged hens, osteoporosis, or “cage layer fatigue” is also significant (Silversides et al. 2012).

Housing systems for laying hens play a significant role in the development or the prevention of bone diseases. The increase in physical activity and mechanical loading during rearing and lay can stimulate bone formation and reduce the severity of osteoporosis in laying hens in alternative housing systems (Rodriguez-Navarro et al. 2018). The reduced mobility in the conventional cage has been shown to impact bone mass adversely (Fleming et al. 2006; Regmi et al. 2016), and switching from conventional cages to active housing systems has been shown to improve bone strength. However, the aviary or free-range systems do not necessarily result in a lower fracture incidence because of the possibility of more traumatic accidents (Fleming et al. 2006). One of the downsides of aviary and free-range housing systems might be the higher prevalence of keel bone fracture. For instance, the rate of keel bone fracture has been reported to be 48% in a floor pen system, while it was 28% in a cage system (Petrik et al. 2015). The higher prevalence of keel bone fractures in furnished cages and aviaries has been associated with trauma caused by flights and landing accidents sustained during the production period (Wilkins et al. 2011).

The underlying mechanism of different skeletal disorders in laying hens such as osteoporosis or keel bone damage might be different in various housing systems and breeds. Housing systems and breeds have been reported to be associated with various types of keel deformities, indicating the intrinsic biomechanical nature of the keel bone might be a contributing factor in causing deformities (Regmi et al. 2016). Similarly, the effect of various housing systems on bone has been reported to be dependent on the hen strain (Silversides et al. 2012). The cortical bone properties in White Leghorn hens have been shown to be not affected by the housing system, but the heavier strains such as ISA Brown and Brown Leghorn line had greater tibia cortical bone mass in the

floor pens (Silversides et al. 2012). However, white leghorns tended to spend more time on perches in the floor pen system than the brown layers, leading to an increase in the area and cortical density of radius in White Leghorns (Silversides et al. 2012). Silversides et al. (2012) concluded that the white leghorns used their space, both horizontal and vertical, more effectively than the brown layers, and that this likely contributed to better bone characteristics when they were kept in floor pens in comparison to conventional systems (Silversides et al. 2012).

Alternative housing systems have been shown to improve tibia and keel bone integrity compared with a conventional systems; however, this effect might not be sufficient to prevent fracture and bone mass loss (Regmi et al. 2016; Casey-Trott et al. 2017a; Casey-Trott et al. 2017b; Neijat et al. 2019). Other problems associated with the alternative housing systems are increased feather pecking (Blokhuis et al. 2007) and eggshell microbial population (Vlčková et al. 2018) compared with conventional systems.

1.3.3. Nutrition

Genetic selection and changes in the housing system have led to an improvement in skeletal health, allowing to extend the lifespan of hens. However, the high demand of Ca for eggshell formation has demonstrated that providing required nutrients for skeletal health is a critical factor from the start of the rearing phase to the end of the production cycle (Korver 2020). Modern hens have shown more resistance to skeletal problems and cage fatigue than in the past; however, their high level of egg production over their extended lay cycle can predispose them to skeletal problems and poor shell quality in the long term (Korver 2020). To ensure that hen's genetic potential is achieved, formulating diets with sufficient nutrients is critical. There are various nutritional strategies in practice to improve skeletal health, such as the correct use and balance of Ca, P, and vitamin D_3 (Whitehead 2004). It has been recommended that providing more Ca by using a pre-

lay diet aids in the development of medullary bone before the onset of lay. However, it should be taken into consideration that medullary bone is not deposited until two weeks before the onset of lay. Therefore, the higher Ca level in the pre-lay diet does not provide any advantage to the bird before this point (Korver 2020), and might adversely impact feed consumption. The range of dietary Ca level has been recommended to be 0.9% to 1.2% during the growth period of the pullet, increasing to 2% to 2.5% just prior to the onset of lay and 3.5% to 4.5% once lay is established (Bain et al. 2016).

Most eggs are laid in the morning after the lights are turned on; therefore, the morning feed probably is not directly involved in eggshell Ca demand since eggshell formation starts approximately 5-h after oviposition and continues throughout the night. Similarly, the afternoon feed is also not fully synchronized with eggshell formation. Introducing midnight-feeding by switching on the light can provide synchronization of dietary Ca intake with shell formation and improves eggshell quality (Whitehead and Fleming 2000; Korver 2020). However, the 8 hours of uninterrupted darkness in every 24-h period, legislated by European directive (1999/74/CE), and National Farm Animal Care Council (Section 4.3), prohibited this type of split lighting program (European Directive 1991, National Farm Animal Care Council, 2017). Therefore, Ca supply for eggshell formation relies on bone mineral content, mainly deposited in medullary bone.

The form of dietary Ca has been reported to influence bone reserves. Throughout the night, the intestine of the hen becomes depleted of Ca when only finely ground limestone (1–2 mm) was used in the diet. At this point of high demand for Ca for eggshell formation, hens entirely rely on bone mass to supply Ca for eggshell formation. Large particle limestone (2–4 mm) is retained in the gizzard for a long period of time and slowly dissolved over time. The gradual release of Ca from the gizzard for absorption by the digestive tract provides an opportunity to supply Ca

requirement overnight and diminishes the mobilization of bone Ca. The combination of 2/3 coarse and 1/3 fine limestone has been reported to supply both readily available Ca and a slowly-released source of Ca (Whitehead 2004; Bain et al. 2016; Korver 2020).

Aging reduces the efficiency of digestion, absorption, and metabolism of Ca, leading to an increase in dietary Ca level and a widening of the Ca: available P ratio (Wilkins et al. 2011); however, over-supply of Ca has been reported have an adverse impact on bone, eggshell quality and kidney (Akbari Moghaddam Kakhki et al. 2019a) and interferes with exogenous phytase activity (Bello and Korver 2019). Dietary vitamin D plays an important role in the absorption of Ca and P, and feeding metabolites of vitamin D has been shown to maintain eggshell quality and skeletal bone mass in aged laying (Akbari Moghaddam Kakhki et al. 2019a).

In the recent decade, there has been increased interest in factors that influence skeletal development in early-life and its impact on leg problems in broilers during the post-hatch period (Torres and Korver 2018). There is growing evidence that nutrition of breeders affects their offspring during the embryonic period and may program later-life metabolism and growth (Uni et al. 2005). Embryo development and growth are affected by both endogenous and exogenous factors. The breeders tightly regulate the egg environment through the supply of nutrition to the egg and the manipulation of incubation conditions in naturally brooding (Tong et al. 2013). Vitamin and mineral supplemented through the breeder diet or *in-ovo* injection have been reported to increase bone size and improve bone mechanical properties in the offspring (Favero et al. 2013; Saunders-Blades and Korver 2015; Yair et al. 2015). A stronger skeletal system allows the hatchlings to be more able to reach feed and water after placement at the farm, resulting in earlier access to nutrients and, thus, increased post-hatch growth (Torres and Korver 2018). Most of the research on the effect of breeder-feeding or *in-ovo* injection of nutrients have been completed in

broilers (Yair et al. 2015) with minimal work in laying hens. It has been demonstrated that the cortical (structural part) continues to lose mineral content starting from sexual maturity and over the lay cycle (Korver 2020, Akbari Moghaddam Kakhki et al. 2019a). Thus, nutritional strategies that aim to minimize osteoporosis should not be focused only on the late-phase of the lay cycle or when there is a high risk of osteoporosis, but rather in the early stages of skeletal development (Korver 2020).

1.4. Role of PUFA in modulating skeletal system

Essential fatty acids (**FA**) such α-linolenic acid (**ALA**; 18:3n-3) and linoleic (**LA**, 18:2n-6) have been shown to have health benefits, but they cannot be synthesized in the body and have to be supplied through the diet (Lee et al. 2018). Dietary inclusion of n-3 polyunsaturated fatty acids (**PUFA**) has attracted attention due to their health benefit (Lee et al. 2018). Enriched animal products such as milk, meat, and eggs can be sources for n-3 PUFA for humans. These products can confer health benefits to the animals as well (Lee et al. 2018). However, the risks of n-3 PUFA use in animal products include lipid peroxidation, reduced shelf-life, sensory quality, and productivity (Lee et al. 2018).

Lipids are one of the major components of fertile eggs and comprise over 30% of the yolk. An average-sized egg of 60g contains around 5.5g of lipid, comprising 65% triacylglycerol and 28% phospholipids (Cherian 2015). Over 95% of phospholipids and 88% of triacylglycerol are taken up by the embryo throughout the incubation period, serving as important structural precursors for membrane lipid bilayers and as a source of energy, respectively (Cherian 2015). Consequently, egg fat is a vital source of energy and essential FAs during embryogenesis and early post-hatch (Noble and Cocchi 1990). The rapid uptake of different lipid components by the embryo starting from the second week of incubation continues until the residual yolk is completely

absorbed (Cherian 2015). In avian species, the yolk acts as the main reservoir for long-chain PUFA. The yolk sac membrane is an extra-embryonic structure growing out from the hindgut of the embryo and it envelops the yolk (Cherian 2015). This structure develops elaborate folds and a microvillus structure during the first week of incubation (Yadgary et al. 2011). Uptake of FA is facilitated by endocytosis of low-density lipoproteins through the inner endodermal columnar cells of the yolk sac membrane to the surrounding blood vessels.

Embryonic exposure to n-3 PUFA has been shown to increased docosahexaenoic acid (**DHA**, 22:6 n-3) content in broiler chickens lasting for 28 days while it kept the hepatic content arachidonic acid (**AA**, 20:4 n-6) low for 40 days (Bullock et al. 2014). Similarly, the same response was observed for the brain FA profile, which reflected the breeder's diet FA profile (Bullock et al. 2014). However, DHA was more responsive to the maternal diet than AA. In mammals, consumption of n-3 PUFA during the prenatal and postnatal period (lactation period) has been shown to affect brain development, body composition and modulates metabolic pathways leading to changes in the risk of developing diseases such as obesity, diabetes, cancer and cardiovascular diseases (Mennitti et al. 2015). However, there are inconsistent effects of mammal maternal feeding of n-3 PUFA on body composition and the expression of genes related to lipolysis. For instance, Sardinha et al. (2017) reported that feeding pregnant mice n-3 PUFA (fish oil) decreased fat mass and insulin resistance in male offspring (Sardinha et al. 2012). In contrast, other researchers found no change in body composition (Ibrahim et al. 2009) and the expression of lipogenic genes (fatty acid synthase, glycerol 3-phosphate dehydrogenase, etc.) and insulin sensitivity in male offspring (Ibrahim et al. 2009). Muhlhausler et al. (2011) observed an increase in fat deposition in male rats exposed to n-3 PUFA through maternal feeding, while there was no difference in the expression of lipogenic genes. Another mechanism by which n-3 PUFA can exert

their long-lasting effect is through epigenetic modification. Prenatal intake of PUFA is involved in epigenetic regulation of the PUFA conversion in offspring through the transcriptional regulation of Δ5 desaturase and Δ6 desaturase, resulting in persistent changes in the PUFA content of cell membranes in the offspring (Mennitti et al. 2015). Reduction in susceptibility to mammary carcinogenesis in 166-days old female rats of dams fed an enriched n-3 PUFA diet was associated with elevated estrogen levels during pregnancy. In Mennitti et al. study, only 38% of the offspring exposed to n-3 PUFA developed a tumor compared with 64% of the offspring exposed to n-6 PUFA (Mennitti et al. 2015).

In a typical corn-soy poultry breeder hen diet, LA constitutes over 50% of total FA compared to ~3–3.5% of ALA due to the predominance of corn and the other sources of dietary fat that are high in n-6 FA (Cherian 2008). Desaturation and elongation enzymes are involved in the conversion of n-6 and n-3 PUFA. Competitive inhibition of these enzymes occurs, depending on which substrate is present at the highest concentration. Thus, the balance between n-6 to n-3 PUFA is essential for the optimal synthesis of long-chain PUFA (Brenner 1971). Currently, little consideration is given to the composition of the breeder hen dietary FA composition and what effect it may have on the physiology of the offspring (Cherian 2011). Furthermore, the current industry practice of feeding breeder flock diets high in n-6 FA due to high corn inclusions limits the supply of n-3 PUFA to the hatchlings.

Like other animals, ALA and LA cannot be synthesized *de novo* in avian species due to the inability to insert double bonds (deficiency in requisite desaturases) beyond the Δ-9 carbon (Brenner 1971). The same desaturase and elongase enzymes are required in the synthesis of n-6 and n-3 PUFA, so the efficacy of the conversion of long-chain n-3 PUFA from ALA is dependent on factors such as the concentration of n-6 FA (Brenner 1971). While both n-3 and n-6 PUFA

share the same metabolic pathway, each family of FA has been found to exert distinctly different and sometimes opposing biological effects. Docosahexaenoic acid and eicosapentaenoic acid (**EPA**, 20:5 n-3) are functionally crucial for many biological processes. Polyunsaturated fatty acids, including n-3 and n-6 PUFA, have been shown to influence cardiovascular disorders, some forms of mental illness, inflammatory diseases, insulin resistance, and bone formation (Boeyens et al. 2014). In addition, it has been reported the type of PUFA consumed during bone modeling is an essential determinant of bone formation (Cohen and Ward 2005). Fatty acids, especially n-3 PUFA, are believed to possess various health benefits such as increasing BMC and attenuating age-related bone loss (Boeyens et al. 2014). It is believed that n-3 PUFA may modify bone resorption and formation (Moon et al. 2012) through decreasing locally produced prostaglandins (**PG**, Mazzuco et al. 2005) and inflammatory cytokines (Moon et al. 2012). Hence, n-3 PUFA may represent a useful non-pharmacological way to attenuate bone loss and osteoporosis.

1.4.1. Effects of dietary PUFA on prostaglandin synthesis

One mechanism by which n-3 PUFA works to influence bone metabolism is modifying the production of eicosanoids, in particular PG. The PG family is made up of fast-acting hormones originating from PUFA (Kruger et al. 2010). The dominant PG in bone, PG2 (Kruger et al. 2010), is derived from AA, while PG3, equipotent to PG2 in bone resorption, is derived from EPA (Kajarabille et al. 2013). Both precursors undergo the same enzymatic reaction in synthesis (Kruger et al. 2010); however, EPA undergoes a less efficient enzymatic reaction than AA, subsequently favoring PG2 production over PG3 production (Kruger et al. 2010). In addition, PG1, another member of the PG family and derived from gamma-linolenic acid (20:3n-6), possesses anti-inflammatory properties and has been shown to reduce PGE2 synthesis (Kruger et al. 2010).

Dietary EPA and gamma-linolenic acid can regulate PGE2 production and subsequently impact bone resorption (Kruger et al. 2010).

Different factors are required for osteoclastogenesis and differentiation, such as receptors for activation of nuclear factor-KB ligand (**RANKL**) and macrophage colony-stimulating factor (**M-CSF,** Nakanishi and Tsukamoto 2015). RANKL, when expressed in both soluble form or membrane-bound by OSB, is activated by T-cells and stromal cells before binding to its receptor for activation of nuclear factor-KB (**RANK**) (Boeyens et al. 2014). In addition, other factors such as osteoprotegerin, a colony-stimulating factor 1 receptor for M-CSF, and a decoy receptor of RANKL, play essential roles in osteoclastogenesis (Kajarabille et al. 2013). The initial step in osteoclastogenesis occurs in hematopoietic cells when M-CSF binds to the colony-stimulating factor 1 receptor, which stimulates the expression of RANK (Nakanishi et al. 2013). Prostaglandin E2 stimulates the expression of RANKL and RANK and down-regulates the expression of osteoprotegerin. Bound RANKL-RANK activates two pathways in generating OSC precursors, including microphthalmia-associated transcription factor and nuclear factor of activated T-cells cytoplasmic 1, and stimulates the differentiation of mononuclear osteoclast-precursors into mature multinucleated OSC (Nakanishi et al. 2013). The mature OSC express tartrate-resistant acid phosphatase (**TRAP**), which is widely used as a mature OSC marker and is essential for collagen breakdown and bone matrix resorption (Boeyens et al. 2014). However, the effect of PG2 on bone is biphasic (Liu and Denbow 2001) and has also been reported to promote bone formation (Kajarabille et al. 2013). The exposure of OSB to PG2 stimulates the secretion of transforming growth factor-beta 1 as an up-regulator for differentiation and proliferation of OSB (Kruger et al. 2010). PGE2 stimulates the local expression of insulin-like growth factor 1 in bone and subsequently increases osteoblastogenesis, new bone-cell formation, and matrix production

(Kajarabille et al. 2013). The possible increase in bone formation in response to PG2 may be associated with prolonged exposure to a lower dose of PGE2 (Kruger et al. 2010).

1.4.2. Effects of PUFA on cytokine production

There is an interplay between bone metabolism and the immune system (Kruger et al. 2010). The function of an inflammatory response is to fight and clear against infection, remove harmful chemicals, and repair damaged tissue and organs (Kajarabille et al. 2013). However, failure to resolve the inflammation and maintain homeostasis in the target tissue can lead to disease (Kajarabille et al. 2013). Synovial tissue produces M-CSF and different cytokines, including interleukin (**IL**)-1, IL-6, IL-17, tumor necrosis factor-α (**TNF-α**), under an inflammatory state (Kajarabille et al. 2013). These cytokines increase osteoclastogenesis and, thereby, bone resorption through the RANKL-RANK pathway (Kruger et al. 2010). One example is the abnormal generation and activity of OSC, which has been found to be a significant factor in inhibited chondrocyte proliferation and induced cartilage degradation attributed to rheumatoid arthritis, osteoarthritis (Kajarabille et al. 2013) and tibial dyschondroplasia in poultry. Lipopolysaccharide challenge has been reported to increase PGE2, inflammatory cytokines, and expression of RANKL concomitant with the down-regulation of osteoprotegerin, subsequently inducing osteoclastogenesis (Kruger et al. 2010).

Polyunsaturated FA are able to modify inflammatory mediators and therefore have the potential to affect bone development and growth (Kruger et al. 2010). Dietary n-6 PUFA exerts the potential to increase inflammation, while n-3 PUFA can decrease inflammatory mediators (Innes and Calder 2018). Dietary n-6 PUFA, mainly AA, are involved in the increased production of M-CSF and cytokines, including IL-1, IL-6, and TNF-α, which may occur through a protein kinase C-dependent mechanism (Kruger et al. 2010). The beneficial effect of dietary n-3 PUFA on

bone development is mainly associated with eicosanoid biosynthesis (Kajarabille et al. 2013). As PG2 and PG3 are both generated through the same enzymatic reaction, PG2 can increase IL-6 production, while PG3 increases it to a lower extent (Kruger et al. 2010). Furthermore, the FA profile in cell membranes shifts toward n-3 PUFA in response to dietary addition of n-3 PUFA, resulting in a lower n-6: n-3 PUFA ratio in membranes and an alteration in their function, which can modulate the production of pro-inflammatory cytokines (Kajarabille et al. 2013).

The use of ovariectomized mice is considered a model for postmenopausal osteoporosis, while the administration of 17β-oestradiol-3-benzoate mimics the estrous cycle (Jin et al. 2017). Supplementation of 45 g/kg of menhaden oil (as a source of n-3 PUFA) in the feed of 9 wk old ovariectomized rats reduced PGE2, TNF-α, and IL-6 compared to that of the corn oil supplemented group (Nakanishi et al. 2013). Rats fed menhaden oil had significantly heavier tibia and femur, thicker tibia cortical, and a lower OSC index; however, the bodyweight of both groups was similar (Nakanishi et al. 2013). These authors also observed lower expression of M-CSF, RANKL, and RANK in ovariectomized rats fed diets supplemented with menhaden oil compared to corn oil (Nakanishi et al. 2013). Jin et al. (2017) observed that the dietary addition of n-3 PUFA at 0.48, 12.36, and 24.28 % of total FA supplied with fish oil for 12 weeks inhibited expression of IL-1 and IL-6, while femur BMC was increased in 12-week old ovariectomized mice injected with 17β-oestradiol-3-benzoate. Cohen and Ward (2005) tested the effect of 10 % flaxseed oil versus 10 % corn oil on bone characteristics and serum cytokines. Dietary treatments were offered from birth until 28 days of age to both male and female mice. The measured serum cytokines, including IL-1, IL-6, TNF-α, and BMC of the femur, did not differ among the two groups (Cohen and Ward 2005). From these results, the authors concluded that a notable effect of n-3 PUFA on bone metabolism was more likely to occur in a later stage of life (Cohen and Ward 2005). Rajaram et

al. (2017) reported modifying the ratio of n-6: n-3 PUFA did not influence bone turnover in the short-term in healthy 42 ± 3-year-old human adults. The discrepancies in the above results might be related to various factors in the experiments, including type, dose, and duration of the intervention as well as the species of in the target population. However, whether the protective effects of n-3 PUFA can translate to bone metabolism remains unknown and must be further explored (Rajaram et al. 2017).

1.4.3. Other potential effects of n-3 PUFA

Reactive oxygen species (**ROS**) are readily produced at a low level during metabolism and eliminated by endogenous antioxidant systems (Kajarabille et al. 2013). Production of ROS at high levels leads to oxidative stress, in which its magnitude is associated with aging, tissue inflammation, and degeneration (Kajarabille et al. 2013). Under normal conditions, the production of ROS, especially superoxide radicals, help OSC to chisel away older bone and eliminate calcified tissue (Kajarabille et al. 2013). Enzymes involved in the antioxidant system (e.g., superoxide dismutase or glutathione peroxidase) control ROS formation by OSC. The OSB produce glutathione peroxidase to protect against ROS. Overproduction of ROS increases apoptosis of OSB and osteocytes, and thus increases osteoclastogenesis by stimulating RANKL expression through NF-KB activation (Kajarabille et al. 2013).

The susceptibility of FA to oxidation is dependent on the degree of unsaturation and chain length. Thus, unsaturated FA, especially n-3 PUFA, are susceptible to oxidation (Kajarabille et al. 2013). However, there are controversial results regarding the effect of dietary PUFA and the level of tissue oxidation (Kajarabille et al. 2013). One factor attributed to these discrepancies is the compartment, where the oxidation reaction occurs. For example, aqueous environments such as

the cell membrane or plasma produce different oxidation profile than the solid ones such as bone and cartilage (Kajarabille et al. 2013).

Lipoxygenase generates various types of lipid mediators, such as resolvin, lipoxins, and protectins. These mediators have anti-inflammatory and pro-resolving activities and capable of promoting the resolution of inflammation and the return to homeostasis (Poulsen et al. 2008). Dietary supplementation of DHA and EPA has been reported to influence the percentage of lipid mediators in bone marrow as lipoxygenase mediators (Poulsen et al. 2008). It has been reported that resolvins decreased OSC growth, differentiation, and pit formation (Kruger et al. 2010).

1.5. Dietary n-3 PUFA and skeletal issues in poultry

Studies on the effect of feeding with n-3 PUFA on bone attributes of layer birds have been reported. For instance, Josling et al. (2019) fed 20 WOA Hy-Line Silver-Brown either control, short-chain FA, medium-chain FA n-9, n-3 PUFA and n-6 PUFA for 58 weeks at a constant inclusion level of 30 g/kg. Femur weight and ash percentage were higher in birds fed with n-3 PUFA compared to short-chain FA. Whereas the dietary treatments did not affect egg production performance and eggshell quality, the n-3 PUFA diet reduced feed intake, showing lower intake of Ca and thereby limiting the positive effect of the PUFA n-3 diet on bone quality. The authors hypothesized that n-3 PUFA might reduce the production of locally produced PG, resulting in lowered OSC activity compared to short-chain FA (Josling et al. 2019). Baird et al. (2008) evaluated the impact of the various dietary ratios of n-6: n-3 PUFA (ranging from 47.8:1 to 4.7:1) on bone quality in White Leghorns from 16 to 58 WOA. The tibia cortical thickness was higher in birds fed a diet containing the 5.9:1 ratio of n-6: n-3 PUFA than hens fed the diet with 47.8:1 ratio of ∑n-6: ∑n-3 PUFA. Over reducing the ratio of n-6: n-3 PUFA reduced the thickness of tibia cortical. Despite the increase in the thickness of cortical bone, the TBS was not influenced by the

diets (Baird et al. 2008). Toscano et al. (2015) compared the effect of long (EPA and DHA) and short (ALA) chain n-3 PUFA on egg production, keel bone, and eggshell quality starting from 27 to 65 WOA. Although the ratio of ∑n-6: ∑n-3 was adjusted at 1.1, the ∑n-3 and ∑n-6 (milligram) were not equal in the tested diets. These results showed that yield stress was greater in the humerus of birds fed either n-3 PUFA treatment compared to control group, independent from age (Toscano et al. 2015). The effect of the two types of n-3 PUFA was different on keel bone load value such that ALA had a positive impact on keel bone load, while DHA had an adverse impact. The different response of each type of n-3 PUFA might be attributed to the fact that chickens received ALA through the diet and then converted it to longer chain n-3 PUFA at a very low conversion rate (< 5%), showing a bottleneck in long-chain PUFA production (Toscano et al. 2015). The high provision of long-chain n-3 PUFA might by-pass what is perhaps a crucial regulatory mechanism and overwhelm the capacity of hens to control important inflammatory and bone regulatory factors. This bypass may result in health detriments, in particular the ability to mount an effective inflammatory response and to regulate bone function, with direct or indirect effects on bone and general health (Toscano et al. 2015). Another explanation for the adverse impact of long-chain n-3 PUFA on bone parameters could be an increase in lipid oxidation due to longer-chain and more double-bonds, which may increase bone resorption (see section 1.4.5). However, the increase in lipid oxidation may be a result of the form in which the DHA was provided. On the other hand, Mazzuco et al. (2005) did not observe any effect on tibia and humerus characteristics in response to reducing the ratio of ∑n-6: ∑n-3 PUFA from 8.0 to 0.6 in the pre and post molt-diets of White Leghorns from 62 to 76 WOA.

A study by Liu and Denbow (2001) reported that *ex-vivo* biosynthesis of PGE2 was higher in the femur of newly hatched quail layers from breeders fed diets supplemented with 50 g/kg

soybean oil and poultry fat ($P < 0.01$) compared to those supplemented with menhaden fish oil, which was correlated with AA content in the femur. However, these researchers did not measure bone characteristics (Liu and Denbow 2001). In their next study, Liu et al. (2003a) investigated long-term supplementation with the same source in the diet of 30-days old male quails for seven months. Their results showed the positive effect of dietary inclusion of fish oil on tibia mineral density, ash, Ca, and P percentage compared to soybean oil. Further on, in another study, Liu et al. (2003b) examined the effect of feeding the same fat and oil sources to quail breeders on tibia developments in the offspring. Dietary addition of fish oil increased tibia ash percentage and deoxypyridinoline, a structural part in collagen type I, in day-old offspring compared to the soybean oil group. Moreover, tibia cortical density and shear force were higher in the 1, and 2 WOA progeny from breeders were fed with fish oil compared to the soybean group (Liu et al. 2003b). Considering all the reviewed papers, the potential effect of n-3 PUFA to change bone properties has been reported to depend on two factors of the supplemental n-3 PUFA; 1) type of n-3 PUFA (Toscano et al. 2015) and 2) the dose of n-3 PUFA (Baird et al. 2008). Most of the aforementioned avian studies were focused on feeding with n-3 PUFA at or after the onset of lay. The only study performed on pre-hatch feeding with n-3 PUFA evaluated the bone properties in offspring (quails) before the onset of lay. As such, there is minimal information on the pre-hatch or pullet feeding with n-3 PUFA on skeletal development in pullets and the subsequent impact on the bone properties and eggshell quality.

1.6. Issues with the dietary supply of PUFA- The case of sustainable sources

Marine organisms (fish, seafood, algae, etc.) are the most important natural sources of n-3 PUFA. These organisms are fed, directly or indirectly, from marine phytoplankton as the primary producer of n-3 PUFA in the trophic chain (Rubio-Rodríguez et al. 2010). On average, fish oils

such as those obtained from sardine, cod, mackerel, and squid contain 22-24% n-3 PUFA (Hoshino et al. 1990). It has been reported that global fish stocks are in danger due to environmental changes, overfishing, and destructive fishing (Domingo et al. 2007). With health-conscious consumers increasingly embracing seafood, securing supplies of fish oil, a source of n-3 PUFA, has been a challenge at a time when supply availability has been volatile. Moreover, heavy metal contamination has been observed in various marine fishes like salmon, sardine, tuna, anchovy, mackerel, or hake, risking human and animal health (Domingo et al. 2007). For these reasons, the benefits of fish as a source of n-3 PUFA are being questioned. Agricultural traders, animal feed groups, and seed makers are scrambling to introduce alternatives to fish oil, turning to algae, microalgae and genetically modified flax and canola.

Although the commercial application (i.e., food, cosmetics, health products, biofuel, etc.) of microalgae has been developed in the last century, the application of algae has evolved with humans (Rubio-Rodríguez et al. 2010). Microalgae have shown great potential in food science, pharmacology, or human health, as sources of PUFA, sterols, or carotenoids, among others (Cardozo et al. 2007). Microalgae can be a potential sustainable source of n-3 PUFA in humans and animals due to possessing the advantage of being easily cultivated and presenting neither an unpleasant odor nor a high amount of cholesterol. At the same time, microalgae contain other beneficial factors such as squalene and phytosterols, antioxidants, etc. (Rubio-Rodríguez et al. 2010). One example of widely used microalgae is *Aurantiochytrium limacinum*, a species of unicellular marine fungi with 50% fat of its total dry weight, and DHA that exceeds 50% of its total FA (Gao et al. 2013). The unique FA content of *Aurantiochytrium limacinum* results from its ability to synthesize the PUFAs directly from acetyl-CoA and malonyl-CoA substrates through a polyketide synthase pathway (Gao et al. 2013).

Flax (*Linum usitatissimum*) has been cultivated for centuries and has found wide use as a food product (Singh et al. 2011). Full-fat flaxseed has been reported to contain 40% oil, 22% crude protein, 28 % dietary fiber, and 7.7% moisture (Canadian Grain Commission 2017), in which 58 % of its total FA is ALA. Comparatively, other common oilseeds such as soybean and canola have approximately 9.6% ALA (Canadian Grain Commission 2017). The application of flaxseed in diets of laying hens for enriching eggs for human consumption has been documented, which is limited to less than 20 % due to the presence of antinutritional factors (Caston et al. 1994; Konieczka et al. 2017). Canada is the largest producer of flaxseed, accounting for almost 80% of the global trade (Goyal et al. 2014) and showing the potential of using flaxseed as a sustainable source of n-3 PUFA in Canada.

Most human studies have shown that conversion of high doses of ALA to EPA occurs, conversion to DHA is severely limited (Gerster 1998). The conversion efficiency of ALA to EPA in humans has been reported to be 6% and 3.8% with a background diet high in saturated fat, while in a background diet high in n-6 PUFA, the conversion ratios dropped by 50% (Gerster 1998). Therefore, it is reasonable to ensure the adequate amount of EPA and DHA delivered through diet since evidence has been increasing that this long-chain metabolite has an autonomous function, e.g., in the brain, retina, and spermatozoa where it is the most prominent FA (Gerster 1998).

1.7. Summary

Selective breeding for productivity traits has led to an increase of Ca required for eggshell production greater than the medullary bone can supply. Therefore, the reservoir of minerals amassed in the hen's skeletal system before puberty is crucial, especially considering Ca output associated with the number of eggs per bird in an extended lay cycle. The inclusion of n-3 PUFA in diets of laying hens has been shown to improve the bone properties such as the mineral density

of structural bone. Fetal and early-life periods are critical for the development and programming of metabolic systems, including the skeleton. The embryonic and pullet periods are essential periods of bone development in which important genetic programs governing metabolism and growth are established. To date, most investigations on the role of feeding n-3 PUFA on skeletal health focused on hens, and some data showed that the impact of n-3 PUFA on bone properties depended on the type of n-3 PUFA. There is thus a gap in the knowledge on the impact of exposing pullets to n-3 PUFA during the embryonic and rearing period on skeletal development at sexual maturity and the subsequent impact on bone properties and eggshell quality in hens.

Chapter 2. Thesis Objectives and Hypothesis

The potential of n-3 PUFA in stimulating bone, brain, and immune cells development at embryonic through to early phases of chicks could significantly improve poultry productivity and health. Previous research in this area had focused on feeding n-3 PUFA to adult birds at the age with high susceptibility to osteoporosis, but limited research has investigated early-life feeding of n-3 PUFA in the skeletal system in different strains of laying hens.

Hypothesis: n-3 PUFA exposure in embryonic or pullet phases or a combination of phases will stimulate skeletal development at sexual maturity and subsequently improve bone strength and eggshell quality in ISA brown and Shaver white hens.

Overall objective: To investigate the role of different n-3 PUFA on embryonic and rearing skeletal development and the subsequent impact on egg production, bone and eggshell quality in ISA brown and Shaver white hens.

Specific objectives:

1. To investigate the effect feeding of ALA and DHA to ISA brown and Shaver white pullet breeders on the hatching egg composition, embryonic uptake of nutrients, and body composition at hatch.

2. To investigate the effect feeding of ALA and DHA to ISA brown and Shaver white pullet breeders and their offspring on skeletal development during rearing.

3. To investigate the effect feeding of ALA and DHA to ISA brown and Shaver white pullet breeders and their offspring on the subsequent impact on egg production, eggshell, and skeletal quality.

Chapter 3. The impact of the feeding of dietary sources of docosahexaenoic and α-linolenic acids to ISA brown and Shaver white layer breeders on hatching egg composition, embryonic uptake of nutrients and body compositions of hatchlings[1]

3.1. Abstract

The effect of the feeding of dietary sources of DHA and ALA to ISA brown and Shaver white layer breeders on hatching egg composition, embryonic uptake of nutrients, and body compositions of hatchlings were studied. Twenty-six-week-old ISA brown and Shaver white breeders were fed either: control diet (CON) or test n-3 PUFA diets supplemented with DHA (microalgae fermentation product) or ALA (co-extruded full-fat flaxseed product). Test diets had similar total n-3 PUFA and n-6: n-3 PUFA ratio. Feeding n-3 PUFA increased yolk DHA concentration to a greater extent in Shaver white compared with ISA brown. n-3 PUFA reduced the embryonic uptake of organic matter and mineral compared to the CON group ($P < 0.05$), resulting in a higher ratio of residual yolk to BW at hatch ($P = 0.002$). Embryos from hens fed n-3 PUFA utilized less total FA in phospholipid fraction ($P < 0.001$), and they preferentially utilized more DHA compared with CON embryos. Feeding ALA increased the body fat deposition and decreased lean percentage in Shaver white hatchlings compared with CON ($P < 0.05$). Although data suggested Shaver white had a higher propensity of depositing DHA than ISA brown, irrespective of strain, feeding n-3 PUFA modified embryonic pattern of FA utilization towards utilization of DHA. The change in body composition in response to feeding n-3 PUFA was associated with the shift in embryonic uptake of dry matter, organic matter, and minerals.

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3.2. Introduction

Unlike mammals, the growth and development of the avian embryos are dependent on nutrients deposited in a fertile egg. Embryonic utilization of the egg's nutrients has a direct effect on embryo growth, hatchability, and post-hatch performance (Uni and Ferket 2004; Uni et al. 2005). Previous research showed that chicken embryos preferentially utilized PUFA, particularly DHA, between d 15 and 19 of incubation as compared to all other examined FA (Yadgary et al. 2013). This suggested that chick embryos may have specific requirements for n-3 PUFA. The available literature is mainly limited to the effect of breeders' diets supplemented with n-3 PUFA sources on the FA profile in eggs (Ao et al. 2015), tissue, and embryonic FA uptake (Yadgary et al. 2013). However, there is little information on the effects of feeding egg-type chick breeder diets with n-3 PUFA sources on the hatching egg composition, embryonic uptake of nutrients other than FA, and body composition of hatchlings at hatch (Uni et al., 2005; Yadgary et al. 2013). The hypothesis in this chapter was that feeding egg-type chick breeders with diets supplemented with n-3 PUFA sources may change the hatching egg composition and patterns of nutrients utilization and subsequently change body composition and quality of hatchlings. Thus, this chapter aimed to evaluate the impact of feeding white and brown egg-type breeders with diets rich in n-3 PUFA and subsequent effects on hatching egg composition, embryonic uptake of nutrients and body compositions of hatchlings.

3.3. Materials and methods

3.3.1. Birds and management

The experimental protocol (#3675) was approved by the University of Guelph Animal Care Committee, and birds were cared for in accordance with the Canadian Council on Animal Care guidelines (CCAC 2009). Day-old breeders of ISA brown and Shaver white (250 ♀ and 40 ♂ per

strain) were procured from Hendrix Genetic Canada (Kitchener, ON, Canada) and kept at the Arkell Poultry Research Station at the University of Guelph. The birds were housed in separate floor pens by strain and gender in groups of 30. At 16 WOA, females of each strain were equally distributed based on the average of their BW and placed in floor pens of 600 × 188 cm equipped with 21 nipple drinkers and 3 round pan feeders. Males of each strain were introduced to each flock to a total of 31 birds (27 ♀ and 4 ♂).

The room temperature was initially set at 34°C on the first day and reduced by 2°C per week until a constant temperature of 21°C was reached. The lighting program started on the first day at 40 lux, 02:00 to 18:00 hrs, and was reduced on the 28th day to 10 Lux, 08:00 to 20:00 hrs up to 16 WOA. During the laying period, birds received 14 hrs of incandescent light (20 lux, 03:00 to 1700 hrs) and 10-h dark. The vaccination program included: Infectious Bronchitis (spray), Marek's disease (injection), and Immucox for coccidiosis control (gel droplet) on the first day followed by Newcastle-Infectious Bronchitis vaccine (spray) at 3 WOA, Infectious Laryngotracheitis Vectormune FP-LT-AE (wing web) at 6 WOA, Newcastle-Infectious Bronchitis at 10 (spray) and 16 (intramuscular) WOA. Birds were fed commercial fine crumble starter (0-6 WOA, 2,900 kcal/kg AME, 21.0 % CP, 1.06 % Ca and 0.77 % P) and coarse crumbles (7-16 WOA, 2,900 kcal/kg AME, 18.0% CP, 1.00% Ca and 0.78% P). During the laying period, birds were fed commercial feed with 2,875 kcal/kg AME, 18.0% CP, 4.24% Ca, and 0.68% P until 25 WOA. All feeds were supplied by Floradale Feed Mill (Floradale, ON, Canada). All eggs produced at week 25 were counted and weighed to establish baseline egg production for each strain.

At 26 WOA, birds were distributed within each strain into three dietary treatments with three replications (Table 3.1): 1) Control (**CON**), a corn, soybean meal, wheat and corn gluten diet, 2) CON plus 1% of dried microalgae (*Aurantiochytrium limacinum)* supplement (**DMA**) as a rich

source of DHA (0.04% ALA; 0.2% EPA; 17.9% DHA as-is basis with 3.1 % moisture (Alltech, Nicholasville, KY), 3) CON plus 2.6 % of co-extruded full-fat flaxseed and pulse (field peas) mixture (**FFF**, 1:1 wt/wt) as a source of ALA (10.5% ALA; 0.0% EPA; 0.0% DHA as-is basis with 5.2 % moisture, LinPRO®, O & T Farms Ltd, Regina, SK, Canada). The inclusion of 1% of DMA was previously shown to double enrichment of n-3 PUFA (DHA) in laying hens (Ao et a, 2015). All diets were formulated to meet or exceed the breeder specification (Table 3.2, Parent Stock Management Guide: ISA Brown, 2018: Parent Stock Management Guide: Shaver White, 2018). The inclusion level of FFF was chosen to ensure diets 2 and 3 were formulated to have a similar concentration of total n-3 PUFA (Table 3.2).

3.3.2. Sampling

Birds were fed the respective diets for 30 days, enough time to reach to the peak of n-3 PUFA deposition in the egg (Neijat et al. 2016). Samples of eggs (n=10) were collected to confirm n-3 PUFA enrichment. A total of 3,109 eggs were collected (~500 eggs per diet), individually marked and subsequently stored at 4ºC until incubation (within 8 d of the collection). Egg production was recorded daily, and feed intake was recorded to the end of egg collection. All eggs produced in the last three consecutive days of egg collection were weighed. Twenty eggs per diet were collected at the beginning and at the end of the egg collection period for egg composition analyses. Eggs were incubated and hatched in a commercial-grade incubator and hatcher (Nature Form, Jacksonville, FL) at the Arkell Poultry Research Station (Guelph. ON). Briefly, eggs were incubated at 37.5°C with 55 % humidity to d 19 and then transferred to the hatcher set at 36.9°C with 66 % humidity. Incubator conditions were the same for all strains by positioning one egg tray (90 eggs) of CON, DMA, and FFF of both strains at one level of the six used levels (central level, two levels above, and three levels below the central level). Eggs were candled on d 19, and eight

eggs per diet were randomly collected, weighed, and embryos were dissected. The small intestine samples were kept at –80°C for further analyses. The separation of different parts of the small intestine obtained at d 19 of the embryonic period was not possible due to the small size. On the day of hatch, chicks were counted and sexed. All males were euthanized by CO_2. Ten birds from each diet were euthanized for tissue sampling. Jejunum segments, starting from the end part of the duodenum and ending to at the yolk sack residue (Meckel's diverticulum), were stored at -80°C. The entire liver and residual yolk sac (**RY**) were taken and stored at -20°C for further analyses.

3.3.3. Sample processing and analyses

Egg components, including yolk and albumen, were separated by a plastic hand-held egg separator (Pierce Chicken IgY Purification Kit # 44918, Thermo Fisher Scientific, Waltham MA). Paper napkins were used to eliminate the adhering albumen from yolks, and then the yolks were weighed. The eggshells were washed in water, dried overnight at 105°C, and weighed (Akbari Moghaddam Kakhki et al. 2016). The albumen weight was calculated by subtracting yolk and shell weights from the whole egg weight.

Both residual yolk (**RY**) and yolk samples were freeze-dried, weighed to measuring dry matter (**DM**). 1 g ± 0.05 dried egg yolk and RY was weighed, ashed at 600˚C for 12 h, then reweighed for measuring ash content as an indicator of total mineral. Then ash samples were stored for Ca and P analysis. The organic matter (**OM**) in yolk and RY was calculated by subtracting the mineral weight from the whole DM weight. The concentration of Ca and P in diet and yolk was measured according to the described method by Khanal et al. (2019). 0.05 ± 0.01 g of ashed samples was weighed, and wet digested with a mixture of 5 mL concentrated hydrochloric and 100 µL nitric acids for 24 h at 120˚C. The digested samples were transferred into 100 mL

volumetric flask, ringed, topped to volume with double deionized water. An aliquot of 15 mL was submitted for reading the concentration of Ca and P using inductively coupled plasma atomic emission absorption spectroscopy (Varian Inc, Palo Alto, CA).

Lipid separation was done based on the method described by Reza-López et al. (2009). Briefly, ~0.05 ± 0.005 g sample of egg yolk or RY were weighed and placed in 15 mL glass tubes followed by adding 1 mL 0.1M KCL, and 4 mL chloroform: methanol (2:1, CHCL3: C298-1, MeOH; A452-4, Thermo Fisher, Waltham MA). Homogenate was vortexed, flushed with nitrogen, and allowed to sit overnight at 4°C. Samples were spun at 1,460 rpm for 10 min, followed by transferring the chloroform layer (lower layer). Chloroform was dried down by nitrogen and reconstituted by adding 250 µL of chloroform. Diluted samples (25 µL sample + 75 µL chloroform) were spotted on a 20×20 cm with 250 µm thickness thin-layer chromatography silica gel plate (heated for 30 min at 100°C; P01011, Sigma Aldrich, St. Louis, MO). Plates were activated at 100^{o}C prior to use. Then, the plate was moved into a glass TLC tank lined with filter paper containing 80 mL petroleum ether (261734, Sigma Aldrich, St. Louis, MO), 20 mL ethyl ether (E492-4, Thermo Fisher, Waltham MA) and 1 mL acetic acid (A35, Thermo Fisher, Waltham MA). The plate was run until the solvent reached approximately 3 cm from the top of the plate. The air-dried plate was coated by a fine mist of 0.1 % (w/v) 8-anilino-1-naphtalene-sulfonic acid using a nebulizer and visualized under UV light. Scored lipid bands were scraped and transferred into glass screw-capped tubes (15 mL) followed by the addition of 2 mL hexane, 2 µL of 0.1 mg/mL C17:0 free fatty acid internal standard and 2 mL 14 % BF3-MeOH (B1252, Sigma Aldrich, St. Louis, MO). Samples were methylated at 100°C for 1.5 hr and then centrifuged at 10 *g* to separate phases. Extracted hexane was transferred into gas chromatography vials and dried down under nitrogen. Fatty acid methyl esters were reconstituted in 50 µL hexane and separated on an

Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a flame ionization detector and separated on a DB-FFAP fused-silica capillary column (15 m, 0.1μm film thickness, 0.1 mm.; # 127-32H2, Technologies, Santa Clara, CA). Samples were injected in 200:1 split mode. The injector and detector ports were set at 250°C. Fatty acid methyl esters were eluted using a temperature program set initially at 150°C and held for 0. 25 min, increased at 35°C /min and held at 170°C for 3 min, increased at 9°C /min to 225°C, and finally increased 80°C/min to 245°C and held for 2.2 min. The run-time per sample was 12 min. The carrier gas was hydrogen, set to a 30 mL/min constant flow rate. Peaks were identified by comparison with authentic FA standards (GLC463; Nu-Chek Prep, Elysian, MN). Peaks were quantified using EZchrom Elite version 3.2.1 software.

Feed samples were ground and analyzed for the FA profile by a commercial lab (Activation Laboratories, Ancaster, ON) based on the described method by O'fallon et al. (2007). Briefly, the digestion of samples was performed with a combination of water, methanol, and sodium hydroxide in a warm water bath followed by neutralization of the sample with sulfuric acid in the warm water bath. After this process was completed, hexane extraction was performed to concentrate the newly produced fatty acid methyl ester in the organic layer. The extract was then injected into a gas chromatography-flame ionization detector on an SP-2560 capillary column where the now separated individual FA methyl esters were identified and quantified.

For tissue protein extraction, the whole small intestine was taken at d-19 (0.11 ± 0.013 g), and jejunum samples were taken at day of hatch (**DOH**, 0.12 ± 0.010 g) were weighed then placed into free-standing microcentrifuge tube (02-682-558, Thermo Fisher, Waltham MA) followed by addition of Tissue Protein Extraction Reagent (sample weight × 15; 78510, Thermo Fisher, Waltham MA) supplemented with Halt™ Protease Inhibitor cocktail (78430, Thermo Fisher,

Waltham MA). For liver samples, 0.1 ± 0.01 g samples were weighed, and Phosphate-buffered saline (28372, Thermo Fisher, Waltham MA) was added to the tubes (samples weight × 15). Then, 0.1 ± 0.01 acid-washed glass beads (≤ 106 μm; G4649-100G, Sigma Aldrich, St. Louis, MO) were added and followed by homogenization with a bead mill for one cycle of 150 sec at 3 m/s (15-340-163; Fisher Brand bead mill-24, Thermo Fisher, Waltham MA). Homogenized samples were then centrifuged at 10,000 × g for 15 min at 4°C. Supernatants were analyzed for protein concentration based on the described method of Smith et al. (1985) by using a Pierce BCA protein assay kit (23225, Thermo Fisher, Waltham MA) and kept at -80°C for further analyses. The concentration of G-protein coupled receptor 120 (**GPR 120**), small intestine fatty acid binding protein (**FABP**) and hepatic malondialdehyde (**MDA**) was measured in duplicate using ELISA kits that followed the recommended assay procedures (GPR 120: ECKG0026 and I-FABP: ECKF0037, ABclonal, Woburn, MA, and MDA: RDR-MDA-Ge, Reddot Biotech, Kelowna, BC).

Body composition including the percentage of BMC, fat and lean in the whole body were analyzed using Prodigy dual-energy X-ray absorptiometry (GE Healthcare, Madison, WI, USA) equipped with enCORE software (version 14.0, GE Healthcare, Madison, WI, USA) set on "small animal analysis" method with measurement features of 196.7 cm and 40.0 cm for length and width, respectively Akbari Moghaddam Kakhki et al. (2019a).

3.3.4. Calculations and statistical analyses

The percentage of RY to egg weight (g: g) was calculated by dividing the recorded weight of RY to the weight of the egg multiplied by 100. Apparent embryonic uptake of DM, FA, mineral, and OM was calculated by subtracting the respective concentration in RY from its averaged concentration in yolk samples of each treatment before incubation. The ratio of daughter FA to parent FA was used for calculating enzyme activity (Nain et al. 2012). The activity of enzymes

involved in the conversion of long-chain n-6 PUFA (Δ6-desaturase + elongase + Δ5-desaturase activity), and the conversion of long-chain n-3 PUFA (Δ6-desaturase + elongase + Δ5-desaturase activity) was calculated based on the following formula as described by Nain et al. (2012).

$$\text{conversion of long-chain n-6 PUFA} = \frac{\text{AA (mg)}}{\text{LA (mg)}}$$

$$\text{conversion of long-chain n-3 PUFA} = \frac{\text{EPA (mg)}}{\text{ALA (mg)}}$$

Data were tested for normality with UNIVARIATE plot normal procedure of SAS 9.4 and subsequently subjected to a two-way ANOVA in a 2 (ISA brown and Shaver white) × 3 (CON, DMA, and FFF) factorial arrangement using TUKEY test by the GLIMMIX procedures of SAS 9.4. Significance was declared at $P < 0.05$.

3.4. Results

3.4.1. Fatty acid concentrations in experimental diets

Supplementation with DMA and FFF modified the FA profile of the diets, as shown in Table 3.3. The dominant FA were LA, oleic (18:1n-9), and palmitic (16:0) acids across all the diets. The diets containing DMA had a higher relative percent composition of DHA (3.8% *vs.* 0.0% of FA) and lower ALA (5.1% *vs.* 6.3% of FA) compared with CON. The FFF had higher relative percent composition of ALA (10.3% *vs.* 6.3% of FA) compared with CON.

3.4.2. Production performance

Feed intake, egg production, mass, and weight were neither affected by the interaction between strains and diets nor the main effect of diets ($P > 0.05$; Table 3.4). Feed intake was higher ($P = 0.039$) for ISA brown hens compared with Shaver white breeders (113 *vs.* 108 g/b/d). Egg production, mass, and weight were not influenced by strain ($P > 0.05$). Average egg production,

mass, and weight was 93.2%, 54.5g/b/d, and 50.8g, respectively, for ISA brown. Corresponding values for Shaver white were 94.8%, 52.89g/b/d, and 50.12g, respectively.

3.4.3. Egg Components

The interactive effect of strain and diets did not affect egg weight and its components (Table 3.5; $P > 0.05$). ISA brown hens had heaver and eggshell weight compared with Shaver white ($P = 0.023$). However, strain did not influence albumen, yolk, and eggshell measurements ($P > 0.05$). Egg weight and its components were not influenced by the main effect of diet ($P > 0.05$).

3.4.4. Hatching Eggs Composition

Yolk weight and concentration of DM, OM, total mineral, Ca, and P were neither affected by the interaction between strain and diet nor the main effects of diet ($P > 0.05$, Table 3.6). The main effect of strain on yolk weight and DM content was such that ISA brown breeders had heavier yolks with higher DM content compared with Shaver white breeders ($P < 0.05$).

3.4.5. Fatty acid profile of yolk phospholipid fraction

There was an interaction between strain and diet on the relative percent composition of gamma-linolenic acid, AA, adrenic acid (22:4n-6), docosapentaenoic acid (22:5n-6), DHA, ∑n-6 PUFA, and ∑n-6: ∑n-3 PUFA in the phospholipid fraction of yolk ($P < 0.05$; Table 3.7). The absolute amount (mg) of LA, ALA, eicosadienoic acid (20:2n-6), gamma-linolenic acid, AA, EPA, docosapentaenoic acid, ∑n-6 PUFA and ∑n-6:∑n-3 PUFA and total FA in phospholipids was influenced by the interaction effect ($P < 0.05$; Table 3.8). However, this interactive effect did not follow the same patterns for all observations. The percent composition and absolute among of gamma-linolenic acid was higher in Shaver white from DMA group compared with Shaver white

from CON and ISA brown from every treatment. The percentage of composition of AA in Shaver white from DMA group and ISA brown from CON group was higher than ISA brown of DMA group. The absolute amount of ISA brown from DMA and FFF groups were lower than the rest of treatments. The percentage composition of adrenic and docosapentaenoic acids was higher in ISA brown from CON group was higher than DMA group in both strains. The absolute amount of docosapentaenoic acid was higher than the rest of the treatments. Supplemental DMA increased the DHA by 28.03% compared with CON in ISA brown, and the corresponding value was 31.19% for Shaver white. Feeding FFF increased DHA by 18.96% and 22.28% in ISA brown and Shaver white hens, respectively. Supplementation with DMA decreased the ratio of ∑n-6: ∑n-3 PUFA (relative percent composition) by 45.33% and 28.7% compared with CON in ISA brown and Shaver white, respectively. Feeding FFF reduced the corresponding values by 31.32% in ISA brown and 21.99% in Shaver white. The interactive effect of strain and diets on the absolute amount of ∑n-6: ∑n-3 PUFA was increased to 46.56% and 22.12% by supplementation with DMA in ISA brown and Shaver white, respectively. These values were 33.72% and 22.12% in ISA brown and Shaver white, respectively, in response to supplementation with FFF.

The FA profile in the yolk phospholipid was influenced by the strain ($P < 0.05$). Shaver white had a higher absolute and relative value of Eicosadienoic acid, gamma-linolenic acid, AA, EPA, docosapentaenoic acid and DHA in the FA content of their yolk phospholipid compared with ISA brown, which resulted in a higher relative percent composition of ∑n-3 PUFA and a lower ratio of ∑n-6: ∑n-3 PUFA ($P < 0.05$). Yolk phospholipid fraction of ISA brown contained a higher relative percent composition of palmitic, oleic, LA, and adrenic acid compared with Shaver white. Only the absolute amount of hexadecenoic acid was higher in ISA brown than Shaver white ($P = 0.024$).

Dietary addition with DMA increased the absolute amount of DHA ($P < 0.001$), reduced absolute amount of adrenic ($P < 0.05$), and reduced the relative percent composition of ALA compared to CON. The relative percent composition of EPA and the absolute amount of DHA were increased ($P = 0.001$) in response to the inclusion of FFF. Supplementation with DMA and FFF increased the absolute and relative amount of ∑n-3 PUFA ($P < 0.05$).

3.4.6. Fatty acid profile of yolk triglyceride fraction

There was an interaction between strain and diet in relative value (percent composition) of DHA ($P = 0.003$) and ∑n-6 PUFA ($P = 0.032$, Table 3.9) as well as absolute amount (mg) of LA, ALA, docosapentaenoic acid, ∑n-6 PUFA and total triglycerides ($P < 0.05$; Table 3.10). Supplementation with DMA increased the relative percent composition of DHA by 123.07% and 175.00% in ISA brown and Shaver white, respectively, compared with CON. The relative percent composition of DHA was increased by 30.77% and 141.66% in response to the inclusion of FFF in ISA brown and Shaver white, respectively. The ∑n-6 PUFA was higher in Shaver white from DMA group compared with Shaver white from FFF group. The absolute amount of LA in Shaver white from DMA group was higher than ISA brown from DMA and FFF groups. The absolute amount of ALA in ISA brown from FFF and Shaver white from CON was higher than the rest of the treatments. The absolute value of docosapentaenoic acid in Shaver white from DMA and FFF was higher than then the rest of treatments.

The total absolute amount of triglycerides was decreased by 16.19% and 28.46% in response to the inclusion of DMA in ISA brown and Shaver white, respectively, compared with CON. The corresponding values were decreased by 1.79% and 28.73% by supplementation with FFF in ISA brown and Shaver white, respectively, compared with CON.

There was higher relative percent composition of stearidonic acid (18:4n-3), eicosadienoic acid, gamma-linolenic acid, eicosatrienoic acid (20:3n-3), docosadienoic acid (22:2n-6), docosapentaenoic acid and DHA ($P < 0.05$) in the yolk triglyceride of Shaver white compared to ISA brown, which resulted in a lower ratio of ∑n-6: ∑n-3 PUFA ($P = 0.003$) compared to ISA brown. These observations were not significant when the comparison was made in terms of the absolute amount (mg) of stearidonic acid and eicosatrienoic acid ($P > 0.05$). Subsequently, there was more absolute amount of ∑n-6 PUFA in triglycerides of ISA brown compared with Shaver white ($P < 0.001$). Supplemental DMA decreased the relative percent composition of ALA and increased the relative percent composition of eicosadienoic acid ($P < 0.05$) compared with CON. Supplementation with DMA reduced the absolute amount of LA and ∑n-6 PUFA ($P < 0.05$). Triglycerides of FFF treatment had a less absolute amount of ∑n-6 PUFA ($P < 0.001$) compared with CON.

3.4.7. Calculated n-3 and n-6 PUFA desaturation and elongation

There was an interaction between strain and diet ($P = 0.009$) in the activity of n-6 PUFA desaturation and elongation (Table 3.11). Supplementation with DMA reduced n-6 PUFA desaturation and elongation activity in the phospholipids in ISA brown. Shaver white had a higher activity of desaturation and elongation of n-3 PUFA and n-6 PUFA in phospholipid fraction and n-3 PUFA in triglyceride fraction ($P < 0.05$) compared with ISA brown. Supplementation with DMA enhanced the activity of n-3 PUFA desaturation and elongation enzymes in phospholipid ($P < 0.001$) and triglyceride ($P = 0.029$) fractions.

3.4.8. Residual yolk weight over the embryonic period

The interaction between strain and diet did not affect the DM of RY, absolute and percentage, and RY: EW and RY: BW ratios during the embryonic period and DOH (Table 3.12). The RY

absolute DM in d-14 of the embryonic period ($P = 0.024$) and DM percentage ($P < 0.001$) in DOH were greater in ISA brown compared with Shaver white. The DM percentage in RY at d 19 was greater in FFF and DMA compared with CON ($P = 0.023$). Compared with CON, breeders fed either source of n-3 PUFA had greater absolute DM in RY ($P < 0.001$) and RY: BW ratio ($P = 0.002$) at hatch.

3.4.9. Apparent uptake of dry matter, organic matter, and mineral during the embryonic period

The percentage of consumed DM, OM, and total mineral relative to their total amount in the yolk are shown in Table 3.13. The interactive effect of strain and diet reduced the percentage of consumed DM by ISA brown embryos from breeders fed with either source of n-3 PUFA ($P = 0.026$), while it did not affect Shaver White ($P > 0.05$). The interaction between strain and diet did not affect the percentage of OM and mineral uptake ($P > 0.05$). The percentage of consumed OM was reduced in response to supplementation with either source of n-3 PUFA ($P = 0.002$). ISA brown embryos consumed 7.13% less proportion of yolk mineral compared with Shaver white embryos ($P = 0.004$). The percentage of consumed mineral content was reduced in embryos from breeders fed with DMA compared with CON ($P = 0.004$).

3.4.10. Apparent embryonic utilization of fatty acids of phospholipid and triglyceride fractions

There was an interaction between strain and diet in the apparent utilization of ALA, gamma-linolenic acid, AA, and total phospholipid FA ($P < 0.05$; Table 3.14). The interactive effect did not follow a pattern in either strain. The ISA brown embryos from DMA and FFF diets utilized less gamma-linolenic acid, AA, and less total phospholipid than Shaver white. Shaver white embryos utilized less ALA and more gamma-linolenic acid, while they maintained equal total phospholipid utilization as CON embryos. Shaver white embryos utilized more ALA, gamma-

linolenic acid, AA, EPA, docosapentaenoic acid, and DHA and subsequently utilized 11.11% more phospholipids than ISA brown embryos ($P < 0.05$). Diet influenced utilization of FA, among n-6 PUFA, adrenic acid utilization was decreased by feeding FFF and DMA relative to CON ($P < 0.05$). Among the n-3 PUFA group, the utilization of DHA was increased in DMA and FFF ($P = 0.001$). The utilization of total phospholipid was lower for embryos from breeders fed DMA and FFF compared with breeders fed CON ($P < 0.001$).

There was an interaction between strain and diet in the utilization of ALA, gamma-linolenic acid, and docosapentaenoic acid of triglyceride ($P < 0.05$; Table 3.15). Shaver white embryos from DMA and FFF utilized less ALA ($P < 0.001$), utilized more docosapentaenoic acid ($P = 0.015$) and tended to utilize more DHA ($P = 0.105$). Shaver white embryos utilized less triglyceride compared with ISA brown ($P = 0.020$). The main effect of diet was significant for the utilization of AA, where embryos from DMA utilized more AA compared to CON and FFF ($P < 0.001$).

3.4.11. Bodyweight, Liver Weight and Body Composition at Hatch

Day-old pullet BW was affected by the interaction between strain and diet with Shaver white pullets from breeders fed CON being 4.89 g heavier than Shaver white pullets from breeders fed FFF ($P = 0.006$; Table 3.16). The relative weight of the liver to the whole BW was decreased by the interaction between strain and diet in ISA brown hatchlings from DMA and FFF compared with CON ($P < 0.001$). The interaction between strain and diet reduced the percentage of lean ($P = 0.001$) and increased the percentage of fat in Shaver white hatchlings from FFF compared with CON ($P = 0.005$). The interaction between strain and diet did not affect BMC ($P > 0.05$). Shaver white pullets had more BMC than ISA brown ($P < 0.05$). Pullets from breeders fed CON had more BMC (%) compared with pullets from breeders fed the FFF diet ($P < 0.001$).

3.4.12. Fatty acid transporters and hepatic malondialdehyde

There was no interaction between strain and diets on intestinal and jejunum GPR 120 and FABP as well as hepatic MDA ($P > 0.05$; Table 3.17). The concentration of GPR 120 was higher in the jejunum of Shaver white embryos compared with ISA brown ($P < 0.05$). The effect of strain was also observed in hepatic MDA, where Shaver white had higher MDA (8.07%; $P = 0.005$) compared with ISA brown embryos. The concentration of GPR 120 on d 19 of the embryonic period and DOH was increased by dietary inclusion of DMA ($P > 0.05$). The concentration of FABP was neither affected by the main effect of strain, nor the dietary treatments ($P < 0.05$). Dietary treatment did not affect hepatic MDA ($P > 0.05$).

3.5. Discussion

Dietary FA profile confirmed that DMA was a rich source of DHA, whereas FFF was a rich source of ALA. Supplementation with DMA and FFF enriched diets with the other FA to a lesser extent compared with DHA and ALA. ISA brown breeders tended to lay heavier eggs compared with Shaver white, resulting in a higher absolute eggshell weight. The differences between white and brown hens were previously reported by Singh et al. (2009), where brown strains had heavier egg and eggshell weight compared with white strains. Similarly, Nain et al. (2012) observed no changes in egg and yolk weight in response to supplementation of up to 15% LinPRO (the same source as FFF in the current study) in the diet of 65 WOA Lohmann White Leghorn hens. It has been reported that production performance (egg production percentage, weight, and feed conversion ratio), eggshell and yolk percentage were not affected by supplementation of up to 3 % microalgae (the same source as DMA in the current study) in diets of 45 WOA Hy-Line W-36 laying for 36 WOA (Ao et al. 2015).

Chick embryos receive all their nutritional requirements during incubation from the shell, albumen, and yolk. Therefore, egg composition is vital for embryonic growth. Also, optimum embryo development reflects the quality, health, production, and welfare of chicks throughout the post-hatch life (Onbaşılar et al. 2017). Egg yolk is the primary source of minerals such as P, iron, zinc, while eggshell is the main compartment of Ca and magnesium for the embryo growth (Yair and Uni 2011). Other egg compartments (allantoic fluid, albumen, etc.) contain low amounts of minerals (Yair and Uni 2011). The breeder-feeding of n-3 PUFA in this study did not influence yolk composition, including organic matter, total mineral, Ca, and P.

Despite the dietary ALA being two times higher in the FFF diet than the DMA diet, the total ALA content was identical in the eggs. Nain et al. (2012) reported a 426% and 769% increase in ALA content (mg/egg) by supplementing 7.5% and 15% LinPRO, respectively. However, based on our confirmatory test for FA deposition in FFF eggs, the corresponding value (based on the percentage of FA to total fat) was 70% and 60% in ISA brown and Shaver white, respectively. In the current study, feeding DMA increased the DHA content by 30.8% and 83.3% in phospholipid and triglycerides, respectively. The corresponding values for FFF fed birds were 21.7% and 50.0% for phospholipid and triglycerides, respectively. This difference in DHA levels in the yolk might be because the concentration of DHA was 17-fold higher in the DMA than the FFF in the current study. Similar to our observations, Ao et al. (2015) observed a linear increase in DHA concentration without any changes in egg fat content when the supplemental levels of microalgae were increased up to 3 % in diets of 45 WOA Hy-line W-36 hens over 36 weeks. Conversely, supplementation with 7.5 % LinPRO for 18-d increased DHA (mg/egg) by 36.6% in 65 WOA Lohmann White Leghorn hens (Nain et al. 2012).

Based on the findings of the current study, Shaver white breeders deposited more n-3 and less n-6 FA in their eggs compared with ISA brown breeders. Similar to the current study, Ao et al. (2015) observed a linear decrease in LA and AA by increasing the inclusion level of microalgae up to 3 % in the diet of 45 WOA W-36 Hy-line over 36 weeks. In the current study, there was no detectable eicosadienoic acid, AA, and docosapentaenoic acid in the CON diet. However, the higher absolute amount of eicosadienoic acid, AA, and docosapentaenoic acid were incorporated into the phospholipid fraction in CON compared with DMA and FFF, indicating the *de novo* desaturation and elongation of eicosadienoic acid, AA and docosapentaenoic acid from the LA. Enzymes such as Δ-6 desaturase, elongase 5, and Δ -5 desaturase have been shown to be involved in the process of elongation and desaturation of LA and ALA (Cherian et al. 2007).

The inhibitory effect of n-3 PUFA on Δ-6 desaturase activity might be one reason for the reduction of eicosadienoic acid, AA, and docosapentaenoic acid in DMA and FFF treatments, along with a lower dietary composition of LA as the precursor in DMA and FFF than in CON. The 18-carbon precursors of n-6 PUFA and n-3 PUFA substrates compete for the same enzymatic machinery of desaturation and elongation to get converted into longer-chain PUFA (Watkins 1991). In the case of equal quantity, n-3 PUFA are metabolized as the preferred substrate to that of n-6 PUFA by these desaturase and elongase enzymes (Cherian 2008). Feeding the FFF diet resulted in a substantial increase in yolk triglyceride ALA content in Isa brown but a decrease in Shaver white which can be explained by the use of ALA in conversion to DHA by Shaver white breeders. The observation of the better ability of Shaver white in retaining n-3 PUFA was consistent with higher activity of n-3 and n-6 PUFA desaturation and elongation enzymes in either fraction in Shaver white than ISA brown breeders. The lower calculated activity of n-6 desaturation and elongation in phospholipid of ISA brown in response to supplementation with DMA, and

higher activity of n-3 PUFA desaturation and elongation in the phospholipid and triglycerides fractions in either strain, may partially explain the reduction of n-6 FA members. Similar to the findings of the current study, despite different dietary levels, a dose-dependent reduction in the activity of desaturation and elongation in n-6 PUFA was reported by the inclusion of 7.5% and 15% LinPRO (Nain et al. 2012) and up to 3% microalgae in hens (calculated based on the provided data; Ao et al. 2015). Regarding the higher potential of triglycerides in retaining long-chain n-3 PUFA than phospholipid, Neijat et al. (2017) observed that the triglyceride fraction had a better ability to deposit n-3 PUFA compared with the phospholipid faction even though these two major lipid classes have been shown to possess the ability to retain long-chain PUFA. The discrepancies in the results of various studies on the effect of dietary FA profile on egg FA profile may be attributed to different ages (Koppenol et al. 2014b), and supplemental level (Nain et al. 2012; Ao et al. 2015), and based on the findings of this study, the bird strain can also be an important factor.

Withdrawal of the RY into the abdominal part of the embryo prior to hatching supplies the demand for nutrients in the newly hatched chicks during the first few days of life (Şahan et al. 2014). About 60% of egg nutrients are absorbed throughout the embryonic period, and the remaining 30% play an essential role in supplying energy for the newly hatched birds over the first few post-hatch days (Nangsuay et al. 2011). However, the remaining RY can also host bacteria leading to yolk sac infection (Khan et al. 2004). In the current study, the results showed that hatchlings from breeders fed diets supplemented with n-3 PUFA had a heavier leftover of RY at hatch, illustrating the possible change in the embryonic uptake of nutrients in response to breeder-feeding n-3 PUFA.

The egg fat acts as a vital source of energy and essential FA, such as LA and ALA, during embryogenesis and early post-hatch (Noble and Cocchi 1990). There is a rapid uptake of different

lipid components by the embryo, which has been shown to start from the second week of incubation (Cherian 2015). Throughout the embryonic period, half of the lipid content in the yolk becomes incorporated into embryonic tissue (Speake et al. 1998). The required energy for embryo development has been shown to be supplied by β-oxidation of the remaining FA, mainly saturated FA (Yalçın et al. 2012). The preference of the embryo to consume FA that is AA and DHA rather than other C-16 and C-18 FA led to higher accumulation of PUFA in several tissues (Speake et al. 1998). The major PUFA in the avian central nervous system is DHA (Cherian 2008), which is incorporated into membrane phospholipids and has been shown to make up around 17 % of the total phospholipids content (Maldjian et al. 1996). In the current study, embryos from DMA and FFF diets utilized more DHA from the phospholipid fraction amounting to 8% and 7% phospholipid, respectively. The less utilization of ALA might be attributed to the lower yolk content and the use of ALA as the precursor for the synthesis of n-3 PUFA (Cherian and Sim 1993). Similarly, Cherian and Sim (1993) and Lin et al. (1991) observed that there was a preferred utilization of DHA in Leghorn white embryos. In the current study, the ability of embryo to consume n-6 PUFA was influenced by diet. The lower utilization of the n-6 PUFA family (mainly LA, AA and adrenic acids) by embryos from DMA and FFF might be attributed to lower yolk content (Koppenol et al. 2014a) and preference of the embryo in utilization of DHA.

Shaver white embryos utilized 11.1% more FA from phospholipids and 10.5% less FA from triglycerides compared with ISA brown. Genetic selection for various traits in different strains on poultry influence not only the post-hatch performance and variables but also pre-hatch characteristics (Everaert et al., 2018). The difference in embryonic metabolism and physiological events such as thyroid hormones, corticosterone, air cell pCO2 (Everaert et al., 2018), and body composition (protein and fat content; Pal et al., 2002) may play a role. In addition, the phenotypic

differences among strains arise from the first days of the embryonic period (Ho et al., 2011). The embryo relies on the egg nutrients for growth, which can provide the potential for maternal impact to influence the development of breed-specific phenotypes (Ho et al., 2011).

The utilization of total phospholipids and triglycerides was lower in embryos from DMA and FFF compared with CON. The retained DM by the embryos has been associated with the yolk FA profile (Peebles et al. 1999). This connection highlights the vital role of yolk FA content in modifying nutrient utilization by the embryo (Yalcin et al. 2008). In avian species, the yolk acts as the main reservoir for long-chain PUFA. The yolk sac membrane eventually develops elaborate folds and a microvillus structure (Yadgary et al. 2011). The yolk sac membrane plays an important role in the delivery of the required nutrients for embryo development (Romanoff 1960). The functional ability of the yolk sac membrane is dependent on numerous factors such as 1) morphological and structural changes (Romanoff 1960), 2) nutrient digestion, and transportation changes (Yadgary et al. 2013). Nutrient transportation in the yolk sac membrane can be conducted via transporters for amino acids or receptor-mediated endocytosis of lipoproteins (Yadgary et al. 2013). Breeder-feeding of conjugated LA reduced the ability of the embryo to take in lipid out of the YS, which resulted in higher embryo mortality due to lack of energy (Yadgary et al. 2013). There is scant information about the effect of nutrients, especially FA on yolk sac membrane characteristics (Leone et al. 2010).

The reduction in embryonic uptake of FA from phospholipid and triglyceride, which was observed in response to breeder-feeding of DMA and FFF in this study, can be attributed to the reduction in the relative weight of the liver in ISA brown pullets from DMA and FFF compared with those from CON. It has been reported that the weight of the liver is associated with the early mobilization of yolk lipids since the liver plays a vital role in the remodeling of RY lipids into

lipoprotein particles that are exported into circulation (Wolanski et al., 2007). The reason for the increase in fat, a decrease in lean and BMC percentages is still unknown. Dietary FA profile could modify the abdominal fat deposition in broilers, such that male broilers fed a diet supplemented with linseed, as a source of ALA, had lower abdominal fat compared with those fed diets supplemented with tallow or olive oil (Crespo and Esteve-Garcia 2001). Supplementation of AA in diet of mice before mating decreased body mass and lean mass, whereas it increased the fat mass in their offspring (Buckley et al. 2005). The role PUFA in changing body composition is assumed to be attributed to the ability of n-3 and n-6 FA in modifying the glucose tolerance, insulin sensitivity, signaling, and lipid metabolism (Buckley et al. 2005).

The absorption and utilization of nutrients by the embryo from various compartments of the egg depend on breeder age and the strain of the broiler or layer (Onbaşılar et al. 2017). However, our results showed that the profile of FA in yolk might affect the uptake of nutrients. In the current study, it is not clear that the change in body composition was originated either from the inability of the embryo to uptake nutrients from RY due to increase/change in FA profile in the egg, a change in programming the putative mechanisms underlying determining body development, subsequently a change in nutrients requirement or both ways.

Hatchability, chick quality, and production are influenced by the hen's age (Latour et al. 1998), egg storage, incubation conditions and strain (Şahan et al. 2014). Wolanski et al. (2007) and Şahan et al. (2014) reported that RY characterization and uptake of nutrients are dependent on the strains. Furthermore, commercial strains can be categorized based on their efficiency in utilizing nutrients from RY (Wolanski et al. 2007; Şahan et al. 2014). For instance, Şahan et al. (2014) calculated the range of RY weight in ten different commercial strains (anonymous strains) from 3.70 g to 5.50 g, accounting for approximately 10% to 14% of chick BW at DOH. Thus, the

effect of strain should be considered as an important criterion in maternal feeding strategies since different strains may have different rates of nutrients utilization from RY.

Jejunum and ileum are the main sites for FA digestion (Tancharoenrat et al. 2014) and absorption (Hurwitz et al. 1973) in poultry. The fatty acid-binding protein family is involved in transporting FA by expediting FA through extra and intracellular membranes (Chmurzyńska 2006). It has been reported that the level of dietary fat is correlated with the expression of FABP in poultry (Katongole and March 1980). In the current study, neither strain nor dietary treatment affected the concentration of FABP. Intestinal GPR 120 has been reported to be regulated by dietary long-chain FA (Mo et al. 2013), which is expressed in the intestine, adipose tissue (Koren et al. 2014), and macrophages (Mo et al. 2013). In the current study, the concentration of intestinal GPR 120 was dependent on strain and dietary treatments. The GPR 120 is a membrane-bound n-3 PUFA sensor (Talukdar et al. 2010). Activated GPR 120 has been shown to initiate a signaling pathway, which interferes with the activation of NF-KB as the transcription factor involved in the up-regulation of proteins associated with inflammatory cytokines. To our knowledge, there is no previous report on GPR 120 in poultry. In the current study, Shaver white embryos fed either DMA or FFF had a higher concentration of intestinal GPR 120 than CON. The expression of GPR 120 has been correlated with the dietary n-3 PUFA content (Talukdar et al. 2010), and in the current study, DMA and FFF had higher dietary n-3 PUFA than CON. When DMA and FFF were added in the diets, the MDA was not affected. MDA is a by-product of lipid peroxidation and acts as an indicator of oxidative stress. When the difference between strains was assessed, the Shaver white breeders had higher hepatic MDA compared with ISA brown breeders, illustrating that Shaver white embryos went through higher levels of oxidative stress during the embryonic period compared with ISA brown embryos.

3.6. Conclusions

Supplementation n-3 PUFA sources in breeder diets did not change the yolk DM, OM, or minerals. However, dietary supplementation with either source of n-3 PUFA modified the yolk FA profile toward more DHA either directly with DMA, or indirectly with FFF as the DHA must be derived from ALA. Supplementation with DMA was more effective in enriching eggs with long-chain n-3 PUFA, such as EPA and DHA. Although the total FA uptake from different lipid fractions was reduced, higher preferential utilization of DHA by embryos of either strain suggests a high requirement of DHA during the embryonic period. The uptake of nutrients, including DM, OM, and minerals, was reduced in response to n-3 PUFA sources, leading to a change in the composition of the body and an increase in the leftover of RY. The effect of supplementing sources of n-3 PUFA on BW and body composition showed the potential of a breeder-feeding strategy on hatchling quality. The quality of the newly hatched chick is a significant factor in determining its livability, growth performance, and health. Thus, the impact of feeding n-3 PUFA should be considered based on the quality of day-old pullets and the subsequent effect of productivity and health. Moreover, due to the difference among strains in deposition and synthesis of n-3 PUFA, the breeder line needs to be taken into consideration, among other factors in studies. Due to the vital role of n-3 PUFA, particularly DHA in metabolic and physiological mechanisms, more research is required to characterize subsequent post-hatch growth and development in egg-type chicks from breeders fed n-3 PUFA rich diets.

3.7. Table and figures

Table 3.1. Composition of the experimental diets for the ISA brown and Shaver white breeders from 26 to 31-week of age, as fed basis.

Item	CON	DMA	FFF
Ingredient, g/kg			
Corn grain	522.35	516.65	509.06
Soybean meal	233.41	234.67	224.70
Wheat	50.00	50.00	50.00
Corn gluten (60.4 % crude protein)	36.85	34.42	35.75
DMA[1]	-	10.00	-
FFF[2]	-	-	26.00
Soybean oil	20.40	17.48	17.49
Limestone coarse	37.93	37.92	37.92
Limestone fine	63.22	63.20	63.21
Monocalcium phosphate	15.03	14.94	14.95
Vitamin and trace mineral premix[3]	12.00	12.00	12.00
DL-Methionine, 99 %	1.59	1.62	1.62
L-Lysine HCl, 78 %	0.74	0.70	0.77
L-Threonine, 98 %	0.09	0.09	0.14
Salt	2.65	2.65	2.69
Sodium bicarbonate	2.07	1.98	2.03
Choline Chloride, 60 %	1.52	1.52	1.52
Ethoxyquin[4]	0.15	0.15	0.15

[1]Microalgae (*Aurantiochytrium limacinum)* fermentation product, as a source of docosahexaenoic acid (DHA), Alltech Canada, Guelph, Ontario, Canada.
[2] Co-extruded full fat flaxseed and pulse of field peas mixture (1:1 wt/wt), as a source of α-linolenic acid (ALA), O & T Farms Ltd., Saskatoon, Saskatchewan, Canada.
[3] Provided in kg of diet: vitamin A (retinol), 10,000 IU; vitamin D_3 (cholecalciferol), 3,000 IU; vitamin E, 100 mg; vitamin K_3 (menadione), 5.0 mg; vitamin B_1 (thiamin), 4.0 mg; vitamin B_2 (riboflavin), 10.0 mg; vitamin B_3 (niacin), 50.0 mg; vitamin B_5 (pantothenic acid), 20.0 mg; vitamin B_6 (pyridoxine), 4.0 mg; vitamin B_9 (folic acid), 2.0 mg; vitamin B_{12} (cyanocobalamin), 30.0 mg; biotin , 200 mcg; choline, 400.0 mg; Mg, 110 mg; Zn, 80 mg; Fe, 40.0 mg; Cu, 10.0 mg ; I, 1 mg, Se, 0.31 mg.
[4] SANTOQUIN®, Novus International Inc., Saint Charles, MO.

Table 3.2. Calculated nutritional composition of the experimental diets for the ISA brown and Shaver white breeders from 26 to 31-week of age, as fed basis.

Item	CON	DMA	FFF
Metabolizable energy, kcal/kg	2,800	2,800	2,800
Crude protein, %	18.20	18.20	18.20
Calcium, %	4.00	4.00	4.00
Analyzed calcium, %	4.05	4.18	4.12
Available phosphorus, %	0.38	0.38	0.38
Analyzed total phosphorus, %	0.70	0.78	0.72
SID[1] Lysine, %	0.80	0.80	0.80
SID Methionine, %	0.42	0.42	0.42
SID Methionine + cystine, %	0.65	0.65	0.65
SID Threonine, %	0.56	0.56	0.56
SID Tryptophan, %	0.18	0.18	0.18
Sodium, %	0.17	0.17	0.17
Chloride, %	0.25	0.25	0.25
∑n-3 FA, %	0.20	0.42	0.43
∑n-6 FA, %	2.47	2.31	2.37
∑n-6: ∑n-3 FA	12.35	5.50	5.51

[1] Standardized ileal digestible

Table 3.3. Analyzed fatty acid profiles of experimental diets, as fed basis.

Items	CON		DMA[1]		FFF[2]	
	% of total fatty acids	% of dry matter	% of total fatty acids	% of dry matter	% of total fatty acids	% of dry matter
14:0	0.00	0.00	0.57	0.03	0.10	0.00
14:1	0.00	0.00	0.00	0.00	0.00	0.00
15:0	0.00	0.00	0.16	0.01	0.04	0.00
15:1	0.00	0.00	0.00	0.00	0.00	0.00
16:0	10.35	0.47	16.37	0.74	10.19	0.44
16:1	0.00	0.00	0.11	0.01	0.15	0.01
17:0	0.00	0.00	0.15	0.01	0.11	0.00
17:1	0.00	0.00	0.00	0.00	0.04	0.00
18:0	1.47	0.07	1.85	0.08	1.93	0.08
18:1n-9	31.62	1.45	23.75	1.08	30.11	1.31
18:2n-6	47.63	2.18	45.68	2.07	41.36	1.80
20:0	0.00	0.00	0.79	0.04	0.34	0.01
18:3n-6	0.00	0.00	0.09	0.00	0.15	0.01
20:1	0.00	0.00	0.28	0.01	0.60	0.03
18:3n-3	6.30	0.29	5.52	0.25	9.26	0.40
21:0	0.00	0.00	0.00	0.00	0.03	0.00
20:2	0.00	0.00	0.00	0.00	0.04	0.00
22:0	0.00	0.00	0.12	0.01	0.17	0.01
20:3n-6	0.00	0.00	0.00	0.00	0.00	0.00
22:1n-9	0.00	0.00	0.00	0.00	0.02	0.00
20:3n-3	0.00	0.00	0.07	0.00	0.02	0.00
20:4n-6	0.00	0.00	0.05	0.00	0.06	0.00
23:0	0.00	0.00	0.00	0.00	0.03	0.00
22:2	0.00	0.00	0.00	0.00	0.00	0.00
24:0	0.00	0.00	0.07	0.00	0.11	0.00
20:5n-3	0.00	0.00	0.08	0.00	0.05	0.00
24:1	0.00	0.00	0.00	0.00	0.06	0.00
22:5	0.00	0.00	0.00	0.00	0.00	0.00
22:6n-3	0.00	0.00	3.80	0.17	0.25	0.01
∑n-3 FA	6.30	0.29	9.48	0.43	9.58	0.46
∑n-6 FA	47.63	2.18	45.89	2.08	45.60	1.81
∑n-6: ∑n-3	7.56		4.84		4.76	
Total	100.00	4.57	100.00	4.56	100.00	4.49

[1]Microalgae (*Aurantiochytrium limacinum)* fermentation product as a source of docosahexaenoic acid.
[2]Co-extruded full-fat flaxseed and pulse mixture (FFF, 1:1 wt/wt), as a source of α-linolenic acid.

Table 3.4. Effects of feeding sources of docosahexaenoic and α-linolenic acids on production performance in ISA brown and Shaver White breeders during offering the dietary treatments[1]

Items		Feed intake	Egg production	Egg weight	Egg mass
		g/bird/day	*Hen-day, %*	*g*	*g/bird/day*
Strain	Diet				
ISA brown	CON	116	95.11	54.82	52.16
ISA brown	DMA[2]	107	90.39	52.72	47.69
ISA brown	FFF[3]	115	94.18	55.88	52.67
Shaver white	CON	107	94.22	52.96	49.92
Shaver white	DMA	110	97.56	52.70	51.39
Shaver white	FFF	107	92.56	53.00	49.04
SEM		2.509	1.744	1.064	1.648
Main effect					
Strain					
ISA brown		113[a]	93.22	54.47	50.84
Shaver white		108[b]	94.78	52.88	50.12
SEM		1.449	1.001	0.614	0.952
Diet					
CON		111	94.67	53.89	51.04
DMA		109	93.97	52.71	49.54
FFF		111	93.37	54.44	50.56
SEM		1.774	1.233	0.752	1.166
Probabilities (*P*-value)					
Strain		0.039	0.297	0.092	0.602
Diet		0.499	0.762	0.289	0.623
Strain × Diet		0.061	0.051	0.421	0.101

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

[1] data are means of three replications (pen) of 27 hens and 4 roosters during 26 to 31 wk of age.

[2] Microalgae (*Aurantiochytrium limacinum)* fermentation product as a source of docosahexaenoic acid.

[3] Co-extruded full-fat flaxseed and pulse mixture (FFF, 1:1 wt/wt), as a source of α-linolenic acid.

Table 3.5. Effects of feeding sources of docosahexaenoic and α-linolenic acids on the egg components of Shaver white and ISA brown breeders[1]

Items		Albumen		Yolk		Eggshell	
		%	g	%	g	%	g
Strain	Diet						
ISA brown	CON[2]	63.45	36.88	26.44	15.39	10.11	5.88
ISA brown	DMA[3]	64.05	39.21	25.77	15.77	10.18	6.23
ISA brown	FFF[4]	64.04	37.08	25.65	14.84	10.30	5.97
Shaver white	CON	63.41	35.98	26.73	15.10	9.87	5.58
Shaver white	DMA	62.82	36.15	27.02	15.52	10.26	5.89
Shaver white	FFF	64.48	36.32	25.36	14.27	10.15	5.72
SEM		0.673	1.022	0.645	0.431	0.187	0.154
Main effect							
Strain							
ISA brown		63.85	37.72	25.95	15.33	10.20	6.03[a]
Shaver white		63.57	36.15	26.37	14.96	10.10	5.73[b]
SEM		0.476	0.565	0.381	0.356	0.108	0.089
Diet							
CON		63.43	36.43	26.58	15.24	9.99	5.73
DMA		63.43	37.68	26.39	15.65	10.22	6.06
FFF		64.26	36.70	25.51	14.56	10.23	5.84
SEM		0.476	0.700	0.472	0.316	0.132	0.109
Probabilities (*P*-value)							
Strain		0.616	0.053	0.441	0.297	0.509	0.023
Diet		0.373	0.411	0.220	0.052	0.352	0.099
Strain × Diet		0.462	0.428	0.503	0.924	0.680	0.963

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

[1] Data are means of 16 egg samples per each treatment.

[2] Control

[3] Microalgae (*Aurantiochytrium limacinum*) fermentation product as a source of docosahexaenoic acid.

[4] Co-extruded full-fat flaxseed and pulse mixture (FFF, 1:1 wt/wt), as a source of α-linolenic acid.

Table 3.6. Effects of feeding sources of docosahexaenoic and α-linolenic acids on egg yolk composition of ISA brown and Shaver white layer breeders [1]

Items		Dried yolk, g	Dry matter, %	Organic matter, % [4]	Mineral, %		
					Total	Ca	P
Strain	Diet						
ISA brown	CON	7.91	51.34	96.76	3.32	0.21	0.63
ISA brown	DMA[2]	7.92	50.16	96.72	3.39	0.21	0.64
ISA brown	FFF [3]	7.26	48.91	97.04	3.28	0.18	0.61
Shaver white	CON	7.23	47.95	96.89	3.34	0.20	0.61
Shaver white	DMA	7.43	47.70	96.58	3.38	0.20	0.63
Shaver white	FFF	7.07	49.58	96.76	3.33	0.20	0.61
SEM		0.220	0.547	0.116	0.033	0.006	0.010
Main effect							
Strain							
ISA brown		7.69[a]	50.14[a]	96.84	3.33	0.20	0.63
Shaver white		7.24[b]	48.44[b]	96.74	3.35	0.20	0.62
SEM		0.127	0.200	0.064	0.019	0.004	0.006
Diet							
CON		7.57	49.65	96.83	3.33	0.20	0.62
DMA		7.67	48.93	96.65	3.39	0.21	0.63
FFF		7.17	49.24	96.90	3.31	0.19	0.61
SEM		0.156	0.372	0.076	0.024	0.005	0.007
Probabilities (*P*-value)							
Strain		0.016	<0.001	0.292	0.503	0.389	0.121
Diet		0.064	0.192	0.085	0.066	0.157	0.130
Strain × Diet		0.533	0.061	0.202	0.661	0.067	0.660

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

[1] n = 10.

[2] Microalgae (*Aurantiochytrium limacinum*) fermentation product as a source of docosahexaenoic acid.

[3] Co-extruded full-fat flaxseed and pulse mixture (FFF, 1:1 wt/wt), as a source of α-linolenic acid.

[4] Organic matter was calculated by subtracting yolk ash content from the dry matter of yolk.

Table 3.7. Effects of feeding sources of docosahexaenoic and α-linolenic acids on the fatty acid profiles of the egg yolk phospholipid fraction (%) in ISA brown and Shaver white breeders.

	ISA brown			Shaver white				Strain			Diet				*P*-value		
Treatment	CON[1]	DMA[2]	FFF[3]	CON	DMA	FFF	SEM	ISA	Shaver	SEM	CON	DMA	FFF	SEM	Strain	Diet	Strain × Diet
16:0	27.28	28.4	28.42	27.02	26.97	27.36	0.488	28.00^{a}	27.12^{b}	0.281	27.13	27.69	27.86	0.345	0.021	0.343	0.930
18:0	16.74	14.91	16.45	12.82	18.2	17.07	1.379	16.03	16.03	0.839	14.78	16.56	16.76	0.975	0.821	0.143	0.120
18:2n-6	15.47	15.09	15.19	14.56	14.62	14.19	0.284	15.25^{a}	14.46^{b}	0.172	15.02	14.86	14.69	0.217	0.002	0.398	0.891
18:3n-6	0.08	0.03	0.05	0.12	0.14	0.12	0.012	0.05^{b}	0.13^{a}	0.007	0.10^{a}	0.09^{b}	0.09^{b}	0.009	<0.001	0.042	0.492
18:3n-3	0.28	0.38	0.36	0.49	0.22	0.27	0.052	0.34	0.33	0.032	0.39^{a}	0.30^{b}	0.32^{ab}	0.040	0.687	0.006	0.076
18:4n-3	0.04	0.05	0.04	0.03	0.03	0.02	0.007	0.04	0.03	0.004	0.04	0.04	0.03	0.005	0.064	0.897	0.205
20:2n-6	0.26	0.23	0.24	0.27	0.29	0.28	0.012	0.24^{b}	0.28^{a}	0.006	0.27	0.26	0.26	0.008	<0.001	0.072	0.519
20:3n-6	0.15^{b}	0.10^{b}	0.10^{b}	0.18^{b}	0.36^{a}	0.28^{ab}	0.018	0.12^{b}	0.27^{a}	0.011	0.17^{b}	0.23^{a}	0.19^{b}	0.013	<0.001	<0.001	0.001
20:4n-6	5.98^{a}	4.00^{b}	5.05^{ab}	5.87^{ab}	6.12^{a}	5.54^{ab}	0.287	5.01^{b}	5.84^{a}	0.167	5.93^{a}	5.06^{b}	5.30^{ab}	0.190	<0.001	0.001	0.017
20:3n-3	0.05	0.05	0.07	0.04	0.04	0.05	0.009	0.06	0.04	0.005	0.05	0.05	0.06	0.006	0.188	0.654	0.281
20:5n-3	0.04	0.08	0.09	0.11	0.10	0.10	0.009	0.07^{b}	0.10^{a}	0.005	0.08^{b}	0.09^{ab}	0.10^{a}	0.007	<0.001	0.001	0.082
22:2n-6	0.06	0.04	0.04	0.05	0.05	0.03	0.008	0.05	0.04	0.005	0.06	0.05	0.04	0.006	0.748	0.075	0.593
22:4n-6	0.50^{a}	0.37^{b}	0.47^{ab}	0.44^{ab}	0.32^{b}	0.38^{ab}	0.029	0.45^{a}	0.38^{b}	0.018	0.47^{a}	0.35^{b}	0.43^{ab}	0.021	0.009	0.023	0.007
22:5n-6	0.77^{a}	0.52^{b}	0.41^{c}	0.65^{ab}	0.42^{c}	0.64^{ab}	0.053	0.57	0.57	0.027	0.71^{a}	0.47^{b}	0.53^{ab}	0.035	0.890	<0.001	0.023
22:5n-3	0.06	0.07	0.03	0.09	0.18	0.12	0.019	0.05^{b}	0.13^{a}	0.012	0.08^{b}	0.13^{a}	0.08^{b}	0.014	<0.001	0.021	0.207
22:6n-3	4.80^{c}	6.67^{ab}	5.71^{b}	5.47^{bc}	7.95^{a}	7.70^{a}	0.323	5.73^{b}	7.04^{a}	0.203	5.14^{c}	7.31^{a}	6.71^{b}	0.230	<0.001	<0.001	<0.001
∑n-3	4.45	7.29	6.30	6.23	8.52	8.26	0.301	6.04^{b}	7.67^{a}	0.183	5.39^{b}	7.91^{a}	7.28^{a}	0.230	<0.001	<0.001	0.499
∑n-6	23.27^{a}	19.90^{c}	21.57^{b}	21.21^{bc}	22.53^{ab}	21.77^{ab}	0.386	21.58	21.84	0.233	22.24^{a}	21.22^{b}	21.67^{ab}	0.383	0.429	0.036	<0.001
∑n-6 :∑n-3	5.14^{a}	2.81^{cd}	3.53^{b}	3.41^{bc}	2.65^{d}	2.66^{d}	0.150	3.83^{a}	2.91^{b}	0.087	4.27^{a}	2.73^{b}	3.10^{b}	0.106	<0.001	<0.001	<0.001

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

[1]Control

[2] Microalgae (*Aurantiochytrium limacinum*) fermentation product as a source of docosahexaenoic acid.

[3] Co-extruded full-fat flaxseed and pulse mixture (FFF, 1:1 wt/wt), as a source of α-linolenic acid.

[4] Expressed as mg of phospholipid per gram of yolk dry matter.

Table 3.8. Effects of feeding sources of docosahexaenoic and α-linolenic acids on fatty acids profiles of phospholipid fraction (mg/ g of DM) of egg yolk in Shaver white and ISA brown layer breeders.

	ISA brown			Shaver white				Strain			Diet				*P*-Value		
Treatment	CON	DMA	FFF	CON	DMA	FFF	SEM	ISA	Shaver	SEM	CON	DMA	FFF	SEM	Strain	Diet	Strain × Diet
16:0	8.47	6.51	7.02	8.07	7.53	7.46	0.387	7.33	7.68	0.235	8.27^{a}	7.02^{b}	7.24^{b}	0.273	0.280	0.006	0.195
18:0	5.05	3.38	3.98	3.87	5.07	4.69	0.421	4.14	4.54	0.256	4.46	4.23	4.34	0.321	0.269	0.856	0.105
18:2n-6	4.68^{a}	3.45^{b}	3.74^{b}	4.28^{ab}	4.08^{ab}	3.82^{b}	0.184	3.96	4.06	0.106	4.48^{a}	3.76^{b}	3.78^{b}	0.141	0.508	<0.001	0.029
18:3n-6	0.03	0.01	0.01	0.04	0.04	0.03	0.003	0.02^{b}	0.04^{a}	0.002	0.03	0.02	0.02	0.003	<0.001	0.064	0.051
18:3n-3	0.08^{b}	0.09^{a}	0.09^{a}	0.15^{a}	0.06^{b}	0.07^{b}	0.013	0.09	0.09	0.008	0.11^{a}	0.07^{b}	0.08^{ab}	0.010	0.638	0.011	0.003
20:2n-6	0.08^{a}	0.05^{b}	0.06^{b}	0.08^{a}	0.08^{a}	0.08^{a}	0.003	0.06^{b}	0.08^{a}	0.002	0.08^{a}	0.07^{b}	0.07^{b}	0.002	<0.001	0.003	<0.001
20:3n-6	0.05^{b}	0.02^{c}	0.03^{c}	0.05^{b}	0.10^{a}	0.07^{b}	0.005	0.03^{b}	0.08^{a}	0.003	0.05^{b}	0.06^{a}	0.05^{b}	0.003	<0.001	0.031	<0.001
20:4n-6	1.81^{a}	0.79^{b}	1.24^{b}	1.51^{a}	1.71^{a}	1.58^{a}	0.131	1.28^{b}	1.60^{a}	0.076	1.66^{a}	1.25^{b}	1.41^{ab}	0.093	0.006	0.011	<0.001
20:5n-3	0.01^{b}	0.02^{b}	0.02^{b}	0.03^{a}	0.03^{a}	0.03^{a}	0.003	0.02^{b}	0.03^{a}	0.001	0.02	0.02	0.03	0.002	<0.001	0.338	0.006
22:2n-6	0.02	0.01	0.01	0.02	0.01	0.01	0.003	0.01	0.01	0.001	0.02	0.01	0.01	0.002	0.891	0.044	0.475
22:4n-6	0.15	0.09	0.12	0.13	0.09	0.10	0.009	0.12	0.11	0.005	0.14^{a}	0.09^{c}	0.11^{b}	0.006	0.106	<0.001	0.265
22:5n-6	0.23^{a}	0.12^{bc}	0.10.^{c}	0.13^{bc}	0.18^{b}	0.18^{b}	0.015	0.15	0.16	0.008	0.18^{a}	0.15^{ab}	0.14^{b}	0.009	0.414	0.009	<0.001
22:5n-3	0.02	0.02	0.01	0.03	0.05	0.03	0.005	0.01^{b}	0.04^{a}	0.003	0.02	0.03	0.02	0.004	<0.001	0.078	0.055
22:6n-3	1.23	1.51	1.42	1.62	2.22	2.07	0.107	1.39^{b}	1.97^{a}	0.065	1.43^{b}	1.87^{a}	1.74^{a}	0.082	<0.001	<0.001	0.286
∑n-3	1.37	1.65	1.56	1.84	2.38	2.22	0.120	1.53^{b}	2.15^{a}	0.063	1.60^{b}	2.02^{a}	1.89^{a}	0.073	<0.001	0.001	0.453
∑n-6	7.04^{a}	4.52c	5.30^{bc}	6.23^{ab}	6.28^{ab}	5.86^{b}	0.249	6.12^{a}	5.62^{b}	0.151	6.63^{a}	5.40^{b}	5.58^{b}	0.190	0.021	<0.001	<0.001
∑n-6:∑n-3	5.13^{a}	2.74^{cd}	3.40^{b}	3.39^{bc}	2.64^{d}	2.64^{d}	0.151	2.81^{b}	2.61^{a}	0.092	4.14^{a}	2.67^{b}	2.95^{b}	0.115	<0.001	<0.001	<0.001
Total	30.27^{a}	22.79^{c}	24.61^{bc}	29.50^{ab}	27.89^{ab}	26.92^{abc}	1.166	28.10^{a}	25.89^{b}	0.710	29.89^{a}	25.34^{b}	25.77^{b}	0.890	0.029	<0.001	0.053

Values with uncommon superscripts within each column are significantly different ($P < 0.05$).
Fatty acids with a quantity of less than 20 µg were not reported; however, they were included in the total amount.

Table 3.9. Effects of feeding sources of docosahexaenoic and α-linolenic acids on the fatty acid profiles of the egg yolk triglyceride fraction (%) in ISA brown and Shaver white breeders.

	ISA brown			Shaver white				Strain			Diet				*P*-value		
Treatment	CON[1]	DMA[2]	FFF[3]	CON	DMA	FFF	SEM	ISA	Shaver	SEM	CON	DMA	FFF	SEM	Strain	Diet	Strain × Diet
16:0	25.21	25.11	24.09	25.25	22.34	21.58	1.668	24.80	23.06	0.986	25.23	23.73	22.84	1.221	0.217	0.737	0.346
16:1n-9	2.84	2.80	2.80	1.68	1.84	1.96	0.310	2.81	1.83	0.183	2.26	2.32	2.38	0.226	<0.001	0.912	0.910
18:0	5.60	7.20	5.18	7.02	7.59	6.98	0.640	5.99	7.20	0.354	6.31	7.40	6.08	0.439	0.021	0.288	0.147
18:1n-9	45.81	42.15	46.30	45.78	43.78	41.54	1.531	44.75	43.70	0.846	45.80^{a}	42.97^{b}	43.92^{ab}	1.04	0.383	0.021	0.845
18:2n-6	16.46	17.07	17.38	15.16	17.8	16.02	0.676	16.97	16.33	0.399	15.81	17.44	16.70	0.495	0.261	0.443	0.070
18:3n-6	0.05	0.03	0.03	0.07	0.16	0.1	0.015	0.04^{b}	0.11^{a}	0.008	0.06b	0.10	0.07	0.010	<0.001	<0.001	0.102
18:3n-3	1.22	1.08	1.84	1.74	1.19	1.09	0.131	1.38	1.34	0.078	1.48^{a}	1.14^{b}	1.47^{a}	0.096	0.709	<0.001	0.930
18:4n-3	0.08	0.07	0.06	0.09	0.11	0.07	0.009	0.07^{b}	0.09^{a}	0.005	0.09^{a}	0.09^{a}	0.07^{b}	0.006	0.023	0.004	0.188
20:2n-6	0.13	0.15	0.12	0.16	0.26	0.22	0.022	0.13^{b}	0.21^{a}	0.012	0.15b	0.21a	0.17^{ab}	0.014	<0.001	0.020	0.055
20:3n-6	0.03	0.03	0.01	0.04	0.17	0.09	0.027	0.02^{b}	0.10^{a}	0.015	0.04b	0.10a	0.05^{ab}	0.018	<0.001	0.016	0.080
20:4n-6	0.21	0.79	0.27	0.25	0.35	0.28	0.246	0.42	0.29	0.136	0.23	0.57	0.28	0.168	0.497	0.449	0.363
20:3n-3	0.09	0.04	0.04	0.08	0.10	0.08	0.018	0.06^{b}	0.09^{a}	0.009	0.09^{a}	0.07^{ab}	0.06^{b}	0.010	0.047	0.022	0.624
20:5n-3	0.03	0.04	0.03	0.05	0.10	0.03	0.027	0.03	0.06	0.016	0.04	0.07	0.03	0.018	0.100	0.061	0.191
22:2n-6	0.06	0.04	0.03	0.06	0.15	0.20	0.044	0.04^{b}	0.14^{a}	0.024	0.06	0.10	0.12	0.030	0.009	0.233	0.301
22:4n-6	0.18	0.21	0.09	0.12	0.13	0.24	0.035	0.16	0.16	0.021	0.15	0.17	0.17	0.025	0.864	0.005	0.408
22:5n-6	0.12	0.14	0.06	0.09	0.19	0.42	0.119	0.11	0.23	0.066	0.11	0.17	0.24	0.081	0.174	0.211	0.530
22:5n-3	0.02	0.03	<0.01	0.02	0.14	0.18	0.028	0.03^{b}	0.11^{a}	0.016	0.02^{c}	0.09^{b}	0.18^{a}	0.020	<0.001	0.007	0.056
22:6n-3	0.13^{b}	0.29^{b}	0.17^{b}	0.24^{b}	0.66^{a}	0.58^{a}	0.066	0.20^{b}	0.49^{a}	0.037	0.19^{b}	0.48^{a}	0.38^{ab}	0.062	<0.001	0.002	0.003
∑n-3	1.56	2.44	2.15	2.03	2.41	2.32	0.391	2.05	2.25	0.221	1.80	2.42	2.23	0.267	0.522	0.249	0.803
∑n-6	17.24^{ab}	18.45^{ab}	17.98^{ab}	17.79^{ab}	15.95^{b}	19.37^{a}	0.737	17.89	17.70	0.435	17.52	17.20	18.68	0.539	0.761	0.140	0.032
∑n-6:∑n-3	11.07	9.97	8.86	9.05	6.65	8.71	0.741	9.97^{a}	8.13^{b}	0.419	10.06	8.31	8.78	0.507	0.003	0.053	0.099
Total [4]	29.57^{a}	24.78^{b}	29.04^{ab}	32.22^{a}	23.05^{b}	22.96^{b}	1.18	27.80	26.08	0.649	30.89^{a}	23.92^{b}	26.00^{ab}	0.831	0.069	<0.001	0.002

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

[1] Control [2] Microalgae (*Aurantiochytrium limacinum*) fermentation product as a source of docosahexaenoic acid. [3] Co-extruded full-fat flaxseed and pulse mixture (FFF, 1:1 wt/wt), as a source of α-linolenic acid. [4] Expressed as mg of triglyceride per gram of yolk dry matter.

Table 3.10. Effects of feeding sources of docosahexaenoic and α-linolenic acids on fatty acids profiles of triglyceride fraction (mg/ g of DM) of egg yolk in Shaver white and ISA brown layer breeders.

Treatment	ISA brown			Shaver-White				Strain			Diet				*P*-Value		
	CON	DMA	FFF	CON	DMA	FFF	SEM	ISA	Shaver	SEM	CON	DMA	FFF	SEM	Strain	Diet	Strain × Diet
16:0	7.49a	6.20^{ab}	7.01^{ab}	8.16^{a}	5.86^{b}	5.50^{b}	0.345	6.90	6.51	0.287	7.82^{a}	6.04^{b}	6.25^{b}	0.355	0.175	<0.001	0.010
18:0	1.66	1.72	1.50	2.28	1.74	1.60	0.138	1.63^{b}	1.87^{a}	0.081	1.97^{a}	1.73^{ab}	1.55b	0.101	0.038	0.018	0.081
18:2n-6	4.84	4.24	5.03	4.83	4.06	3.65	0.166	4.70^{a}	4.17^{b}	0.09	4.83^{a}	4.15^{b}	4.34^{ab}	0.114	<0.001	<0.001	0.101
18:3n-6	0.02^{ab}	0.01^{b}	0.01^{b}	0.02^{ab}	0.04^{a}	0.02^{ab}	0.004	0.01^{b}	0.03^{a}	0.002	0.02	0.02	0.02	0.003	<0.001	0.122	0.013
18:3n-3	0.36^{b}	0.28^{b}	0.53^{a}	0.56^{a}	0.27^{b}	0.25^{b}	0.036	0.39	0.36	0.022	0.46^{a}	0.27^{b}	0.39^{ab}	0.027	0.302	<0.001	<0.001
18:4n-3	0.02	0.02	0.02	0.03	0.03	0.02	0.002	0.02	0.02	0.001	0.02	0.02	0.02	0.001	0.106	0.086	0.503
20:2n-6	0.04	0.04	0.03	0.05	0.06	0.05	0.005	0.04^{b}	0.05^{a}	0.003	0.05	0.05	0.04	0.003	<0.001	0.349	0.507
20:4n-6	0.06	0.17	0.08	0.08	0.08	0.06	0.049	0.10	0.07	0.027	0.07	0.12	0.07	0.033	0.441	0.413	0.512
20:3n-3	0.03	0.01	0.01	0.03	0.02	0.02	0.005	0.02	0.02	0.003	0.03	0.02	0.02	0.003	0.283	0.052	0.247
22:2n-6	0.02	0.01	0.01	0.02	0.03	0.04	0.002	0.01^{b}	0.02^{a}	0.001	0.02	0.02	0.01	0.001	0.005	0.736	0.253
22:4n-6	0.05	0.05	0.03	0.04	0.06	0.10	0.021	0.04	0.06	0.012	0.05	0.06	0.07	0.017	0.241	0.734	0.172
22:5n-6	0.03	0.03	0.02	0.09	0.04	0.03	0.025	0.03	0.03	0.018	0.06	0.04	0.02	0.014	0.194	0.663	0.290
22:5n-3	<0.01^{b}	0.01^{b}	0.00^{b}	0.01^{b}	0.03^{a}	0.04^{a}	0.007	<0.01^{b}	0.03^{a}	0.004	0.01^{b}	0.02^{a}	0.02^{a}	0.005	<0.001	0.036	0.024
22:6n-3	0.04	0.07	0.05	0.08	0.15	0.13	0.081	0.05^{b}	0.12^{a}	0.008	0.06^{b}	0.11^{a}	0.09^{ab}	0.010	<0.001	0.002	0.266
∑n-3	0.46	0.57	0.62	0.70	0.53	0.46	0.081	0.55	0.56	0.045	0.58	0.55	0.54	0.053	0.866	0.857	0.067
∑n-6	5.06^{ab}	4.55^{bc}	5.21^{a}	5.08^{ab}	4.42^{c}	4.04^{c}	0.141	4.94^{a}	4.51^{b}	0.083	5.07^{a}	4.48^{b}	4.62^{b}	0.107	<0.001	<0.001	<0.001
∑n-6: ∑n-3	11.07	9.97	8.86	8.34	8.70	9.04	0.986	9.97	8.70	0.583	9.71	9.33	8.95	0.698	0.132	0.764	0.371
Total	29.57^{a}	24.78^{b}	29.04^{ab}	32.22^{a}	23.05^{b}	22.96^{b}	1.18	27.80	26.08	0.649	30.89^{a}	23.92^{b}	26.0^{ab}	0.831	0.069	<0.001	0.002

Values with uncommon superscripts within each column are significantly different ($P < 0.05$).
Fatty acids with a quantity of less than 20 µg were not reported. However, they were included in the total amount.

Table 3.11. Effects of feeding sources of docosahexaenoic and α-linolenic acids on calculated conversion of fatty acids in Shaver white and ISA brown breeders.[1]

Items		n-6 PUFA conversion[2]		n-3 PUFA conversion[3]	
		PH[4]	TG[5]	PH	TG
Strain	Diet				
ISA brown	CON[6]	0.387[a]	0.014	0.126	0.020
ISA brown	DMA[7]	0.242[b]	0.014	0.286	0.033
ISA brown	FFF[8]	0.334[ab]	0.015	0.219	0.015
Shaver white	CON	0.361[ab]	0.016	0.237	0.023
Shaver white	DMA	0.419[a]	0.020	0.461	0.039
Shaver white	FFF	0.412[a]	0.017	0.394	0.026
SEM		0.031	0.001	0.031	0.006
Main effect					
Strain					
ISA brown		0.321[b]	0.015[b]	0.211[b]	0.023
Shaver white		0.397[a]	0.018[a]	0.364[a]	0.029
SEM		0.018	0.0007	0.019	0.003
Diet					
CON		0.374	0.015	0.181[b]	0.022[b]
DMA		0.331	0.017	0.373[a]	0.036[a]
FFF		0.373	0.016	0.307[ab]	0.021[b]
SEM		0.023	0.0009	0.025	0.004
Probabilities (*P*-value)					
Strain		0.006	0.004	<0.001	0.187
Diet		0.300	0.419	<0.001	0.029
Strain × Diet		0.009	0.135	0.514	0.850

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

[1] data are means of 16 egg samples per each treatment.

[2] n-6 PUFA conversion pathway enzyme activity (Δ6-desaturase, elongase, and Δ5-desaturase) was calculated as ratio of 20:4 n-6 to 18:2 n-6.

[3] n-3 polyunsaturated fatty acid conversion pathway enzyme activity (Δ6-desaturase, elongase, and Δ5-desaturase) was calculated as ratio of 20:5 n-3 to 18:3 n-3.

[4] Phospholipid.

[5] Triglycerides.

[6] Control

[7] Microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid.

[8] Co-extruded full fat flaxseed and pulse mixture (FFF, 1:1 wt/wt), as a source of α-linolenic acid.

Table 3.12. Effects of feeding sources of docosahexaenoic and α-linolenic acids on residual yolk dried weight during the embryonic period in ISA brown and Shaver white.[1]

Items		d-14 of embryonic period			d-19 of embryonic period			Day of hatch		
		DM, g	DM, %	RY: EW[4]	DM, g	DM, %	RY: EW[5]	DM, g	DM, %	RY: BW[6]
Strain	Diet									
ISA brown	CON	2.76	47.76	11.41	5.03	48.92	21.14	1.05	53.03	6.67
ISA brown	DMA[2]	2.91	47.23	11.09	4.30	49.49	16.80	1.93	54.32	8.89
ISA brown	FFF[3]	3.21	47.06	12.67	5.21	49.77	20.07	1.99	53.56	9.02
Shaver white	CON	2.25	44.56	11.54	4.66	46.54	18.86	1.27	51.82	7.63
Shaver white	DMA	2.50	46.80	10.13	4.62	48.20	19.45	1.63	51.91	8.87
Shaver white	FFF	2.49	46.25	10.79	5.06	50.74	19.92	1.71	51.06	8.73
SEM		0.290	1.258	1.110	0.461	1.02	1.454	0.127	0.600	0.530
Main effect										
Strain										
ISA brown		2.96[a]	47.35	11.72	4.85	49.40	19.33	1.65	53.64[a]	8.19
Shaver white		2.41[b]	45.87	10.82	4.78	48.49	19.41	1.54	51.59[b]	8.41
SEM		0.164	0.711	0.628	0.243	0.536	0.840	0.073	0.346	0.306
Diet										
CON		2.50	46.16	11.47	4.84	47.73[b]	20.00	1.16[b]	52.42	7.15[b]
DMA		2.70	47.02	10.61	4.46	48.84[ab]	18.13	1.78[a]	53.11	8.88[a]
FFF		2.85	46.66	11.73	5.14	50.26[a]	19.99	1.85[a]	52.31	8.88[a]
SEM		0.199	0.861	0.760	0.305	0.673	1.028	0.090	0.424	0.374
Probabilities (*P*-value)										
Strain		0.024	0.148	0.317	0.847	0.229	0.949	0.273	<0.001	0.620
Diet		0.489	0.787	0.556	0.279	0.023	0.341	<0.001	0.353	0.002
Strain × Diet		0.853	0.487	0.658	0.711	0.167	0.247	0.082	0.493	0.471

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

[1] data are means of 8 samples per each treatment for d 14 and 19 of embryonic period and 10 samples on the day of hatch.

[2] Microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid. [3] Co-extruded full fat flaxseed and pulse mixture (FFF, 1:1 wt/wt), as a source of α-linolenic acid. [4] Percentage of residual yolk to egg weight at d-14 of embryonic period (g:g).[5] Percentage of residual yolk to egg weight at d-19 of embryonic period (g:g). [6] Percentage of residual yolk to body weight at hatch (g:g).

Table 3.13. Effects of feeding sources of docosahexaenoic and α-linolenic acids on apparent uptake of dry matter, mineral and organic material by ISA brown and Shaver white embryos.[1]

Items		Dry matter %[4]	Organic material %[5]	Mineral %[6]
Strain	Diet			
ISA brown	CON	86.55[a]	87.19	73.25
ISA brown	DMA[2]	73.59[bc]	74.02	65.76
ISA brown	FFF[3]	71.20[c]	72.20	67.83
Shaver white	CON	81.27[ab]	77.53	80.80
Shaver white	DMA	76.49[bc]	78.74	70.95
Shaver white	FFF	78.23[abc]	80.69	76.50
SEM		2.221	2.383	2.837
Main effect				
Strain				
ISA brown		77.11	77.85	68.95[b]
Shaver white		78.66	78.89	76.08[a]
SEM		1.282	1.317	1.638
Diet				
CON		83.91[a]	82.50[a]	77.02[a]
DMA		75.04[b]	76.24[b]	68.35[b]
FFF		74.72[b]	76.46[b]	72.17[ab]
SEM		1.570	1.632	2.006
Probabilities (*P-value*)				
Strain		0.398	0.258	0.004
Diet		<0.001	0.002	0.015
Strain × Diet		0.026	0.105	0.824

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

[1] n = 10.

[2] Microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid. [3] Co-extruded full fat flaxseed and pulse mixture (FFF, 1:1 wt/wt), as a source of α-linolenic acid. [4] Percentage of consumed dry matter during embryonic period to the total dry matter of yolk. [5] Percentage of consumed organic matter during embryonic period to the total organic matter of yolk. [6] Percentage of consumed ash during embryonic period to the total ash of yolk.

Table 3.14. Effects of feeding sources of docosahexaenoic and α-linolenic acids on the apparent utilization of phospholipid throughout the embryonic period in Shaver white and ISA brown breeders. mg/embryo

Treatment		18:3n-6	18:3n-3	20:3n-6	20:4n-6	20:5n-3	22:4n-6	22:5n-3	22:6n-3	Total[1]
Strain	Diet									
ISA brown	CON[2]	0.17^{ab}	0.62^{a}	0.34^{b}	13.54^{a}	0.08	1.05	0.11	9.49	226.38^{a}
ISA brown	DMA[3]	0.03^{c}	0.63^{a}	0.15^{c}	4.81^{c}	0.13	0.45	0.08	11.51	151.13^{b}
ISA brown	FFF[4]	0.06^{bc}	0.50^{b}	0.17^{c}	7.67^{bc}	0.13	0.67	0.02	9.87	151.57^{b}
Shaver white	CON	0.25^{a}	0.99^{a}	0.36^{b}	10.67^{b}	0.24	0.88	0.17	11.37	206.55^{a}
Shaver white	DMA	0.27^{a}	0.39^{b}	0.68^{a}	11.74^{ab}	0.20	0.42	0.28	15.86	193.79^{a}
Shaver white	FFF	0.20^{a}	0.47^{b}	0.53^{a}	11.21^{ab}	0.21	0.61	0.23	15.22	187.52^{a}
SEM		0.018	0.105	0.041	0.999	0.020	0.066	0.039	0.809	9.178
Main effect										
Strain										
ISA brown		0.09^{b}	0.58^{b}	0.22^{b}	8.67^{b}	0.11^{b}	0.72	0.07^{b}	10.29^{b}	176.36^{b}
Shaver white		0.24^{a}	0.62^{a}	0.52^{a}	11.18^{a}	0.21^{a}	0.63	0.23^{a}	14.14^{a}	195.96^{a}
SEM		0.004	0.064	0.024	0.608	0.012	0.040	0.024	0.493	5.585
Diet										
CON		0.21^{a}	0.81^{a}	0.35^{b}	12.10^{a}	0.16	0.97^{a}	0.14	10.43^{b}	216.46^{a}
DMA		0.15^{b}	0.51^{b}	0.41^{a}	8.28^{b}	0.16	0.44^{c}	0.18	13.69^{a}	172.46^{b}
FFF		0.13^{b}	0.49^{b}	0.35^{b}	9.44^{b}	0.17	0.63^{b}	0.13	12.55^{a}	169.55^{b}
SEM		0.005	0.078	0.031	0.764	0.015	0.050	0.030	0.573	7.010
Probabilities (*P*-value)										
Strain		<0.001	0.021	<0.001	0.005	<0.001	0.102	<0.001	<0.001	0.020
Diet		0.033	<0.001	0.002	0.002	0.950	<0.001	0.356	0.001	<0.001
Strain × Diet		0.032	<0.001	<0.001	<0.001	0.065	0.533	0.130	0.117	0.003

Values with uncommon superscripts within each column are significantly different ($P < 0.05$).
[1] Including all phospholipid of all the measured fatty acids.
[2] Control [3] Microalgae (*Aurantiochytrium limacinum*) fermentation product as a source of docosahexaenoic acid. [4] Co-extruded full-fat flaxseed and pulse mixture (FFF, 1:1 wt/wt), as a source of α-linolenic acid.

Table 3.15. Effects of feeding sources of docosahexaenoic and α-linolenic acids on the apparent utilization of triglyceride throughout the embryonic period in Shaver white and ISA brown breeders. mg\embryo

Treatment		18:3n-6	18:3n-3	20:3n-6	20:4n-6	20:5n-3	22:4n-6	22:5n-3	22:6n-3	Total[1]
Strain	Diet									
ISA brown	CON[2]	0.11[b]	2.69[ab]	0.07[b]	0.38	0.06	0.34	0.02[b]	0.25	217.67
ISA brown	DMA[3]	0.03[b]	1.90[b]	0.04[b]	1.16	0.07	0.30	0.04[b]	1.78	164.22
ISA brown	FFF[4]	0.02[b]	3.52[a]	0.00[b]	0.22	0.06	0.12	0.00[b]	0.23	185.70
Shaver white	CON	0.14[b]	3.71[a]	0.07[b]	0.45	0.10	0.24	0.05[b]	0.50	215.73
Shaver white	DMA	0.25[a]	1.83[b]	0.26[a]	0.43	0.17	0.26	0.21[a]	0.97	149.84
Shaver white	FFF	0.13[b]	1.59[b]	0.12[ab]	0.31	0.05	0.68	0.29[a]	0.87	147.93
SEM		0.027	0.289	0.039	0.086	0.042	0.184	0.044	0.661	8.033
Main effect										
Strain										
ISA brown		0.06[b]	2.70	0.03[b]	0.59	0.06	0.25	0.02[b]	0.75	189.20[a]
Shaver white		0.18[a]	2.38	0.15[a]	0.40	0.11	0.40	0.18[a]	0.78	171.17[b]
SEM		0.017	0.160	0.023	0.022	0.025	0.102	0.026	0.374	3.099
Diet										
CON		0.13	3.20[a]	0.07	0.41[b]	0.08	0.29	0.03	0.37	216.70[a]
DMA		0.14	1.86[b]	0.15	0.80[a]	0.12	0.28	0.13	1.37	157.03[b]
FFF		0.08	2.56[a]	0.05	0.27[b]	0.05	0.41	0.14	0.55	166.81[b]
SEM		0.022	0.191	0.029	0.030	0.032	0.130	0.033	0.486	4.298
Probabilities (*P*-value)										
Strain		<0.001	0.139	0.001	0.558	0.167	0.301	<0.001	0.960	0.020
Diet		0.129	<0.001	0.060	0.003	0.233	0.720	0.053	0.264	<0.001
Strain × Diet		0.007	<0.001	0.028	0.065	0.506	0.123	0.015	0.505	0.289

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

[1] Including all triglyceride of all the measured fatty acids.

[2] Control [3] Microalgae (*Aurantiochytrium limacinum*) fermentation product as a source of docosahexaenoic acid [4] Co-extruded full-fat flaxseed and pulse mixture (FFF, 1:1 wt/wt), as a source of α-linolenic acid.

Table 3.16. Effects of feeding sources of docosahexaenoic and α-linolenic acids on body weight, liver and body composition of day-old pullets of ISA brown and Shaver white.[1]

Items		BW, g	Liver %	Body Composition, %		
				Lean[2]	Fat[2]	BMC[2]
Strain	Diet					
ISA brown	CON	33.22^{ab}	0.17^{a}	77.53^{ab}	21.39^{ab}	1.08
ISA brown	DMA[3]	33.18^{ab}	0.12^{c}	75.31^{b}	23.71^{a}	0.98
ISA brown	FFF[4]	35.44^{ab}	0.12^{c}	77.41^{ab}	21.81^{a}	0.78
Shaver white	CON	37.16^{a}	0.13^{bc}	79.76^{a}	18.97^{b}	1.27
Shaver white	DMA	34.43^{ab}	0.14^{abc}	77.55^{ab}	21.42^{ab}	1.03
Shaver white	FFF	32.27^{b}	0.16^{ab}	75.20^{b}	23.86^{a}	0.94
SEM		1.050	0.008	0.613	0.595	0.059
Main effect						
Strain						
ISA brown		33.95	0.14	76.75	22.30	0.82^{b}
Shaver white		34.62	0.14	77.50	21.42	1.09^{a}
SEM		0.631	0.004	0.368	0.357	0.032
Diet						
CON		35.19	0.15^{a}	80.43^{a}	20.18^{b}	1.17^{a}
DMA		33.81	0.13^{b}	78.17^{b}	22.56^{ab}	1.01^{ab}
FFF		33.85	0.14^{ab}	76.87^{b}	22.83^{a}	0.86^{b}
SEM		0.788	0.005	0.433	0.421	0.053
Probabilities (*P*-value)						
Strain		0.447	0.428	0.146	0.080	0.005
Diet		0.362	0.022	<0.001	<0.001	<0.001
Strain × Diet		0.006	<0.001	0.001	0.005	0.397

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

[1] data are means of 10 samples per each treatment.

[2] Bone mineral content

[3]Microalgae (*Aurantiochytrium limacinum*) fermentation product as a source of docosahexaenoic acid.[4] Co-extruded full-fat flaxseed and pulse mixture (FFF, 1:1 wt/wt), as a source of α-linolenic acid.

Table 3.17. Effects of feeding sources of docosahexaenoic and α-linolenic acids on ileum G-protein-coupled receptor 120 (GPR 120), intestinal fatty acid-binding protein (FABP) and hepatic malondialdehyde in 19-d of the embryonic period (E19) and day of hatch (DOH) of Shaver white and ISA brown breeders.[1]

Items		E19				DOH					
		Intestinal GPR 120[2]		Intestinal FABP[3]		Jejunum GPR 120		Jejunum FABP		Hepatic MDA[4]	
		ng/mg	ng/mg protein	ng/mg	ng/mg protein	ng/mg	ng/mg protein	ng/mg	ng/mg protein	ng/mg	ng/mg protein
Strain	Diet										
ISA brown	CON[5]	24.09	3.52	0.28	0.04	20.56	1.82	1.28	0.14	222	4.45
ISA brown	DMA[6]	69.42	6.49	0.30	0.03	34.42	3.06	1.28	0.14	229	4.24
ISA brown	FFF[7]	50.81	4.90	0.28	0.03	19.49	1.73	1.28	0.14	228	4.70
Shaver white	CON	31.24	3.15	0.32	0.03	23.82	2.12	1.29	0.15	224	4.71
Shaver white	DMA	86.37	8.70	0.34	0.04	34.36	3.05	1.26	0.12	233	4.94
Shaver white	FFF	70.83	7.31	0.34	0.04	33.28	2.96	1.27	0.13	228	4.90
SEM		13.394	1.540	0.043	0.005	3.413	0.303	0.012	0.001	4.900	0.162
Main effect											
Strain											
ISA brown		48.10	4.97	0.28	0.03	24.82^{b}	2.20^{b}	1.28	0.14	226	4.46^{b}
Shaver white		62.82	6.38	0.33	0.03	30.48^{a}	2.71^{a}	1.27	0.13	228	4.85^{a}
SEM		7.732	0.889	0.025	0.003	1.970	0.175	0.007	0.0006	2.829	0.094
Diet											
CON		27.66^{b}	3.33^{b}	0.30	0.03	22.19^{b}	1.97^{b}	1.29	0.14	223	4.58
DMA		77.90^{a}	7.59^{a}	0.32	0.03	34.39^{a}	3.05^{a}	1.27	0.13	231	4.59
FFF		60.82^{a}	6.10^{ab}	0.31	0.03	26.39^{ab}	2.34^{ab}	1.28	0.13	228	4.80
SEM		9.471	1.089	0.030	0.004	2.413	0.214	0.009	0.0008	3.465	0.115
Probabilities (*P*-value)											
Strain		0.186	0.267	0.160	0.288	0.047	0.046	0.360	0.417	0.665	0.005
Diet		0.002	0.027	0.913	0.859	0.003	0.002	0.485	0.486	0.121	0.331
Strain × Diet		0.882	0.604	0.943	0.574	0.116	0.110	0.471	0.452	0.951	0.251

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

[1] Data are means of 10 samples per each treatment. [2] The sensitivity of the assay was 0.1 ng/mL[3] the sensitivity of the assay was 0.1 ng/mL [4] malondialdehyde [5] Control [6] Microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid.[7] Co-extruded full fat flaxseed and pulse mixture (FFF, 1:1 wt/wt), as a source of α-linolenic acid.

Chapter 4. Impact of feeding sources of docosahexaenoic and α-linolenic acid to ISA brown and Shaver white layer breeders and their offspring on skeletal attributes during the rearing phase[1]

4.1. Abstract

In chapter 3, embryos from breeders fed n-3 PUFA had preferential consumption of DHA but lower uptake of DM, OM, and minerals during the embryonic period. The intent of this chapter was to investigate bone development. During rearing, pullets from breeders fed the control diet were fed control or supplemented diets, and pullets from breeders fed supplemented diets either continued with respective n-3 PUFA diets or the control diet. A total of 60 pullets per post-hatch diet were reared in cages (12 pullets/cage, n=5) with free access to feed and water; bled at 6, 12, and 18 WOA for bone turnover markers; and necropsied at 18 WOA for tibia and femur bone samples. Day-old pullets from breeders not fed n-3 PUFA had greater body mineral content ($P < 0.001$). There was an interaction between strain and breeder diet on tibia breaking strength (**TBS**) and cortical ash content in tibia and femur at 18 WOA such that, diet responses were only observed in Shaver white pullets ($P < 0.05$). In this context, TBS and cortical ash content in tibia and femur were greater in pullets from breeders fed DMA compared to those from breeders fed CON. In conclusion, the strain effects were strong on skeletal attributes. Breeder feeding of DMA was effective in supporting the tibia strength and cortical bone development in Shaver white pullets, while pullet feeding of either source did not have any effect. Further investigations are warranted to establish the subsequent impact of these strategies on skeletal properties during the laying cycle.

[1] Published in part: Akbari Moghaddam Kakhki, R., Price, J. Moats, Bédécarrats, G. Y., Karrow, N. A. and E.G. Kiarie. 2020. Impact of feeding microalgae (*Aurantiochytrium limacinum*) and co-extruded mixture of full-fat flaxseed as sources of n-3 fatty acids to ISA brown and Shaver white breeders and progeny on pullet skeletal attributes at hatch through to 18 weeks of age. Poult. Sci.99: 2087-2099 https://doi.org/10.1016/j.psj.2019.12.016.

4.2. Introduction

In chapter 3, feeding breeders with sources of n-3 PUFA increased the concentration of n-3 PUFA, while DM, OM, and mineral content of eggs were not affected. Embryos of both n-3 PUFA treatments showed different patterns of nutrients uptake compared to embryos from the CON group. Uptake of DM, OM, minerals, and total FA was lower in DMA and FFF groups, while the uptake of DHA was increased compared to the CON group. The change in the uptake of nutrients can fortify the possible role of n-3 PUFA on skeletal growth during embryonic and pullet phases. Specific embryonic environmental conditions have been shown to provoke the adaptive responses of the fetus in mammals (Mennitti et al., 2015). These adaptive responses in the fetus may alter gene expression and have a permanent effect on the structure and function of several organs and tissues (Mennitti et al., 2015). In the mammalian fetus, a change in the PUFA composition of the offspring's cell membranes was observed when the mother was exposed to n-3 PUFA during pregnancy (Mennitti et al., 2015). This change highlights the possibility of modification of the offspring phenotype when they are exposed to n-3 PUFA during their embryonic and early-life periods. Therefore, FA profiling might be an indicator of how the exposure to n-3 PUFA during embryonic and early-life periods can influence the body FA profile and the conversion of n-3 PUFA and n-6 PUFA (Cherian, 2015). The hypothesis of this study was that feeding with n-3 PUFA in either embryonic and rearing periods would improve the development of the bone properties and FA profile during the rearing phase. This study thus investigated the effect of feeding with the source of ALA and DHA in embryonic, rearing, or both periods on the bone development and FA during the pullet phase.

4.3. Materials and methods

4.3.1. Bird Management and sampling

Female hatchlings from chapter 3 were procured for this experiment. Ten female hatchlings per breeder treatment were euthanized for right tibia samples, which were stored at -80˚C for future analyses. The rest of birds were weighed and distributed into post-hatch treatments (Figure 4.1) with respect to breeder diet as follows: 1) breeder CON-pullet CON (CON-CON), 2) breeder CON-pullet DMA (CON-DMA), 3) breeder CON-pullet FFF (CON-FFF), 4) breeder DMA-pullet CON (DMA-CON), 5) breeder DMA-pullet DMA (DMA-DMA), 6) breeder FFF-pullet CON (FFF-CON), and 7) breeder FFF-pullet FFF (FFF-FFF). Post-hatch diets were formulated to meet or exceed nutritional requirements from hatch to pre-lay (ISA-brown and Shaver white, 2018) and are presented in Tables 4.1 and 4.2. Just like breeder diets, rearing diets supplemented with DHA and ALA had the same total amount of total n-3 PUFA and the ratio of n-6: n-3 PUFA (Table 4.2).

Twelve birds of the same breeder treatments combinations were placed in a cage of 20″ × 30″ (Ford Dickinson Inc., Mitchell, ON, Canada) to give five replications per post-hatch diet and kept until 18 WOA. Pullets were allowed *ad-libitum* access to feed and water. The temperature was initially set at 34°C on the first day and reduced by 2°C/ per week to a constant of 21°C. The lighting program started off on the first day at 40 lux, 02:00 to 18:00 hrs, and was reduced on the 28th day to 10 Lux, 08:00 to 20:00 hrs to 16 WOA. The vaccination program included: bronchitis (spray), Marek's disease (injection), Immucox (gel droplet) on the first day; Newcastle-bronchitis vaccine (spray) and ILT Vectormune FP-LT-AE (wing web) at 6 WOA; Newcastle-bronchitis at 10 (spray) and 16 WOA (intramuscular).

Blood samples were taken from 6 WOA birds, spun at 2,500 × g for 15 min at 4°C and plasma kept at -80 °C. Those birds used for blood sampling at 6 WOA were tagged for repeated

blood sampling at 12 and 18 WOA and palpation at 18 WOA. All tagged birds were palpated before lighting at 07:00 hr, and those found with a hard eggshell in the shell gland were selected (Akbari Moghaddam Kakhki et al. 2019a). Seven tagged birds of Shaver white and two birds of ISA brown were found without a hard shell in the shell gland and were replaced with untagged birds. The blood samples were taken from the selected birds and centrifuged at 2,000 × g for 30 min at 5°C and serum stored at -20°C until required for analysis. Left and right tibias and femurs were dissected, de-fleshed, and stored at -20˚C for further analysis. After the sampling, the population of birds was adjusted. Based on the Canadian National Farm Animal Care Council, each bird must be provided with a minimum space allowance of 67.0 square inches for white birds and 75.0 square inches for brown birds after the age of maturity (NFACC, 2017).

4.3.2. Sample analyses

For protein extraction from tibia samples taken at DOH, the whole tibia was crushed in liquid nitrogen using a mortar and pestle. After this process, a 0.1 ± 0.012 g sample was placed in a free-standing microcentrifuge tube (02-682-558, Thermo Fisher, Waltham MA) followed by addition of Tissue Protein Extraction Reagent (sample weight × 15; 78510, Thermo Fisher, Waltham MA) supplemented with protease inhibitor (Halt™ Protease Inhibitor cocktail, CAT# 78430, Thermo Fisher, Waltham MA). Then, 0.2 g ± 0.01 of acid-washed glass beads (2 mm; Z273627-1EA, Sigma Aldrich, St. Louis, MO) were added into the tubes and homogenized using a bead mill for two cycles of 15 sec at 5 m/s and one cycle of 150 sec at 3 m/s. Samples were then spun at 10,000 × *g* for 15 min at 4°C. Supernatants were analyzed for protein concentration based on the method of Smith et al. (1985) using an assay kit (Pierce BCA protein assay kit, #23225, Thermo Fisher, Waltham MA) and kept at -80°C until further analyses.

The whole body of day-old pullets used for measuring body composition in chapter 3, were freeze-dried, weighed, ground, and ashed for measuring total mineral and Ca and P. The concentration of Ca and P in the diet and body of pullets was measured according to the described method by Khanal et al. (2019) and described in the previous chapter. Left tibia and femur samples were taken at 18 WOA and used for measuring the dry weight, ash content, and ash percentage in epiphysis, medullary and cortical sub-parts. Sub-parts were separated, according to Akbari Moghaddam Kakhki et al. (2019b). The epiphysis, cortical, and medullary sections were dried at 105˚C for 24 h and reweighed. Subsequently, dried sub-parts were ashed at 600˚C for 12 h (Akbari Moghaddam Kakhki et al. 2019b) and reweighed for measuring total bone ash content, ash content, and ash percentage in sub-part and total bone ash concentration (Akbari Moghaddam Kakhki et al. 2019b). It has been demonstrated that bone attributes are correlated with BW (Erdal et al. 2012). Considering the difference in BW of the strains and possible significant differences among strains originating from the variation in BW among strains, all the bone attributes were normalized to BW, allowing a better picture of physiological difference among strains, not the difference in BW. Bone attributes, including length, dry weight, and ash content of tibia and femur were normalized by dividing the weight to BW (Akbari Moghaddam Kakhki et al. 2019b).

The tibia is one of the main long bones in hens, where osteoporotic fractures occur (Whitehead and Fleming, 2000; Whitehead, 2004). Therefore, the breaking strength test was done only on the tibia than the femur. Right tibia samples of 18 WOA were thawed for 48-hr at 4˚C and used for measuring breaking strength, according to Khanal et al. (2019). Breaking strength was measured using a 3-point bending test with an Instron material tester automated with the material test system software BlueHill 3.0 version 3.7.7 (Model: Instron crop, Canton, MA). Briefly, the maximum distance between the upper and lower anvil was fixed to 35 mm. Each tibia was

underpinned by a fulcrum with a span of 4 cm, connecting to the tibia at the beginning of the epiphyses (metaphysis region). The crosshead speed was set at 2 mm/s. All bones were kept in the same orientation, and the maximum load on mid-shaft of the tibia to break the bone was considered the value of TBS.

For measuring the profile of FA in plasma, briefly, 50 µL of plasma was mixed with potassium chloride, chloroform, and methanol. Homogenized samples were spun at 836 × g for 10 min, followed by the transfer of the chloroform layer. Then chloroform was dried off using nitrogen gas and followed by the addition of methanol for saponification at 100^{o}C for 1 hour. The rest of the analysis was followed based on the described method in chapter 3. In brief, after the addition of hexane, BF_3-MeOH and C17:0 free FA internal standard, the samples were methylated at 100°C for 1 hour and spun at 836 × g for 10 min. Extracted hexane was transferred into gas chromatography vials and analyzed by Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a flame ionization detector and separated on a DB-FFAP fused-silica capillary column (127-32H2, Technologies, Santa Clara, CA). Samples were injected in 200:1 split mode by injector set at 250°C. Fatty acid methyl esters were eluted using a temperature program set initially at 150°C and held for 0. 25 min, increased to 35°C /min and held at 170°C for 3 min, increased to 9°C /min to 225°C, and finally increased to 80°C/min to 245°C and held for 2.2 min. The carrier gas was hydrogen, set to a 30 mL/min constant flow rate. The plasma concentration of LA, ALA, AA, EPA, DHA, and total FA were determined.

The concentration of collagen types I, II in the tibia, osteocalcin and TRAP in plasma was measured in duplicate using competitive ELISA kits following recommended assay procedures (collagen type I: ECKC0021, collagen type II: CKC0786, osteocalcin: ECKO0008 and TRAP: ECKT0544; ABclonal, Woburn, MA). Antibodies for ELISA kits were rabbit polyclonal and

validated using an immunogen of the specific chicken protein. The intra-assay CV was 8.35%, 2.19%, 17.06%, 20.31%, and 5.08% for collagen type II, I, osteocalcin of 6, 12 WOA, and TRAP, respectively.

4.3.3. Calculations and statistical analyses

The normality of data was tested using the UNIVARIATE plot normal procedure (SAS 9.4). Data of day-old pullets were subjected to a two-way ANOVA in a two (ISA brown and Shaver white) and three (CON, DMA, and FFF) factorial arrangement. The rest of the data were subjected to nested factorial arrangement for including a two-way ANOVA in two strains (ISA brown and Shaver white) and three breeder diets factorial arrangement as well as a two-way ANOVA in two strains and seven post-hatch diets factorial arrangement using the GLIMMIX procedure (SAS 9.4). The correlation between bone-turnover markers (osteocalcin at 6 and 12 WOA and TRAP) and bone attributes was performed using the CORR procedure. Significance was declared at $P < 0.05$.

4.4. Results

4.4.1. Fatty Acid Concentrations in the Diets

Supplementation with DMA increased concentration of DHA and EPA in total fat across the starter to pre-lay diets by an average of 3.37 ± 0.13% and 0.05 ± 0.01%, respectively (Table 4.3). Supplementation with FFF increased the concentration of ALA and DHA in the starter to pre-lay diets by an average of 9.21 ± 0.21% and 0.17 ± 0.05%, respectively.

4.4.2. Whole Bone Mineral Content and Tibia Collagen in Day-Old Pullets

There was no interaction of stain and diet on the percentage of total mineral, and P in the body ($P > 0.05$; Table 4.4). Total mineral content was higher in Shaver white hatchlings compared to ISA brown hatchlings ($P = 0.045$), while it did not affect Ca and P content in hatchlings ($P >$

0.05). Hatchlings from breeders fed with DMA had a lower percentage of mineral, Ca, and P in their body compared to CON ($P < 0.05$). The collagen type II content in tibias was not affected by the main effect of strain ($P > 0.05$). Tibia collagen type II was increased ($P < 0.05$) in pullets from breeders fed FFF compared with those from breeders fed CON, while pullets from breeders fed DMA were numerically intermediate between CON and FFF ($P > 0.05$). The collagen type I was neither affected by the interaction of strain and diet nor the main effects of strain and diet ($P < 0.05$).

4.4.3. Plasma Bone Turnover Markers During Rearing

The effects of strain and diet on plasma bone turnover markers are shown in Table 4.5. The interactive effect of strain and breeder diet affected the concentration of osteocalcin at 6 WOA and TRAP at 18 WOA ($P < 0.05$). Pullets of ISA brown breeders fed FFF, and Shaver white breeders fed DMA had a higher concentration of osteocalcin compared with ISA brown pullets from breeders fed CON, DMA, and Shaver white breeders fed FFF ($P < 0.001$). The concentration of TRAP was higher in ISA brown pullets from breeders fed FFF than pullets from ISA brown breeders fed with FFF and all the Shaver white pullets from all three breeder diets ($P = 0.009$). There was no interaction between strain and breeder diet on plasma osteocalcin at 12 WOA ($P = 0.413$). In addition, there was no interaction between strain and offspring diets on the concentration of osteocalcin and TRAP ($P > 0.05$). Shaver white pullets had a 43.1% greater concentration of plasma osteocalcin compared with ISA brown pullets at 12 WOA ($P < 0.001$), while ISA brown pullets had 86.77% greater TRAP concentration compared with Shaver white pullets ($P = 0.001$). Breeder and offspring diets influenced the plasma concentration of osteocalcin at 12 WOA ($P < 0.001$). Breeder-feeding of DMA reduced the concentration of osteocalcin in 12 WOA offspring

compared to CON and FFF, while those pullets of CON-DMA and FFF-FFF had higher osteocalcin compared to the rest of the treatments ($P < 0.001$).

4.4.4. Body Weight of Pullets

The BW of 18 WOA pullets was not affected by the interaction between strain and breeder diet, the interaction between strain and offspring diet, or the main effects of breeder and offspring diets ($P > 0.05$, Figure 4.2). ISA brown pullets were 349.1 g heavier than Shaver white pullets at 18 WOA ($P < 0.001$).

4.4.5. Offspring Egg Production at The Time of Sampling

The age of laying the first egg was 121 d and 125 d for ISA brown and Shaver white, respectively. The age of maturity, the age of 50% egg production, was 141 d and 137 d for ISA brown and Shaver white, respectively. The egg production percentage was 2.00% and 0.86% at the time of sampling of 18 WOA ISA brown and shaver white birds, respectively.

4.4.6. Plasma Fatty Acid Profiles In 18-Week-Old Pullets

There was no interaction between strain and diets on plasma percent composition of LA, ALA, AA, EPA, DHA, ∑n-3 PUFA, ∑n-6 PUFA, ∑n-6: ∑n-3 PUFA ratio, and total FA at 18 WOA ($P > 0.05$, Table 4.6). ISA brown pullets had a higher percent composition of LA, ALA, AA, EPA, DHA, ∑n-3 PUFA and ∑n-6 PUFA compared with Shaver white pullets ($P < 0.05$). The main effect of the breeder diet was such that pullets from CON and FFF had a higher percent composition of LA, ∑n-3 PUFA, ∑n-6 PUFA, and lower total fat compared with DMA pullets ($P < 0.05$). The breeder diet did not affect the percent composition of ALA, AA, EPA, DHA, and ∑n-6: ∑n-3 PUFA ratio ($P > 0.05$). Pullets in CON-CON and FFF-CON groups maintained a higher percent composition of LA compared with the rest of the treatments ($P = 0.005$). Pullets in CON-

FFF and FFF-FFF groups maintained a higher percent composition of ALA compared with the rest of the treatments ($P < 0.001$). The greatest plasma percent composition of AA was observed in pullets of CON-CON, FFF-CON and FFF-FFF groups, whereas pullets of CON-DMA and DMA-DMA had the lowest percent composition of AA ($P < 0.001$). The percent composition of EPA was higher in pullets of FFF-FFF relative to pullets of FFF-CON, DMA-CON, DMA-DMA, and CON-CON groups ($P < 0.001$). The percent composition of DHA was higher in pullets of CON-DMA, DMA-DMA and FFF-FFF relative to pullets of CON-CON, DMA-CON, and FFF-CON groups ($P < 0.001$). Pullets of CON-DMA, CON-FFF and DMA-DMA and FFF-FFF groups had a higher ∑n-3 PUFA compared to other treatments ($P < 0.001$). The greatest plasma ∑n-6 PUFA was observed in pullets of CON-CON, FFF-CON, whereas pullets of DMA-DMA had the lowest plasma ∑n-6 PUFA ($P = 0.007$). The ∑n-6: ∑n-3 PUFA ratio was lower in the CON-DMA, CON-FFF, DMA-DMA, FFF-FFF groups compared to the rest of the treatments ($P < 0.001$). The greatest total plasma fat was observed in pullets of DMA-CON and DMA-DMA, followed by CON-CON, FFF-CON, DMA-CON compared to the rest of the treatments ($P = 0.001$).

4.4.7. Calculated n-3 and n-6 PUFA desaturation and elongation

There was no interaction between strain and diet on the activity of n-6 and n-3 PUFA desaturation and elongation at 18 WOA ($P > 0.05$; Table 4.6). ISA brown had greater n-3 PUFA conversion compared to Shaver white pullets ($P = 0.001$). Feeding breeders FFF increased n-6 PUFA conversion in their offspring at 18 WOA ($P < 0.001$). The n-6 PUFA conversion at 18 WOA varied among different offspring diets. The FFF-FFF had a higher conversion than CON-CON, CON-DMA, CON-FFF, DMA-CON ($P < 0.001$). The n-3 PUFA conversion was higher in pullets of CON-DMA compared to DMA-CON ($P < 0.001$).

4.4.8. Tibia and Femur Attributes in 18 Weeks of Age Pullets

The effect of strain and diet on the whole tibia and femur attributes in 18 WOA pullets are shown in Table 4.7. The interaction between strain and breeder diet showed improved TBS in Shaver white pullets from breeders fed with DMA compared with Shaver white from breeders fed CON ($P = 0.001$). In addition, ISA brown from breeder fed with DMA had higher TBS than ISA brown pullets from every dietary treatment. There was no interaction between strain and breeder diet on tibia weight, ash content, or ash percentage ($P > 0.05$). Shaver white pullets had lighter tibia with greater ash concentration compared with tibia from ISA brown pullets ($P < 0.001$). There was no interaction between strain and offspring diet, the main effect of offspring diets, nor the main effect of offspring in tibia weight, ash content, and ash percentage in pullets ($P > 0.05$).

The interaction between strain and breeder diet influenced femur ash content, in which Shaver white pullets from breeder fed with DMA had higher femur ash content than the rest of the treatment in brown and white pullets ($P = 0.001$). Femur weight, ash content, or ash percentage were not influenced by the interaction between strain and offspring diet nor the main effect of the offspring diet ($P > 0.05$). Shaver white pullets had lighter femurs with higher ash content and ash concentration compared with ISA brown pullets ($P < 0.001$). The main effect of the breeder diet on femur ash percentage was such that breeder feeding of DMA increased femur ash percentage in offspring compared to the FFF group ($P = 0.038$).

The attributes of tibia epiphysis and medullary were not affected by the interaction between strain and offspring diet, the main effect breeder diet, and offspring diet ($P > 0.05$; Table 4.8). The interactive effect of strain and breeder diet increased the tibia ash content in Shaver white pullets from breeders fed with DMA compared to Shaver white from breeders fed with CON and ISA brown pullets from breeders fed with DMA and FFF ($P = 0.001$). The main effect of strain was such that the tibia epiphysis and medullary region of Shaver white pullets had lower dry weight (*P*

< 0.001), as well as higher ash percentage in the epiphysis, medullary, and cortical regions compared with ISA brown pullets ($P < 0.05$).

The interaction between strain and breeder diet influenced cortical ash content of femur, such that Shaver white pullets from breeders fed with DMA had higher ash content than Shaver white and ISA brown pullets fed with any breeder diets ($P < 0.001$; Table 4.9). However, this interactive effect was not observed in the rest of the parameters of femur epiphysis, medullary, and cortical ($P > 0.05$). The characterizations of femur epiphysis, medullary, or cortical were not influenced by the interaction between strain and offspring diet and the main effect of the offspring diet ($P > 0.05$). The main effect of the breeder diet was such that Shaver white pullets from breeders fed with DMA had higher dry weight and ash content in their femur compared to the rest of the treatments ($P < 0.001$). However, the interaction between strain and breeder diet did not affect the weight, ash content, nor ash percentage of femur epiphysis and medullary as well as cortical ash percentage. The femurs of Shaver white pullets had higher ash weight and ash percentage in their medullary and cortical bone but lower epiphysis and medullary weight compared with ISA brown ($P < 0.05$). The main effect of breeder diets only affected the cortical weight and ash weight, in which cortical weight and ash content were improved in pullets from breeders fed with DMA compared to the CON and FFF groups ($P < 0.001$).

4.4.7. Correlation among parameters

There was a correlation between osteocalcin concentration measured at 6 WOA and osteocalcin measured at 12 WOA (correlation coefficient = 0.409), tibia ash weight (correlation coefficient = 0.441) and ash concentration (correlation coefficient = 0.421) in ISA brown pullets ($P < 0.05$, Table 4.10). The TRAP concentration had no correlation with any bone attributes in ISA brown pullets ($P > 0.05$). The osteocalcin measured at 6 WOA correlated with osteocalcin

measured at 12 WOA in Shaver white pullets (correlation coefficient = 0.563, $P < 0.001$). The concentration of TRAP correlated with ash concentration in whole tibia (correlation coefficient = - 0.305), whole femur (correlation coefficient = - 0.387) and femur cortical (correlation coefficient = - 0.305) in Shaver white pullets.

4.5. Discussion

The composition of hatching eggs may modify the skeletal morphological features of developing embryos and hatchlings (Yair et al. 2012). Bone development during the embryonic period has been shown to peak after the d-14 of incubation in broilers (Yair et al. 2012). Feeding n-3 PUFA to breeders reduced the concentration of total mineral, Ca, and P in hatchlings, which might be associated with the impact of the increased concentration of n-3 PUFA in the eggs (Chapter 3), resulting in greater DHA uptake but lower uptake of DM, OM, and minerals from the RY. This connection highlights the vital role of yolk FA content in modifying nutrient utilization by the embryos (Yalcin et al. 2008). Another possible factor involved in the change in body mineral content may be the difference in egg component percentages because egg yolk and eggshell are the main sources of nutrients and minerals for bone development (Yalcin et al. 2008; Neunzehn et al. 2015). However, the absolute value and percentage of yolk, albumen, and eggshell were not different among the dietary treatments. In addition, the dietary concentration of Ca was 4.0%, 4.2% and 4.1% and the dietary concentration of P was 0.7%, 0.8% and 0.7% for CON, DMA, and FFF, respectively. The inadequate embryonic uptake of mineral has been associated with the low bone mineral content at hatch, leading to impairment in the development of the skeletal system and other critical organs (Yair and Uni, 2011).

Endochondral ossification ensures that the process of longitudinal growth occurs, which is initiated by the secretion of the extracellular matrix via chondrocytes (Whitehead 2004). The

extracellular matrix has been shown to contain a high content of collagen type II (Whitehead 2004). As chondrocytes become enlarged in the hypertrophic zone, the extracellular matrix is surrounded by collagen type X (Whitehead 2004). After chondrocytes undergo apoptosis and are resorbed, OSB secrete collagen type I and form a scaffold for hydroxyapatite crystals (Rath et al. 2000). The greater concentration of collagen type II in embryos of the FFF compared with CON and equal concentration of collagen type I among treatments could explain why the reduced tibia mineralization in embryos of DMA and FFF was not associated with a delay in collagen synthesis/development. On the other hand, it can be understood that embryos of FFF had more collagen type II, which might provide more area for OSB to secrete collagen type I, meaning more scaffold to host hydroxyapatite crystals and more potential for greater BMC in later stages of life. These observations emphasize the alteration in functions of OSB by the change in the availability of PUFA (Kushwaha et al. 2018).

In the current study, the concentration of osteocalcin in plasma at 6 and 12 WOA showed that breeder-feeding of FFF resulted in a higher plasma level of osteocalcin and TRAP than the DMA group. However, the level of osteocalcin at 12 WOA was not correlated with bone characteristics. One reason for this discrepancy might be the high variability of the observations as the intra-assay coefficient variation was 20.31%. The differences in the concentration of biomarkers for the activity of OSB (osteocalcin at 6 and 12 WOA) and OSC (TRAP at 18 WOA) demonstrated differences in bone turnover rate in the two strains. Pottgueter (2015) reported there is a peak of bone formation in pullet starting from 6 until 12 WOA, explaining the importance of measuring the biomarkers of bone formation at these ages. After sexual maturity, the role of OSB in structural bone is important as structural bone start to lose its mineral content. Khanal et al. (2019) observed differences in femur physical attributes between Lohmann Brown and Lohmann

LSL-Lite at different times in the lay cycle. Lohmann LSL-Lite had greater femur mineral density and content compared with Lohmann Brown (Khanal et al. 2019). Similarly, in the current study, 18 WOA Shaver white pullets had greater tibia and femur ash concentration compared with ISA brown pullets, which might be associated with higher bone formation than ISA brown pullets.

In the current study, ISA brown pullets maintained higher ∑n-3 PUFA, and ∑n-6 PUFA in their plasma compared to Shaver white pullets. The ISA brown pullets consumed 187 g more feed throughout the rearing period compared to Shaver white pullets, which partially contribute to the differences in FA profile among strains (data not presented). The differences in plasma FA profiles of various strains of layer hens are unknown. As mentioned in chapter one, Shaver white layer breeders had higher efficiency of depositing n-3 PUFA in their eggs and higher n-3 PUFA conversion than ISA brown layer breeders.

Fetal programming is included in fetus adaptive responses to specific prenatal environmental conditions, which may result in the alteration of gene expression and permanent effects on the structure and function of several organs and tissues (Mennitti et al., 2015). One mechanism by which n-3 PUFA can exert their long-lasting effect is through modification in the pattern of DNA methylation. Prenatal exposure of PUFA is involved in epigenetic regulation of PUFA conversion in offspring through the transcriptional regulation of elongase, Δ-6, and Δ-5 desaturase enzymes, resulting in persistent changes in the PUFA content of cell membrane in the offspring (Mennitti et al., 2015). The effect of feeding breeders n-3 PUFA on plasma FA profile in 18 WOA pullets was dependent on the sources of n-3 PUFA with DMA being more effective in reducing n-3 PUFA, n-6 PUFA, and n-6 PUFA conversion, while it increased the total fat in plasma. However, feeding DMA or FFF to offspring was effective in increasing the level of n-3 PUFA and reducing n-6 PUFA in plasma regardless of their breeder diet.

Different strains possess different traits and characteristics such as feed intake, body growth and development, sexual maturity, egg production efficiency, and skeletal health (Singh et al. 2009; Khanal et al. 2019). The phenotypic differences among strains start during the embryonic period (Ho et al. 2011). The differences in bone metabolism among strains may explain why bone attributes of 18 WOA ISA brown pullets were not affected by feeding n-3 PUFA; this difference highlighted the importance of the impact of genetics on bone turnover and phenotypic differences. Breeder-feeding of DMA to Shaver white breeders increased the TBS, tibia cortical ash content, total femur ash content, its cortical ash content, and ash percentage compared to the CON and FFF groups, while offspring-feeding of sources of n-3 PUFA did not affect any of the mentioned parameters. These improvements in bone attributes suggested that feeding n-3 PUFA to layer breeders was more effective than feeding n-3 PUFA to their offspring. In addition, the effect of breeder-feeding of n-3 PUFA sources is dependent on the type of FA. Although there is no report on the impact of embryonic-feeding and pullet-feeding of n-3 PUFA on bone properties in laying hens, a body of studies evaluated the impact of feeding with n-3 PUFA at or after the onset of lay. These studies have shown the potential of n-3 PUFA in supporting skeletal growth in layer hens (Josling et al., 2019; Baird et al., 2008), showing the distinct impact of each type of n-3 PUFA (Toscano et al., 2015) and dosage of n-3 PUFA (Baird et al. 2008).

As mentioned in chapter 1, two main mechanisms are involved in the impact of n-3 PUFA on bone metabolism through modifying the production of eicosanoids, in particular, PGE2 (Kruger et al. 2010) and pro-inflammatory cytokines (Nakanishi and Tsukamoto 2015). These two groups of inflammatory mediators increase the expression of stimulatory factors such as RANKL and initiate a cascade of downstream pathways, which results in an increase in osteoclastogenesis, activation of OSC, and bone resorption (Boeyens et al. 2014).

4.6. Conclusions

In this chapter, feeding with n-3 PUFA decreased mineral content and delayed pre-hatch bone mineralization, which was not associated with collagen synthesis/development. ISA brown offspring had a higher plasma percent composition of ∑n-3 and ∑n-6 PUFA compared with Shaver white birds. Breeder feeding of DMA showed the potential of decreasing the percent of n-3 and n-6 PUFA compared to FFF in 18 WOA offspring, while pullet-feeding of either DMA or FFF was effective in increasing n-3 and n-6 PUFA in plasma of 18 WOA pullets. The impact of embryonic and rearing exposure to n-3 PUFA on rearing bone development was strain-dependent. Embryonic exposure to n-3 PUFA through feeding breeders DHA was an effective strategy in supporting the development of tibia and femur structural (cortical) bone in Shaver White pullets. The strains showed differences in their bone characterization, highlighting the importance of considering the strain effect in designing and interpreting the results of nutritional and management studies.

4.7. Tables and figures

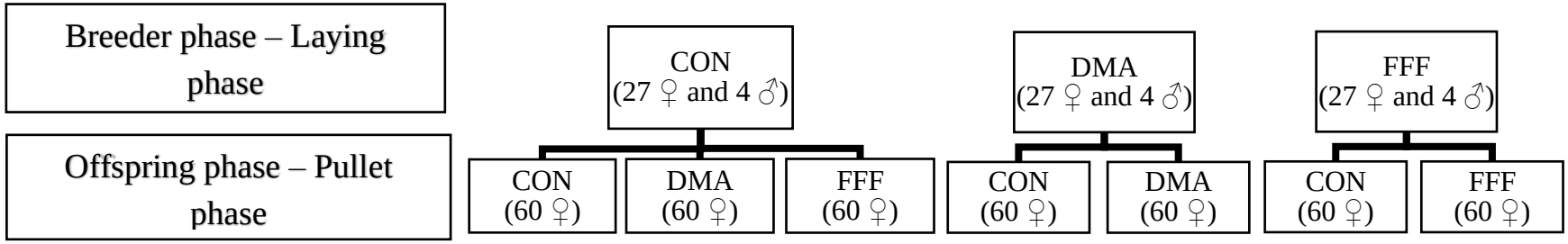

Figure 4. 1. Dietary treatment layout for the breeder and offspring phases.

Day-old female breeder pullets were divided into three dietary treatments: 1) control (CON); 2) micro-Algae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid (DMA); and 3) co-extruded full-fat flaxseed and pulses mixture (50/50, wt/wt), as a source of α-linolenic acid (FFF). Offspring from CON treatment was further divided into three post-hatch treatments (CON, DMA and FFF) while offspring from the DMA and FFF treatments were divided into two post-hatch treatments, CON and DMA or CON and FFF. The concentration of total n-3 FA and ratio of n-6: n-3 were identical among DMA and FFF diets in both phases.

Table 4. 1. Composition of the experimental diets for the ISA brown and Shaver white pullets, as fed basis.

Item	Starter			Grower			Developer			Pre-lay		
	CON	DMA	FFF	CON	DMA	FFF	CON	DMA	FFF	CON	DMA	FFF
Ingredient, g/kg												
Corn grain	611.13	598.39	590.44	478.89	473.00	464.40	419.42	435.13	417.64	474.52	490.23	472.74
Soybean meal	311.16	327.00	317.09	253.76	251.80	243.80	175.92	176.16	167.82	209.80	209.90	201.57
Wheat	-	-	-	70.00	70.00	70.00	60.00	50.00	60.00	60.00	50.00	60.00
Corn gluten	18.83	6.45	7.59	-	-	-	-	-	-	-	-	-
DMA[1]	-	10.00	-	-	10.00	-	-	10.00	-	-	10.00	-
FFF[2]	-	-	26.50	-	-	26.00	-	-	24.50	-	-	24.50
Wheat middlings	-	-	-	130.00	130.00	130.00	275.20	265.57	265.69	147.40	137.77	137.89
Soybean oil	1.50	1.50	1.50	10.00	7.00	7.40	9.00	3.00	4.00	9.00	3.00	4.00
Limestone fine	17.98	17.90	17.90	19.50	20.00	20.00	20.15	20.06	20.09	58.41	58.33	58.36
Mono Ca phosphate	19.25	19.09	19.10	15.16	15.50	15.50	16.42	16.41	16.40	17.58	17.57	17.56
Poultry premix[3]	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
DL-Methionine, 99 %	2.38	2.48	2.48	2.83	2.80	2.90	3.18	3.18	3.20	3.23	3.24	3.25
L-Lysine HCl, 78 %	0.89	0.51	0.57	2.05	2.10	2.10	2.82	2.83	2.87	2.35	2.35	2.39
L-Threonine, 98 %	0.24	0.19	0.24	0.94	0.90	1.00	1.20	1.19	1.24	1.03	1.43	1.46
Salt	1.86	1.95	1.99	0.69	0.70	0.70	1.45	1.45	1.48	1.43	1.02	1.06
Sodium bicarbonate	3.75	3.50	3.55	5.16	5.10	5.10	4.21	4.13	4.18	4.21	4.13	4.18
Choline Chloride	0.89	0.89	0.89	0.89	0.90	0.90	0.89	0.89	0.89	0.89	0.89	0.89
Ethoxyquin[4]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15

[1]Microalgae (*Aurantiochytrium limacinum)* fermentation product, as a source of docosahexaenoic acid (DHA), Alltech Canada, Guelph, Ontario, Canada.
[2] Co-extruded full fat flaxseed and pulse mixture (1:1 wt/wt), as a source of α-linolenic acid (ALA), O & T Farms Ltd., Saskatoon, Saskatchewan, Canada.
[3] Provided in kg of diet: vitamin A (retinol), 10,000 IU; vitamin D_3 (cholecalciferol), 3,000 IU; vitamin E, 100 mg; vitamin K_3 (menadione), 5.0 mg; vitamin B_1 (thiamin), 4.0 mg; vitamin B_2 (riboflavin), 10.0 mg; vitamin B_3 (niacin), 50.0 mg; vitamin B_5 (pantothenic acid), 20.0 mg; vitamin B_6 (pyridoxine), 4.0 mg; vitamin B_9 (folic acid), 2.0 mg; vitamin B_{12} (cyanocobalamin), 30.0 mg; biotin , 200 mcg; choline, 400.0 mg; Mg, 110 mg; Zn, 80 mg; Fe, 40.0 mg; Cu, 10.0 mg ; I, 1 mg, Se, 0.31 mg.
[4] SANTOQUIN®, Novus International Inc., Saint Charles, MO

Table 4. 2. Calculated nutritional composition of the experimental diets for the ISA brown and Shaver white pullets, as fed basis.

Item	Starter			Grower			Developer			Pre-lay		
	CON	DMA	FFF	CON	DMA	FFF	CON	DMA	FFF	CON	DMA	FFF
Metabolizable energy, kcal/kg	2,900	2,900	2,900	2,800	2,800	2,800	2,700	2,700	2,700	2,700	2,700	2,700
Crude protein, %	21.00	21.00	21.00	19.10	19.10	19.10	17.20	17.20	17.20	17.20	17.20	17.20
Calcium, %	1.10	1.10	1.10	1.10	1.10	1.10	1.10	1.10	1.10	2.50	2.50	2.50
Analyzed calcium, %	1.15	1.15	1.17	1.20	1.18	1.77	1.20	1.20	1.21	2.52	2.51	2.49
Available phosphorus, %	0.48	0.48	0.48	0.40	0.40	0.40	0.44	0.44	0.44	0.45	0.45	0.45
Analyzed phosphorus, %	0.79	0.79	0.79	0.71	0.71	0.71	0.80	0.80	0.79	0.83	0.83	0.84
SID[1] Lysine, %	1.00	1.00	1.00	0.98	0.98	0.98	0.90	0.90	0.90	0.90	0.90	0.90
SID Methionine, %	0.53	0.53	0.53	0.53	0.53	0.53	0.54	0.54	0.54	0.54	0.54	0.54
SID Methionine + cystine, %	0.78	0.78	0.78	0.76	0.76	0.76	0.76	0.76	0.76	0.76	0.76	0.76
SID Threonine, %	0.67	0.67	0.67	0.66	0.66	0.66	0.60	0.60	0.60	0.60	0.60	0.60
SID Tryptophan, %	0.23	0.23	0.23	0.21	0.21	0.21	0.18	0.18	0.18	0.18	0.18	0.18
Sodium, %	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18
Chloride, %	0.23	0.23	0.23	0.16	0.16	0.16	0.20	0.20	0.20	0.20	0.20	0.20
∑n-3, %	0.07	0.31	0.33	0.13	0.35	0.36	0.11	0.30	0.31	0.11	0.31	0.31
∑n-6, %	1.63	1.64	1.69	1.82	1.66	1.73	1.54	1.26	1.35	1.69	1.41	1.50
∑n-6: ∑n-3	23.29	5.29	5.12	14.00	4.74	4.81	14.00	4.20	4.35	15.36	4.55	4.84

[1] Standardized ileal digestible

Table 4. 3. Analyzed fatty acid profile in post-hatch experimental diets, % of total fat [1]

Item	18:2n-6[2]	18:3n-3[3]	20:4n-6[4]	20:5n-3[5]	22:6n-3[6]	∑n-3	∑n-6	∑n-6: ∑n-3	Total fat[7]
Starter									
CON	55.50	4.18	0.00	0.00	0.00	4.18	55.50	13.28	2.95
DMA	50.16	5.46	0.00	0.05	3.45	9.07	50.16	5.53	3.08
FFF	50.53	9.50	0.00	0.02	0.15	9.67	50.62	5.23	3.01
Grower									
CON	55.27	6.32	0.00	0.00	0.00	6.32	55.47	8.78	3.53
DMA	51.42	5.86	0.00	0.06	3.24	9.20	51.53	5.60	3.62
FFF	51.16	9.01	0.00	0.00	0.11	9.12	51.35	5.63	3.56
Developer									
CON	57.16	5.44	0.00	0.00	0.00	5.48	57.39	10.48	4.17
DMA	50.09	5.58	0.02	0.07	3.28	9.00	50.19	5.58	4.31
FFF	50.15	9.15	0.01	0.06	0.19	9.42	50.42	5.36	4.25
Pre-lay									
CON	58.06	4.14	0.00	0.00	0.00	4.14	58.06	14.03	3.64
DMA	52.05	5.25	0.00	0.04	3.52	8.87	52.05	5.87	3.89
FFF	52.50	9.18	0.00	0.00	0.23	9.41	52.50	5.58	3.71

[1] CON, control; DMA, micro-Algae (*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.

[2] Linoleic

[3] α-linolenic acid

[4] Arachidonic acid

[5] Eicosapentaenoic acid

[6] Docosahexaenoic acid

[7] Expressed as a gram per 100 grams of feed, *as fed basis.*

Table 4. 4. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown and Shaver White breeders and/offspring on body mineral content and tibia collagen of day-old pullets[1]

Items		Body Mineral, %			Tibia Collagen II		Tibia Collagen I	
		Total	Ca[2]	P[3]	ng/mg	ng/µg protein	ng/mg	ng/µg protein
Strain	Diet[4]							
ISA brown	CON	7.86	0.93^{ab}	0.74	3487	4.712	96.41	0.130
ISA brown	DMA	7.73	0.89^{b}	0.70	3493	4.713	97.48	0.131
ISA brown	FFF	8.19	0.96^{ab}	0.75	3498	4.723	97.62	0.132
Shaver white	CON	8.48	1.01^{a}	0.77	3490	4.693	95.40	0.128
Shaver white	DMA	7.86	0.90^{b}	0.70	3495	4.714	96.88	0.131
Shaver white	FFF	8.14	0.91^{b}	0.71	3505	4.736	96.39	0.130
SEM		0.139	0.021	0.015	3.581	0.006	1.022	0.001
Main effect								
Strain								
ISA brown		7.93^{b}	0.93	0.73	3492	4.716	97.17	0.13
Shaver white		8.16^{a}	0.94	0.73	3497	4.715	96.22	0.13
SEM		0.080	0.012	0.009	2.067	0.004	0.590	0.0007
Diet								
CON		8.17^{a}	0.97^{a}	0.75^{a}	3488^{b}	4.702^{b}	95.90	0.129
DMA		7.80^{b}	0.90^{b}	0.70^{b}	3494^{ab}	4.714^{ab}	97.18	0.131
FFF		8.16^{a}	0.94^{ab}	0.73^{ab}	3502^{a}	4.729^{a}	97.01	0.131
SEM		0.098	0.015	0.011	2.532	0.005	0.722	0.001
Probabilities (*P*-value)								
Strain		0.045	0.408	0.953	0.142	0.806	0.263	0.211
Diet		0.013	<0.005	0.003	0.002	<0.001	0.407	0.401
Strain × Diet		0.055	0.011	0.108	0.792	0.051	0.952	0.915

Values with uncommon superscripts within each column are significantly different ($P < 0.05$).
[1]n=10. [2] Calcium [3] Phosphorus [3]CON, control; DMA, micro-Algae (*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.

Table 4. 5. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown and Shaver White breeders and/offspring on body mineral content and tibia collagen of day-old pullets, ng/mL[1]

Items		Osteocalcin		Tartrate resistant acid phosphatase
		6-week	12-week	18-week
Strain	Breeder diet			
ISA brown	CON	19.41^{bc}	9.09	8.06^{ab}
ISA brown	DMA	13.04^{d}	6.22	4.42^{b}
ISA brown	FFF	29.25^{a}	9.21	10.08^{a}
Shaver white	CON	20.93^{bc}	11.88	5.64^{b}
Shaver white	DMA	23.20^{ab}	10.30	4.44^{b}
Shaver white	FFF	16.65^{cd}	12.95	4.08^{b}
SEM		1.367	0.545	1.420
Main effect				
Strain				
ISA brown		20.57	8.18^{b}	8.52^{a}
Shaver white		20.26	11.71^{a}	4.72^{b}
SEM		0.839	0.303	0.880
Breeder diet				
CON		20.17^{ab}	10.49^{a}	6.86^{ab}
DMA		18.12^{b}	8.26^{b}	4.43^{b}
FFF		22.95^{a}	11.08^{a}	8.58^{a}
SEM		1.319	0.326	0.836
Offspring diet				
CON-CON		20.17^{bc}	10.49^{b}	6.86
CON-DMA		29.24^{a}	14.55^{a}	7.74
CON-FFF		15.21^{c}	8.36^{b}	3.32
DMA-CON		18.12^{bc}	8.26^{b}	4.43
DMA-DMA		16.69^{c}	8.23^{b}	3.82
FFF-CON		20.71^{bc}	11.08^{b}	8.58
FFF-FFF		25.19^{ab}	13.49^{a}	8.36
SEM		1.567	0.545	1.661
Probabilities (*P*-value)				
Strain		0.835	<0.001	0.001
Breeder diet		0.009	<0.001	0.023
Offspring diet		0.001	<0.001	0.058
Strain × breeder diet		<0.001	0.413	0.009
Strain × offspring diet		0.081	0.107	0.924

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

[1]n=10.

[2]The day-old female pullets from breeders fed CON, DMA and FFF were divided into three (CON, DMA and FFF), two (CON and DMA) and two (CON, FFF) post-hatch treatments, respectively. CON, control; DMA, micro-Algae (*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.

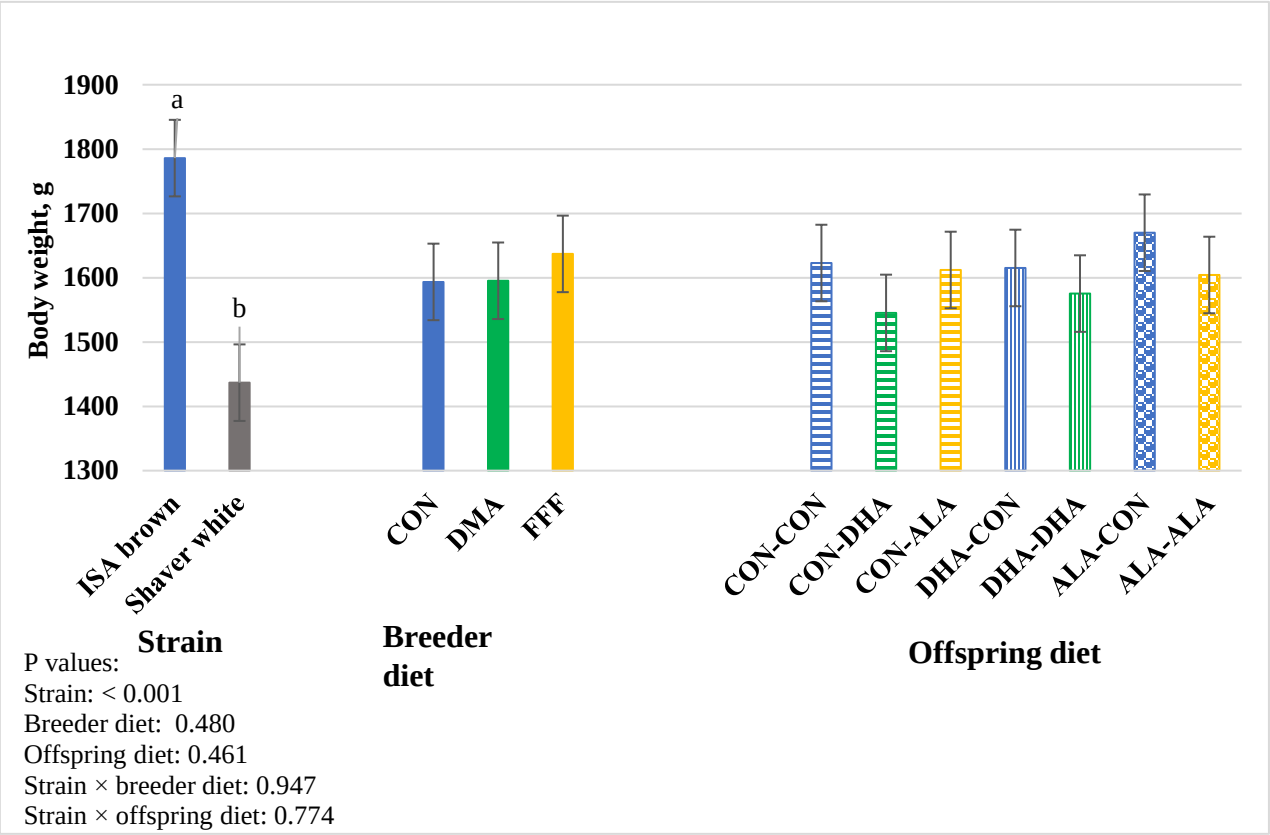

Figure 4. 2. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown and Shaver White breeders and/offspring on body weight of 18 wks old pullets. The day-old female pullets from breeders fed CON, DMA and FFF were divided into three (CON, DMA and FFF), two (CON and DMA) and two (CON, FFF) post-hatch treatments, respectively. The concentration of total n-3 PUFA and ratio of n-6: n-3 were identical among DMA and FFF diets in both breeder and offspring phases.

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

Table 4. 6. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown and Shaver white breeders and offspring on plasma concentration (%) and calculated conversion of fatty acids in 18-week-old pullets[1]

Item[2]	LA	ALA	AA	EPA	DHA	∑n-3	∑n-6	∑n-6: ∑n-3	Total fat	n-6 PUFA conversion	n-3 PUFA conversion
Main effects											
Strain											
ISA brown	21.07^{a}	0.75^{a}	5.86^{a}	0.38^{a}	2.86^{a}	4.49^{a}	29.33^{a}	6.53	388.82^{b}	0.27	0.52^{a}
Shaver white	16.21^{b}	0.64^{b}	4.27^{b}	0.13^{b}	1.97^{b}	3.06^{b}	21.87^{b}	7.15	454.11^{a}	0.27	0.19^{b}
SEM	0.858	0.030	0.312	0.019	0.101	0.304	0.942	0.478	24.521	0.013	0.040
Breeder diet[3]											
CON	21.82^{a}	0.64	6.07	0.13	1.57	2.70^{a}	29.79^{a}	11.03	418.95^{b}	0.25^{b}	0.42
DMA	17.57^{b}	0.54	5.17	0.10	1.01	1.96^{b}	24.30^{b}	12.40	663.94^{a}	0.23^{b}	0.28
FFF	21.06^{a}	0.58	6.00	0.13	1.47	2.51^{a}	29.29^{a}	11.68	415.08^{b}	0.33^{a}	0.36
SEM	1.241	0.055	0.406	0.041	0.155	0.574	1.112	0.621	41.541	0.018	0.054
Offspring diet[4]											
CON-CON	21.82^{a}	0.64^{b}	6.07^{a}	0.13^{b}	1.57^{b}	2.70^{b}	29.79^{a}	11.03^{a}	418.96^{b}	0.25^{bc}	0.42^{ab}
CON-DMA	18.60^{ab}	0.48^{bc}	3.44^{b}	0.28^{ab}	3.85^{a}	4.99^{a}	24.12^{b}	4.83^{b}	295.56^{c}	0.18^{c}	0.69^{a}
CON-FFF	17.32^{b}	0.99^{a}	5.19^{ab}	0.44^{ab}	2.35^{ab}	4.33^{a}	24.39^{b}	5.63^{b}	363.95^{bc}	0.30^{b}	0.40^{ab}
DMA-CON	17.57^{b}	0.54^{bc}	5.17^{ab}	0.10^{b}	1.01^{b}	1.96^{b}	24.30^{b}	12.40^{a}	664.10^{a}	0.23^{bc}	0.28^{b}
DMA-DMA	16.69^{b}	0.45^{c}	3.05^{b}	0.24^{b}	3.28^{a}	4.40^{a}	21.59^{c}	4.90^{b}	522.02^{a}	0.18^{c}	0.42^{ab}
FFF-CON	21.06^{a}	0.58^{b}	6.00^{a}	0.13^{b}	1.47^{b}	2.52^{b}	29.30^{a}	11.68^{a}	415.06^{b}	0.33^{ab}	0.36^{ab}
FFF-FFF	17.40^{b}	1.12^{a}	6.54^{a}	0.47^{a}	3.38^{a}	5.53^{a}	25.73^{b}	4.64^{b}	270.76^{c}	0.39^{a}	0.40^{ab}
SEM	1.551	0.074	0.680	0.057	0.363	0.607	1.421	0.942	66.287	0.025	0.085
Probabilities											
Strain	<0.001	<0.001	0.004	<0.001	<0.001	<0.001	<0.001	0.139	0.003	0.677	<0.001
Breeder diets	0.008	0.241	0.096	0.754	0.153	0.001	0.041	0.412	<0.001	<0.001	0.165
Offspring diet	0.005	<0.001	0.046	<0.001	<0.001	<0.001	0.007	<0.001	0.001	<0.001	<0.001
Strain × breeder diet	0.720	0.967	0.473	0.139	0.787	0.688	0.874	0.404	0.247	0.181	0.255
Strain × offspring diet	0.460	0.092	0.733	0.082	0.195	0.964	0.761	0.198	0.574	0.560	0.084

Values with different superscripts within each column are significantly different ($P < 0.05$). [1]Data are means of 10 birds per treatment (2 birds per cage). [2] LA: linoleic acid; ALA: Alpha-linolenic acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid are expressed as the proportion of total fatty acids. Total fatty acids are in milligram/100 mL [3] CON: Control; DMA: Microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid; FFF: Co-extruded full-fat flaxseed and pulse mixture (1:1 wt/wt), as a source of α-linolenic acid. [4]The day-old female pullets from breeders fed CON, DMA and FFF were divided into three (CON, DMA, and FFF), two (CON and DMA) and two (CON, FFF) post-hatch treatments, respectively. The order shows breeder diet and pullet diet (e.g., breeder diet-pullet diet, CON-CON).

Table 4. 7. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown and Shaver White breeders and/offspring on attributes of whole tibia and femur in 18-week-old pullets.[1]

Items		Tibia				Femur		
		BS[2], N/kg BW	Wt, g/kg BW	Ash, g/kg BW	Ash, %	Wt, g/kg BW	Ash, g/kg BW	Ash, %
Strain	Breeder diet							
ISA brown	CON	132.5^{bcd}	4.89	1.74	35.75	3.65^{a}	1.38^{b}	38.01
ISA brown	DMA	119.7^{cd}	4.69	1.67	35.62	3.51^{ab}	1.31^{b}	37.53
ISA brown	FFF	105.8^{d}	4.99	1.68	33.69	3.47^{ab}	1.20^{b}	34.77
Shaver white	CON	141.3^{bc}	4.27	1.63	38.35	3.15^{b}	1.31^{b}	41.83
Shaver white	DMA	183.3^{a}	4.18	1.71	41.03	3.50^{ab}	1.68^{a}	48.32
Shaver white	FFF	163.9^{ab}	4.42	1.72	39.03	3.34^{ab}	1.39^{b}	41.55
SEM		6.943	0.106	0.051	0.908	0.078	0.049	1.497
Main effect								
Strain								
ISA brown		119.3^{b}	4.86^{a}	1.70	35.12^{b}	3.55^{a}	1.29^{b}	36.95^{b}
Shaver white		162.8^{a}	4.29^{b}	1.68	39.31^{a}	3.30^{b}	1.44^{a}	43.60^{a}
SEM		4.629	0.069	0.034	0.594	0.051	0.032	0.980
Breeder diet								
CON		136.9	4.58	1.69	37.05	3.40	1.35^{b}	39.92^{ab}
DMA		151.5	4.44	1.69	38.32	3.50	1.49^{a}	42.92^{a}
FFF		134.9	4.70	1.70	36.36	3.41	1.29^{b}	38.16^{b}
SEM		4.909	0.075	0.045	0.642	0.067	0.035	1.058
Offspring diet[3]								
CON-CON		136.9	4.58	1.68	37.05	3.40	1.35	39.92
CON-DMA		142.7	4.65	1.73	37.48	3.51	1.40	40.34
CON-FFF		140.0	4.65	1.68	36.24	3.37	1.30	38.75
DMA-CON		151.5	4.44	1.69	38.32	3.51	1.49	42.92
DMA-DMA		160.1	4.39	1.72	39.37	3.49	1.54	44.16
FFF-CON		134.9	4.70	1.70	36.36	3.41	1.29	38.16
FFF-FFF		145.1	4.84	1.70	35.49	3.52	1.32	37.78
SEM		8.503	0.130	0.06	1.111	0.095	0.060	1.833
Probabilities (*P*-value)								
Strain		<0.001	<0.001	0.768	<0.001	<0.001	0.001	<0.001
Breeder diet		0.101	0.130	0.954	0.207	0.473	0.004	0.038
Offspring diet		0.170	0.426	0.870	0.443	0.293	0.102	0.183
Strain × breeder diet		0.001	0.906	0.305	0.273	0.016	0.001	0.124
Strain × offspring diet		0.372	0.444	0.845	0.535	0.068	0.998	0.791

Values with uncommon superscripts within each column are significantly different ($P < 0.05$).
[1]n=10. [2] Breaking strength. [3]The day-old female pullets from breeders fed CON, DMA and FFF were divided into three (CON, DMA and FFF), two (CON and DMA) and two (CON, FFF) post-hatch treatments, respectively. CON, control; DMA, micro-Algae (*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.[5] CON: Control

Table 4. 8. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown and Shaver White breeders and/offspring on attributes of tibia subparts in 18-week-old pullets[1]

Items		Epiphysis			Medullary			Cortical		
		Wt, 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, %
Strain	Breeder diet[2]									
ISA brown	CON	2.69	0.82	30.56	0.60	0.004	0.77	1.60	0.92^{abc}	58.12
ISA brown	DMA	2.55	0.78	30.83	0.62	0.004	0.70	1.53	0.88b^{c}	57.91
ISA brown	FFF	2.72	0.84	28.24	0.74	0.004	0.57	1.53	0.84^{c}	57.08
Shaver white	CON	2.38	0.74	31.33	0.43	0.005	1.22	1.47	0.89^{bc}	60.64
Shaver white	DMA	2.21	0.71	32.08	0.41	0.004	1.24	1.56	1.00^{a}	63.83
Shaver white	FFF	2.41	0.75	31.12	0.47	0.005	1.15	1.54	0.97^{ab}	62.76
SEM		0.080	0.049	0.824	0.076	0.0007	0.197	0.049	0.020	1.818
Main effect										
Strain										
ISA brown		2.66^{a}	0.82^{a}	29.87^{b}	0.64^{a}	0.004	0.68^{b}	1.56	0.88^{b}	57.70^{b}
Shaver white		2.34^{b}	0.73^{b}	31.51^{a}	0.44^{b}	0.005	1.21^{a}	1.52	0.94^{a}	62.41^{a}
SEM		0.043	0.026	0.615	0.041	0.0004	0.105	0.032	0.013	1.012
Breeder diet										
CON		2.53	0.78	30.94	0.51	0.004	1.00	1.54	0.90	59.38
DMA		2.38	0.74	31.46	0.51	0.004	0.97	1.55	0.94	60.87
FFF		2.56	0.80	29.68	0.60	0.004	0.86	1.54	0.90	59.82
SEM		0.056	0.349	0.583	0.054	0.0004	0.139	0.043	0.018	1.285
Offspring diet										
CON-CON		2.53	0.78	30.94	0.51	0.004	1.00	1.54	0.90	58.38
CON-DMA		2.52	0.80	31.71	0.56	0.005	1.22	1.56	0.93	59.98
CON-FFF		2.56	0.76	29.98	0.54	0.003	0.64	1.55	0.91	59.49
DMA-CON		2.38	0.74	31.46	0.51	0.004	0.97	1.55	0.94	60.87
DMA-DMA		2.37	0.76	31.96	0.49	0.005	1.35	1.56	0.96	61.55
FFF-CON		2.56	0.80	29.68	0.60	0.004	0.86	1.54	0.90	59.82
FFF-FFF		2.60	0.75	29.09	0.63	0.004	0.74	1.61	0.94	60.91
SEM		0.080	0.049	1.151	0.076	0.0007	0.197	0.061	0.025	1.833
Probabilities (*P*-value)										
Strain		<0.001	0.031	0.015	<0.001	0.144	0.001	0.432	<0.001	0.002
Breeder Diet		0.105	0.565	0.099	0.408	0.824	0.723	0.978	0.257	0.669
Offspring Diet		0.968	0.784	0.413	0.571	0.081	0.057	0.476	0.064	0.894
Strain × breeder diet		0.977	0.960	0.383	0.758	0.998	0.927	0.243	<0.001	0.505
Strain × offspring diet		0.558	0.841	0.630	0.984	0.997	0.800	0.675	0.678	0.827

Values with uncommon superscripts within each column are significantly different ($P < 0.05$).
[1]n=10. [2]The day-old female pullets from breeders fed CON, DMA and FFF were divided into three (CON, DMA and FFF), two (CON and DMA) and two (CON, FFF) post-hatch treatments, respectively. CON, control; DMA, micro-Algae (*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. of α-linolenic acid.

Table 4. 9. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown and Shaver White breeders and/offspring on attributes of femur subparts in 18 wks old pullets[1]

Items		Epiphysis			Medullary			Cortical		
		Wt, 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, %
Strain	Breeder diet[2]									
ISA brown	CON	2.08	0.72	34.69	0.37	0.003	0.99	1.20[b]	0.65[b]	55.16
ISA brown	DMA	1.95	0.67	34.92	0.41	0.003	0.92	1.15[b]	0.63[b]	55.18
ISA brown	FFF	1.96	0.61	31.59	0.46	0.003	0.65	1.06[b]	0.58[b]	55.47
Shaver white	CON	1.81	0.69	38.34	0.27	0.004	1.63	1.08[b]	0.62[b]	57.61
Shaver white	DMA	1.70	0.68	40.21	0.24	0.004	1.70	1.56[a]	1.00[a]	63.83
Shaver white	FFF	1.91	0.72	37.76	0.29	0.004	1.69	1.14[b]	0.67[b]	58.37
SEM		0.064	0.032	1.518	0.043	0.0005	0.224	0.051	0.028	1.457
Main effect										
Strain										
ISA brown		2.00[a]	0.67	33.87[b]	0.41[a]	0.003[b]	0.87[b]	1.14[b]	0.63[b]	55.25[b]
Shaver white		1.80[b]	0.70	38.71[a]	0.27[b]	0.004[a]	1.67[a]	1.23[a]	0.74[a]	59.60[a]
SEM		0.034	0.020	0.994	0.023	0.0002	0.146	0.027	0.015	0.954
Breeder diet										
CON		1.94	0.71	36.52	0.32	0.003	1.31	1.14[b]	0.64[b]	56.38
DMA		1.82	0.68	37.56	0.32	0.003	1.31	1.36[a]	0.81[a]	59.51
FFF		1.93	0.67	34.67	0.38	0.003	1.17	1.10[b]	0.62[b]	56.92
SEM		0.045	0.028	1.315	0.031	0.0003	0.194	0.036	0.020	1.262
Diet										
CON-CON		1.94	0.71	36.52	0.32	0.003	1.31	1.14	0.64	56.38
CON-DMA		1.99	0.73	37.33	0.33	0.003	1.23	1.20	0.66	55.80
CON-FFF		1.92	0.67	35.11	0.34	0.004	1.37	1.11	0.62	56.72
DMA-CON		1.82	0.68	37.56	0.32	0.003	1.31	1.36	0.81	59.51
DMA-DMA		1.82	0.69	38.41	0.28	0.004	1.63	1.39	0.84	59.85
FFF-CON		1.93	0.67	34.67	0.38	0.003	1.17	1.10	0.62	54.92
FFF-FFF		1.98	0.67	34.02	0.43	0.004	1.11	1.10	0.65	59.06
SEM		0.064	0.039	1.859	0.044	0.0005	0.274	0.052	0.029	1.785
Probabilities (*P*-value)										
Strain		<0.001	0.428	<0.001	<0.001	0.036	<0.001	0.004	<0.001	0.001
Breeder diet		0.101	0.477	0.295	0.303	0.944	0.831	<0.001	<0.001	0.152
Offspring iet		0.748	0.817	0.822	0.211	0.096	0.580	0.559	0.107	0.545
Strain × breeder diet		0.130	0.149	0.746	0.595	0.967	0.726	<0.001	<0.001	0.139
Strain × offspring diet		0.573	0.990	0.796	0.372	0.594	0.835	0.561	0.111	0.412

Values with different superscripts within each column are significantly different ($P < 0.05$).
[1]n=10.[2]The day-old female pullets from breeders fed CON, DMA and FFF were divided into three (CON, DMA and FFF), two (CON and DMA) and two (CON, FFF) post-hatch treatments, respectively. CON, control; DMA, micro-Algae (*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.

Table 4. 10. Coefficient of correlation analysis between serum bone-turnover markers and bone attributes in ISA brown and Shaver white pullets [1]

Item	OST[2] - 6 week	OST[3] - 12 week	TRAP[4]-18 week
ISA brown			
OST-12wk	0.409*		
TRAP-18wk	0.102	-0.031	
Tibia ash content (g: g BW[5])	0.441**	0.237	0.044
Tibia ash percentage (%)	0.421*	0.008	-0.057
Femur ash content (g: g BW)	0.006	-0.145	0.040
Femur ash percentage (%)	0.077	-0.148	-0.074
Tibia cortical ash content (g: g BW)	0.047	0.005	0.101
Tibia cortical ash percentage (%)	0.010	0.007	-0.123
Femur cortical ash content (g: g BW)	0.011	-0.077	0.188
Femur cortical ash percentage (%)	-0.021	0.143	-0.198
Shaver white			
OST-12wk	0.563**		
TRAP-18wk	0.072	-0.045	
Tibia ash content (g: g BW)	0.112	0.087	0.074
Tibia ash percentage (%)	-0.038	-0.054	-0.305*
Femur ash content (g: g BW)	-0.028	-0.089	-0.206
Femur ash percentage (%)	-0.071	-0.155	-0.387*
Tibia cortical ash content (g: g BW)	0.167	0.177	-0.089
Tibia cortical ash percentage (%)	0.113	-0.207	-0.037
Femur cortical ash content (g: g BW)	-0.088	-0.093	-0.305*
Femur cortical ash percentage (%)	-0.123	-0.374	-0.024

[1] * indicates <0.05 probability and ** indicates <0.001 probability.
[2] Osteocalcin measured at 6-wk.
[3] Osteocalcin measured at 12-wk.
[4] Tartrate resistant acid phosphatase measured at 18-wk.
[5] Bodyweight

Chapter 5. Impact of feeding sources of docosahexaenoic and α-linolenic acid to ISA brown and Shaver white layer breeders and their pullets on the subsequent impact on egg production, eggshell, and skeletal quality during laying phase[1]

5.1. Abstract

In chapter 4, breeder-feeding with DMA increased TBS and cortical mineral content in tibia and femur in Shaver white pullet at 18 WOA. This chapter follows up the subsequent impact of either breeder feeding, pullet-feeding, or both on egg production, plasma FA profile, eggshell quality, and bone attributes in 42 WOA hens. The remaining birds of chapter 4 were fed with a commercial diet without a supplemental source of n-3 PUFA. There was no interaction between strain and either breeder or offspring diets and no main effects of breeder and offspring diets on egg production, egg mass, and eggshell quality ($P > 0.05$). However, the strain effect on egg weight was such that ISA brown had heavier eggs and lower eggshell % than Shaver white ($P < 0.001$). Shaver white had higher TBS and tibia ash concentration compared with ISA brown ($P < 0.001$). There was no interaction between strain and breeder diet, no interaction between strain and offspring diet, and no main effects of breeder nor offspring diets on TBS, tibia epiphysis, and cortical parts and keel bone ($P > 0.05$). Breeder feeding of DMA and pullet feeding of either n-3 PUFA source increased tibia medullary ash concentration compared with other diets ($P < 0.001$). In conclusion, independent of breeder strain, provision of ALA and DHA to breeders and their offspring did not change egg production, egg quality traits, tibia structural bone, or keel bone at 42 WOA.

[1] Published in part: Akbari Moghaddam Kakhki, V.L. Shouldice, K. R., Price, J. Moats, and E.G. Kiarie. 2020. n-3 fatty acids fed to ISA brown and Shaver white breeders and their female progeny during rearing: Impact on egg production, eggshell and select bone attributes from 18 to 42 weeks of age. Poult. Sci. https://doi.org/10.1016/j.psj.2020.03.061.

5.2. Introduction

Although there are some reports on the potential effect of feeding with n-3 PUFA on bone attributes in laying hens (Baird et al., 2008; Josling et al., 2019), there is no report on the subsequent impact on skeletal properties after the removal of dietary n-3 PUFA. The positive effects of breeder feeding with fish oil on ash percentage and cortical density in offspring have been reported in day-old, 1, and 2 WOA quails (Liu et al. 2003b). In chapter 4, bone mass in the structural part of tibia and femur in 18 WOA Shaver white pullets was increased in response to breeder-feeding with DMA. The positive impact of n-3 PUFA on skeletal attributes after the removal of dietary sources of n-3 PUFA can originate from the optimized bone mineral density that can last long after the removal of n-3 PUFA, or long-lasting increase in the proportion of n-3 PUFA in FA profile, which can reduce the production of inflammatory mediators. Therefore, FA profiling might be an indicator of how the exposure to n-3 PUFA during embryonic and early-life periods can influence the body FA profile and the conversion of n-3 PUFA and n-6 PUFA (Cherian, 2015; Cherian and Sims, 2001). In addition, the possible increase in bone density might be reflected in eggshell quality since the skeletal system supplies around 40% of eggshell Ca (Elaroussi et al. 1994). Eggshell strength is one of several key elements in ensuring the safety and integrity of the egg contents. About 10% of downgraded eggs at grading stations are rejected due to inferior eggshell quality (Kemps et al. 2006). It was hypothesized that the positive effect of feeding n-3 PUFA on bone properties in pullets would last in the laying phase and might influence the eggshell quality as well.

5.3. Materials and methods

5.3.1. Birds and management

After the onset of lay, all the birds of each dietary treatment from the previous chapter were fed with a commercial diet without a supplemental source of n-3 PUFA containing 39.16% LA, 2.79% ALA (of total fat), and 4.55% total fat throughout the laying phase (Floradale Feed Mill, Floradale, ON, Canada). Based on the Canadian National Farm Animal Care Council, each bird must be provided with a minimum space allowance of 67.0 square inches for white birds and 75.0 square inches for brown birds after the age of maturity (NFACC, 2017). After 18 WOA, the population of each cage was reduced to eight birds for ISA brown and nine birds for Shaver white, giving 75 and 67 square inches for ISA brown and Shaver white hens.

5.3.2. Sampling

ISA brown and Shaver white pullets were kept separately in two cage aisles in one house. Based on the original schedule, the eggshell quality analysis was planned to start from 22 WOA. However, a mechanical malfunction of the watering system and subsequent water shortage occurred on 21 WOA in the cage aisle of Shaver white birds, resulting in mortality and drop in egg production. Before the incident, the egg production was 61.4%, which dropped to 26.8% due to the water shortage. The same cage density (seven Shaver white and six ISA brown) was maintained for both strains starting at 22 WOA. The BW was measured at 22, 23, and 24 WOA as an indicator of the recovery. Birds were given two weeks of recovery time to reach the same egg production rate as before the incident. Starting at 25 WOA egg numbers were recorded daily, and half of the produced eggs were randomly collected, labeled, weighed, and kept at 4°C for eggshell quality analysis every four weeks. The last eggshell quality measurement was performed on the last day of the 41st week. The eggshell quality testing was performed within the 48-h post egg collection.

At 42 WOA, birds were weighed, then euthanized for tibia and keel bone sampling. Hens with a hard eggshell in the shell gland were chosen as described by Akbari Moghaddam Kakhki et al. (2019a). Keel bone, left and right tibias were dissected, de-fleshed, and stored at -20˚C for further analysis.

Blood samples were taken at 42 WOA to measure the FA profile in the plasma. Blood samples were centrifuged at 2,500 × g for 15 min at 4°C and stored at -80°C until required for analysis, as described in chapter 4. The plasma concentration of LA, ALA, AA, EPA, DHA, and total FA were determined. The concentrations of other n-3 and n-6 PUFA were below the detection limit or lower than 0.50 % of total FA.

5.3.3. Sample analyses

Eggshell thickness (**EST**) and eggshell breaking strength (**ESBS**) were measured, according to Mwaniki et al. (2018). Eggshell thickness (mm) was measured using a high-resolution non-destructive device with precision ultrasound (ORKA Food Technology Ltd., Ramat HaSharon, Israel), and ESBS was measured by Force Reader (ORKA Food Technology Ltd. Ramat HaSharon, Israel). Eggs then were cracked open, and the eggshells were washed with water, dried for 24 hours at 105°C, and weighed as described by Akbari Moghaddam Kakhki et al. (2019a). The left tibia was used for measuring the dry weight, ash content, and ash percentage in epiphysis, medullary and cortical sub-parts. Sub-parts were separated, according to Akbari Moghaddam Kakhki et al. (2019b) and followed by measuring dry weight, ash content, and ash percentage of tibia subparts as well as keel bone as described in chapter 4. The breaking strength test was performed on the right tibia, as described in chapter 4.

5.3.4. Calculations and statistical analyses

The normality of data was tested using the UNIVARIATE plot normal procedure (SAS 9.4). To demonstrate how dietary treatments modified bone attributes through the lay cycle compared with 18 WOA, the measured bone attributes (total bone and subparts) at 42 WOA were divided by previously reported values from the pullet phase, multiplied by 100 and reported as a change index (**CI**, Akbari Moghaddam Kakhki., 2018b). Data were subjected to nested factorial arrangement for including a two-way ANOVA in two strains (ISA brown and Shaver white) and three breeder diets factorial arrangement as well as a two-way ANOVA in strains and post-hatch diets factorial arrangement using GLIMMIX procedure (SAS 9.4). Egg weight was considered as a covariate for analyzing data of EST and ESBS. Significance was declared at $P < 0.05$.

5.4. Results

5.4.1 Egg production and eggshell quality

Hen-day egg production was not affected by strain, breeder diet, offspring diet, or interactions ($P > 0.05$; Table 5.1). Egg weight was affected by the interaction between diet and strain ($P = 0.008$). This interactive effect did not show any pattern within each strain, however, Shaver white hens from every post-hatch treatment with the exception of CON-FFF produced lighter weight eggs compared with ISA brown hen from all the dietary treatments. The ISA brown hens had 5.41 g heavier eggs compared with Shaver white ($P < 0.001$). Egg mass was not affected by the interaction between strain and breeder diet, the interaction of strain and offspring diet, or the main effect of breeder and offspring diets ($P > 0.05$). ISA brown hens had 4.76g heavier egg mass compared with Shaver white ($P < 0.001$). The eggshell thickness and absolute eggshell weight were not affected by the interaction between strain and breeder diet, the interaction between strain and offspring diet or the main effect of strain, breeder diet, and offspring diet (Table 5.2; *P*

> 0.05). Shaver white had greater ESBS and percentage of eggshell to egg weight compared with ISA brown ($P < 0.001$). However, the interaction between strain and breeder diet, the interaction between strain and offspring diet, or the main effect of breeder diet and offspring diet did not affect ESBS and the percentage of the eggshell ($P > 0.05$).

5.4.2. Plasma Fatty Acid Profiles

The percent composition of LA, ALA, AA, DHA, as well as ∑n-3 PUFA, ∑n-6 PUFA, ∑n-6: ∑n-3 PUFA ratio, and total fat at 42 WOA were not affected by the interaction between strain and breeder diets ($P > 0.05$; Table 5.3). ISA brown hens had lower total fat compared with Shaver white hens ($P < 0.001$). The breeder and offspring diet did not influence the percent composition of FA and total fat in plasma at 42 WOA.

5.4.3. Calculated n-3 and n-6 PUFA desaturation and elongation

There was no interaction between strains and diets on the activity of n-6 and n-3 PUFA desaturation and elongation ($P > 0.05$; Table 5.3). Shaver white hens showed higher n-6 PUFA conversion compared to ISA brown hens ($P < 0.001$), while there was no difference among strains in n-3 PUFA conversion ($P = 0.115$). The interaction between strain and breeder diet affected the n-6 PUFA conversion such that breeder feeding with DMA reduced and breeder-feeding with FFF increased n-6 PUFA conversion in Shaver white hens. At 42 WOA, hens of the CON-DMA and FFF-FFF had a higher n-6 PUFA conversion compared with the rest of the treatments ($P < 0.001$). The n-3 PUFA conversion was higher in pullets of CON-DMA compared to CON-CON and DMA-CON ($P < 0.001$). However, these effects in n-3 PUFA conversion were not observed in 42 WOA hens ($P > 0.05$).

5.4.4. Tibia and keel bones attribute at 42 WOA

The BW and the majority of the tibia attributes were not influenced by the interaction between strain and breeder diet nor the main effect of breeder and offspring diets ($P > 0.05$; Tables 5.4 and 5.5). ISA brown hens were 402.3 g heavier than Shaver white hens ($P < 0.001$; Table 27). Shaver white hens had 18.5% and 1.3% higher whole TBS and ash concentration, respectively, compared with ISA brown hens ($P < 0.05$; Table 27). In addition, Shaver white hens had 14.9 and 12.1 % lower whole tibia and ash weight ($P < 0.05$). Tibia breaking strength, weight, and ash weight were affected by the interaction between strain and offspring diets, in which ISA brown of breeder fed with CON had higher tibia weight ISA brown hens from breeder fed with DMA and Shaver white hens from every treatment ($P < 0.05$). Ash weight of ISA brown from breeder fed with CON was higher than Shaver white hens from breeders with CON or DMA.

ISA brown hens had heavier tibia epiphysis compared with Shaver white ($P < 0.001$) Although ISA brown hens showed heavier tibia medullary bone than Shaver whites (0.46 *vs.* 0.39 g/kg BW, $P < 0.01$), Shaver white hens had greater tibia medullary ash concentration than ISA brown hens (21.4 *vs.* 17.4%, $P < 0.01$). ISA brown hens had 14.2% and 11.4% greater tibia cortical weight and ash content compared with Shaver white, whereas Shaver white hens had 2.7 % higher tibia cortical ash concentration than ISA brown hens ($P < 0.001$). The main effect of breeder diets on tibia subparts was such that ash weight and ash percentage in offspring were increased by breeder-feeding of DMA relative to CON and FFF ($P < 0.005$). The main effect of the offspring diet on medullary ash content and ash concentration was such that hens from CON-CON had the lowest values compared with other dietary treatments ($P < 0.001$). Hens fed DMA-CON had higher ($P < 0.001$) tibia medullary ash concentration than hens in FFF-CON and FFF-FFF.

There was no interaction between strain and breeder diet, the interaction between strain and offspring diet, nor the main effects of breeder and offspring diets on keel bone attributes ($P > 0.05$; Table 5.6). The strain effect was such that ISA brown hens had 23.5%, 29.1%, and 1.1% more keel weight, ash content, and ash concentration, respectively, compared with Shaver white hens ($P < 0.05$).

5.4.5. Evolution of tibia attributes from 18 to 42 WOA

There was no strain, breeder diet, offspring diet, or interaction effects on CI of body and whole tibia weight between 18 and 42 WOA ($P > 0.05$; Table 5.7). There was neither interaction between strain and breeder diet, strain and offspring diet, nor the main effect of breeder and offspring diets on CI of whole tibia BS, ash content, and ash concentration ($P > 0.05$). However, the strain effect was such that Shaver white hens showed a greater decline in whole tibia BS (83.7% *vs.* 96.3%) and ash content (84.1% *vs.* 94.3%) at 42 WOA relative to 18 WOA than ISA brown hens. Moreover, the CI of whole tibia ash concentration was lower in Shaver white hens than ISA brown hens (117% *vs.* 128%). There was no strain, breeder and offspring diets, and interaction effects on CI of tibia epiphysis and medullary weight, ash content, and ash concentration ($P > 0.05$; Table 5.8). The only strain effect was observed on CI of tibia cortical attributes, in this context, Shaver whites exhibited lower 42 WOA decline in cortical weight (70. 1% *vs.* 78.7%), ash content (75.1% *vs.* 89.1%), and ash percentage (107.1% *vs.* 113.9%) compared with ISA brown ($P < 0.05$).

5.5. Discussion

In this study, the water system malfunction only occurred in the Shaver white hens, which could be associated with the difference among strains by effecting BW. Bodyweight measurement before the incident was 1436.8 g. The BW after the incident was 1655.5 g, or 15.22% higher than

BW of 18 WOA. The average BW in those found dead was only 4.9% higher than BW of 18 WOA. After the recovery period (23 WOA), the BW was 1719.8 g, or 19.7% higher than BW of 18 WOA values. At 24 WOA, the BW increase relative to 18 WOA was calculated as 20.1%. In addition, the production performance was reached to recommended values presented in Shaver white breed guideline. Therefore, the effect of the water system malfunction was negligible on measured parameters.

The quality of the eggshell has a considerable impact on the profitability of the egg industry (Lichovnikova, 2007). In the current study, supplementing either breeder, offspring, or both diets with n-3 PUFA but providing an unsupplemented layer diet did not affect egg production and eggshell quality. This finding was consistent with previous reports regarding the impact of feeding n-3 PUFA on egg production and eggshell quality (Amini and Ruiz-Feria, 2007; Nain et al., 2012; Wu et al., 2018). Supplementation of a diet based on corn, oat, soybean, and canola meals with up to 15% of flaxseed products (same source as FFF in the current study) did not influence the egg weight and eggshell thickness (Nain et al. 2012). Supplementation of pearl millet and soybean meal diet with up to 12% flaxseed did not affect egg production or quality (i.e., egg mass, feed intake, eggshell weight, and thickness) of 62 WOA White Leghorn hens (Amini and Ruiz-Feria 2007). Egg production and egg weight were not influenced when 37 WOA Lohmann brown hens were fed diets supplemented with up to 8% microalgae (*Nannochloropsis Sp.*; Wu et al. 2018). Ao et al. (2016) found that egg weight, eggshell percentage, and ESBS were not impacted by 1 or 2% inclusion of DMA to corn and soy diets of 17 to 70 WOA Hy-Line W 36 layers. Koppenol et al. (2014b) observed no effect on egg production, egg mass, eggshell weight, and thickness when Ross 308-broiler breeders were fed diets supplemented 1.5% of either soybean oil, tuna fish oil, or fish oil with EPA/DHA ratio of 0.8, 0.4, and 2.1, respectively. However, supplementation with

tuna fish oil or fish oil decreased the egg weight compared to soybean oil. Supplementing corn, wheat middlings, and soybean meal diet with 10% flaxseed did not affect egg production, egg mass, egg weight, and eggshell thickness in 24 WOA ISA brown hens (Hayat et al. 2009). Conversely, Cachaldora et al. (2006) observed a linear reduction in egg production and eggshell thickness in response to increasing marine fish oil level from 15 to 60 g/kg in the corn-barley-soybean meal diet of 44 WOA Warren laying hens. However, feed intake and egg weight were not affected by the level of marine fish oil.

Feed intake, body growth and development, sexual maturity, egg production efficiency, skeletal health (Khanal et al., 2019), and eggshell quality (Ketelaere et al., 2002) differs between layer hen strains. In the current study, the strains possessed different characteristics in egg production and eggshell quality. ISA brown hens showed higher egg weight and egg mass, but lower eggshell quality. In the current study, ISA brown hens maintained a lower ratio of ∑n-6: ∑n-3 PUFA in their plasma compared to Shaver white hens. The differences in plasma FA profiles of various strains of layer hens are unknown. In chapter 4, ISA brown pullets maintained a higher ∑n-6 and lower ratio of ∑n-6: ∑n-3 PUFA compared to Shaver white pullets. In chapter 3, the results showed that Shaver white layer breeders had higher efficiency of depositing n-3 PUFA in their eggs and higher n-3 PUFA conversion than ISA brown layer breeders.

EPA and DHA concentration were 0.26% and 2.42%, respectively as a percent composition of total FA at 18 WOA, which were dropped to 0.03% and 0.87 % at 42 WOA (chapter 4). Over the embryonic period, the embryos of tested diets consumed more n-3 PUFA than the CON birds (chapter3). The first destination for lipoproteins in a young bird is the liver, and it has been reported that n-3 PUFA can last approximately 30 days after the removal of dietary sources (Cherian, 2015).

Consequently, it is possible that had the birds been tested at an earlier age, either the pullet effect, breeder effect, or both on the FA profile in hens may have been noticed.

As previously suggested, in chapter 4, n-3 PUFA can exert their long-lasting effect through modification in the pattern of DNA methylation. An increase in expression of elongase, Δ-6, Δ-5 desaturase enzymes, and persistent changes in the PUFA content of cell membrane has been reported in response to prenatal exposure to n-3 PUFA (Mennitti et al., 2015). There was no effect of breeder-diet on n-3 PUFA in 42 WOA hens, showing the effect of breeder-feeding with n-3 PUFA on the FA profile of 18 WOA pullets did not last after the removal of dietary supplemental n-3 PUFA. It has been reported that feeding breeders n-3 PUFA increased DHA content in broiler chickens lasting for 28 days while it kept the hepatic content arachidonic acid low for 40 days (Koppenol et al., 2014).

After sexual maturity, OSB-bone formation switches from the formation of the cortical bone layer towards the development of the medullary bone (Whitehead 2004). As the laying cycle progresses, OSC resorption of structural bone continues mainly within the long bones, which leads to a decrease in structural bone content; this weakens the bones (Whitehead 2004). Progressive deterioration of structural bone throughout the laying cycle increases the susceptibility of fractures and osteoporotic mortality (Whitehead 2004).

Changes in dietary concentration of Ca and vitamin D3 after the onset of lay have been reported not to be effective in alleviating the adverse effect of aging on structural bones (Akbari Moghaddam Kakhki et al. 2019b). It has been suggested that nutritional strategies aiming to minimize osteoporosis should not be focused on late-phase of the lay cycle or when there is a high

risk of osteoporosis and that they should preferably be implemented in early stages of skeletal development (Akbari Moghaddam Kakhki et al. 2018b).

All birds were fed with a commercial diet without a supplemental source of n-3 PUFA, after the onset of lay, to examine the impact of the breeder and pullet feeding of n-3 PUFA on the tibia and keel attributes in the post-peak of egg production. Hens from breeders fed with DMA had greater ash content and ash percentage in their tibia medullary. In addition, feeding of DMA and FFF to pullets from either breeders fed with tested diets or CON increased the ash content and ash percentage compared to pullets from CON-CON. However, there was no change in structural parts in response to the breeder and pullet-phase feeding of n-3 PUFA, showing that the positive impact of n-3 PUFA on the structural bone did not last after the removal of dietary source of n-3 PUFA.

There are contradictory results in mammalian studies regarding the effect of prenatal and early-life feeding of n-3 on bone development in later-life. Korotkova et al. (2004) tested the effect of feeding rats in their late pregnancy diets containing either 70 g linseed oil (as a source of n-3 PUFA), soybean oil (as a source of n-6 and n-3 PUFA), or sunflower-seed oil (as a source of n-6 FA) on bone development in their offspring at 30-d. Their results showed that maternal feeding a diet containing soybean oil increased BW, femur length, cortical density, thickness, and area (Korotkova et al. 2004). Although the PUFA levels in diets were not presented, the authors concluded the lower total amount of PUFA in the diet of linseed oil was responsible for the lower bone parameters compared with the soybean oil group (Korotkova et al. 2004). Korotkova et al. (2005) observed that feeding essential FA-deficient diets to rats during late gestation and throughout lactation increased BW, cortical content, area, and thickness while it decreased the trabecular bone density of femur in 44-d old pups. However, the bone attributes were not normalized based on the BW (Korotkova et al. 2005).

It has been reported that n-3 PUFA supplemented diets can reduce keel bone fracture likely without a detrimental effect on production depending on the quantities of n-3 PUFA as a high level of n-3 PUFA may lead to health and production detriments (Toscano et al., 2015). However, whether the protective effects of n-3 PUFA can translate to bone metabolism remains unknown and must be further explored (Toscano et al., 2015).

Differences in feed intake, body growth, and development, sexual maturity, egg production efficiency, skeletal health differ between strains of the layer hen (Khanal et al. 2019). In the laying phase, the strains possessed different traits in the tibia and keel bone. The tibia in Shaver white hens was stronger compared with ISA brown hens, while ISA brown hens maintained higher ash content and ash percentage in their keel bone. The findings of chapter 4 showed that 18 WOA Shaver white pullets had higher tibia and femur ash concentration compared with ISA brown pullets. In addition, the difference in bone metabolism among strains was demonstrated not only by the lack of effect of feeding of n-3 PUFA on tibia characteristics in ISA brown pullets versus an effect in the Shaver white pullets but also higher activity of OSC and lower activity of OSB in ISA brown compared with Shaver white (chapter 4). However, the rate of mineral mass loss among treatments and strains was measured by calculating CI value based on the difference between sampling at 42 WOA and 18 WOA. Lower CI values for TBS, ash content, ash percentage, in cortical bones in Shaver white compared with ISA brown hens demonstrated the faster erosion rate and depletion of mineral in the tibia of Shaver white hens compared with ISA brown. This difference emphasizes the importance of considering the role of genetic effect in studying nutritional strategies to improve bone quality in poultry.

5.6. Conclusions

Embryonic and rearing exposure to n-3 PUFA did not affect egg production, eggshell and bone quality in the unsupplemented layer phase of 42 WOA ISA brown, and Shaver white hens. The strains showed differences in their production performance, eggshell quality, and bone properties. These differences highlighted the importance of considering the strain effect in designing and interpreting the results of nutritional and management studies. Embryonic or pullet exposure to either type of n-3 PUFA did not influence plasma FA profile in 42 WOA hens. Either embryonic exposure, pullet exposure, or both did not affect TBS and its structural parts in unsupplemented 42 WOA hens. However, the effect of the n-3 PUFA source on tibia medullary characteristics was dependent on the phase of feeding and type of n-3 PUFA source. Only breeder-feeding with DMA increased the tibia medullary ash content and percentage while feeding either sources of n-3 PUFA during the pullet phase increased ash content and percentage in tibia medullary compared to the CON-CON group in 42 WOA ISA brown and Shaver white hens.

5.7. Table and figures

Table 5. 1. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown and Shaver white breeders and their pullets on laying hen egg production from 25 to 42 weeks of age.[1,2]

Items		Hen-day egg production, %	Egg weight, g	Egg mass, g/bird/day
Strain	Diet[3]			
ISA brown	CON-CON[4]	91.59	65.44[a]	59.77
ISA brown	CON-DMA	93.96	64.02[ab]	60.22
ISA brown	CON-FFF	94.31	63.64[ab]	60.04
ISA brown	DMA-CON	95.89	63.81[ab]	61.21
ISA brown	DMA-DMA	92.23	66.54[a]	61.32
ISA brown	FFF-CON	90.77	65.11[a]	59.18
ISA brown	FFF-FFF	95.48	64.66[ab]	61.77
Shaver white	CON-CON	93.40	59.00[c]	55.20
Shaver white	CON-DMA	94.51	59.55[c]	56.36
Shaver white	CON-FFF	94.29	61.39[bc]	58.03
Shaver white	DMA-CON	90.31	59.25[c]	53.50
Shaver white	DMA-DMA	94.24	59.17[c]	55.81
Shaver white	FFF-CON	94.90	58.41[c]	55.51
Shaver white	FFF-FFF	95.71	59.58[c]	57.07
SEM		1.809	0.681	1.248
Main effect				
Strain				
ISA brown		94.12	64.81[a]	60.58[a]
Shaver white		94.35	59.40[b]	55.82[b]
SEM		0.393	0.262	0.480
Breeder diet				
CON		94.17	62.17	58.27
DMA		93.88	62.19	57.96
FFF		94.82	61.94	58.38
SEM		0.514	0.341	0.624
Offspring diet				
CON-CON		94.17	62.17	58.27
CON-DMA		94.24	61.78	58.29
CON-FFF		94.30	62.52	59.04
DMA-CON		93.88	62.19	57.96
DMA-DMA		93.23	62.86	58.56
FFF-CON		94.82	61.94	58.38
FFF-FFF		95.59	62.12	59.42
SEM		0.705	0.482	0.882
Probabilities (*P*-value)				
Strain		0.686	<0.001	<0.001
Breeder diets		0.396	0.839	0.882
Offspring diet		0.381	0.276	0.276
Strain × breeder diet		0.662	0.121	0.152
Strain × offspring diet		0.216	0.008	0.723

Values with uncommon superscripts within each column are significantly different ($P < 0.05$). [1] Data are means of 5 replications per each treatment. [2] Laying hens during 25 to 42 weeks of age were not fed the experimental diets. These diets were only fed in the breeder and offspring pullet stage. [3] CON: Control; DMA: Microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid; FFF: Co-extruded full-fat flaxseed and pulse mixture (1:1 wt/wt), as a source of α-linolenic acid. [4] The day-old female pullets from breeders fed CON, DMA and FFF were divided into three (CON, DMA, and FFF), two (CON and DMA) and two (CON, FFF) post-hatch treatments, respectively.

Table 5. 2. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown and Shaver white breeders and their pullets on laying hen eggshell quality from 25 to 42 weeks of age.[1,2]

Items	Eggshell thickness, mm	Eggshell breaking strength, Kgf	Eggshell	
			Weight, g	%
Main effect				
Strain				
ISA brown	0.429	4.24[b]	6.40	9.9[b]
Shaver white	0.430	5.01[a]	6.50	11.1[a]
SEM	0.002	0.051	0.039	0.069
Breeder diet[3]				
CON	0.428	4.63	6.45	10.5
DMA	0.433	4.63	6.42	10.5
FFF	0.427	4.62	6.48	10.5
SEM	0.002	0.068	0.053	0.091
Offspring diet[4]				
CON-CON	0.428	4.63	6.45	10.5
CON-DMA	0.430	4.64	6.42	10.4
CON-FFF	0.426	4.57	6.47	10.6
DMA-CON	0.432\3	4.63	6.42	10.5
DMA-DMA	0.435	4.63	6.46	10.6
FFF-CON	0.427	4.62	6.48	10.5
FFF-FFF	0.426	4.57	6.51	10.5
SEM	0.003	0.097	0.075	0.129
Probabilities (*P*-value)				
Strain	0.901	<0.001	0.094	<0.001
Breeder diets	0.088	0.989	0.772	0.971
Offspring diet	0.886	0.921	0.854	0.395
Strain × breeder diet	0.516	0.240	0.606	0.928
Strain × offspring diet	0.863	0.620	0.281	0.432

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

[1] Data are means of 5 replications per each treatment.

[2] Laying hens during 25 to 42 weeks of age were not fed the experimental diets. These diets were only fed in the breeder and offspring pullet stage.

[3] CON: Control; DMA: Microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid; FFF: Co-extruded full-fat flaxseed and pulse mixture (1:1 wt/wt), as a source of α-linolenic acid.

[4] The day-old female pullets from breeders fed CON, DMA and FFF were divided into three (CON, DMA, and FFF), two (CON and DMA), and two (CON, FFF) post-hatch treatments, respectively.

Table 5. 3. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown and Shaver white breeders and offspring during rearing on plasma concentration (%) of fatty acids in 42-week-old hens[1]

Item[2]		LA	ALA	AA	DHA	∑n3	∑n6	∑n6: ∑n3	Total fat	n-6 conversion	n-3 conversion
Strain	Breeder diet										
ISA brown	CON	15.98	0.44	2.56	0.86	1.46	19.86	13.57	509.79	0.16^{bc}	0.04
ISA brown	DMA	16.18	0.43	2.65	0.87	1.50	20.11	13.45	482.88	0.15^{bc}	0.03
ISA brown	FFF	16.66	0.44	2.37	0.87	1.48	20.47	13.86	421.41	0.14^{c}	0.04
Shaver white	CON	15.78	0.41	2.38	0.82	1.44	19.37	13.44	758.10	0.17^{b}	0.06
Shaver white	DMA	16.64	0.47	2.56	0.92	1.64	20.53	12.49	678.13	0.15^{c}	0.04
Shaver white	FFF	15.47	0.44	2.41	0.80	1.42	19.23	13.54	733.46	0.18^{a}	0.06
SEM		0.354	0.029	0.066	0.031	0.064	0.300	0.724	24.407	0.005	0.013
Main effect											
Strain											
ISA brown		15.75	0.44	2.63	0.90	1.51	19.76	13.08	493.18^{b}	0.15^{b}	0.04
Shaver white		15.60	0.45	2.56	0.83	1.49	19.49	13.06	755.24^{a}	0.17^{a}	0.06
SEM		0.992	0.016	0.037	0.036	0.098	0.825	0.519	13.149	0.003	0.009
Breeder diet[3]											
CON		15.88	0.42	2.47	0.84	1.45	19.61	13.50	633.95	0.16	0.05
DMA		16.41	0.45	2.61	0.89	1.57	20.32	12.95	580.51	0.15	0.04
FFF		16.06	0.44	2.39	0.83	1.45	19.85	13.70	577.44	0.16	0.05
SEM		1.152	0.021	0.053	0.053	0.115	0.998	0.641	16.811	0.003	0.010
Offspring diet[4]											
CON-CON		15.88	0.42	2.47	0.84	1.45	19.61	13.50	633.95	0.15^{b}	0.08
CON-DMA		15.02	0.43	2.69	0.82	1.47	18.99	12.95	720.07	0.18^{a}	0.04
CON-FFF		15.99	0.48	2.36	0.81	1.47	19.62	13.34	606.07	0.15^{b}	0.04
DMA-CON		16.40	0.45	2.63	0.89	1.58	20.33	12.95	581.00	0.15^{b}	0.04
DMA-DMA		15.45	0.41	2.31	0.93	1.53	19.46	12.73	592.05	0.15^{b}	0.03
FFF-CON		16.09	0.44	2.39	0.83	1.45	19.85	13.70	577.44	0.14^{b}	0.04
FFF-FFF		14.92	0.46	2.63	0.93	1.57	19.50	12.40	659.37	0.18^{a}	0.06
SEM		1.449	0.026	0.068	0.079	0.184	1.300	0.901	25.573	0.005	0.015
Probabilities											
Strain		0.885	0.600	0.086	0.091	0.134	0.810	0.909	<0.001	<0.001	0.115
Breeder diets		0.241	0.797	0.109	0.418	0.521	0.074	0.754	0.654	0.072	0.673
Offspring diet		0.270	0.855	0.630	0.527	0.715	0.106	0.877	0.091	<0.001	0.227
Strain × breeder diet		0.154	0.441	0.321	0.748	0.796	0.487	0.650	0.078	<0.001	0.890
Strain × offspring diet		0.782	0.906	0.711	0.871	0.815	0.821	0.986	0.674	0.101	0.414

Values with different superscripts within each column are significantly different ($P < 0.05$). [1]Data are means of 10 birds per treatment (2 birds per cage). [2] LA: linoleic acid; ALA: Alpha-linolenic acid; AA: arachidonic acid; EPA: eicosapentaenoic acid are expressed as proportion of total fat acids. [3] CON: Control; DMA: Microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid; FFF: Co-extruded full fat flaxseed and pulse mixture (1:1 wt/wt), as a source of α-linolenic acid. [4] The day-old female pullets from breeders fed CON, DMA and FFF were divided into three (CON, DMA and FFF), two (CON and DMA) and two (CON, FFF) post-hatch treatments, respectively. All birds were fed a standard laying diet from 19 WOA.

Table 5. 4. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown and Shaver white breeders and their pullets on attributes of the whole tibia from 42-week-old laying hens.[1,2]

Items		BW, g	Tibia			
			Breaking strength [3]	Weight, g/kg BW	Ash weight, g/kg BW	Ash concentration, %
Strain	Breeder diet					
ISA brown	CON	2,272.5	117.7^{abc}	3.71^{a}	1.63^{a}	44.02
ISA brown	DMA	2,309.2	109.8^{bc}	3.38^{bc}	1.55^{ab}	44.97
ISA brown	FFF	2,267.7	101.2^{c}	3.57^{ab}	1.55^{ab}	44.40
Shaver white	CON	1,894.1	122.9^{ab}	3.02^{d}	1.35^{c}	44.70
Shaver white	DMA	1,894.0	132.2^{ab}	3.04^{cd}	1.40^{bc}	46.08
Shaver white	FFF	1,854.4	134.6^{a}	3.21^{cd}	1.49^{abc}	46.38
SEM		43.756	5.618	0.066	0.042	0.527
Main effect						
Strain						
ISA brown		2,283.1^{a}	109.6^{b}	3.55^{a}	1.58^{a}	44.46^{b}
Shaver white		1,880.8^{b}	129.9^{a}	3.09^{b}	1.41^{b}	45.72^{a}
SEM		28.64	3.058	0.047	0.027	0.351
Breeder diet[4]						
CON		2,083.3	120.3	3.37	1.49	44.36
DMA		2,101.6	121.0	3.21	1.48	45.52
FFF		2,061.1	117.9	3.39	1.52	45.39
SEM		37.89	3.973	0.058	0.031	0.457
Offspring diet[5]						
CON-CON		2,120.2	114.6	3.29	1.47	44.46
CON-DMA		2,069.9	121.1	3.42	1.51	44.19
CON-FFF		2,059.9	125.1	3.39	1.50	44.43
DMA-CON		2,060.5	121.2	3.25	1.48	45.52
DMA-DMA		2,142.6	120.8	3.24	1.47	45.53
FFF-CON		2,036.6	115.9	3.41	1.53	45.04
FFF-FFF		2,085.6	119.9	3.30	1.51	45.74
SEM		53.59	5.618	0.088	0.053	0.646
Probabilities (*P*-value)						
Strain		<0.001	<0.001	<0.001	<0.001	0.017
Breeder diets		0.752	0.841	0.071	0.614	0.096
Offspring diet		0.678	0.731	0.732	0.943	0.952
Strain × breeder diet		0.909	0.025	0.035	0.022	0.543
Strain × offspring diet		0.188	0.383	0.642	0.338	0.103

Values with uncommon superscripts within each column are significantly different ($P < 0.05$).
[1] n=5, [2] Laying hens during 25 to 42 weeks of age were not fed the experimental diets. These diets were only fed in the breeder and offspring pullet stage. [3] Breaking strength N/ kg BW.
[5] CON: Control; DMA: Microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid; FFF: Co-extruded full-fat flaxseed and pulse mixture (1:1 wt/wt), as a source of α-linolenic acid.

Table 5. 5. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown and Shaver white breeders and their pullets on attributes of tibia subparts from 42-week-old laying hens.[1,2]

Items	Epiphysis			Medullary			Cortical		
	Wt, 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, %
Main effect									
Strain									
ISA brown	1.90[a]	0.72[a]	38.06	0.46[a]	0.08	17.37[b]	1.21[a]	0.78[a]	64.62[b]
Shaver white	1.64[b]	0.62[b]	37.92	0.39[b]	0.08	21.36[a]	1.06[b]	0.70[b]	66.34[a]
SEM	0.031	0.013	0.509	0.007	0.002	0.399	0.022	0.014	0.293
Breeder diet[3]									
CON	1.80	0.67	37.10	0.42	0.08[b]	18.48[b]	1.15	0.75	65.13
DMA	1.73	0.67	38.59	0.42	0.09[a]	21.42[a]	1.09	0.72	65.93
FFF	1.77	0.68	38.74	0.43	0.08[b]	18.63[b]	1.16	0.76	65.56
SEM	0.041	0.017	0.662	0.009	0.003	0.528	0.029	0.019	0.381
Offspring diet									
CON-CON	1.77	0.68	38.27	0.41	0.06[c]	13.68[c]	1.11	0.73	65.63
CON-DMA	1.80	0.66	36.27	0.43	0.09[ab]	20.71[ab]	1.19	0.77	64.72
CON-FFF	1.82	0.66	36.76	0.41	0.09[ab]	21.04[ab]	1.16	0.75	65.04
DMA-CON	1.73	0.67	38.53	0.43	0.10[a]	22.58[a]	1.08	0.71	66.02
DMA-DMA	1.73	0.67	38.64	0.41	0.08[ab]	20.26[ab]	1.10	0.72	65.85
FFF-CON	1.81	0.69	38.55	0.44	0.08[ab]	18.07[b]	1.17	0.76	65.26
FFF-FFF	1.73	0.67	38.93	0.42	0.08[ab]	19.20[b]	1.15	0.75	65.85
SEM	0.059	0.024	0.936	0.013	0.004	0.747	0.040	0.026	0.539
Probabilities (*P*-value)									
Strain	<0.001	<0.001	0.878	<0.001	0.250	<0.001	<0.001	<0.001	<0.001
Breeder diets	0.462	0.771	0.102	0.602	0.004	<0.001	0.191	0.278	0.277
Offspring diet	0.877	0.891	0.633	0.543	<0.001	<0.001	0.751	0.884	0.712
Strain × breeder diet	0.104	0.110	0.948	0.834	0.930	0.748	0.065	0.088	0.762
Strain × offspring diet	0.713	0.085	0.075	0.640	0.286	0.062	0.627	0.811	0.432

[1] Values with uncommon superscripts within each column are significantly different ($P < 0.05$). n=5

[2] Laying hens during 25 to 42 weeks of age were not fed the experimental diets. These diets were only fed in the breeder and offspring pullet stage.

[3] CON: Control; Microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid (DMA); Co-extruded full fat flaxseed and pulse mixture (FFF, 1:1 wt/wt), as a source of α-linolenic acid.

Table 5. 6. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown and Shaver white breeders and their pullets on keel bone attributes from 42-week-old laying hens.[1,2]

Items	Weight, g/kg BW	Ash weight, g/kg BW	Ash concentration, %
Main effect			
Strain			
ISA brown	2.68[a]	1.11[a]	41.93[a]
Shaver white	2.17[b]	0.86[b]	39.79[b]
SEM	0.066	0.022	0.752
Breeder diet[3]			
CON	2.47	1.00	40.48
DMA	2.32	0.97	41.91
FFF	2.48	0.98	40.40
SEM	0.086	0.029	0.995
Offspring diet			
CON-CON	2.33	0.97	41.50
CON-DMA	2.44	1.04	42.69
CON-FFF	2.63	0.98	37.25
DMA-CON	2.42	1.05	43.25
DMA-DMA	2.22	0.90	40.56
FFF-CON	2.55	0.97	39.50
FFF-FFF	2.41	0.99	41.30
SEM	0.123	0.041	1.408
Probabilities (*P*-value)			
Strain	<0.001	<0.001	0.049
Breeder diets	0.345	0.804	0.468
Offspring diet	0.291	0.095	0.071
Strain × breeder diet	0.137	0.080	0.453
Strain × offspring diet	0.418	0.963	0.340

Values with uncommon superscripts within each column are significantly different ($P < 0.05$).
[1]n=5
[2] Laying hens during 25 to 42 weeks of age were not fed the experimental diets. These diets were only fed in the breeder and offspring pullet stage.
[3] CON: Control; DMA: Microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid; FFF: Co-extruded full fat flaxseed and pulse mixture (1:1 wt/wt), as a source of α-linolenic acid.

Table 5. 7. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown and Shaver white breeders and their pullets on the change of the whole tibia attributes in the 42-week-old laying hen relative to their 18-week-old pullet attributes. % [1,2,3]

Items	Body weight	Tibia			
		Breaking strength	Weight	Ash weight	Ash concentration
Main effect					
Strain					
ISA brown	129.0	96.3[a]	74.1	94.3[a]	127.9[a]
Shaver white	132.4	83.7[b]	72.3	84.1[b]	116.6[b]
SEM	2.650	4.304	1.477	2.219	2.132
Breeder diet[4]					
CON	131.4	86.4	73.6	87.3	118.7
DMA	132.9	96.7	72.8	90.2	124.3
FFF	127.5	88.7	73.0	91.3	125.6
SEM	3.503	5.694	1.921	2.885	2.771
Offspring diet					
CON-CON	133.7	90.9	71.7	87.1	121.3
CON-DMA	130.9	89.0	73.8	87.4	118.7
CON-FFF	129.4	79.4	75.3	87.2	116.0
DMA-CON	131.8	94.1	69.7	89.2	128.5
DMA-DMA	134.1	99.2	75.9	91.1	120.2
FFF-CON	124.8	91.0	77.0	93.5	120.7
FFF-FFF	130.3	86.5	68.9	89.0	130.4
SEM	4.958	8.051	2.717	4.080	3.919
Probabilities (*P*-value)					
Strain	0.382	0.043	0.508	0.003	<0.001
Breeder diets	0.531	0.374	0.937	0.527	0.118
Offspring diet	0.897	0.821	0.119	0.951	0.210
Strain × breeder diet	0.988	0.305	0.195	0.441	0.398
Strain × offspring diet	0.112	0.754	0.706	0.819	0.742

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

[1] n=5

[2] Laying hens during 25 to 42 weeks of age were not fed the experimental diets. These diets were only fed in the breeder and offspring pullet stage.

[3]Calculated by dividing week 42 values by week 18 values and multiplied by 100. The data of 18 WOA values were obtained from Akbari Moghaddam Kakhki., et al (2019b).

[4] CON: Control; DMA: Microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid; FFF: Co-extruded full-fat flaxseed and pulse mixture (1:1 wt/wt), as a source of α-linolenic acid.

Table 5. 8. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown and Shaver white breeders and their pullets on the change of tibia subpart attributes in the 42-week-old laying hen relative to their 18-week-old pullet attributes. % [1,2,3]

Items	Epiphysis			Medullary			Cortical		
	Wt	Ash	Ash percentage	Wt	Ash	Ash percentage	Wt	Ash	Ash percentage
Main effect									
Strain									
ISA brown	72.4	91.4	126.6	88.9	3,287.8	4,099.8	78.28[a]	89.0[a]	114.2[a]
Shaver white	70.7	85.7	121.3	104.7	3,051.3	3,204.5	70.47[b]	75.7[b]	107.3[b]
SEM	1.716	2.774	2.632	8.633	647.90	585.0	2.103	2.022	1.998
Breeder diet[4]									
CON	72.1	86.3	120.1	101.1	2,782.3	2,855.2	74.46	80.7	109.1
DMA	70.3	88.4	125.9	101.0	3,849.2	4,300.9	72.86	82.0	112.9
FFF	72.0	92.0	127.6	86.89	2,877.0	3,800.2	75.81	84.2	110.2
SEM	1.82	3.783	2.842	10.421	841.65	773.90	2.781	2.627	2.632
Offspring diet									
CON-CON	70.0	86.8	124.3	97.7	3,659.8	2,993.2	72.2	81.9	114.3
CON-DMA	71.0	82.9	116.8	94.3	2,095.1	2,699.1	77.4	82.4	106.7
CON-FFF	75.2	89.3	119.1	108.2	2,591.9	2,873.4	73.7	78.0	106.5
DMA-CON	68.5	88.9	130.4	95.9	5,853.3	6,676.2	68.2	78.9	115.5
DMA-DMA	72.2	88.0	121.5	106.2	1,845.0	1,925.7	77.5	85.1	110.3
FFF-CON	76.9	94.3	120.6	89.6	2,544.7	3,182.3	79.2	87.8	111.3
FFF-FFF	67.1	89.7	134.7	84.2	3,209.5	4,418.1	72.4	80.6	109.2
SEM	3.154	5.190	4.923	16.151	1,190.3	1,094.5	3.934	3.713	3.830
Probabilities (*P*-value)									
Strain	0.560	0.192	0.170	0.168	0.725	0.261	0.011	<0.001	0.019
Breeder diets	0.822	0.496	0.201	0.605	0.587	0.331	0.762	0.598	0.534
Offspring diet	0.150	0.877	0.160	0.956	0.167	0.051	0.297	0.409	0.402
Strain × breeder diet	0.252	0.624	0.782	0.783	0.199	0.492	0.337	0.264	0.911
Strain × offspring diet	0.725	0.424	0.184	0.989	0.538	0.824	0.637	0.494	0.944

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

[1]n=5 [2] Laying hens during 25 to 42 weeks of age were not fed the experimental diets. These diets were only fed in the breeder and offspring pullet stage.

[3] Calculated by dividing week 42 values by week 18 values and multiplied by 100. [4]CON: Control; Microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid (DMA); Co-extruded full-fat flaxseed and pulse mixture (FFF, 1:1 wt/wt), as a source of α-linolenic acid.

Chapter 6. General discussion and conclusions

Long-term maintenance of organs and tissues associated with egg production is a prerequisite for extending the laying cycle of commercial flocks (Rossi et al. 2013). Modern hens showed more resistance to skeletal problems predecessors; however, their high level of egg production throughout their extended laying cycle can predispose them to eggshell and skeletal quality problems (Korver 2020). There are various nutritional strategies in practice to improve skeletal health and avoid skeletal disorders. Dietary n-3 PUFA have been reported to improve the skeletal properties and strength in laying hens (Josling et al., 2019; Baird et al., 2008). Previous studies in this area focused on feeding n-3 PUFA to adult birds, and there is a lack of information on the impact of feeding with n-3 PUFA before the onset of lay on bone properties in laying hens. The overall objective of this thesis was to study the effect of embryonic and pullet exposure to DHA and ALA on skeletal development before puberty and subsequent impact on egg production, eggshell and bone quality in ISA brown and Shaver white hens.

In chapter 3, the effects of feeding DHA and ALA sources on hatching egg composition, embryonic uptake of nutrients, and body composition were investigated. The enrichment of egg with n-3 PUFA was effective, which was in agreement with the findings of Ao et al. (2015) and Nain et al. (2012). Chick embryos receive all their nutritional requirements during incubation from the shell, albumen, and yolk. Therefore, egg composition is vital for embryonic growth (Nangsuay et al. 2011). Feeding ALA and DHA reduced the uptake of RY due to the reduced embryonic uptake of OM and mineral compared to the CON group. Embryos from hens fed with n-3 PUFA sources preferentially utilized more DHA compared with CON embryos that utilized less total FA in the phospholipid fraction. The changes in embryonic uptake of nutrients resulted in increased fat deposition and decreased lean mass percentage in Shaver white hatchlings of ALA breeder

compared with CON. It has been reported that yolk FA influence DM uptake by the embryos (Peebles et al. 1999, Yalcin et al. 2008), highlighting the potential of yolk FA content in modifying embryonic nutrient utilization. The body composition changes, and heavier RY bring more questions on the possible adverse impact of embryonic exposure to ALA and DHA on hatchlings quality.

Quantitative values for bone properties to predict bone disorders such as osteoporosis or cage fatigue have not been reported in laying hens. Illustration of changes in skeleton attributes over the course of these trials shows how bone properties altered. The proportion of structural bone in total tibia was similar from 12 (see appendices, Tables 8.1 and 8.2) to 18 WOA, whereas it dropped after the onset of lay (Figure 6.1), which might be attributed to the increased proportion of medullary bone. Meanwhile, the ash content of the structural part in tibia continuously diminished from 12 to 42 WOA, showing the progressive resorption from structural bone. On the other hand, TBS increased from 12 to 18 WOA, followed by a reduction after the onset of lay. These patterns of change in bone attributes demonstrate a reduction in bone quality after the onset of lay, which could lead to skeletal problems by the end of the laying cycle. In chapter 4, the effect of embryonic and rearing exposure to n-3 PUFA on skeletal attributes from hatching to 18 weeks was investigated. Hatchlings from breeders fed with DMA had a lower percentage of mineral in their body compared to CON, which is associated with a decreased uptake of minerals during the embryonic period. Tibia breaking strength and ash content in the cortical part of tibia and femur were increased in Shaver white pullets from breeders fed with DMA compared with Shaver white from breeders fed CON.

In this study, the effect on bone development was observed in the Shaver white breed only, showing that strains have different bone metabolism as supported by differences of bone properties

and plasma biomarkers of bone formation and resorption among strains. These differences highlighted the importance of genetics on bone turnover and phenotypic differences and can establish the groundwork for larger-scale study to be executed with different strains and types of egg-laying birds. If a strain has more capacity in terms of skeletal development in response to nutritional stimuli, it may eventually alter the choice of laying hen strains to optimize bird health and egg production for extended laying cycle (Riczu et al. 2004).

In chapter 5, the effect of embryonic and pullet exposure to DHA and ALA on production performance, eggshell quality, and bone attributes in ISA brown and Shaver white laying hens was studied. A healthy skeletal system has a direct impact on eggshell quality since skeleton supplies around 40% of Ca for eggshell formation (Kemps et al. 2006). However, the effect of n-3 PUFA on pullet's skeleton was not observed at 42 WOA, demonstrating the impact of feeding n-3 PUFA did not last 24 weeks after the removal of the n-3 PUFA supplementary sources. The majority of egg production and eggshell quality traits were not affected by the interaction between strain and diets, nor the main effect of strain and diet, showing embryonic and pullet exposure to ALA and DHA cannot influence the production performance in ISA brown and Shaver white hens when supplemental feeding of n-3 PUFA is removed from 24 to 42 WOA. The efficacy of DHA versus ALA was compared among bone properties at 18 and 42 WOA. At 18 WOA, there were no differences between DHA and ALA in tibia parameters. On the other hand, DHA showed a positive effect on femur attributes compared to ALA. However, there were no differences between DHA and ALA in structural parts of the bone in 42 WOA hens, showing that there were no differences in the efficacy of DHA and ALA in terms of bone quality in 42 WOA hens that were exposed to n-3 PUFA either during embryonic, pullet phase or both.

6.1. Limitations of the Current Study

There were various limitations to the studies presented in this thesis, which include: the housing system, the dosage of n-3 PUFA, bone analysis, the interaction between the skeletal and immune systems.

Water shortage incidence: The incidence of water shortage only happened in Shaver white hens. There might be unknown long-term effect on Shaver white hens, which did not influence ISA brown hens. Therefore, the comparison among white and brown strains in chapter 5 might need to be interpreted with caution.

Housing system: Alternative housing systems (e.g., enriched cage, aviary, and free-range) have been reported to improve skeletal properties by increasing bone turnover compared to conventional systems (Silversides et al. 2012). Many countries in EU have moved towards alternative housing systems and many restaurant chains have declared goals of moving to alternative systems by 2025 or later. The Egg Farmers of Canada has made the decision to move toward 100% alternative system production (i.e., enriched, aviary, free-run, and free-range) by 2036. This switch to alternative systems might change the impact of feeding n-3 PUFA due to allowing birds to practice more physical activity and, subsequently, higher bone turnover. From the present data, it is difficult to predict the impact of exposure to n-3 PUFA in different housing systems; therefore, further studies are needed to investigate it.

Dosage of n-3 PUFA: The dosage of n-3 PUFA has been reported to influence the potential of n-3 PUFA on skeletal attributes (Baird et al. 2008). An enriched egg in Canada should contain 0.3 g or more of ∑n-3 PUFA per reference 100 g based on the Canadian Food Inspection Agency. Since this was a proof-of-concept research on the impact of embryonic and pullet exposure to n-3 PUFA on bone properties, the inclusion level of n-3 PUFA sources were maintained to enrich eggs

with n-3 PUFA at the recommended level. Further studies should investigate the impact of exposure to various levels of n-3 PUFA.

Bone analyses: The application of new technologies for studying bone properties such as quantitative computed tomography can provide a better demonstration of skeleton attributes in hens and possibly in live hens, which was not available at the time of sample analysis of the current study. In addition, bone analysis at the later laying cycle might give a better picture of the likely effect on skeleton and eggshell quality. However, keeping hens for an extended period (80 or more WOA) requires attention for other factors such as budget, and availability of housing.

The interaction between immune and skeletal systems: In the present study, 72 hours before the final sampling at 42 WOA, a limited number of spare ISA brown hens were challenged with 4 mg/ kg of BW with lipopolysaccharide according to the model described by Mireles et al. (2005). The administration with LPS adversely affected eggshell weight, tibia, and keel bone parameters (Tables 8-5 to 8-10). However, the exposure to n-3 PUFA did not affect the majority of bone properties in challenged hens. The immune system challenge could not be applied earlier during the pullet phase for both strains because of delays in approval from the animal care committee. Future studies are needed to investigate the relationship between the skeletal and immune systems in hens and how immune system challenges can increase the chance of observing the effect of n-3 PUFA on bone quality.

6.2. Figure

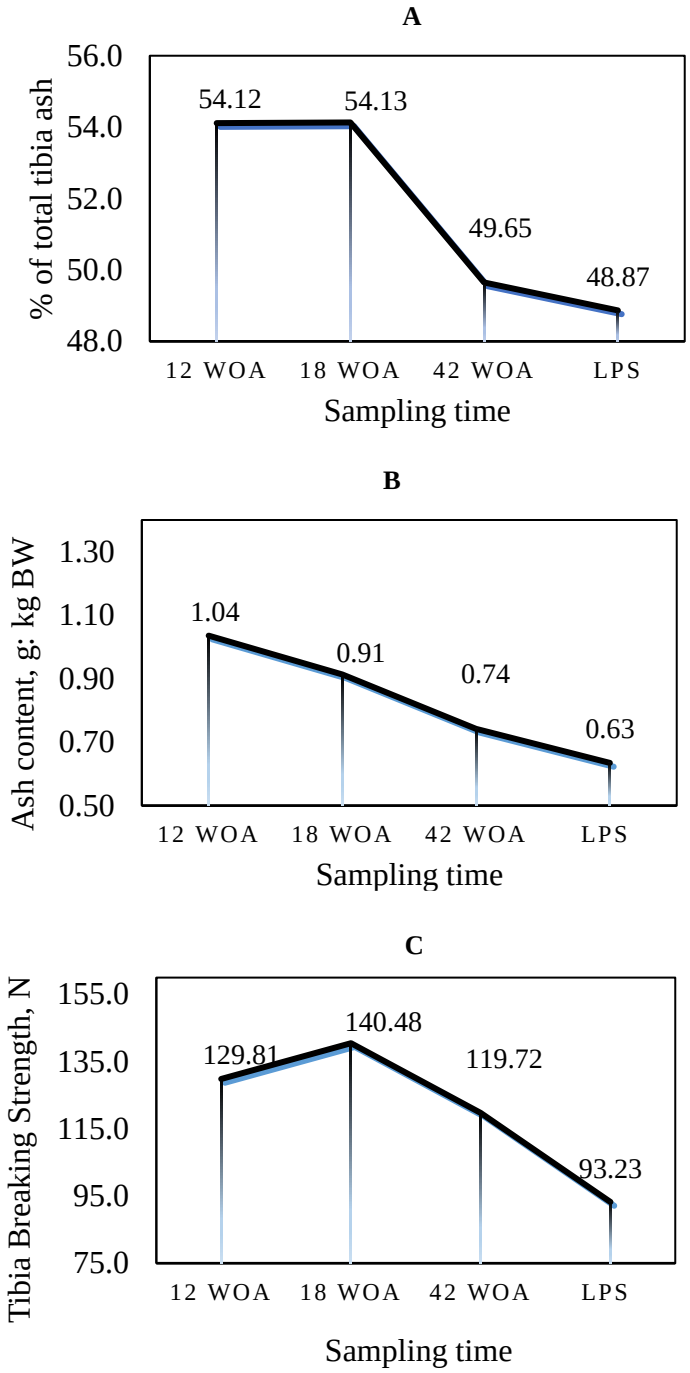

Figure 6. 1. The changes in the percentage of tibia ash (A), ash content (B), and tibia breaking strength (C) in the offspring at different ages. The sampling procedure and analysis of 12 WOA and LPS (4 mg/kg BW at 42 WOA) were only done in ISA brown hens and not reported separately in any chapter but were similar to the sampling procedure mentioned in chapter 4. The findings of sampling at 12 WOA and LPS were not presented in any chapter, however they are presented in appendices.

Chapter 7. References[1]

Akbari Moghaddam Kakhki, R., Golian, A., and Zarghi, H. 2016. Effect of dietary digestible lysine concentration on performance, egg quality, and blood metabolites in laying hens. Journal of Applied Poultry Research **25**: 506-517.

Akbari Moghaddam Kakhki, R., Mousavi, Z., and Anderson, K. 2018. An appraisal of moulting on post-moult egg production and egg weight distribution in white layer hens; meta-analysis. British Poultry Science **59**: 278-285.

Akbari Moghaddam Kakhki, R., Heuthorst, T., Mills, A., Neijat, M., and Kiarie, E. 2019a. Interactive effects of calcium and top-dressed 25-hydroxy vitamin D_3 on egg production, egg shell quality, and bones attributes in aged Lohmann LSL-lite layers. Poultry Science **98**: 1254-1262.

Akbari Moghaddam Kakhki, R., Heuthorst, T., Wornath-Vanhumbeck, A., Neijat, M., and Kiarie, E. 2019b. Medullary bone attributes in aged Lohmann LSL-lite layers fed different levels of calcium and top-dressed 25-hydroxy vitamin D_3. Canadian Journal of Animal Science **99**: 138-149.

Akbari Moghaddam Kakhki, R., Lu, Z., Thanabalan, A., Leung, H., Mohammadigheisar, M., and Kiarie, E. 2019c. Eimeria challenge adversely affected long bone attributes linked to increased resorption in 14-day-old broiler chickens. Poultry Science **98**: 1615-1621.

Amini, K., and Ruiz-Feria, C. 2007. Evaluation of pearl millet and flaxseed effects on egg production and n-3 fatty acid content. British Poultry Science **48**: 661-668.

Anderson, K., Havenstein, G., Jenkins, P., and Osborne, J. 2013. Changes in commercial laying stock performance, 1958-2011: thirty-seven flocks of the North Carolina random sample and subsequent layer performance and management tests. World's Poultry Science Journal **69**: 489-514.

Anez-Bustillos, L., Cowan, E., Cubria, M.B., Villa-Camacho, J.C., Mohamadi, A., Dao, D.T., Pan, A., Fell, G.L., Baker, M.A., and Nandivada, P. 2018. Effects of dietary omega-3 fatty acids on bones of healthy mice. Clinical Nutrition. **38**: 2145–2154.

Ao, T., Macalintal, L., Paul, M., Pescatore, A., Cantor, A., Ford, M., Timmons, B., and Dawson, K. 2015. Effects of supplementing microalgae in laying hen diets on productive

[1] The format of the references is based on the Canadian Journal of Animal Science

performance, fatty-acid profile, and oxidative stability of eggs. Journal of Applied Poultry Research **24**: 394-400.

Bain, M., Nys, Y., and Dunn, I. 2016. Increasing persistency in lay and stabilising egg quality in longer laying cycles. What are the challenges? British Poultry Science **57**: 330-338.

Baird, H., Eggett, D., and Fullmer, S. 2008. Varying ratios of omega-6: omega-3 fatty acids on the pre-and postmortem bone mineral density, bone ash, and bone breaking strength of laying chickens. Poultry Science **87**: 323-328.

Bar, A., and Hurwitz, S. 1987. Vitamin D metabolism and calbindin (calcium-binding protein) in aged laying hens. The Journal of Nutrition **117**: 1775-1779.

Bello, A., and Korver, D. 2019. Long-term effects of *Buttiauxella sp*. phytase on performance, eggshell quality, apparent ileal Ca and P digestibility, and bone properties of white egg layers. Poultry Science **98**: 4848-4859.

Bishop, S., Fleming, R., McCormack, H., Flock, D., and Whitehead, C. 2000. Inheritance of bone characteristics affecting osteoporosis in laying hens. British Poultry Science **41**: 33-40.

Boeyens, J., Deepak, V., Chua, W.-H., Kruger, M., Joubert, A., and Coetzee, M. 2014. Effects of ω3-and ω6-polyunsaturated fatty acids on RANKL-induced osteoclast differentiation of RAW264. 7 cells: a comparative in vitro study. Nutrients **6**: 2584-2601.

Blokhuis, H., Van Niekerk, T.F., Bessei, W., Elson, A., Guémené, D., Kjaer, J., Levrino, G.M., Nicol, C., Tauson, R., and Weeks, C. 2007. The LayWel project: welfare implications of changes in production systems for laying hens. World's Poultry Science Journal **63**: 101-114.

Braniste, V., Leveque, M., Buisson-Brenac, C., Bueno, L., Fioramonti, J., and Houdeau, E. 2009. Oestradiol decreases colonic permeability through oestrogen receptor β-mediated up-regulation of occludin and junctional adhesion molecule-A in epithelial cells. The Journal of Physiology **587**: 3317-3328.

Brenner, R.R. 1971. The desaturation step in the animal biosynthesis of polyunsaturated fatty acids. Lipids **6**: 567-575.

Buckley, A.J., Keserü, B., Briody, J., Thompson, M., Ozanne, S.E., and Thompson, C.H. 2005. Altered body composition and metabolism in the male offspring of high fat–fed rats. Metabolism **54**: 500-507.

Budgell, K., and Silversides, F. 2004. Bone breakage in three strains of end-of-lay hens. Canadian Journal of Animal Science **84**: 745-747.

Bullock CJ, Bobe G, Cherian G. 2014. Gastrointestinal and hepatic tissue fatty acid composition and interleukin-6 concentration in broiler chickens: effect of maternal dietary n-3 fatty acids. Journal of Animal Science **92**:414. # 0827.

Buss, E., and Guyer, R. 1984. Bone parameters of thick and thin eggshell lines of chickens. Comparative biochemistry and physiology A, Comparative physiology **78**: 449-452.

Cachaldora, P., García-Rebollar, P., Alvarez, C., Blas, J.D., and Méndez, J. 2006. Effect of type and level of fish oil supplementation on yolk fat composition and n-3 fatty acids retention efficiency in laying hens. British Poultry Science **47**: 43-49.

Canadian Grain Commission. 2017. https://www.grainscanada.gc.ca/en/.

Cardozo, K.H., Guaratini, T., Barros, M.P., Falcão, V.R., Tonon, A.P., Lopes, N.P., Campos, S., Torres, M.A., Souza, A.O., and Colepicolo, P. 2007. Metabolites from algae with economical impact. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **146**: 60-78.

Casey-Trott, T., Guerin, M., Sandilands, V., Torrey, S., and Widowski, T. 2017a. Rearing system affects prevalence of keel-bone damage in laying hens: a longitudinal study of four consecutive flocks. Poultry Science **96**: 2029-2039.

Casey-Trott, T., Korver, D., Guerin, M., Sandilands, V., Torrey, S., and Widowski, T. 2017b. Opportunities for exercise during pullet rearing, Part II: Long-term effects on bone characteristics of adult laying hens at the end-of-lay. Poultry Science **96**: 2518-2527.

Caston, L., Squires, E., and Leeson, S. 1994. Hen performance, egg quality, and the sensory evaluation of eggs from SCWL hens fed dietary flax. Canadian Journal of Animal Science **74**: 347-353.

CCAC. 2009. The care and use of farm animals in research, teaching and testing. In: Canadian Council on Animal Care. Ottawa, Canada.

Cherian, G. 2008. Egg quality and yolk polyunsaturated fatty acid status in relation to broiler breeder hen age and dietary n-3 oils. Poultry Science **87**: 1131-1137.

Cherian, G. 2011. Essential fatty acids and early life programming in meat-type birds. World's Poultry Science Journal **67**: 599-614.

Cherian, G. 2015. Nutrition and metabolism in poultry: role of lipids in early diet. Journal of Animal Science and Biotechnology **6**: 28-36.

Cherian, G., and Sim, J.S. 1993. Net transfer and incorporation of yolk n-3 fatty acids into developing chick embryos. Poultry Science **72**: 98-105.

Cherian, G., Traber, M., Goeger, M., and Leonard, S. 2007. Conjugated linoleic acid and fish oil in laying hen diets: effects on egg fatty acids, thiobarbituric acid reactive substances, and tocopherols during storage. Poultry Science **86**: 953-958.

Chmurzyńska, A. 2006. The multigene family of fatty acid-binding proteins (FABPs): function, structure and polymorphism. Journal of Applied Genetics **47**: 39-48.

Cohen, S.L., and Ward, W.E. 2005. Flaxseed oil and bone development in growing male and female mice. Journal of Toxicology and Environmental Health, Part A **68**: 1861-1870.

National Research Council. 1994. Nutrient Requirements of Poultry: Ninth Revised Edition, 1994. The National Academies Press, Washington, DC. 176-178.

Crespo, N., and Esteve-Garcia, E. 2001. Dietary fatty acid profile modifies abdominal fat deposition in broiler chickens. Poultry Science **80**: 71-78.

Cruz, C.E.B., Freitas, E.R., Farias, N.N.P., Xavier, R.P.d.S., Lima, J.d.S.C., Sá, N.L., Braz, N.d.M., and Bezerra, R.M. 2012. Bone quality of laying hens fed different levels of fiber in the growth phase (7 to 17 weeks of age). Revista Brasileira de Zootecnia **41**: 2032-2038.

Domingo, J.L., Bocio, A., Falcó, G., and Llobet, J.M. 2007. Benefits and risks of fish consumption: Part I. A quantitative analysis of the intake of omega-3 fatty acids and chemical contaminants. Toxicology **230**: 219-226.

Elaroussi, M.A., Forte, L.R., Eber, S.L., and Biellier, H.V. 1994. Calcium homeostasis in the laying hen. 1. Age and dietary calcium effects. Poultry Science **73**: 1581-1589.

Erdal, R., Richardson, I., Ljøkjel, K., and Haug, A. 2012. Sensorial quality and bone strength of female and male broiler chickens are influenced by weight and growth rate. British Poultry Science **53**: 616-622.

European Directive, E. 1999. Council Directive 99/74/EC of 19 July 1999 laying down minimum standards for the protection of laying hens. Official journal of the European Communities **203**: 53-57.

Everaert, N., Willemsen, H., De Smit, L., Witters, A., De Baerdemaeker, J., Decuypere, E., and Bruggeman, V. 2008. Comparison of a modern broiler and layer strain during embryonic development and the hatching process. British Poultry Science **49**: 574-582.

Fan, C.-M., Su, Y.-W., Howe, P., and Xian, C. 2018. Long chain omega-3 polyunsaturated fatty acid supplementation protects against adriamycin and cyclophosphamide chemotherapy-induced bone marrow damage in female rats. International Journal of Molecular Sciences **19**: 484-499

FAO. 2009. How to Feed the World in 2050. Rome, Italy

Favero, A., Vieira, S., Angel, C., Bos-Mikich, A., Lothhammer, N., Taschetto, D., Cruz, R., and Ward, T. 2013. Development of bone in chick embryos from Cobb 500 breeder hens fed diets supplemented with zinc, manganese, and copper from inorganic and amino acid-complexed sources. Poultry Science **92**: 402-411.

Feng, Y., Ding, Y., Liu, J., Tian, Y., Yang, Y., Guan, S., and Zhang, C. 2015. Effects of dietary omega-3/omega-6 fatty acid ratios on reproduction in the young breeder rooster. BMC Veterinary Research **11**: Article: 73.

Fernyhough, M., Nicol, C.J., van de Braak, T., Toscano, M.J., and Tønnessen, M. 2020. The ethics of laying hen genetics. Journal of Agricultural and Environmental Ethics **33**: 15-36.

Fleming, R., McCormack, H., McTeir, L., and Whitehead, C. 2006. Relationships between genetic, environmental and nutritional factors influencing osteoporosis in laying hens. British Poultry Science **47**: 742-755.

Foley, J. 2019. Feeding 9 Billion: A five step plan to feed the world. National Geographic Magazine. National Geographic. Washington, D.C.USA.

Fonolla-Joya, J., Reyes-García, R., García-Martín, A., López-Huertas, E., and Muñoz-Torres, M. 2016. Daily intake of milk enriched with n-3 fatty acids, oleic acid, and calcium improves metabolic and bone biomarkers in postmenopausal women. Journal of the American College of Nutrition **35**: 529-536.

Forte, L.R., Langeluttig, S.G., Biellier, H.V., Poelling, R.E., Magliola, L., and Thomas, M.L. 1983. Upregulation of kidney adenylate cyclase in the egg-laying hen: role of estrogen. American Journal of Physiology-Endocrinology and Metabolism **245**: E273-E280.

Gao, M., Song, X., Feng, Y., Li, W., and Cui, Q. 2013. Isolation and characterization of Aurantiochytrium species: high docosahexaenoic acid (DHA) production by the newly isolated microalga, Aurantiochytrium sp. SD116. Journal of Oleo Science **62**: 143-151.

Gerster, H. 1998. Can adults adequately convert a-linolenic acid (18: 3n-3) to eicosapentaenoic acid (20: 5n-3) and docosahexaenoic acid (22: 6n-3)? International Journal for Vitamin and Nutrition Research **68**: 159-173.

Gilbert, A. 1983. Calcium and reproductive function in the hen. Proceedings of the Nutrition Society **42**: 195-212.

Goyal, A., Sharma, V., Upadhyay, N., Gill, S., and Sihag, M. 2014. Flax and flaxseed oil: an ancient medicine & modern functional food. Journal of Food Science And Technology **51**: 1633-1653.

Hayat, Z., Cherian, G., Pasha, T., Khattak, F., and Jabbar, M. 2009. Effect of feeding flax and two types of antioxidants on egg production, egg quality, and lipid composition of eggs. Journal of Applied Poultry Research **18**: 541-551.

Hoshino, T., Yamane, T., and Shimizu, S. 1990. Selective hydrolysis of fish oil by lipase to concentrate n-3 polyunsaturated fatty acids. Agricultural and Biological Chemistry **54**: 1459-1467.

Hyline International. 2014. www.hyline.com/USERDocs/Pages/PUB_INVEST_ENG.pdf Accessed September 2019

Ho, D.H., Reed, W.L., and Burggren, W.W. 2011. Egg yolk environment differentially influences physiological and morphological development of broiler and layer chicken embryos. Journal of Experimental Biology **214**: 619-628.

Hocking, P., Bain, M., Channing, C., Fleming, R., and Wilson, S. 2003. Genetic variation for egg production, egg quality and bone strength in selected and traditional breeds of laying fowl. British Poultry Science **44**: 365-373.

Hurwitz, S., Bar, A., Katz, M., Sklan, D., and Budowski, P. 1973. Absorption and secretion of fatty acids and bile acids in the intestine of the laying fowl. The Journal of Nutrition **103**: 543-547.

Ibrahim, A., Basak, S., and Ehtesham, N.Z. 2009. Impact of maternal dietary fatty acid composition on glucose and lipid metabolism in male rat offspring aged 105 d. British Journal of Nutrition **102**: 233-241.

Innes, J.K., and Calder, P.C. 2018. Omega-6 fatty acids and inflammation. Prostaglandins, Leukotrienes and Essential Fatty Acids **132**: 41-48.

Jin, Y., Lee, M., and Park, Y. 2017. Synergistic attenuation of ovariectomy-induced bone loss by combined use of fish oil and 17β-oestradiol. British Journal of Nutrition **117**: 479-489.

Josling, G., Hugo, A., Fair, M., and de Witt, F. 2019. Long term effect of dietary lipid saturation on eggshell quality and bone characteristics of laying hens. Poultry Science **98**: 3593-3601.

Kajarabille, N., Díaz-Castro, J., Hijano, S., López-Frías, M., López-Aliaga, I., and Ochoa, J.J. 2013. A new insight to bone turnover: Role of-3 polyunsaturated fatty acids. The Scientific World Journal. Article: 589641

Katongole, J., and March, B. 1980. Fat utilization in relation to intestinal fatty acid binding protein and bile salts in chicks of different ages and different genetic sources. Poultry Science **59**: 819-827.

Kameda, T., Mano, H., Yuasa, T., Mori, Y., Miyazawa, K., Shiokawa, M., Nakamaru, Y., Hiroi, E., Hiura, K., and Kameda, A. 1997. Estrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts. Journal of Experimental Medicine **186**: 489-495.

Kemps, B.J., Govaerts, T., De Ketelaere, B., Mertens, K., Bamelis, F.R., Bain, M.M., Decuypere, E.M., and De Baerdemaeker, J.G. 2006. The influence of line and laying period on the relationship between different eggshell and membrane strength parameters. Poultry Science **85**: 1309-1317.

Kerschnitzki, M., Zander, T., Zaslansky, P., Fratzl, P., Shahar, R., and Wagermaier, W. 2014. Rapid alterations of avian medullary bone material during the daily egg-laying cycle. Bone **69**: 109-117.

Khan, K.A., Khan, S.A., Aslam, A., Rabbani, M., and Tipu, M.Y. 2004. Factors contributing to yolk retention in poultry: a review. Pakistan Veterinary Journal **24**: 46-51.

Khanal, T., Widowski, T., Bédécarrats, G., and Kiarie, E. 2019. Effects of pre-lay dietary calcium (2.5 vs. 4.0%) and pullet strain (Lohmann Brown vs. Selected Leghorn LSL-Lite) on calcium utilization and femur quality at 1st through to the 50th egg. Poultry Science. **98**:4919-4928

Kim, W., Donalson, L., Bloomfield, S., Hogan, H., Kubena, L., Nisbet, D., and Ricke, S. 2007. Molt performance and bone density of cortical, medullary, and cancellous bone in laying hens during feed restriction or alfalfa-based feed molt. Poultry Science **86**: 1821-1830.

Konieczka, P., Czauderna, M., and Smulikowska, S. 2017. The enrichment of chicken meat with omega-3 fatty acids by dietary fish oil or its mixture with rapeseed or flaxseed—Effect of feeding duration: Dietary fish oil, flaxseed, and rapeseed and n-3 enriched broiler meat. Animal Feed Science and Technology **223**: 42-52.

Koppenol, A., Buyse, J., Everaert, N., Willems, E., Wang, Y., Franssens, L., and Delezie, E. 2014a. Transition of maternal dietary n-3 fatty acids from the yolk to the liver of broiler breeder progeny via the residual yolk sac. Poultry Science **94**: 43-52.

Koppenol, A., Delezie, E., Aerts, J., Willems, E., Wang, Y., Franssens, L., Everaert, N., and Buyse, J. 2014b. Effect of the ratio of dietary n-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid on broiler breeder performance, egg quality, and yolk fatty acid composition at different breeder ages. Poultry Science **93**: 564-573.

Koren, N., Simsa-Maziel, S., Shahar, R., Schwartz, B., and Monsonego-Ornan, E. 2014. Exposure to omega-3 fatty acids at early age accelerate bone growth and improve bone quality. The Journal of Nutritional Biochemistry **25**: 623-633.

Korotkova, M., Ohlsson, C., Hanson, L., and Strandvik, B. 2004. Dietary n-6: n-3 fatty acid ratio in the perinatal period affects bone parameters in adult female rats. British Journal of Nutrition **92**: 643-648.

Korotkova, M., Ohlsson, C., Gabrielsson, B., Hanson, L.Å., and Strandvik, B. 2005. Perinatal essential fatty acid deficiency influences body weight and bone parameters in adult male rats. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids **1686**:

248-254.

Korver, D. 2020. Calcium nutrition, bone metabolism, and eggshell quality in longer-persisting layer flocks. Proc. 31st Annual Australian Poultry Science Symposium. Sydney.

Kruger, M.C., and Schollum, L.M. 2005. Is docosahexaenoic acid more effective than eicosapentaenoic acid for increasing calcium bioavailability? Prostaglandins, Leukotrienes and Essential Fatty Acids **73**: 327-334.

Kruger, M.C., Coetzee, M., Haag, M., and Weiler, H. 2010. Long-chain polyunsaturated fatty acids: selected mechanisms of action on bone. Progress In Lipid Research **49**: 438-449.

Kuroda, T., Ohta, H., Onoe, Y., Tsugawa, N., and Shiraki, M. 2017. Intake of omega-3 fatty acids contributes to bone mineral density at the hip in a younger Japanese female population. Osteoporosis International **28**: 2887-2891.

Kushwaha, P., Wolfgang, M.J., and Riddle, R.C. 2018. Fatty acid metabolism by the osteoblast. Bone **115**: 8-14.

Latour, M.A., Peebles, E., Doyle, S., Pansky, T., Smith, T., and Boyle, C.R. 1998. Broiler breeder age and dietary fat influence the yolk fatty acid profiles of fresh eggs and newly hatched chicks. Poultry Science **77**: 47-53.

Lau, B.Y., Ward, W.E., Kang, J.X., and Ma, D.W. 2009. Femur EPA and DHA are correlated with femur biomechanical strength in young fat-1 mice. The Journal of Nutritional Biochemistry **20**: 453-461.

Lee, S.A., Whenham, N., and Bedford, M.R. 2018. Review on docosahexaenoic acid in poultry and swine nutrition: Consequence of enriched animal products on performance and health characteristics. Animal Nutrition **5**: 11-21

Leinonen, I., Williams, A., Wiseman, J., Guy, J., and Kyriazakis, I. 2012. Predicting the environmental impacts of chicken systems in the United Kingdom through a life cycle assessment: Broiler production systems. Poultry Science 91: 8-25.

Leone, V., Worzalla, S., and Cook, M. 2010. Evidence that maternal conjugated linoleic acid negatively affects lipid uptake in late-stage chick embryos resulting in increased embryonic mortality. Poultry Science **89**: 621-632.

Lichovnikova, M. 2007. The effect of dietary calcium source, concentration and particle size on calcium retention, eggshell quality and overall calcium requirement in laying hens. British Poultry Science **48**: 71-75.

Lin, D.S., Connor, W.E., and Anderson, G.J. 1991. The incorporation of n-3 and n-6 essential fatty acids into the chick embryo from egg yolks having vastly different fatty acid compositions. Pediatric Research **29**: 601-605.

Liu, D., and Denbow, D. 2001. Maternal dietary lipids modify composition of bone lipids and ex vivo prostaglandin E2 production in early postnatal Japanese quail. Poultry Science **80**: 1344-1352.

Liu, D., Veit, H., Wilson, J., and Denbow, D. 2003a. Long-term supplementation of various dietary lipids alters bone mineral content, mechanical properties and histological characteristics of Japanese quail. Poultry Science **82**: 831-839.

Liu, D., Veit, H., Wilson, J., and Denbow, D. 2003b. Maternal dietary lipids alter bone chemical composition, mechanical properties, and histological characteristics of progeny of Japanese quail. Poultry Science **82**: 463-473.

Luck, M., and Scanes, C. 1979. Plasma levels of ionized calcium in the laying hen (*Gallus domesticus*). Comparative Biochemistry and Physiology Part A: Physiology **63**: 177-181.

Maldjian, A., Cristofori, C., Noble, R.C., and Speake, B.K. 1996. The fatty acid composition of brain phospholipids from chicken and duck embryos. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **115**: 153-158.

Mazzuco, H., McMurtry, J., Kuo, A., and Hester, P. 2005. The effect of pre-and postmolt diets high in n-3 fatty acids and molt programs on skeletal integrity and insulin-like growth factor-I of White Leghorns. Poultry Science **84**: 1735-1749.

Mennitti, L.V., Oliveira, J.L., Morais, C.A., Estadella, D., Oyama, L.M., do Nascimento, C.M.O., and Pisani, L.P. 2015. Type of fatty acids in maternal diets during pregnancy and/or lactation and metabolic consequences of the offspring. The Journal of Nutritional Biochemistry **26**: 99-111.

Mireles, A., Kim, S., and Klasing, K. 2005. An acute inflammatory response alters bone homeostasis, body composition, and the humoral immune response of broiler chickens. Poultry Science **84**: 553-560.

Mo, X.-L., Wei, H.-K., Peng, J., and Tao, Y.-X. 2013. Free fatty acid receptor GPR120 and pathogenesis of obesity and type 2 diabetes mellitus. Progress in Molecular Biology and Translational Science. **114**:251-276.

Moon, H.-J., Kim, T.-H., Byun, D.-W., and Park, Y. 2012. Positive correlation between erythrocyte levels of n–3 polyunsaturated fatty acids and bone mass in postmenopausal Korean women with osteoporosis. Annals of Nutrition and Metabolism **60**: 146-153.

Muhlhausler, B.S., Miljkovic, D., Fong, L., Xian, C.J., Duthoit, E., and Gibson, R.A. 2011. Maternal omega-3 supplementation increases fat mass in male and female rat offspring. Frontiers in Genetics **2**: Article 48.1-10

Mazzuco, H., McMurtry, J., Kuo, A., and Hester, P. 2005. The effect of pre-and postmolt diets high in n-3 fatty acids and molt programs on skeletal integrity and insulin-like growth factor-I of White Leghorns. Poultry Science **84**: 1735-1749.

Mwaniki, Z., Neijat, M., and Kiarie, E. 2018. Egg production and quality responses of adding up to 7.5% defatted black soldier fly larvae meal in a corn–soybean meal diet fed to Shaver White Leghorns from wk 19 to 27 of age. Poultry Science **97**: 2829-2835.

Nain, S., Renema, R., Korver, D., and Zuidhof, M. 2012. Characterization of the n-3 polyunsaturated fatty acid enrichment in laying hens fed an extruded flax enrichment source. Poultry Science **91**: 1720-1732.

Nakanishi, A., and Tsukamoto, I. 2015. n-3 polyunsaturated fatty acids stimulate osteoclastogenesis through PPARγ-mediated enhancement of c-Fos expression, and suppress osteoclastogenesis through PPARγ-dependent inhibition of NFKB activation. The Journal of Nutritional Biochemistry **26**: 1317-1327.

Nakanishi, A., Iitsuka, N., and Tsukamoto, I. 2013. Fish oil suppresses bone resorption by inhibiting osteoclastogenesis through decreased expression of M-CSF, PU. 1, MITF and RANK in ovariectomized rats. Molecular Medicine Reports **7**: 1896-1903.

NAFCC. 2017. National Farm Animal Care Council: Code of practice for the care and handling of pullets and laying hens. Accessed September 2018. https://www.nfacc.ca/poultry-layers-code-of-practice

Nangsuay, A., Ruangpanit, Y., Meijerhof, R., and Attamangkune, S. 2011. Yolk absorption and embryo development of small and large eggs originating from young and old breeder hens. Poultry Science **90**: 2648-2655.

Neijat, M., Ojekudo, O., and House, J. 2016. Effect of flaxseed oil and microalgae DHA on the production performance, fatty acids and total lipids of egg yolk and plasma in laying hens. Prostaglandins, Leukotrienes and Essential Fatty Acids **115**: 77-88.

Neijat, M., Eck, P., and House, J. 2017. Impact of dietary precursor ALA versus preformed DHA on fatty acid profiles of eggs, liver and adipose tissue and expression of genes associated with hepatic lipid metabolism in laying hens. Prostaglandins, Leukotrienes and Essential Fatty Acids **119**: 1-17.

Neijat, M., Casey-Trott, T., Robinson, S., Widowski, T., and Kiarie, E. 2019. Effects of rearing and adult laying housing systems on medullary, pneumatic and radius bone attributes in 73-wk old Lohmann LSL lite hens. Poultry Science **98**: 2840-2845.

Neunzehn, J., Szuwart, T., and Wiesmann, H.-P. 2015. Eggshells as natural calcium carbonate source in combination with hyaluronan as beneficial additives for bone graft materials, an in vitro study. Head & Face Medicine **16**: 11-12

Noble, R., and Cocchi, M. 1990. Lipid metabolism and the neonatal chicken. Progress in Lipid Research **29**: 107-140.

O'fallon, J., Busboom, J., Nelson, M., and Gaskins, C. 2007. A direct method for fatty acid methyl ester synthesis: application to wet meat tissues, oils, and feedstuffs. Journal of Animal Science **85**: 1511-1521.

Onbaşılar, E., Kahraman, M., Ahlat, O., Güngör, Ö., Çalık, A., Taban, S., and Yalçın, S. 2017. Differences in egg nutrient availability and embryo development in white layer breeder genotypes. Poultry Science **96**: 3600-3607.

Parent Stock Management Guide: ISA brown. 2018. Edition L7140. Hendrix Genetic, Boxmeer, The Netherlands.

Parent Stock Management Guide: Shaver White. 2018. Edition L7140. Hendrix Genetic, Boxmeer, The Netherlands.

Pechak, D., Kujawa, M., and Caplan, A. 1986. Morphology of bone development and bone remodeling in embryonic chick limbs. Bone **7**: 459-472.

Peebles, E.D., Li, L., Miller, S., Pansky, T., Whitmarsh, S., Latour, M.A., and Gerard, P.D. 1999. Embryo and yolk compositional relationships in broiler hatching eggs during incubation. Poultry Science **78**: 1435-1442.

Pelletier, N., Doyon, M., Muirhead, B., Widowski, T., Nurse-Gupta, J., and Hunniford, M. 2018. Sustainability in the Canadian Egg Industry—Learning from the Past, Navigating the Present, Planning for the Future. Sustainability 10: 3524-3548.

Petrik, M.T., Guerin, M.T., and Widowski, T.M. 2015. On-farm comparison of keel fracture prevalence and other welfare indicators in conventional cage and floor-housed laying hens in Ontario, Canada. Poultry Science **94**: 579-585.

Poulsen, R.C., Gotlinger, K.H., Serhan, C.N., and Kruger, M.C. 2008. Identification of inflammatory and proresolving lipid mediators in bone marrow and their lipidomic profiles with ovariectomy and omega-3 intake. American Journal of Hematology **83**: 437-445.

Powers, W., and Angel, R. 2008. A review of the capacity for nutritional strategies to address environmental challenges in poultry production. Poultry Science **87**: 1929-1938.

Preisinger, R. 2018. Innovative layer genetics to handle global challenges in egg production. British Poultry Science **59**: 1-6.

Raja, R., and Hemaiswarya, S. 2010. Microalgae and immune potential. Dietary Components and Immune Function. 515-527. Springer. Berlin, Germany.

Rajaram, S., Yip, E., Reghunathan, R., Mohan, S., and Sabaté, J. 2017. Effect of Altering Dietary n-6: n-3 Polyunsaturated Fatty Acid Ratio with Plant and Marine-Based Supplement on Biomarkers of Bone Turnover in Healthy Adults. Nutrients **9**: 1162-1171.

Rath, N., Huff, G., Huff, W., and Balog, J. 2000. Factors regulating bone maturity and strength in poultry. Poultry Science **79**: 1024-1032.

Regmi, P., Nelson, N., Steibel, J., Anderson, K., and Karcher, D. 2016. Comparisons of bone properties and keel deformities between strains and housing systems in end-of-lay hens. Poultry Science **95**: 2225-2234.

Reza-López, S.A., Anderson, G.H., Szeto, I.M., Taha, A.Y., and Ma, D.W. 2009. High vitamin intake by Wistar rats during pregnancy alters tissue fatty acid concentration in the offspring fed an obesogenic diet. Metabolism **58**: 722-730.

Riber, A.B., Casey-Trott, T.M., and Herskin, M.S. 2018. The influence of keel bone damage on welfare of laying hens. Frontiers in Veterinary Science **5**: 1-6.

Riczu, C., Saunders-Blades, J., Yngvesson, H., Robinson, F., and Korver, D. 2004. End-of-cycle bone quality in white-and brown-egg laying hens. Poultry Science **83**: 375-383.

Rodriguez-Navarro, A., McCormack, H., Fleming, R., Alvarez-Lloret, P., Romero-Pastor, J., Dominguez-Gasca, N., Prozorov, T., and Dunn, I. 2018. Influence of physical activity on tibial bone material properties in laying hens. Journal of Structural Biology **201**: 36-45.

Rogers, A., and Eastell, R. 2001. The effect of 17β-estradiol on production of cytokines in cultures of peripheral blood. Bone **29**: 30-34.

Romanoff, A.L. 1960. The avian embryo. Structural and functional development. The avian embryo. Structural and functional development. Science **131**: 1219-1220.

Rossi, M., Nys, Y., Anton, M., Bain, M., De Ketelaere, B., De Reu, K., Dunn, I., Gautron, J., Hammershøj, M., and Hidalgo, A. 2013. Developments in understanding and assessment of egg and egg product quality over the last century. World's Poultry Science Journal **69**: 414-429.

Rubio-Rodríguez, N., Beltrán, S., Jaime, I., Sara, M., Sanz, M.T., and Carballido, J.R. 2010. Production of omega-3 polyunsaturated fatty acid concentrates: a review. Innovative Food Science & Emerging Technologies **11**: 1-12.

Rufener, C., Baur, S., Stratmann, A., and Toscano, M.J. 2019. Keel bone fractures affect egg laying performance but not egg quality in laying hens housed in a commercial aviary system. Poultry Science **98**: 1589-1600.

Poli, V., Balena, R., Fattori, E., Markatos, A., Yamamoto, M., Tanaka, H., Ciliberto, G., Rodan, G.A., and Costantini, F. 1994. Interleukin-6 deficient mice are protected from bone loss caused by estrogen depletion. The EMBO Journal **13**: 1189-1196.

Pottgueter, R. 2015. 20th European Symposium on Poultry Nutrition. 24–27 August 2015. Prague, Czech Republic.

Saunders-Blades, J., and Korver, D. 2015. Effect of hen age and maternal vitamin D source on performance, hatchability, bone mineral density, and progeny in vitro early innate immune function. Poultry Science **94**: 1233-1246.

Şahan, U., Ipek, A., and Sozcu, A. 2014. Yolk sac fatty acid composition, yolk absorption, embryo development, and chick quality during incubation in eggs from young and old broiler breeders. Poultry Science **93**: 2069-2077.

Sardinha, F.L., Fernandes, F.S., do Carmo, M.G.T., and Herrera, E. 2012. Sex-dependent nutritional programming: fish oil intake during early pregnancy in rats reduces age-dependent insulin resistance in male, but not female, offspring. American Journal of Physiology-Heart and Circulatory Physiology **304**:313-320.

Silversides, F., Singh, R., Cheng, K., and Korver, D. 2012. Comparison of bones of 4 strains of laying hens kept in conventional cages and floor pens. Poultry Science **91**: 1-7.

Singh, K., Mridula, D., Rehal, J., and Barnwal, P. 2011. Flaxseed: a potential source of food, feed and fiber. Critical Reviews in Food Science and Nutrition **51**: 210-222.

Singh, R., Cheng, K., and Silversides, F. 2009. Production performance and egg quality of four strains of laying hens kept in conventional cages and floor pens. Poultry Science **88**: 256-264.

Smith, P.E., Krohn, R.I., Hermanson, G., Mallia, A., Gartner, F., Provenzano, M., Fujimoto, E., Goeke, N., Olson, B., and Klenk, D. 1985. Measurement of protein using bicinchoninic acid. Analytical Biochemistry **150**: 76-85.

Smith, A., Contreras, C., Ko, K.H., Chow, J., Dong, X., Tuo, B., Zhang, H.-h., Chen, D.., and Dong, H. 2008. Gender-specific protection of estrogen against gastric acid-induced duodenal injury: stimulation of duodenal mucosal bicarbonate secretion. Endocrinology **149**: 4554-4566.

Sparke, A., Sims, T., Avery, N., Bailey, A., Fleming, R., and Whitehead, C. 2002. Differences in composition of avian bone collagen following genetic selection for resistance to osteoporosis. British Poultry Science **43**: 127-134.

Speake, B.K., Murray, A.M., and Noble, R.C. 1998. Transport and transformations of yolk lipids during development of the avian embryo. Progress in Lipid Research **37**:1-32.

Talukdar, S., Bae, E.J., Imamura, T., Morinaga, H., Fan, W., Li, P., Lu, W.J., Watkins, S.M., and Olefsky, J.M. 2010. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell **142**: 687-698.

Tanaka, Y., Castillo, L., and DeLuca, H. 1976. Control of renal vitamin D hydroxylases in birds by sex hormones. Proceedings of the National Academy of Sciences **73**: 2701-2705.

Tancharoenrat, P., Ravindran, V., Zaefarian, F., and Ravindran, G. 2014. Digestion of fat and fatty acids along the gastrointestinal tract of broiler chickens. Poultry Science **93**: 371-379.

Tong, Q., Romanini, C., Exadaktylos, V., Bahr, C., Berckmans, D., Bergoug, H., Eterradossi, N., Roulston, N., Verhelst, R., and McGonnell, I. 2013. Embryonic development and the physiological factors that coordinate hatching in domestic chickens. Poultry Science **92**: 620-628.

Torres, C., and Korver, D. 2018. Influences of trace mineral nutrition and maternal flock age on broiler embryo bone development. Poultry Science **97**: 2996-3003.

Toscano, M.J., Booth, F., Wilkins, L., Avery, N., Brown, S., Richards, G., and Tarlton, J. 2015. The effects of long (C20/22) and short (C18) chain omega-3 fatty acids on keel bone fractures, bone biomechanics, behavior, and egg production in free-range laying hens. Poultry Science **94**: 823-835.

Trebble, T.M. 2005. Bone turnover and nutritional status in Crohn's disease: relationship to circulating mononuclear cell function and response to fish oil and antioxidants. Proceedings of the Nutrition Society **64**: 183-191.

Trebble, T.M., Stroud, M.A., Wootton, S.A., Calder, P.C., Fine, D.R., Mullee, M.A., Moniz, C., and Arden, N.K. 2005. High-dose fish oil and antioxidants in Crohn's disease and the response of bone turnover: a randomised controlled trial. British Journal of Nutrition **94**: 253-261.

Uni, Z., and Ferket, R. 2004. Methods for early nutrition and their potential. World's Poultry Science Journal **60**: 101-111.

Uni, Z., Ferket, P., Tako, E., and Kedar, O. 2005. In ovo feeding improves energy status of late-term chicken embryos. Poultry Science **84**: 764-770.

van Abel, M., Hoenderop, J.G., Dardenne, O., Arnaud, R.S., van Os, C.H., van Leeuwen, H.J., and Bindels, R.J. 2002. 1, 25-Dihydroxyvitamin D_3-independent stimulatory effect of estrogen on the expression of ECaC1 in the kidney. Journal of the American Society of Nephrology **13**: 2102-2109.

Vlčková, J., Tůmová, E., Ketta, M., Englmaierová, M., and Chodová, D. 2018. Effect of housing system and age of laying hens on eggshell quality, microbial contamination, and penetration of microorganisms into eggs. Czech Journal of Animal Science **63**: 51-60.

Watkins, B.A. 1991. Importance of essential fatty acids and their derivatives in poultry. The Journal of Nutrition **121**: 1475-1485.

Ward, T., Watkins, K., and Southern, L. 1990. Interactive effects of sodium zeolite A (Ethacal®) and monensin in uninfected and *Eimeria acervulina*-infected chicks. Poultry Science **69**: 276-280.

Whitehead, C. 2004. Overview of bone biology in the egg-laying hen. Poultry Science **83**: 193-199.

Whitehead, C., and Fleming, R. 2000. Osteoporosis in cage layers. Poultry Science **79**: 1033-1041.

Wilkins, L., McKinstry, J., Avery, N., Knowles, T., Brown, S., Tarlton, J., and Nicol, C. 2011. Influence of housing system and design on bone strength and keel bone fractures in laying hens. Veterinary Record **169**: 414-415.

Wolanski, N., Renema, R., Robinson, F., Carney, V., and Fancher, B. 2007. Relationships among egg characteristics, chick measurements, and early growth traits in ten broiler breeder strains. Poultry Science **86**: 1784-1792.

Wu, Y., Li, L., Wen, Z., Yan, H., Yang, P., Tang, J., Xie, M., and Hou, S. 2018. Dual functions of eicosapentaenoic acid-rich microalgae: enrichment of yolk with n-3 polyunsaturated fatty acids and partial replacement for soybean meal in diet of laying hens. Poultry Science **98**: 350-357.

Yadgary, L., Yair, R., and Uni, Z. 2011. The chick embryo yolk sac membrane expresses nutrient transporter and digestive enzyme genes. Poultry Science **90**: 410-416.

Yadgary, L., Kedar, O., Adepeju, O., and Uni, Z. 2013. Changes in yolk sac membrane absorptive area and fat digestion during chick embryonic development. Poultry Science **92**: 1634-1640.

Yair, R., and Uni, Z. 2011. Content and uptake of minerals in the yolk of broiler embryos during incubation and effect of nutrient enrichment. Poultry Science **90**: 1523-1531.

Yair, R., Uni, Z., and Shahar, R. 2012. Bone characteristics of late-term embryonic and hatchling broilers: Bone development under extreme growth rate. Poultry Science **91**: 2614-2620.

Yair, R., Shahar, R., and Uni, Z. 2015. In ovo feeding with minerals and vitamin D_3 improves bone properties in hatchlings and mature broilers. Poultry Science **94**: 2695-2707.

Yair, R., Cahaner, A., Uni, Z., and Shahar, R. 2017. Maternal and genetic effects on broiler bone properties during incubation period. Poultry Science **96**: 2301-2311.

Yalcin, S., Bağdatlioğlu, N., Bruggeman, V., Babacanoğlu, E., Uysal, İ., Buyse, J., Decuypere, E., and Siegel, P. 2008. Acclimation to heat during incubation. 2. Embryo composition and residual egg yolk sac fatty acid profiles in chicks. Poultry Science **87**: 1229-1236.

Yalçın, S., Bağdatlıoğlu, N., Yenisey, C., Siegel, P., Özkan, S., and Akşit, M. 2012. Effect of manipulation of incubation temperature on fatty acid profiles and antioxidant enzyme activities in meat-type chicken embryos. Poultry Science **91**: 3260-3270.

Chapter 8. Appendices

Table 8. 1. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown and Shaver White breeders and offspring on feed intake throughout the pullet phase, g/bird/day [1]

Items[2]	Starter		Grower		Developer		Pre-lay		Total	
	ISA (1-4 wk)	Shaver (1-8 wk)	ISA (5-10 wk)	Shaver (9-12 wk)	ISA (11-16 wk)	Shaver (13-16 wk)	ISA (16-2% EP[3])	Shaver (16-2% EP)	ISA	Shaver
Breeder diets										
CON	21.1	25.1	58.1	61.8	91.7	84.4	102.1	81.9	54.5	54.0
DMA	20.5	24.9	55.3	69.9	94.9	78.7	108.7	89.5	56.3	54.9
FFF	20.9	24.7	55.7	65.0	90.8	70.3	103.6	90.7	54.2	51.5
SEM	0.487	0.320	2.125	2.638	2.960	2.137	2.531	3.512	1.244	1.032
Offspring diets										
CON-CON	21.1	25.1	58.1	61.8	91.7	84.4	102.1	81.9	54.5	54.0
CON-DMA	21.1	25.5	57.5	65.6	90.9	79.8	98.3	83.3	54.0	53.9
CON-FFF	21.2	25.1	63.9	62.3	84.3	85.5	101.9	79.4	51.8	54.1
DMA-CON	20.5	24.8	55.3	69.9	94.9	78.7	108.7	89.5	56.3	54.9
DMA-DMA	20.0	25.0	57.8	73.8	98.3	81.4	112.0	92.1	58.0	56.7
FFF-CON	20.9	24.7	55.7	65.0	90.8	70.3	103.6	90.7	54.2	51.5
FFF-FFF	20.7	24.7	58.1	69.6	84.8	67.1	104.1	86.1	52.0	51.4
SEM	0.689	0.453	3.006	3.730	4.187	3.023	3.579	4.967	1.759	1.459
Probabilities (*P*-value)										
Breeder diets	0.644	0.604	0.520	0.080	0.586	0.100	0.142	0.116	0.449	0.173
Offspring diets	0.886	0.851	0.087	0.136	0.103	0.140	0.409	0.630	0.150	0.515

[1] data are means of 5 replications (cages) per each treatment.
[2] CON: Control; DMA: Microalgae (*Aurantiochytrium limacinum*) fermentation product, as a source of docosahexaenoic acid; FFF: Co-extruded full fat flaxseed and pulse mixture (1:1 wt/wt), as a source of α-linolenic acid.
[3] 2% egg production.

Table 8. 2. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown breeders and offspring on the whole tibia and femur attributes in 12-week-old pullets, [1,2]

Items	Body weight, g	Tibia				Femur		
		Breaking strength, N/ Kg BW	Wt, g/kg BW	Ash, g/kg BW	Ash, %	Wt, g/kg BW	Ash, g/kg BW	Ash, %
Breeder diets								
CON	1,281.6	125.97	5.55	1.90	34.24	3.92	1.19	30.59
DMA	1,259.7	130.61	5.59	1.90	34.14	3.79	1.19	31.36
FFF	1,291.0	134.77	5.73	1.94	34.02	4.00	1.22	30.58
SEM	26.193	5.379	0.117	0.033	0.617	0.089	0.022	0.546
Offspring diets[2]								
CON-CON	1,281.6	125.97	5.55	1.90	34.24	3.92	1.19	30.59
CON-DMA	1,270.6	137.48	5.44	1.87	34.52	3.70	1.15	31.00
CON-FFF	1,303.3	139.99	5.73	1.96	34.32	4.00	1.20	30.05
DMA-CON	1,259.7	130.61	5.59	1.90	34.14	3.79	1.19	31.36
DMA-DMA	1,248.7	123.74	5.73	1.93	33.77	3.88	1.23	31.73
FFF-CON	1,291.0	134.77	5.73	1.94	34.02	4.00	1.22	30.58
FFF-FFF	1,278.7	129.55	5.73	1.92	33.72	3.99	1.24	31.12
SEM	37.043	7.607	0.166	0.046	0.872	0.125	0.031	0.773
Probabilities (*P*-value)								
Breeder diets	0.685	0.449	0.491	0.533	0.960	0.273	0.519	0.495
Offspring diets	0.951	0.337	0.531	0.717	0.666	0.347	0.190	0.509

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

[1]n=5.

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

Table 8. 3. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown breeders and offspring on tibia subpart attributes in 12-week-old pullets, [1,2]

Items	Epiphysis			Medullary			Cortical		
	Wt, 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, %
Breeder diets									
CON	3.02	0.87	29.12	0.93	0.008	0.90	1.60	1.01	63.34
DMA	3.02	0.87	28.78	0.96	0.010	0.98	1.61	1.03	64.00
FFF	3.02	0.86	28.60	1.03	0.009	0.83	1.68	1.08	64.31
SEM	0.079	0.014	0.739	0.043	0.001	0.122	0.044	0.024	0.791
Offspring diets									
CON-CON	3.02	0.87	29.12	0.93	0.008	0.90	1.60	1.01	63.34
CON-DMA	2.91	0.84	28.96	0.92	0.007	0.76	1.60	1.03	63.92
CON-FFF	3.04	0.87	28.73	1.00	0.008	0.75	1.70	1.09	64.16
DMA-CON	3.02	0.87	28.78	0.96	0.010	0.98	1.61	1.03	64.00
DMA-DMA	3.14	0.89	28.60	0.99	0.012	1.19	1.61	1.03	64.09
FFF-CON	3.02	0.86	28.60	1.03	0.009	0.83	1.68	1.08	64.31
FFF-FFF	3.01	0.85	28.46	1.07	0.010	0.90	1.66	1.07	64.47
SEM	0.113	0.020	1.045	0.061	0.002	0.173	0.063	0.035	1.119
Probabilities (*P*-value)									
Breeder diets	0.999	0.668	0.849	0.181	0.685	0.689	0.399	0.141	0.613
Offspring diets	0.401	0.257	0.863	0.397	0.365	0.388	0.975	0.879	0.850

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

[1]n=5.

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

Table 8. 4. Effects of feeding sources of docosahexaenoic and α-linolenic acids to ISA brown breeders and offspring on femur subpart attributes in 12-week-old pullets, [1,2]

Items	Epiphysis			Medullary			Cortical		
	Wt, 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, %
Breeder diets									
CON	2.29	0.59	26.20	0.61[ab]	0.006	0.99	1.02	0.59	59.04
DMA	2.24	0.59	26.49	0.56[b]	0.005	0.96	0.99	0.59	60.05
FFF	2.22	0.58	26.11	0.70[a]	0.007	0.93	1.07	0.64	59.65
SEM	0.070	0.013	0.558	0.037	0.001	0.128	0.050	0.019	1.476
Offspring diets									
CON-CON	2.29	0.59	26.20	0.61	0.006	0.99	1.02	0.59	59.04
CON-DMA	2.16	0.57	26.31	0.57	0.004	0.64	0.98	0.58	59.54
CON-FFF	2.20	0.56	25.46	0.72	0.006	0.80	1.07	0.64	59.81
DMA-CON	2.24	0.59	26.49	0.56	0.005	0.96	0.99	0.59	60.05
DMA-DMA	2.32	0.62	26.66	0.55	0.006	1.28	1.00	0.60	60.56
FFF-CON	2.22	0.58	26.11	0.70	0.007	0.93	1.07	0.64	59.65
FFF-FFF	2.24	0.60	26.77	0.68	0.007	1.06	1.08	0.64	59.49
SEM	0.098	0.018	0.789	0.053	0.001	0.181	0.070	0.027	2.087
Probabilities (*P*-value)									
Breeder diets	0.733	0.549	0.883	0.033	0.284	0.921	0.482	0.169	0.863
Offspring diets	0.127	0.065	0.457	0.978	0.502	0.137	0.968	0.894	0.989

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

[1]n=5.

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

Table 8. 5. 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-week-old laying hens upon challenge with lipopolysaccharide, [1,2]

Items	BW change	
	g	%
Challenge		
LPS	19.33	0.93
Sham	0.68	0.03
SEM	15.253	0.785
Breeder diets		
CON	19.33[a]	0.93[a]
DMA	-79.80[b]	-3.65[b]
FFF	-231.10[c]	-10.02[c]
SEM	32.636	1.297
Offspring diets		
CON-CON	19.33[a]	0.93[a]
CON-DMA	-399.81[b]	-16.90[b]
CON-FFF	-56.59[a]	-2.20[a]
DMA-CON	-79.80[b]	-3.65[b]
DMA-DMA	-84.33[a]	-3.71[a]
FFF-CON	-231.10[c]	-10.02[c]
FFF-FFF	-83.95[a]	-4.26[a]
SEM	46.154	1.834
Probabilities (*P*-value)		
Challenge	0.409	0.399
Breeder diets	0.010	0.006
Offspring diets	<0.001	<0.001

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

[1] n=5.

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

Table 8. 6. 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-week-old laying hens upon challenge with lipopolysaccharide,[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 (*P*-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

Values with uncommon superscripts within each column are significantly different ($P < 0.05$).
[1]The day-old female pullets from breeders fed CON, DMA and FFF were divided into three (CON, DMA and FFF), two (CON and DMA) and two (CON, FFF) post-hatch treatments, respectively. 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.
[2] Unchallenged CON-CON produced 13 eggs during 72-hour post-challenge. 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.

Table 8. 7. Effects of a lipopolysaccharide challenge on egg weight and eggshell quality at 24, 48 and 72-h post challenge.[1]

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
P-value	0.852	0.043	0.003	0.008

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

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

Table 8. 8. 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-week-old laying hens challenged with lipopolysaccharide, [1,2]

Items	Wt, 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 (*P*-value)			
Challenge	0.202	0.431	0.008
Breeder diets	0.059	0.411	0.017
Offspring diets	0.099	0.357	0.007

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

[1]n=5.

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

Table 8. 9. 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-week-old layer hens challenged with lipopolysaccharide, [1,2]

Items	Wt, 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 (*P*-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

Values with uncommon superscripts within each column are significantly different ($P < 0.05$).
[1]n=5.
[2]The day-old female pullets from breeders fed CON, DMA and FFF were divided into three (CON, DMA and FFF), two (CON and DMA) and two (CON, FFF) post-hatch treatments, respectively. 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.

Table 8. 10. 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-week-old layer hens challenged with lipopolysaccharide, [1,2]

Items	Epiphysis			Medullary			Cortical		
	Wt, 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 (*P*-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

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

[1]n=5.

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