EFFECT OF ω-3 LIPID SOURCES ON LAYER PERFORMANCE AND EGG QUALITY DURING PEAK PRODUCTION

by

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DECLARATION

I hereby declare that this dissertation submitted by me to the University of the Free State for the degree, **Magister Scientiae Agriculturae**, is my own independent work and has not previously been submitted by me at another University/Faculty. I further cede copyright of the dissertation in favour of the University of the Free State.

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GLORIA IN EXCELSIS DEO

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ACRONYMS AND ABBREVIATIONS

ADF	Acid detergent fibre
ALA	α-Linolenic acid
AME	Apparent metabolizable energy
ANOVA	Analysis of variance
C	Carbon
Ca	Calcium
CP	Crude protein
CV	Coefficient of variation
CVD	Cardiovascular disease
Δ	Delta
DHA	Docosahexaenoic acid
DM	Dry matter
DPA	Docosapentaenoic acid
EFA	Essential fatty acid
EFB	Feed bucket weight at the end of the week
EL	Extruded linseed
EO	Echium oil
EPA	Eicosapentaenoic acid
ESA	Eggshell surface area
EW	Egg weight
EU	European union
FA	Fatty acid
FAME	Fatty acid methyl esters
FAP	Fatty acid profile
FCR	Feed conversion ratio
FE	Feed efficiency
FFDM	Fat free dry matter
FO	Fish oil
GE	Gross energy
GIT	Gastrointestinal tract
GL	Ground linseed
HCL	Hydrochloric acid
HDL	High density lipoprotein
HHEP	Hen housed egg production

HO	High oleic acid
HSD	Honest significant difference
HU	Haugh units
IFBW	Initial feed bucket weight
IU	International Units
K	Potassium
LA	Linoleic acid
LC	Long chain
LDL	Low density lipoprotein
LO	Linseed oil
LSD	Least significant difference
ME	Metabolizable energy
Mg	Magnesium
MIU	Moisture, insoluble impurities and unsaponifiable matter
MUFA	Monounsaturated fatty acid
MUFA : SFA	Monounsaturated fatty acid to saturated fatty acid ratio
n-3	Omega-3 fatty acids
n-6	Omega-6 fatty acids
n-6/n-3	Omega-6 fatty acids to omega-3 fatty acids ratio
n-9	Omega-9 fatty acids
Na	Sodium
NaOH	Sodium hydroxide
NCD	Non-communicable diseases
NDF	Neutral detergent fibre
Non-GMO	Non genetically modified organism
OM	Organic matter
P	Phosphorus
PUFA	Polyunsaturated fatty acid
PUFA : SFA	Polyunsaturated fatty acid to saturated fatty acid ratio
Res	Residual feed
RH	Relative humidity
SC	Short chain
SDA	Stearidonic acid
SFA	Saturated fatty acid
SO	Soybean oil
SW	Shell weight
SWUSA	Shell weight per unit surface area

TAG	Triacylglycerol
TP	Total phosphorus
UFA	Unsaturated fatty acid
USA	United states of America
WFI	Weekly feed intake

Chapter 1

General Introduction

Poultry is globally the leading source of animal protein consumed by humans, because chicken meat and eggs both supply essential nutrients with high vitamin bioavailability in a cost-effective manner (Nkukwana, 2018; Chatterjee *et al.*, 2022). Worldwide research on the significant challenges on both food security and food safety are required (Chatterjee *et al.*, 2022). As part of increasing access to affordable and nutritious animal protein products, Molnár & Szőllősi (2020) indicated that egg consumption should be increased. However, to meet the ever-increasing demands for poultry products, significant improvements in the genetics, nutrition and husbandry practices of chickens should be implemented (El-Sabrout *et al.*, 2022). Globally, the development and expansion of the egg industry depend on creating practical, cost-effective ways to enhance egg output and quality (Thirumalaisamy *et al.*, 2016). While the improvement in nutritional properties of eggs and the development of functional foods contributing to health benefits are under consideration, modern consumers also show interest in the wellbeing of laying hens in battery cages (Di Rosa *et al.*, 2020; El-Sabrout *et al.*, 2022). Given the priority of food security on a global basis, it should be emphasised that the growing demands of health-conscious consumers place a higher demand on the development of designer eggs with a higher nutritional composition (Alagawany *et al.*, 2018).

Due to their excellent nutritional value, low price, and diversity in meal preparation, chicken eggs have long been a favourite food globally (Cherian, 2017; Mohamed *et al.*, 2021). In addition, consumers from developing countries favour egg consumption within different meal options (lunch & dinner), whereas consumers from developed countries tend to link egg consumption with breakfast meals. Chicken eggs are one of the most extensively researched animal protein sources that could be enriched with omega-3 polyunsaturated fatty acids (n-3 PUFAs) (Beheshti Moghadam & Cherian, 2017). Scientists have investigated multiple approaches to adding health-promoting elements into eggs in an attempt to promote egg consumption as a health benefit for consumers (Cherian, 2017).

Omega-3 and omega-6 (n-6) PUFAs play a role in many cellular processes, including cell signalling, maintaining the structural integrity and fluidity of cell membranes, controlling blood pressure, blood glucose levels, nerve function, inflammatory responses, insulin sensitivity, lipid metabolism and blood coagulation (Calder, 2012; Djuricic & Calder, 2021; Kapoor *et al.*, 2021; Ngo Njembe *et al.*, 2021). The essential n-3 and n-6 PUFAs that cannot be synthesized in the body and should be consumed through food are α-linolenic acid (ALA) and linoleic acid (LA), respectively (Ahmad *et al.*, 2012; Kapoor *et al.*, 2021). During the process of desaturase

and elongase, it is possible for humans to convert ALA into long chain (LC) n-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Since only a small amount of ALA may be converted into EPA and DHA, these LC n-3 PUFAs are primarily obtained through the dietary consumption (Burdge, 2006; Takic *et al.*, 2022) of fish and fish oil products that are known for being good sources of EPA and DHA (Zhang *et al.*, 2021).

Despite the COVID-19 pandemic, which claimed more than 3.4 million people due to acute respiratory syndrome on a global basis, chronic non-communicable diseases (NCD) continue to be the leading cause of human deaths on a worldwide basis (Djuricic & Calder, 2021). The World Health Organization (WHO, 2022) estimates that NCDs cause approximately 41 million deaths annually, which amounts to a staggering 74% of all deaths globally. The majority of NCD deaths are linked to cardiovascular diseases (17.9 million), followed by cancer (9.3 million), chronic respiratory diseases (4.1 million), and diabetes (2.0 million, including diabetes-caused kidney disease deaths). These four disease types cause over 80% of all premature NCD-related deaths (WHO, 2022), while oxidative stress and inflammation have been identified as significant risk factors for chronic NCDs (Djuricic & Calder, 2021). Because of the high cholesterol content and potential impact on the cardio-metabolic outcome, egg consumption has generated much debate about its impact on human health (Currenti *et al.*, 2022). However, Marventano *et al.* (2020) failed to establish any relationship between egg consumption and human health aspects such as cancer, cardiovascular disease, and other metabolic disorders. Cerone & Smith (2022) reported that omega-3 and omega-6 (n-6) type polyunsaturated fatty acids fulfil a vital role in maintaining human health and that the dietary intake of these fatty acids (FAs) should also be monitored as part of the strategies to reduce the negative impact of CVD in human health.

One of the most common health issues affecting adolescents and children is obesity (Baumann *et al.*, 2018). Overweight and obese children are prevalent in underdeveloped countries, where they typically become obese adults (Grace *et al.*, 2021). In 2015, there were 1.9 billion obese individuals and 609 million overweight adults, nearly doubling the frequency of obesity and overweight people since 1980, thus affecting more than a third (39%) of the global population (Engwa *et al.*, 2022). In South Africa 31% of males and 68% of females were considered obese or overweight by the end of 2016 (Boachie *et al.*, 2022). Ngo Njembe *et al.* (2021) performed a 3-month study with 24 men and women randomly divided into two groups, who had to consume two eggs daily - either enriched with oleic acid (control group) or enriched with ALA, DHA, rumenic acid and punicic acid (test group). The report (Ngo Njembe *et al.*, 2021) indicates a significant decrease (-3.17 cm) in the waist circumference of the test group individuals who had to consume the eggs enriched with long chain (LC) n-3 PUFAs.

Furthermore, high egg intake resulted in increased ($P< 0.001$) plasma levels of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol, despite having no change in the LDL/HDL ratio between the two groups (Ngo Njembe *et al.*, 2021), suggesting that obesity in the abdominal region could be reduced without increasing the risk of other metabolic disorders.

Diets of the modern, fast-paced consumers are marked by increased consumption of sugar and n-6 PUFAs, which is linked with little exercise, thereby resulting in the rise in chronic diseases such as obesity, diabetes, heart disease and cancer (Baker *et al.*, 2022). According to Stover *et al.* (2020), maintaining regular physiological performance through proper nutrition may help prevent chronic diseases linked to diets. Functional "designer foods" were created to enhance consumer health and well-being, lower healthcare costs and lower the risk of chronic diseases (Chhikara & Panghal, 2022). Designer eggs are sometimes classified as a "vegetarian foodstuff" with a reduced n-6/n-3 PUFA ratio, an increase in antioxidant pigments like carotenoids and/or higher levels of vitamins and minerals such as Se (Alagawany *et al.*, 2018; Réhault-Godbert *et al.*, 2019). The increased dietary intake of vegetable oils high in n-6 PUFAs within western societies is characterized by an n-6/n-3 ratio of 16:1 or higher, whereas the ratio used to be around 1:1 during evolutionary times (Simopoulos, 2019). Therefore, boosting the intake of n-3 PUFA is crucial, resulting in some scope for using dietary fatty acid manipulation strategies to change the FA content of frequently consumed foods in an attempt to improve the n-3 intake (Corrales-Retana *et al.*, 2020). Despite many reports (Basmacioglu *et al.*, 2003; Ansari *et al.*, 2006; Oliveira *et al.*, 2010; Antruejo *et al.*, 2011; Aguillón-Páez *et al.*, 2020) confirming the successful enrichment of eggs with n-3 PUFAs through dietary supplementation using n-3 lipid sources such as linseed oil, chia seeds and -oil, fish oil and soybean oil, limited research has been conducted on the efficiency of echium oil as an alternative lipid source to improve the n-3 PUFAs of eggs and chicken meat.

While it is possible to increase the concentration of DHA and decrease the n-6/n-3 ratio of PUFAs in eggs by dietary inclusion of linseed oil (high in ALA), the endogenous synthesis of DHA and EPA from ALA seems to be slow and inefficient – depending on the dietary concentration of the ALA (Zhao *et al.*, 2021). Despite these inefficiencies in converting ALA into LC n-3 PUFAs, the reliable supply of plant-based n-3 lipid sources (such as linseed) have gained considerable importance recently. Given the primary focus of seed oils as a source of ALA-rich n-3 PUFAs for human consumption, the evaluation of stearidonic acid (SDA) rich plant oils are emerging as a sustainable supply of n-3 PUFAs (Prasad *et al.*, 2021). Echium oil is a non-GMO vegetable oil made from the seeds of the *Echium plantagineum* plant and contains sizable amounts of SDA, which is also categorised as an n-3 PUFA with much

potential since it could be produced very cost-effectively (Miquel, 2008). This plant is native to South Africa's Western Cape area, where it thrives by growing in pastures (Hugo *et al*., 2022).

When comparing the n-3 PUFA content of egg yolks from laying hens fed treatments containing 5% soybean oil, SDA-enriched soybean oil, linseed oil or fish oil, Elkin *et al.* (2015) reported the yolks of the fish oil (305 mg) to have the highest levels of LC n-3 PUFAs, followed by the SDA enriched soybean oil (125 mg), linseed (91 mg) and conventional soybean oil (51 mg) treatments. These findings illustrate the potential of SDA to be converted into LC n-3 PUFAs such as EPA, DPA and DHA. Dey *et al*. (2021) reported that a dietary inclusion level of 1.5 – 2.5% will result in an increase in egg size as well as egg production and internal egg quality traits of laying hens. Both Cachaldora *et al*. (2007) and Zhao *et al*. (2021) reported that an increase in dietary fish oil inclusion resulted in an increased concentration of LC n-3 PUFAs in egg yolk, while it also seems that EPA, DPA and DHA from fish oil are better deposited in comparison with krill oil supplementation. Furthermore, Petrović *et al.* (2012) reported that despite the fact that dietary supplemental linseed oil (1 - 4% inclusion levels) had no effect ($P > 0.05$) on egg production and internal quality traits, a linear decrease ($P < 0.001$) in the n-6/n-3 ratio of egg yolk were recorded, while no negative influence ($P > 0.05$) were recorded on yolk cholesterol linked with dietary lipid inclusion levels.

This study aimed to determine the effect of different n-3 lipid sources, varying in their n-3 carbon chain lengths on (i) the production performance of laying hens during peak production, (ii) external and internal egg quality parameters and (iii) the fatty acid methyl esters (FAME) of egg yolk. Despite substantial research on the impact of supplemental lipid sources and inclusion levels on egg production and quality traits, a lack of information regarding the usage of SDA obtained from echium oil on laying hen performance and egg quality traits exists. In order to reflect the different type of n-3 PUFAs, five experimental diets were formulated as follows: (i) control treatment containing no additional lipid source and serving as basal diet for lipid source inclusion, (ii) fish oil [EPA (C20:5; n-3); DHA (C22:6; n-3)], (iii) soybean oil [ALA (C18:3; n-3)], (iv) linseed oil [ALA (C18:3; n-3)] and (v) echium oil [SDA (C18:4, n-3)]. The four different lipid sources were added to the basal control diet at 1.5%, without compensating for differences in total dietary lipid concentration or apparent metabolizable energy (AME) differences.

Although much attention was given to reducing the repetition of information and procedures within the various chapters, some repetition may occur between the chapters.
The layout of this dissertation is as follows:

- Chapter 1 is a general introduction that overviews the study's objectives and problem description.
- Chapter 2 is a literature review, which will give the reader a fundamental understanding of the subject field under investigation.
- Chapters 3 and 4 will address the primary aims of the study as follows:
  Firstly, Chapter 3 evaluates the effect of different n-3 PUFA lipid sources on the production performance of laying hens during peak production (24 to 40 weeks of age).
  Secondly, Chapter 4 investigates the effect of different n-3 PUFA lipid sources on the internal and external egg quality of laying hens and the yolk FAME during peak production (24 to 40 weeks of age).
- Lastly, Chapter 5 presents the entire dissertation's general conclusion and recommendations.

Chapter 2

Literature review

2.1 Introduction

The South African poultry industry stated that around 75% of the birds in South Africa are raised for meat production, and the other 25% are laying hens for egg production (SAPA, 2019). In 2021, the number of laying hens in the country was reduced by 7.1%, from 28.89 to 26.85 million hens, resulting from the estimated 2.18 million laying hens that were culled during the highly pathogenic avian influenza outbreak (SAPA, 2021). The supply of eggs in 2021 declined in accordance with the number of hens. The massive increase in egg demand in 2020 during the early COVID-19 lockdown helped to ease the surplus in eggs produced. While in 2021 total egg production was 7.5% lower than in 2020 and amounted to about 706.7 million dozen eggs (SAPA, 2021). According to the South African Department of Agriculture, Land Reform and Rural Development (DALRRD, 2022) eggs continue to rank as the fourth-largest animal product sector in South African agriculture, after poultry meat (R50.96 billion), beef (R43.01 billion), and milk (R21.17 billion), with a gross turnover of R11.44 billion at the producer level. SAPA (2021) further stated that with an average price of R22.92/kg in 2021, eggs continued to be the least expensive source of animal protein when compared to beef, pork, and chicken.

Eggs are a wholesome food source that provides particular health benefits to people. It is commonly known that eggs contain nutrients that include highly digestible proteins, fats, vitamins and minerals (Lee & Paik, 2019). Consumer demand for a healthier lifestyle supports the interest in enhancing chicken meat and eggs' fatty acid (FA) profile (Al-Khalaifah *et al.*, 2020a). Omega-3 (n-3) FAs are essential for normal development and growth, but it also contributes to preventing coronary heart disease, inflammation, hypertension, cancer and autoimmune diseases (Omidi *et al.*, 2015). Fish is the food source with the highest concentration of long-chain (LC) n-3 polyunsaturated fatty acid (PUFA) in the human diet, but since the consumption of fish is low in South Africa, the dietary intake of LC n-3 PUFA is lower than recommended for man (Rymer *et al.*, 2010; Omidi *et al.*, 2015). The FA profile of yolk lipids can be modified through the laying hen's diet, whereby n-3 enriched eggs can contain almost four times more DHA than commercially produced eggs (Singh *et al.*, 2012; Khan *et al.*, 2017). As an alternative to fish, FA enriched eggs could form part of a wholesome source of n-3 FAs in human diets. Khan *et al.* (2017) indicated that n-3 FAs in egg yolks are absorbed efficiently in the body and increase the bioavailability of docosahexaenoic acid (DHA) and high-density lipoprotein resulting in nutritional benefits for consumers. The n-3 FAs are

necessary for normal growth and health, and it has also been related to improved cardiovascular health, reduction of inflammation and the normal development of the brain, eyes and nervous system (Gogus & Smith, 2010; Kerr *et al.*, 2015).

The primary aim of this literature review is to evaluate available literature sources regarding the usage of different n-3 lipid sources on (i) the production performance of laying hens and (ii) internal and external egg quality characteristics (iii) as well as the extent to which egg yolk FAs can be manipulated through the laying hen diet to contain a higher concentration of different n-3 PUFAs. Supportive to the primary aim, literature with specific reference to the effect of intermittent chain length n-3 FAs coming from Echium oil will be evaluated for the inclusion in animal diets.

2.2 Lipid use in poultry diets

Formulation of poultry diets that include supplementary lipid sources (either fats or oils) are usually done to increase the energy density of the specific diet by substituting part of the carbohydrates with fats or lipids that have a lower metabolic heat increment. Despite other nutritional or economic considerations, a minimum of 1% of supplementary fat is required in poultry diets to ensure sufficient levels of essential fatty acids such as linoleic acid (LA), decreased dustiness of diets and to improve the palatability of the feed (Omidi *et al.*, 2015). Lipids are, therefore, an important feed component, not just for the high energy content but also for its essential fatty acids (EFAs) and fat-soluble vitamin content (Hudečková *et al.*, 2012).

2.3 Characteristics of lipids

Lipids are a group of organic compounds with a variety of diverse structures. It is not soluble in water, but it is soluble in organic solvents (Kerr *et al.*, 2015). Although fats and oils have similar structural characteristics, they differ in terms of their physical and chemical properties. Unlike fats, oils are a liquid at room temperature and tend to be more chemically reactive than solid fats; however, both groups are generally included in the term ‘fat’ (McDonald *et al.*, 2011).

Lipids provide energy for metabolic processes, but it also provides a supply pool for a variety of signalling molecules and forms part of cellular membrane structures (German, 2011; Jaishy & Abel, 2016). Lipids are the most concentrated energy source per unit of weight, meaning it has a higher energy value than carbohydrates (Kleyn, 2013). The categorization of lipids are as follows namely; (i) simple-, (ii) complex- and (iii) derived lipids. Simple lipids consist of LC FAs, which are either free or attached to an alcohol with an ester linkage, that includes

triacylglycerols (TAG) and waxes (Cole & Eastoe, 1988; McDonald *et al.*, 2011; Blanco & Blanco, 2017). Phospholipids, glycolipids and lipoproteins belong to the complex lipid category because they contain additional groupings like phosphoric acid, sugars, proteins or nitrogenous bases (Cole & Eastoe, 1988; Feingold, 2000; Asokapandian *et al.*, 2021). A derived lipid is produced by hydrolysis of simple or complex lipids and consists of fatty acids, glycerol and other alcohols (Baião & Lara, 2005; Fahy *et al.*, 2011).

2.4 Lipid oxidation

Lipid oxidation is the primary reaction that causes the deterioration of food during processing and storage. The oxidation of food not only reduces shelf life but can produce potentially toxic products that negatively impact human and animal health (Malheiro *et al.*, 2013; Johnson & Decker, 2015; Ahmed *et al.*, 2016). Basically, all foods that contain fat is likely to undergo lipid oxidation even if the unsaturated fatty acid (UFA) content is low. Omega-3 fatty acids are unsaturated and therefore foods containing high concentrations of n-3 FAs are at risk of lipid oxidation (Malheiro *et al.*, 2013; Vieira *et al.*, 2017). The oxidation of UFAs is a complex occurrence that takes place in the presence of oxygen and is brought on or accelerated by the presence of light, photosensitizers, heat, metals and nitrogen reactive species, while the degree of UFAs also contribute to the rate of oxidation (Møller *et al.*, 2007; Mariutti & Bragagnolo, 2017; Papuc *et al.*, 2017). Non-enzymatic oxidation can take place through two processes, namely: auto-oxidation and photo-oxidation (Mariutti & Bragagnolo, 2017). The process of auto-oxidation causes lipids to break down and form a wide range of oxidation products as a result (Ahmed *et al.*, 2016). Under moderate conditions, auto-oxidation requires the direct reaction of molecular or triplet oxygen with organic compounds. Triplet oxygen is a di-radical, meaning one of its even number of electrons has parallel spins, which is why it can react with radicals. Auto-oxidation can be portrayed as a free-radical-mediated oxidation chain-reaction that is generally described by three phases, namely: initiation, propagation and termination (Mariutti & Bragagnolo, 2017; Rontani & Belt, 2020). When initiation takes place, hydroperoxides are formed when oxygen and unsaturated fatty acids bind in the presence of a catalyst (e.g., heat, light, and metals like iron and copper). In the propagation phase, the reactive hydroperoxides bind with other fats to form additional reactive products. During the termination phase, hydrocarbons, ketones and aldehydes are formed, they are volatile which means that the substance easily evaporates at normal temperatures, but are somewhat unreactive (Kleyn, 2013).

Lipids like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are highly unsaturated and prone to oxidation and, therefore must be handled in a manner that will prevent the development of off-flavours that will impede feed intake. The susceptibility of eggs

to undergo oxidation increases when PUFAs are included in the layer diet. Alpha-tocopherol is an antioxidant that may be added to layer diets in order to reduce oxidation (Faitarone *et al.*, 2016). Adding antioxidants and limiting oxygen exposure while processing is effective ways to reduce oxidation and are generally practised while manufacturing n-3 products (Ismail *et al.*, 2016).

2.5 Fatty acids

Hydrocarbon chains form a significant part of the chemical structure of lipids, with FAs being one of the primary types of hydrocarbons. Fatty acids are linear, aliphatic monocarboxylic acids and generally have an even number of carbons with the carboxyl group at one end and a methyl group at the other end (Kerr *et al.*, 2015; Selvaraj, 2017). Fatty acids differ depending on the length of the hydrocarbon chain as well as the presence, number and position of double bonds (Burdge & Calder, 2015). Typical FA chain lengths range from 14 to 22 carbons, but can occasionally reach up to 36 carbon atoms or more in animal tissue. Carbon chain length is generally used to describe fatty acids as short-chain (<C6), medium-chain (C6-12) and long-chain (>C12), while FAs with a chain length of longer than 22 carbon atoms are described as very long-chain FAs (Wang *et al.*, 2013; Selvaraj, 2017; Nguo *et al.*, 2018).

Saturated fatty acids (SFA) do not have double bonds present in the hydrocarbon chain, while unsaturated fatty acids (UFA) have one or more double bonds (Rustan & Drevon, 2005; Wang *et al.*, 2013). The UFAs are further subdivided into two groups, monounsaturated fatty acids (MUFAs) and PUFAs, with MUFAs only containing a single double bond and PUFAs having two or more double bonds (Wang *et al.*, 2013). The fatty acids present in animal tissues have between one and six double bonds, with the FAs in higher plants rarely having more than three double bonds (Selvaraj, 2017). When a FA molecule has a double bond, the acid can take one of two forms, depending on the spatial arrangement of the hydrogen atoms connected to the double bond's carbon atoms. The FA is in the *cis* form when the hydrogen atoms are on the same side of the double bond, and in the *trans* form when the atoms are on opposing sides, as can be seen in Figure 2.1 (Rustan & Drevon, 2005; McDonald *et al.*, 2011; Oteng & Kersten, 2020). According to McDonald *et al.* (2011) and Wynn (2011), most FAs that occur naturally have the *cis* configuration. The majority of trans FAs are formed through hydrogenation of oils rich in PUFAs during industrial processing, while small quantities are also generated in the rumen of ruminants during fermentation (Oteng & Kersten, 2020). When referring to the position of the double bond with regards to the methyl end, omega (n) is used, and when reference is made to the carboxyl end, delta (Δ) is used (Rustan & Drevon, 2005). The FA series that is most common is the omega series, namely n-3, n-6 and n-9 (Pike, 1999).

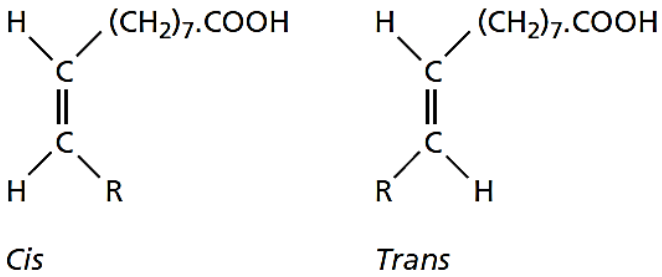

Figure 2.1 Visual representation of *cis* and *trans* configuration of a fatty acid (McDonald *et al.*, 2011)

Immediately after food is ingested, the fat cells absorb the neutral fats as well as the free fatty acids. In the cells, free fatty acids bind to glycerol to form the neutral fat that is stored with the other neutral fats. When glucose is abundant, it is very actively converted to glycerol and is also used to form triglycerides. These are stored in the fat depots just as the fats that were consumed with food (Meyer *et al.*, 1983). The synthesis of triglycerides is called lipogenesis, and insulin promotes the process. If the glucose supply of the body decreases, the fat in the fat cells is broken down by lipase into fatty acids and glycerol (Pirahanchi & Sharma, 2019). This process of fat breakdown is known as lipolysis and is promoted by the hormones adrenaline and glucagon (Lessan & Ali, 2019). Both the FAs and glycerol are taken up in the bloodstream and transported to the target tissue, where they are metabolized to meet the energy needs. Glycerol binds to the glycolysis pathway and converts glucose to pyruvic acid, while the fatty acids are catalysed step by step with the help of coenzyme A, and every time this happens a two-carbon fragment is split off from the fatty acid and oxidized (Meyer *et al.*, 1983). Fatty acids can thus be stored in the adipose tissue of the body, while it can also be converted to energy through beta-oxidation when needed.

2.6 Digestion and absorption of lipids and fatty acids in poultry

In comparison with the other macronutrients, the digestion and absorption of lipids is complex and requires a sequence of physiochemical events involving the breakdown to lipid droplets, emulsification, lipolysis and micelle formation (Ravindran *et al.*, 2016). It is generally known that digestion and absorption of lipids are influenced by dietary factors such as the degree of FA saturation, chain length and animal factors such as age, gender and breed of bird (Ravindran *et al.*, 2016; Rodriguez-Sanchez *et al.*, 2019). Dietary supplied FAs are usually absorbed efficiently from the gastrointestinal tract with absorption efficiencies up to 96% for ALA (Burdge, 2006; Stark *et al.*, 2008).

Digestion and absorption of dietary fat is complex because of the hydrophobic nature of fats (Bielawska & Somlaw, 2020). Since water and fat do not mix, bile is used to decrease the

tension at the oil-water interface to allow the mixing process to take place. This step aids in the emulsification and activation of pancreatic lipase and also prevents the denaturation of lipase when it leaves the surface of emulsified lipid droplets (Ravindran *et al.*, 2016; Lai *et al.*, 2018). Bile salts are rigid amphiphilic molecules, with a non-polar hydrophobic surface on one side and a polar hydrophilic surface on the other side (Galantini *et al.*, 2015). Dietary ingested lipids enter the intestine as large coagulated particles, while the presence of bile causes the coagulated particles to break down into small, stable droplets. This event happens to increase the surface area for lipase to interact (Bauer *et al.*, 2005). After gastric pre-treatment, most of the ingested lipids are hydrolysed in the small intestine.

Triacylglycerol (TAG) is a significant component of lipids and is a molecule of glycerol and three fatty acids (Xu *et al.*, 2018). When digestion takes place, two of the FA molecules are detached from the TAG, leaving a single monoglyceride. Therefore, the hydrolysis of triglycerides results in mono-glyceride and two FAs that are the absorbable units of lipids. Birds developed the distinctive ability of gut refluxes in order to minimise gut size, while reflux action is controlled by the gizzard. The disadvantage of a short digestive tract in birds is therefore overcome through the reflux mechanism, and the retention time is increased, which provides more time for digestion (Tancharoenrat *et al.*, 2014; Ravindran *et al.*, 2016). The reflux process in chickens is ongoing, and this allows for the entering of duodenal contents into the gizzard when the gizzard contracts - a process that has both positive and negative effects on digestion (Sklan *et al.*, 1978). Fat emulsification does not normally take place in the gizzard, but is initiated by bile salts and monoglycerides present in refluxed digesta from the duodenum. Bile secretion is stimulated by the presence of fat in the intestinal chyme, which is influenced by the concentration and type of ingested fat. Lipids are further dispersed into a coarse emulsion by the acidic conditions, mechanical activity of the gizzard and peptic digestion of protein since pepsin's proteolytic activity in the proventriculus and gizzard further facilitates this by causing lipids to be released from cell wall matrices (Tancharoenrat *et al.*, 2014; Ravindran *et al.*, 2016).

Hepatocytes of the liver form bile, which is excreted and transported to the gallbladder for storage or directly delivered into the intestine through the bile duct in the duodenum (Zhu *et al.*, 2020). The main components of bile that is needed for lipid digestion are phospholipids and bile salts (Maldonado-Valderrama *et al.*, 2011). Bile salts are conjugated with taurine in the liver, which increases their solubility in water as well as reduces the cellular toxicity of bile salts (Horáčková *et al.*, 2018). The interfacial composition can be determined through the interactions between bile salts and emulsifiers at the oil-water interface, and this can then have an impact on the rate of lipolysis in the small intestine. Bile salt usually adsorbs onto the

oil droplet, displacing the existing surface-active materials and encourage the adsorption of pancreatic lipase and co-lipase. The lipase then hydrolyses triglycerides into monoglycerides and free fatty acids, which remain at the interface and compete with bile salt adsorption. Lastly, the bile salts facilitate the expulsion of lipolysis products from the interface by forming micelles that solubilise those products (Macierzanka *et al.*, 2019).

The absorption of FAs from the gastrointestinal tract (GIT) differs in terms of site of digestion and absorption efficiency. In poultry, the main site of lipid digestion and absorption is the jejunum (Tancharoenrat *et al.*, 2014). According to Rodriguez-Sanchez *et al.* (2019), the hydrolysis of TAG starts in the duodenum, and the high quantity of lipolysis products (combination of intermediate and end products) in the duodenum indicate that this is the main site of lipid hydrolysis. The primary site of FA absorption is the jejunum, which accounts for 70-80% of FA apparent absorption while approximately 6-26% of FAs are absorbed in the ileum (Rodriguez-Sanchez *et al.*, 2019). Lipid absorption in the GIT after the ileum is reported to be insignificant (Konieczka *et al.*, 2018; Rodriguez-Sanchez *et al.*, 2019). Tancharoenrat *et al.* (2014) reported that the absorption of linoleic acid took place throughout the intestinal tract; however, digestion in the ileum was higher for FAs like LA in comparison with palmitic, stearic and oleic acid which begins in the jejunum.

The removal of FAs and monoglycerides from the oil-water interface happen spontaneously. Inside the micelles, molecules are grouped in a way that the polar groups are on the outer side and in contact with the aqueous phase, while the core is made up of the non-polar groups. Absorption is enabled by micelles that provide a high concentration of lipids in the unstirred water layer next to the mucosal cells. When the micelles come into contact with microvilli, they get disrupted and passively absorbed into the cells. Monoglycerides have an essential role in fat absorption because monogastric animals cannot absorb many FAs in the absence of monoglycerides (Ravindran *et al.*, 2016). Monoglycerides, as well as bile salts, have pieces of their molecular structure that are able to interact with aqueous systems and lipids, which forms an interface between water and lipids (Ravindran *et al.*, 2016). After short chain FAs and monoglycerides are hydrolysed it is passively absorbed via the enterocytes from the intestinal lumen. However, medium and long chain FAs, fat soluble vitamins, di-glycerides and cholesteryl esters need to be solubilised in the hydrophobic core of mixed micelles (Moran, 2017), which then enables the lipids to be transported through the aqueous environment of the intestine (Woollett *et al.*, 2006).

Monoglycerides and long chain FAs are re-esterified inside the enterocytes and combined with free and esterified cholesterol, lipoprotein as well as phospholipids to form chylomicrons which

are then secreted into lymphatic vessels. In poultry, the lymphatic system is poorly developed; therefore, the chylomicrons are secreted directly into the portal circulation and are called portomicrons. The portomicrons are distributed to different tissues, especially the liver, where lipids are used in the synthesis of lipoprotein and phospholipids, alternatively used for energy and/or stored in tissue as fat depositories. Altogether, the digestion and absorption of lipids are a complicated process that requires sufficient amounts of bile salts, pancreatic lipase and co-lipase. A shortage of any of these essential components will compromise the digestion and absorption systems (Ravindran *et al.*, 2016).

2.7 The ratio of omega-6 to omega-3 fatty acids (n-6/n-3)

Significant changes in dietary patterns have led to an alteration in the consumption of n-3 and n-6 FAs in human diets, normally associated with an increase in n-6 and a decrease in n-3 FA intake (Alagawany *et al.*, 2019). This alteration has resulted in an imbalance of the n-6/n-3 ratio, with a current ratio of 20:1 that is being considered significantly different from the dietary ratio of 1:1 of our ancestors (Simopoulos, 2016). The Western-style diet is particularly deficient in LC n-3 FAs such as DHA and EPA. Even health-conscious people have trouble meeting their n-3 dietary needs, mainly due to the scarcity and cost of quality LC n-3 sources, such as fatty fish (Sheppard & Cheatham, 2018). Therefore, enriching food sources such as eggs and meat with higher concentrations of LC n-3 FAs are a good alternative to increase the daily intake of n-3 in order to meet requirements (Alagawany *et al.*, 2019; Huerta-Yepez *et al.*, 2020). It further seems that the optimal ratio of n-6/n-3 should not exceed 2:1 to approximately 4:1 in human diets. The current imbalance in dietary n-6/n-3 ratio is an underlying cause of many chronic diseases that include inflammatory diseases, cardiovascular disease (CVD), cancers and other physiological disturbances (Omidi *et al.*, 2015).

Innes & Calder (2018) reported that consumers of Western-type diets recorded higher levels of arachidonic acid (n-6) in the membrane phospholipids of cells involved in inflammation in humans. It is therefore generally believed that a heightened intake of arachidonic acid or its precursor linoleic acid will increase inflammation in humans. In contrast, Kakutani *et al.* (2011) did not record an increase in inflammatory markers of healthy human adults that had an increase in arachidonic acid intake. However, there is evidence that the inflammation resolving effect of n-3 FAs are inhibited by a diet high in n-6 FAs; indicating that the relationship between n-3 and n-6 FA and their lipid mediators in the field of inflammation is intricate and still not adequately understood (Harris, 2018; Innes & Calder, 2018).

The evidence that eicosapentaenoic acid and arachidonic acid compete as substrates for some enzymes critical to homeostasis, inflammation and vascular reactivity implies that the

dietary n-6/n-3 FA ratio as well as that in the blood could be a suitable way to conceptualise the general omega status of an individual (Harris, 2018). It is further believed that the metabolic pathway through which ALA synthesises DHA is inefficient, suggesting that the shortage of LC n-3 FAs cannot be compensated for by higher levels of SC n-3 FAs. Evidently, the n-3 and n-6 pathways, which share a limited, genetically controlled reserve of desaturases and elongases, are only efficient if LA and ALA intake are balanced. Therefore, it has been implied that the best way to increase n-3 availability within the body is to decrease n-6 intake (Sheppard & Cheatham, 2018).

2.8 Omega-3 lipid sources

Vegetable and animal oils are the two broadly defined types of oils commonly used in feeds. Soybean-, linseed-, rapeseed- and palm oil are the most regularly used vegetable oils in feeds, whereas lard, poultry fat, tallow, and fish oil are the most commonly used animal oils. The concentration of SFA and UFA as well as the ratio of these FAs vary amongst oil sources. Vegetable oils have generally more UFAs than animal oils (having higher concentrations of SFAs), which result in vegetable oils being used at higher dietary inclusion levels than animal oils. Omega-3 FAs can be found in a variety of foods, including plants, seafood, fish oils, and marine algae. Alpha-linolenic, SDA, EPA, docosapentaenoic acid (DPA) and DHA are all n-3 PUFAs, while ALA is an essential fatty acid (EFA) (Shahidi & Ambigaipalan, 2019). In Figure 2.2 the chemical structures of SDA, EPA and DHA can be seen. The most important derivatives of ALA are DHA and EPA, with SDA being the ∆-6 desaturase product of ALA before being converted to DHA and EPA (Bourre & Galea, 2006; Gibson *et al.*, 2013; Calder, 2021).

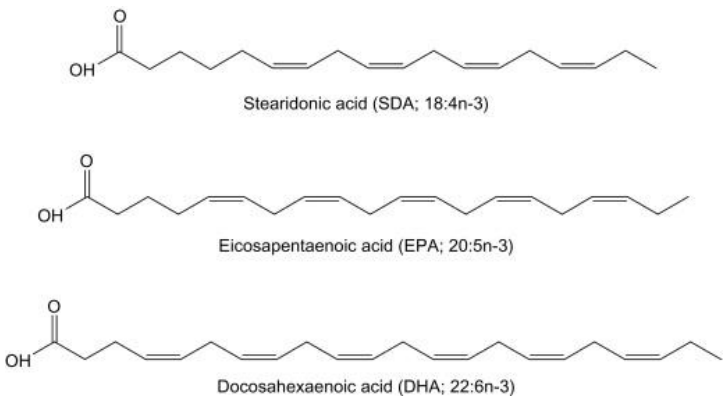

Figure 2.2 Chemical structures of the omega-3 fatty acids, stearidonic acid, eicosapentaenoic acid and docosahexaenoic acid (Kerrihard & Pegg, 2015).

2.8.1 Fish oil

Natural sources of EPA and DHA such as fish oils and a few types of seafood are scarce and quite expensive (Barceló-Coblijn *et al.*, 2008; Eltweri *et al.*, 2017). Between 8% and 44% of the total fatty acid profile (FAP) of fish oil consists of long-chain (LC) EPA and DHA (Konieczka *et al.*, 2017). Cachaldora *et al.* (2008) showed that the modification of LC n-3 FAs in laying hen eggs is dependent on the source and dietary inclusion level of the fish oil. Reasonably high levels of DHA could be deposited in the egg by feeding fish oil without negatively impacting odours (Pike, 1999). In contrast, sensory panellists found that eggs from hens fed a diet with 3% fish oil inclusion, are unacceptable (Ceylan *et al.*, 2011). Many authors (Elswyk, 1997; Lawlor *et al.*, 2010; Coorey *et al.*, 2015; Feng *et al.*, 2020) found that an increase in fish oil inclusion level is associated with an increase in egg taint and the development of a fishy taste.

Feng *et al.* (2020) found that enriching eggs with DHA by supplementing diets with fish oil increased the level of n-3 FAs inside the egg yolk in a dose-dependent manner and also caused a reduction in the n-6/n-3 fatty acid ratio. The control group with no supplemental oil had an n-6/n-3 ratio of 13.55 in the egg yolk, while the diet including 5 g/kg fish oil had a ratio of 2.25 (Feng *et al.*, 2020).

Dong *et al.* (2018) showed that hens fed a diet containing 8% fish oil had the lowest egg production in comparison to the usage of diets containing 8% coconut or soybean oil. The study found that with regards to performance, including egg weight, feed intake and feed conversion, hens fed fish oil performed the poorest, while shell thickness was unaffected by dietary treatment. The lower feed intake in hens fed fish oil played a part in the poor performance of hens, and was ascribed to the high level (8%) of oil used (Dong *et al.*, 2018).

2.8.2 Linseed oil

Linseed oil, similar to other plant-based oil sources, contains both linoleic acid (LA; n-6 source) and alpha-linolenic acid (ALA; n-3 source), which are both essential fatty acids (EFAs) required by animals (Hudečková *et al.*, 2012; Ehr *et al.*, 2017). Linseed contains seven times more ALA than soybean and maize oil, while the concentration of LA is three times lower than that of the mentioned oils – therefore it is used as an n-3 supplement in poultry diets (Ehr *et al.*, 2017).

Both Souza *et al.* (2008) and Oliveira *et al.* (2010) found that diets enriched with linseed oil increased the ALA content of eggs when compared to the control diet, whereas compared to

soybean and sunflower oil diets, EPA in eggs was detected only in the linseed oil treatment. Ibrahim *et al.* (2018) conducted a study to evaluate the effect of modified dietary ratios of n-6/n-3 and reported that by narrowing the n-6/n-3 ratio, by adding fish oil or linseed oil, improved the performance and immune response of broilers, while also increasing the n-3 PUFA concentration of the meat (Ibrahim *et al.*, 2018; Al-Khalaifah *et al.*, 2020a). In a study to evaluate the effect of linseed oil and DHA from microalgae origin on production performance in laying hens, Neijat *et al.* (2016) reported no differences between treatments. Petrović *et al.* (2012) found that when comparing dietary linseed oil treatments (inclusion: 0%, 1%, 2%, 3% and 4%), egg weight, yolk weight and yolk percentage was unaffected; however, the n-6/n-3 ratio decreased.

2.8.3 Soybean oil

Soybean oil is one of the most frequently used lipid sources that is added to monogastric diets over the world (Hudečková *et al.*, 2012). With an apparent metabolizable energy between 35.7 MJ/kg and 46.5 MJ/kg, soybean oil is a well-established oil in the poultry industry to increase the energy concentration of feeds (Ravindran *et al.*, 2016). It is a highly polyunsaturated vegetable oil that is a source of n-3 FAs, such as ALA, which is why it is highly accepted with regards to its fatty acid composition (Table 2.1). However, this characteristic is also the leading cause for the low oxidative stability of soybean oil (Ivanov *et al.*, 2011; Rodrigues *et al.*, 2012). As shown in Table 2.1, soybean oil is high in the n-6 FA, LA (49% – 56%) and also contains the n-3 FA, ALA (Omidi *et al.*, 2015; Li *et al.*, 2016; Deol *et al.*, 2017).

Batkowska *et al.* (2020) found that the ALA n-3 FA content of eggs almost doubled, from 0.58% in the control group to 0.94% if the diet is supplemented with 25 g/kg soybean oil. Additionally, they also recorded a considerable reduction in palmitoleic (C16:1) and oleic (C18:1) acids and an increase in n-6 PUFA in the eggs of the soybean oil supplemented treatment. In a study comparing linseed oil and soybean oil at a 30 g/kg dietary inclusion level, the egg yolks of the linseed oil treatment were higher ($P \leq 0.01$) in oleic-, myristoleic- and palmitoleic acid than the soybean oil treatment (Hudečková *et al.*, 2012).

2.8.4 Echium oil

Echium (*Echium plantagineum*) oil contains stearidonic acid (SDA, 18:4 n-3), which could be converted into EPA with moderate to high levels of efficiency in animals as well as in humans (Al-Khalaifah *et al.*, 2020a). During the conversion of ALA to EPA, DPA and DHA (Figure 2.3), stearidonic acid is the first product and is usually also a minor component of fish oils (Surette *et al.*, 2004).

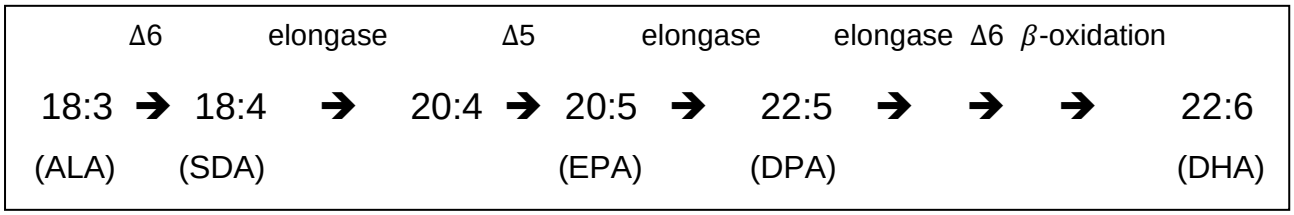

Figure 2.3 Biosynthesis pathway of alpha-linolenic acid (ALA) to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Above each step the corresponding catalysing enzyme is listed (Surette *et al.*, 2004; Eltweri *et al.*, 2017)**.**

Surette *et al.* (2004) found that the delta-6-desaturase rate-limiting step produces SDA and that the consumption of echium oil may lead to the enrichment of body tissue with longer chain PUFAs, therefore resembling the beneficial outcome associated with dietary n-3 PUFA present in fish oils. The findings of Calder (2021) show that, in humans, SDA is converted to EPA but not DHA and that SDA reached a more considerable rise of EPA in blood lipids or blood cells than ALA when given the same intake. As a result, SDA is expected to have a more beneficial effect on health related aspects than ALA, but like ALA, SDA cannot substitute preformed EPA and DHA (Calder, 2021).

Echium oil is remarkably unique because the typical FA composition is as follows: SDA 13%, oleic acid (18:1 n-9) 16%, linoleic acid (LA, 18:2 n-6) 19%, γ-linolenic acid (18:3, n-6) 10% and 30% of ALA (C18:3; n-3) (Surette *et al.*, 2004; Miquel, 2008). Through metabolism, the natural ratio of FAs in echium oil enhanced tissue concentrations of EPA and DHA without increasing concentrations of arachidonic acid (20:4 n-6) in humans (Miquel, 2008). James *et al.* (2003) found that when comparing the consumption of purified ethyl esters of SDA and ALA on plasma and cellular concentrations of n-3 PUFAs in humans, SDA was more effective in enriching body tissues with EPA than ALA. Kitessa & Young (2008) reported that ALA, SDA, EPA and DPA increased significantly in breast and thigh meat of broilers when echium oil was fed compared to rapeseed oil (Kitessa & Young, 2008). It further seems that SDA is incorporated into adipose tissue more readily than into the egg yolk (Elkin *et al.*, 2015). Since the FA profile of egg yolk generally reflects the hen's dietary intake, these differences in specific tissue types indicate that alternative pathway(s) for the hepatic secretion and transport of SDA could exist in the laying hen (Elkin *et al.*, 2015).

Table 2.1 Summary of literature regarding the fatty acid methyl ester composition (%) of various lipid sources used in poultry diets

		Myristic acid (C14:0)	**Palmitic acid (C16:0)**	**Stearic acid (C18:0)**	**Oleic acid (C18:1)**	**Linoleic acid (C18:2)**	**α-Linolenic acid (C18:3)**	**Stearidonic Acid (C18:4)**	**EPA (C20:5)**	**DPA (C22:5)**	**DHA (C22:6)**
Fish oil	Bell *et al.* (2010)	5.80	15.00	3.00	10.10	3.50	1.60	-	8.50	0.80	10.10
	Omidi *et al.* (2015)	5.61	27.43	4.44	32.84	1.59	0.23	-	5.59	-	14.20
	Elkin *et al.* (2015)	7.44	14.86	3.10	8.26	1.22	0.41	-	17.24	2.35	10.93
	Konieczka *et al.* (2017)	-	11.07	0.98	24.00	4.80	1.10	-	11.22	-	11.83
	Al-Khalaifah *et al.* (2020)	8.67	17.15	3.59	7.01	1.12	0.69	-	16.48	-	9.42
Linseed oil	Cachaldora *et al.* (2008)	-	5.50	3.10	19.20	16.80	47.70	-	-	-	-
	Bayrak *et al.* (2010)	-	5.07	4.78	22.30	13.98	53.46	-	-	-	-
	Hudečková *et al.* (2012)	0.04	4.70	3.39	18.82	16.27	56.26	-	-	-	-
	Al-Khalaifah *et al.* (2020)	0.04	5.08	3.42	18.95	16.50	54.44	-	-	-	-
Soybean oil	Hudečková *et al.* (2012)	0.08	10.39	4.19	22.10	53.64	8.17	-	-	-	-
	Tancharoenrat *et al.* (2014)	0.14	10.86	4.65	22.29	48.89	6.54	-	-	-	-
	Omidi *et al.* (2015)	0.34	15.57	3.97	23.50	55.53	1.10	-	-	-	-
	Dong *et al.* (2018)	0.08	10.47	3.92	21.30	51.24	6.31	-	-	-	-
Echium oil	Surette *et al.* (2004)	-	7.10	3.70	15.40	18.80	28.40	12.50	-	-	-
	(Bilgiç & Yeşilçubuk, 2012)	-	7.90	4.80	16.20	15.30	32.90	12.20	-	-	-
	Bilgiç-Keleş *et al.* (2019)	-	7.30	4.80	16.20	14.50	33.50	14.20	-	-	-

2.9 Effect of dietary fatty acid composition on production parameters

2.9.1 Egg production

The onset of lay and egg production of laying hens are significant indicators of the reproductive health of the birds (Ottinger *et al.*, 2015). Promila *et al.* (2017) studied the effect of six dietary inclusion levels (1, 2, 2.5, 3, 3.5 and 4%) of linseed oil added to the control diet and found that the 2.5% linseed oil diet resulted in the highest ($P< 0.05$) hen day egg production. In another study, Soliman & El-Afifi (2020) reported that when compared to 3% palm oil inclusion, 3% flaxseed oil increased ($P< 0.05$) egg production (42.52% vs. 51.90%) and the n-3 alpha-linolenic fatty acid content (0.03% vs. 5.03%) of the egg of Saudi local strain laying hens. This is in agreement with Ehr *et al.* (2017), who found an increased egg production with an increase in dietary flaxseed oil inclusion until a plateau is reached at 2% oil inclusion, where the non-supplemented control resulted in 95% hen housed egg production (HHEP), 0.5% oil inclusion had 91.4% HHEP, 1% oil inclusion had 96.9% and 2% oil inclusion resulted in 98.3% HHEP. Omar *et al.* (2014) reported no effect ($P> 0.05$) on feed intake, feed conversion and egg production; despite recording an increase ($P< 0.05$) in the n-3 PUFA concentrations of eggs associated with an increase in dietary fish oil inclusion levels. Elkin *et al.* (2015) reported no effect ($P> 0.05$) on egg production and egg weight using diets containing 5% supplemental conventional soybean oil, stearidonic acid-enriched soybean oil, flaxseed oil, or fish oil. However, the long-chain n-3 FA content of eggs was markedly higher for the fish oil treatment (305 mg/yolk) than for the conventional soybean oil (51 mg/yolk), flaxseed oil (91 mg/yolk) and stearidonic acid-enriched soybean oil (125 mg/yolk) treatments. Wei *et al.* (2021) also did not find a 3% dietary soybean oil inclusion to have a significant effect on egg production. However, Ekine *et al.* (2020) observed a higher egg production for hens fed a diet with 10% supplementary soybean oil than for the other treatments (0%, 2.5%, 5%, or 7.5% soybean oil inclusion). Ekine *et al.* (2020) concluded that when lipids are added to laying hens' diets, they improve feed conversion, raise the density of the food, make it more palatable, improve metabolic energy efficiency, and increase egg production.

2.9.2 Egg weight

Egg weight has become an important determinant of profitability, leading to management techniques that promote larger eggs. Heavier egg weights can be achieved by either extending the time birds stay in lay since the egg size of laying hens increase as they age and/or increasing egg size through nutritional manipulation (Galea, 2011; Travel *et al.*, 2011).

Ahmad *et al.* (2012) reported that although feed intake was reduced when supplementary linseed oil was fed, the feed efficiency of laying hens increased. Ceylan *et al.* (2011) found no differences ($P > 0.05$) in egg weights when feeding laying hens diets containing rapeseed-, sunflower-, linseed- and fish oil at inclusion levels of 15 g/kg and 30 g/kg for 12 weeks. This is in agreement with many authors (Bean & Leeson, 2003; Zou & Wu, 2005; Petrović *et al.*, 2012; Omidi *et al.*, 2015; Kralik *et al.*, 2018) who found that dietary supplementary lipid sources did not affect egg weight. However, Dong *et al.* (2018) reported that dietary fish oil inclusion (8%) resulted in lower egg weights compared to soybean- and coconut oil inclusion (8%). This is in agreement with Saleh (2013) who reported that egg weight decrease when more than 1.25% fish oil are included in diets. In contrast, Mariod *et al.* (2015) found that dietary supplemented fish oil resulted in heavier eggs (56.4 g vs. 51.1 g) when compared to supplemental palm olein oil.

Although there are no definite explanations for the differences in experimental outcomes regarding the influence of lipid source on egg weights, it can be attributed to variances in the experimental setup, hen age, hen strain and composition of the basal diet (Fraeye *et al.*, 2012). Another factor could be that not all the diets compared in a trial are isocaloric (Ahmad *et al.*, 2017).

2.9.3 Feed intake

Ideally, the profitability of a layer farm would improve if the nutrient requirements of birds were met with lower feed intake requirements of birds while maintaining egg weights. With this taken into consideration, appetite, egg weight, and bodyweight dynamics are the main traits involved in predicting feed intake of hens using multiple linear regression (Clark *et al.*, 2019).

Wu *et al.* (2005) and Barzegar *et al.* (2020) found that the feed intake and feed conversion ratio (FCR) decreased, while the egg weight increased due to dietary supplemented fats. In a five-week period the daily feed intake was found to be nearly identical in a broiler study comparing echium oil to rapeseed oil in an isoenergetic diet, with lipid inclusion levels of 5% and 5.3%, respectively (Kitessa & Young, 2008). Omidi *et al.* (2015) conducted a study to compare different oil sources, namely fish-, olive-, grapeseed-, canola-, and soybean oil (3% dietary inclusion), when modifying egg yolk FA profile and found that feed intake and FCR were not significantly affected by treatments. In contrast, another study (Alagawany *et al.*, 2019) has shown that feed efficiency of laying hens decreased linearly with increased dietary FA unsaturation levels during an 8-week experimental period between 40 and 48 weeks of age. There is variation in the results of different studies which could likely be due to the

differences in dietary FA chain lengths and/or level of lipid inclusion, and whether the dietary treatments are isoenergetic or not.

2.9.4 Feed efficiency

Feed efficiency is of significant importance since feed accounts for 60 -70% of the costs within a layer production unit (Barzegar *et al.*, 2020). A study (Ehr *et al.*, 2017) found that the feed efficiency of laying hens increases linearly with an increase in dietary supplemented linseed oil concentration, while feeding milled flaxseed led to a decrease in feed efficiency. A possible side effect of antinutrients present in flaxseed is decreased digestibility (Ehr *et al.*, 2017; Berenjian *et al.*, 2021). Linatine and cyanogenic glycosides are antinutrients found in flaxseed, but has been shown to have a lower antinutrient impact on human health when compared to soybean and canola (Ganorkar & Jain, 2013). Dong *et al.* (2018) reported that feed intake was higher ($P < 0.05$) at an 8% dietary inclusion of soybean oil compared to that of fish oil, although there were no differences ($P > 0.05$) in the FCR between the two lipid sources.

2.9.5 Laying rhythm

On average, the process of egg formation in laying hens takes about 25 to 27 hours (Gillespie & Flanders, 2010). The laying rhythm of an individual hen is defined by a specific pattern of laying clutches (number of consecutive days with eggs laid), as well as the defined breaks or interruptions between them. When a clutch is completed, a hen would naturally stop laying eggs to become broody for sitting on the nest. For commercial egg production conditions, it is desirable to avoid the brooding behaviour by the selection of hens that have prolonged laying clutches. Clutch size is considered a trait for genetic selection and is used to describe individual laying patterns (Wolc *et al.*, 2019). The individual clutch lengths are used to determine the total egg production of a flock of hens at a certain age (Sakomura *et al.*, 2019). The first egg in a clutch is usually laid early in the morning, and the following eggs are laid later in the succeeding days. The time between consecutive ovipositions minus 24 hours is called the “lag period” and is longer in short clutches and shorter (sometimes even negative) in long clutches (Tůmová *et al.*, 2017).

Tůmová & Gous (2012) found that an environmental temperature of 28ºC did not have a negative effect on egg production in birds with shorter clutch lengths and that lower feed intake did not constrain egg production at this temperature. Nevertheless, because of the lower variance linked to egg weight than with the rate of lay, a reduced feed intake had a negative effect on egg weight. In a study conducted on Lohman Brown laying hens, Petrović *et al.*

(2012) reported that the dietary inclusion of linseed oil (1%, 2%, 3% and 5%) did not alter the laying pattern of the hens.

2.10 Egg quality characteristics

Eggs are a rich source of essential fatty acids, choline, vitamins A and B_{12}, as well as bioavailable zinc, iron and iodine (Iannotti *et al.*, 2014; Lima & Souza, 2018; Papanikolaou & Fulgoni, 2020). Eggs have an affordable economic value and are a good source of animal protein. Due to their lower cost compared to other animal proteins, they have become a popular consumer item worldwide (Gao *et al.*, 2021). A growing number of people are becoming more aware of the internal nutritional composition of eggs, such as the n-3 and n-6 FA content of the egg yolk (Gao *et al.*, 2021). The content of FAs in the egg directly relate to the FA content of the feed while relating to the FA that is the majority in the respective plant oils used, thus it is important to consider the role of the hen's diet on the ratio of both saturated and unsaturated fatty acids inside the egg yolk (Hudečková *et al.*, 2012). The shell, albumen and yolk are the three basic components of a chicken egg, the approximate chemical composition of these components is shown in Table 2.2 (Ajala *et al.*, 2018).

Table 2.2 Approximate chemical composition (g/100g) of the whole egg and egg components

Egg component		Moisture	Protein	Lipid	Ash
Egg shell	(Ajala *et al.*, 2018)	1.0	3.3	0.4	95.1
	Brown eggshell powder (Waheed *et al.*, 2019)	0.2	5.0	0.08	94.3
	White eggshell powder (Waheed *et al.*, 2019)	0.5	3.9	0.4	94.6
Albumen	(Réhault-Godbert *et al.*, 2019)	87.7	10.8	0.2	0.4
	(Gao *et al.*, 2021)	88.0	11.0	0.2	0.8
Yolk	(Réhault-Godbert *et al.*, 2019)	55.0	15.5	26.7	1.7
	(Gao *et al.*, 2021)	48.0	17.5	32.5	2.0
Whole egg	(Chepkemoi *et al.*, 2017)	75.6	15.4	2.34	0.9
	Cage eggs (Zotte *et al.*, 2021)	76.7	12.4	10.1	0.9

2.10.1 Eggshell quality

Eggshell quality is important in the commercial production and marketing of table eggs, as cracked and broken eggs directly influence economic losses. Since the primary role of the eggshell is to keep bacteria and other microorganisms from penetrating the egg cell, it becomes more important to ensure that the eggshells remain defect-free to ensure the hygiene and safety of eggs for human consumption (Pavlovski *et al.*, 2012; Waheed *et al.*, 2019). Cachaldora *et al.* (2008) found that eggshell thickness was not affected when comparing diets containing various levels (0%, 1.5%, 3.0% & 5.0%) of fish oil, soybean oil, linseed oil or lard with a diet with no added fat. These findings concur with Josling *et al.* (2019), who found no effect ($P > 0.05$) on eggshell quality when comparing 3% lipid inclusion of linseed- and fish oil blend, fish oil, sunflower oil, high oleic acid sunflower oil and tallow. When comparing dietary treatments containing 15% canola seed, flaxseed or Linpro, it was found that feeding flaxseed over the long term caused eggshell quality and egg production to be reduced (Jia *et al.*, 2008). Jia *et al.* (2008) stated that shell strength has often been gauged by using egg specific gravity, while eggs from chickens given flaxseed consistently had the lowest specific gravity in the abovementioned investigation. As a result, it's probable that the increased digesta viscosity brought on by the flax mucilage contributed to the reduced calcium absorption (Jia *et al.*, 2008).

2.10.2 Albumen quality

Egg albumen consists primarily of water (84% - 89%) and proteins (10% – 11%), while other components, such as carbohydrates, lipids, and minerals make up a minor part of the albumen content (Sharif *et al.*, 2018). Since albumen quality is easily measured and relates to egg freshness, the height of the thick albumen when an egg is broken onto a flat surface has greatly defined the quality of eggs by expressing it in Haugh units. It adjusts the albumen height to the weight of the egg and utilizes a log scale to account for the fact that the height of albumen decreases logarithmically with storage time (Silversides & Budgell, 2004).

Javed *et al.* (2019) reported no effect of different dietary oil sources on the quality of albumen. In agreement with this, Batkowska *et al.* (2020) reported that 2.5% dietary soybean or linseed oil inclusion did not have an effect on albumen or other egg quality parameters. However, Gao *et al.* (2021) reported a difference ($P < 0.05$) in albumen heights when laying hens were fed 1.5% lipid inclusion of soybean oil (6.90 mm), lard (7.59 mm), mixed vegetable oils (7.37 mm) and 3% of soybean oil (7.20 mm), lard (7.37 mm) or mixed vegetable oils (7.12 mm) compared to the non-supplemented control (7.27 mm).

2.10.3 Yolk colour and quality

The sensory perception of 30 panellists showed that a higher score for farm eggs was mainly due to the paler yellow yolk of industrial eggs (Berkhoff *et al.*, 2020). The variation in the yolk colour of farm eggs can be attributed to several factors such as the pigmentation source and efficient utilization thereof, as well as the combination of feed ingredients, genetics, stress and the availability and stability of xanthophylls (Berkhoff *et al.*, 2020). Yolk colour differences are caused by the profile and concentration of carotenoids in the feed, as well as their absorption (Mugnai *et al.*, 2009; Berkhoff *et al.*, 2020). According to many authors (Van Den Brand *et al.*, 2004; Mugnai *et al.*, 2009; Berkhoff *et al.*, 2020; Mwaniki *et al.*, 2020), free range eggs tend to have darker yolks due to access to diets high in carotenoid pigments, such as grasses and insects.

Cachaldora *et al.* (2008) found that yolk colour increased with dietary inclusion (5%) of soybean or linseed oil when compared to a non-supplemented control diet, whereas an increase in fish oil inclusion (0%, 2.5% and 3.0%) relates to a linear reduction in yolk colour. Petrović *et al.* (2012) found no effect of dietary linseed oil inclusion (0%, 1%, 2%, 3% or 4%) on yolk fat content, yolk weight or yolk percentage but that the n-6/n-3 ratio decreased (P< 0.001) (13.28, 6.38, 4.29, 3.75 and 3.06 respectively).

2.11 Enrichment of chicken eggs

Knowledge regarding the health and clinical benefits of n-3 PUFAs have improved the public's acceptance and preference for food products enriched with these desirable fatty acids (Feng *et al.*, 2020). In the laying hen, n-3 FAs are readily absorbed and transported from dietary sources for deposition into the yolk. It takes an average of fourteen days for a laying hen to adapt to an n-3 FA enriched diet and reach a plateau of dietary n-3 FA incorporation into developing follicles (Ehr *et al.*, 2017). Dietary oil sources such as linseed oil, soybean oil and fish oil have long been used to enrich table eggs with n-3 PUFAs (Batkowska *et al.*, 2020; Feng *et al.*, 2020). Research on using echium oil to improve eggs is limited, but studies in enriching chicken meat with n-3 FAs by using echium oil have been conducted (Kitessa & Young, 2008; Al-Khalaifah *et al.*, 2020b).

2.12 Conclusion

According to the present literature evaluated, there are several ways to increase the nutritional qualities of eggs as a human food source through dietary intervention. These strategies not only meet the demand for healthier food by wealthier, health-conscious consumers with

specific dietary preferences, but can also provide a valuable nutrient source for people with specific food allergies. However, there have been inconsistent findings in the literature about the favourable and/or unfavourable impact of dietary fatty acid profile alteration on production performance and egg quality characteristics. Literature around the influence of echium oil as a source of n-3 stearidonic acid on laying hen performance is also limited. As a result, the inconsistent findings in the literature must be addressed and clarified by additional research, particularly on the effects of different lipid sources (including echium oil) varying in n-3 fatty acid chain length and how it affects laying hen production, egg quality and egg fatty acid content under typical South African climatic conditions.

Chapter 3
Effect of omega-3 lipid sources on production performance of laying hens at peak production

Abstract

The aim of this study was to investigate the effect of dietary omega-3 lipid sources on the production performance of laying hens during the peak laying period. Experimental treatments consisted of a control diet containing 0% supplementary lipid and four lipid sources (varying in omega-3 fatty acid chain length) mixed into the basal diet at 1.5%, respectively. The lipid sources used were fish- (EPA, C20:5 & DHA, C22:6), soybean- (ALA, C18:3), linseed- (ALA, C18:3) and echium oil (SDA, 18:4). A total of 150 Hy-Line Silver-Brown hens (21 weeks of age) were allocated to five experimental diets (n=30 birds/treatment). During weeks 24, 28, 32, 36 & 40, all eggs produced were recorded and weighed individually, while feed intake and body weight of birds were determined. Data were pooled to calculate the average production performance of the peak laying period. A one-way ANOVA procedure was used to analyse the data. Results indicated that omega-3 lipid sources had no effect on feed intake, feed efficiency or egg output. Birds from the control treatment recorded lower (P< 0.05) body weights (1729 g). Although hen-day egg production was lower for the soybean oil treatment compared to linseed oil (94.3% vs. 96.7%), sellable eggs did not differ between treatment means. No clear trends were observed for the effect of omega-3 lipid source on peak production performance of laying hens. The omega-3 lipid source had minor effects on peak production performance of laying hens.

Keywords: Fish oil, soybean oil, linseed oil, echium oil, stearidonic acid, egg production

3.1 Introduction

The importance of dietary oils in animal and poultry feed has come to the attention of breeders and producers due to ongoing advancements in poultry performance potential. Therefore, one of the poultry industry's main areas of focus is finding ways to optimize egg production performance and improve egg quality in order to satisfy the needs of consumers (Gao *et al.*, 2021). Omega-3 fatty acid enriched egg products have become increasingly popular on the global market, particularly in developed countries (Dong *et al.*, 2018). The significance of enhancing the nutritional qualities of animal products (meat, milk, and eggs), particularly through compositional changes that promote health, has grown in recent years (Świątkiewicz *et al.*, 2020). Complementary to their physiological functioning within the body, dietary long-chain omega-3 poly-unsaturated fatty acids (n-3 PUFAs), specifically eicosapentaenoic acid

(EPA) and docosahexaenoic acid (DHA), have positive impacts on human health (Lee *et al.*, 2019; Świątkiewicz *et al.*, 2020; Rouhanipour *et al.*, 2022).

The most commonly used energy source to increase energy density in layer diets is oils, which has a variety of additional benefits including improved palatability, feed intake, animal immunity and lowering morbidity (Oliveira *et al.*, 2011; Stevanović *et al.*, 2018; Lee *et al.*, 2019). Although many authors (Omar *et al.*, 2014; Omidi *et al.*, 2015; Świątkiewicz *et al.*, 2020) found no significant impact on production performance parameters when supplemental lipid sources are added to the diets of laying hens in order to enhance the n-3 FA content of the egg yolk, others (Basmacioglu *et al.*, 2003; Zou & Wu, 2005; Ekine *et al.*, 2020; Wei *et al.*, 2021) did report an impact ($P< 0.05$) on production performance. There is a lot of controversy about the favourable and unfavourable impacts of different oil sources on the production performance parameters of laying hens, which could be ascribed to genotype, age, phase of lay (Tůmová *et al.*, 2017), lipid sources (Dong *et al.*, 2018; Dey *et al.*, 2021), as well as the inclusion level of the different oil sources (Ahmad *et al.*, 2012; Gao *et al.*, 2021), and the difference in fatty acid (FA) composition of different oil sources (Najib & Al-Yousif, 2014; Dong *et al.*, 2018).

Many studies (Bean & Leeson, 2003; Ahmad *et al.*, 2012; Alagawany *et al.*, 2018; Kartikasari *et al.*, 2021) have been conducted to determine how n-type fatty acids in layer hen diets can enhance the nutritional quality of the egg yolk, especially n-3 PUFA. Stearidonic acid (SDA, n-3; C18:4) is a prominent n-3 PUFA in echium oil and has a higher conversion efficiency to longer chain n-3 PUFA such as eicosapentaenoic- (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) compared to α-linoleic acid (ALA, C18:3). Additionally, SDA is less prone to lipid oxidation than EPA from fish oil sources. The use of echium oil illustrates some benefits for dietary n-3 enrichment (Elkin *et al.*, 2015), but contradictions in literature regarding the effect of dietary n-3 lipid source on the production performance of laying hens warrant further clarification. Currently there is no studies available that examines the possible effect of echium oil, rich in n-3 PUFA stearidonic acid, on laying hen performance. Therefore, this chapter will explore the effects of different omega-3 polyunsaturated fatty acid sources on the production performance of laying hens.

3.2 Materials and methods

3.2.1 Experimental site

The experiment took place at Bloemfontein, in the Free State, on a small-scale commercial layer farm with coordinates: 29°04’52.19” S 26°10’42.34” E. Birds were housed in individual

battery cages within a naturally ventilated poultry house which were equipped with curtain sides and artificial light supply.

3.2.2 Experimental birds and husbandry

A total of 150 Hy-Line Silver Brown pullets (± 17 weeks of age) were obtained from a commercial pullet producer near Parys in the Free State Province, South Africa. Birds received a commercial layer mash from arrival until the introduction of the experimental diets at 21 weeks of age. Water and feed provision were on an *ad libitum* basis during this time (17 to 21 weeks of age). At 21 weeks of age, the five experimental diets were randomly assigned to individual birds (n=30 birds/treatment), while feed was given to a maximum of 120 g/bird/day to ensure that the nutrient requirements of birds were met. Feed portioning was done twice daily to ensure adequate amount of feed was available throughout the day and to facilitate feeder trough cleanout, in order to prevent selective eating of coarser dietary particles.

Birds were kept individually in metabolic battery cages of 1600 cm^3/cage (Figure 3.1). Each cage had an individual nipple drinker and feeder tray, while all cages were enriched with an individual wooden perch which enables the chickens to have more locomotion and to ensure easier feed and water consumption. The cages were numerically numbered for identification purposes, while individual birds were also leg tagged with the corresponding cage number to ensure correct placement.

At arrival of the birds (17 weeks of age) a photoperiod of 13 hours light with 11 hours darkness (13L:11D) were regulated with a timer set in accordance with the breed requirements for each phase of development. This was then stepped up to 14 hours of light at 20 weeks of age with 10 hours dark (14L:10D), at 26 weeks of age it was stepped up to 16 hours of daylight and 8 hours of darkness (16L:8D) as indicated by the Hy-Line Guide (Hy-Line Guide, 2016).

Figure 3.1 Experimental birds housed individually in metabolic battery cages

3.2.3 Hy-Line Silver Brown laying hens

The Hy-Line Silver Brown layer genotype is amongst the most productive layer breeds, producing over 330 rich brown eggs up to 74 weeks of age (Tyasi *et al.*, 2020). Together with a heavier mature weight, superior liveability and a strong adaptable temperament these characteristics give the Hy-Line Silver Brown strain an ideal balance in both intensive and alternative production systems (Okoro *et al.*, 2017; Tyasi *et al.*, 2020)

3.2.4 Husbandry management practises

Certain husbandry activities were routinely performed with regard to the experimental birds. The environmental temperature and relative humidity inside the laying house were recorded at five different locations throughout the house. The individual temperature was used to calculate the average daily house temperature, which were summarised on a weekly basis. Every morning at 07:00, the cages were cleaned, excreta and contaminated feed were removed from the feeders before the birds were fed individually from a pre-weighed feed bucket. Excreta underneath the cages were removed on a weekly basis. Number of eggs produced were recorded daily at 8:00 and collected into pre-numbered egg trays, while eggs were individually weighed (0.01 g) during specified data collection weeks (week 24, 28, 32, 36 and 40 of age). During egg collection, the physical appearance of each hen was assessed in order to observe any abnormal behaviour and/or injuries that might be externally visible.

3.2.5 Experimental dietary treatments

A standard commercially registered (Act 36/1947 Registration no. V17775 / NFF3419) layer mash was used as the control basal diet. The experimental treatments consisted of four different omega-3 fatty acids (n-3 FAs) varying in FA chain length, which were mixed “on top” of the control diet at a 15 g/kg inclusion level. The four supplementary lipid sources that were used included fish oil (FO), soybean oil (SO), linseed oil (LO) and echium oil (EO). The mean physical composition and mean chemical composition of the layer mash can be seen in Table 3.1 and Table 3.2 respectively.

3.2.6 Mixing of experimental diets

Blending of oils into the basal diet took place on the Paradys experimental farm of the University of the Free State. To limit the effects of lipid oxidation after mixing of the experimental diets, only limited quantities of feed were mixed to ensure that that it will be consumed within an eight-week period before a new batch of feed was mixed. A horizontal paddle feed mixer machine was used to mix the oils into the basal diet. The mixed feed was then bagged and relabelled according to the specific fatty acid treatment.

3.2.7 Chemical analysis

A representative sample from each dietary treatment was taken after the blending of the oils into the basal diet. These samples were then vacuum packed and frozen for preservation until the trial was complete. The feed samples from all of the mixed batches were then used to perform the different chemical analysis of each dietary treatment and the results were then pooled to give the average for each treatment.

3.2.7.1 Dry matter

The dry matter (DM) content of the feed samples from each dietary treatment was determined according to procedure number 934.01 (AOAC, 2000). A 2 g feed sample was weighed accurately (0.0001 g) into a pre-weighed dried glass vessel before drying it in a drying oven at 100 ºC for a minimum of five hours. The samples were removed from the oven and placed in a desiccator to cool to room temperature, whereafter the weight was recorded again. The weight of the individual crucibles was subtracted to determine the weight loss during drying. The following calculations were used for the determination of DM (Brusewitz *et al.*, 1993):

$$\text{DM (\%)} = \frac{\text{Crucible with dried sample - empty crucible}}{\text{Crucible with sample as is - empty crucible}} \times 100$$

Table 3.1 The mean ingredient composition (%) of the basal diet fed to the layers from 21 to 40 weeks of age (as is basis)

Yellow Maize	54.91
Maise germ cake	1.90
Prime gluten 60	1.75
Wheat bran	12.00
Full fat soybean meal	1.75
Canola oilcake meal	6.00
Soybean meal	5.50
Sunflower oilcake	5.00
Limestone – coarse[1]	2.00
Limestone – fine[2]	7.70
Monocalcium phosphate	0.46
Salt	0.36
Axtra XAP layer[3]	0.04
Choline liquid	0.04
Biolysine 70	0.13
Methionine	0.05
Premix	0.40

[1] Coarse limestone with particle sizes: ≥ 2.0 mm = 70%; > 1.0 mm = 25% & < 1 mm = 5%.

[2] Fine limestone with particle sizes: ≥ 2.0 mm = 34%; >1.0 mm = 30% & < 1 mm = 36%.

[3] An enzyme combination of xylanase, amylase, and protease

Table 3.2 Mean formulated chemical composition (g/kg DM) of basal diet fed during the experimental period (21 to 40 weeks of age)

Dry matter	904.63
Ash	140.68
AME[1] (MJ/kg)	12.49
Crude fat	34.88
Crude protein	165.78
Fibre	48.74
NDF[2]	151.92
ADF[3]	57.37
Calcium	40.14
Magnesium	2.79
Potassium	6.00
Chloride	3.01
Sodium	1.66
Sulphur	2.27
Total phosphorus	5.53
Ca:TP[4]	7.26
AvP[5]	2.47
Ca:AvP[6]	16.25
Arginine	9.95
Histidine	4.42
Isoleucine	6.32
Lysine	7.76
Methionine	3.80
Threonine	5.92
Tryptophan	1.75
Methionine & Cystine	7.36
Valine	8.03

[1] Apparent metabolizable energy in MJ AME/kg DM.

[2] Neutral detergent fibre.

[3] Acid detergent fibre.

[4] Ratio of calcium to total phosphorus.

[5] Available phosphorus

[6] Ratio of calcium to available phosphorus

3.2.7.2 Organic matter and ash

The organic matter (OM) and ash content of the samples were determined by using a muffle furnace. A 2.5 g feed sample was dried for a minimum of 5 hours, cooled in a desiccator and weighed into a pre-weighed and dried porcelain crucible. The crucibles with the samples were placed into a muffle furnace and incinerated at 550 ºC for four (4) hours. After incineration the furnace was allowed to cool to 100 ºC, before crucibles were removed and placed in a desiccator until reaching room temperature before being weighed. Due to the weight variation across crucibles, the weight of the ash and the sample were calculated by subtracting the weight of individual crucibles. This was done according to procedure number 942.05 (AOAC, 2000). The following calculations were used to determine the OM and ash content of each individual sample:

Ash (%) = (weight of ash(g)/weight of sample(g)) x 100
OM (%) = 100% - ash (%)

3.2.7.3 Gross energy

Gross energy (GE) of the feed samples was determined as described by Cantrell *et al.* (2010) using a Leco® AC500 Isoperibol Calorimeter (Leco Corp., St. Joseph, MI) following ASTM standard D5865 (ASTM, 2009). Around 0.35 g of dry feed sample was accurately weighed (0.0001 g) into a dry steel crucible and the weight entered into the computer. A platinum fuse wire was attached to the two electrodes of the bomb. The crucible with the sample was carefully placed in the electrode under the fuse wire, making sure the wire is just above the sample and not touching the crucible. The sample was placed into the bomb vessel and closed with the screw top lid before filling the bomb with oxygen to a pressure of 420 psi. The vessel bucket was filled with 2000 ml of distilled water from the pipet of the calorimeter, and then placed into the calorimeter. The bomb vessel was carefully placed into the vessel bucket, with the water level just above the bomb, the electrodes were connected to the bomb and the lid of the calorimeter closed. Results were expressed as mega joule per kilogram (MJ/kg).

3.2.7.4 Crude fat

The crude fat content of the feed samples was determined according to method No 920.39 (AOAC, 2000), through the use of Soxhlet extraction with petroleum ether (40 – 60°C) as solvent. A 2.5 g feed sample was dried for a minimum of five hours and placed in a desiccator to cool down. The sample was then placed in a 125 mm Whatman® filter paper that was set to zero on the scale. The dry sample weight was recorded and the filter paper with the sample was folded to prevent spillage of the sample before being placed into an extraction thimble. A

piece of clean cotton wool was used to close the thimble and keep the sample in place. The thimble was placed into the extractor that was connected to a pre-weighed round glass flask and 150 ml. of petroleum ether was carefully poured into the thimble. The extraction unit was connected to the water-cooling unit and placed into separate heating units. The samples were boiled for 6 hours with the heating set to ensure a droplet speed of roughly 5 to 6 drops of solvent per second. After fat extraction, the flasks were removed from the heating unit and the remaining petroleum ether was damped off by placing the flask on a warm water bath. The flasks were then dried overnight in a drying oven. After removal from the oven, the flasks were placed in a desiccator and cooled to room temperature before weighing.

3.2.7.5 Crude protein

The AOAC method number 990.03 (AOAC, 2000) was used for the determination of the crude protein (CP) content of feed with a LECO® (CHNS628 Series) nitrogen analyzer. Approximately 0.13 g of each pre-dried feed sample was weighed into an aluminium foil cup that was sealed and placed into the automated carousel of the instrument, providing continual analysis of samples without interruption. The Dumas method works on the concept that nitrogen is liberated by pyrolysis and subsequent combustion, and then swept into the nitrometer by carbon dioxide as a carrier gas. Potassium hydroxide absorbs the carbon dioxide, and the residual nitrogen volume is measured. By multiplying the percentage nitrogen by a factor of 6.25, the nitrogen content is converted to a protein equivalent. The electronic scale as well as the LECO® instrument was connected to the computer on which the protein values were recorded.

3.2.7.6 Neutral detergent fibre

The neutral detergent fibre (NDF) content of the feed was determined according to the method from Van Soest & Robertson (1979). About one 1 g of feed sample was accurately weighed (0.0001 g) into clean pre-weighed sintered glass crucibles. The crucibles were placed into a Fibertech™ extraction unit, where-after 50 cm^3 neutral detergent solution (NDS) was added and the solution was brought to a boil. After 30 minutes of boiling another 50 cm^3 of NDS was added along with 2 cm^3 alpha-amylase solution and brought to a boil for a further 30 minutes. After an effective boiling time of 60 minutes the heat was turned off and the NDS was filtered out by suction and the samples washed with hot water by soaking for 2 minutes and then filtering by vacuum. The washing was repeated until there was no more foam left, before it was washed three times with acetone. The crucibles were removed and placed in an oven at 100°C to dry overnight, then placed in a desiccator to cool. After the weight of the crucibles were recorded the crucibles were placed in a muffle furnace and incinerated at 500°C for 4

hours. After incineration, the furnace was cooled to 100°C and the crucibles placed in a desiccator to cool. The weight of the crucibles was recorded and the NDF determined with the following calculations (Goering & Van Soest, 1970):

$$NDF\ (\%) = \frac{W_2 - W_3}{W_1} \times 100$$

Where:

W_2 = Residue in crucible after drying (g)

W_3= Residue in crucible after incineration (g)

W_1= Mass of the sample (g)

3.2.7.7 Acid detergent fibre

The method of Goering & Van Soest (1970) was used to determine the acid detergent fibre (ADF) content of the feed samples. About one 1 g of each sample was accurately weighed (0.0001 g) before being dried overnight and weighed into pre-dried sintered glass crucibles. The crucibles were placed into a Fibertech™ extraction unit and 100 cm^3 of cold acid detergent solution (ADS) was added to each crucible. The heating element was turned to high until the solution started to boil, where-after which the heat was turned to 70 – 75°C, and the solution was boiled for exactly 60 minutes. The ADS was then filtered out by suction and washed with warm water of 50°C, after which it was rinsed twice with acetone. The crucibles were then dried overnight at 100°C before being cooled in a desiccator and weighed, and placed into a furnace for incineration for 4 hours at 500°C. After incineration, the furnace was cooled to 100°C and the crucibles placed in a desiccator to cool and then weighed. The following formula was used to calculate the ADF (Goering & Van Soest, 1970):

$$ADF\ (\%) = \frac{W_2 - W_3}{W_1} \times 100$$

Where:

W_2 = Residue in crucible after drying (g)

W_3= Residue in crucible after incineration (g)

W_1= Mass of the sample (g)

3.2.7.8 Mineral analysis

The calcium (Ca), magnesium (Mg), potassium (K) and sodium (Na) determination were done with a Savant atomic absorption spectrophotometer (Savant AAS) according to method 968.08 (AOAC, 2000). The feed samples were dried overnight and 3 g was weighed (0.0001

g) and placed in a porcelain crucible. The crucibles were placed in a muffle furnace and incinerated overnight at 550 ºC. The furnace was cooled to 100 ºC and the crucibles were placed in a desiccator to cool down before being weighed. The sample was then dissolved in 5 cm^3 hydrochloric acid (HCL), 6M, and then placed on a water bath to evaporate to dryness. Once dry it was removed to cool down, and then 5 cm^3 nitric acid (HNO_3), 6M, was added. The crucibles were placed on the water bath again and brought to boiling point. The solution was then filtered through filter paper into volumetric flasks (100 ml) and washed through the filter with warm distilled water and mixed well. The required parameters for each element were set on the Savant AAS before analysis. The Savant AAS was then calibrated for each element individually. The sample solution in the 100 mL volumetric flask was then used to determine the mineral concentration (Price, 1972; Athanasopoulos, 1993).

The same prepared sample solution was used for the determination of total phosphorus (TP) spectrophotometrically as the yellow phospho-vanado-molybdate complex. A phosphorus stock standard solution was prepared and used to calibrate the spectrophotometer.

3.2.7.9 Dietary fatty acid methyl esters

The extracted fat from feed material was stored frozen at -20°C in a polytop (glass vial with a push-in top) covered with a blanket of nitrogen. A disposable glass Pasteur pipette, was used to transfer a lipid aliquot (20mg) of the feed into a screw top test tube that is Teflon-lined. Using 0.5 N NaOH in methanol and 14% boron trifluoride in methanol (Park and Goins, 1994), fatty acids were trans-esterified to form methyl esters. A Varian 430 flame ionization GC with a fused silica capillary column, Chrompack CPSIL 88 (100 m length, 0.25 mm ID, 0.2 µm film thicknesses) was used to quantify FAMEs from the feed fat. Starting with an isothermic period of 40°C for 2 minutes, the analysis was performed. The temperature was then increased at 4°C/minute to a maximum of 230°C. After that an isothermic period of 230°C for 10 minutes followed. A Varian CP 8400 Autosampler was then used to inject FAMEs n-hexane (1µl) into the column. Both the injection port and detector were maintained as 250°C. Functioning as the carrier gas, was hydrogen at 45 psi and as the makeup gas nitrogen was used. The chromatograms were recorded by Galaxy Chromatography Software.

By comparing the retention times of FAME peaks from samples with the standards acquired from Supelco (Supelco 37 Component Fame Mix 47885-U, Sigma-Adric Aston Manor, Pretoria, South Africa), fatty acid methyl ester samples were identified. The internal standard used was nonadecanoic acid (C19:0) (SIGMA N553377-IG) to improve quantitative FAME estimation. Acquired form Merck Chemicals (Pty Ltd, Halfway House, Johannesburg, South

Africa), all the other reagents and solvents were of analytical grade. The fatty acid composition was depicted as each individual fatty acid to the proportion of all fatty acids present in the sample. A calculation of the following fatty acid combinations was done: omega-3 (n-3) fatty acids, Omega-6 (n-6) fatty acids, total saturated fatty acids (SFA), total monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), PUFA/SFA ratio and n-6/n-3 ratio.

3.2.8 Record keeping

3.2.8.1 Egg production

Eggs were collected daily before 8:00 and individually recorded for each hen. The visual inspection of the eggs for intact eggshells were performed during daily collection in the morning. During the specific data collection weeks (24, 28, 32, 36 and 40 weeks of age), individual eggs were accurately weighed to the nearest 0.01 g. To record the total egg production, the number of eggs that were produced were divided by the number of live birds in each group and expressed as a percentage as indicated in the formula below (Ahmad & Balander, 2003; Yilmaz Dikmen *et al.*, 2016).

$$\text{Mean egg weight (g)} = \frac{\text{Total egg weight/week}}{\text{Number of eggs laid/week}}$$

$$\text{Total egg production (\%)} = \frac{\text{Weekly hen-day egg production}}{\text{Number of live birds per treatment}} \times 100$$

3.2.8.2 Sellable egg production

The sellable egg production was calculated by subtracting all the cracked and abnormal eggs, (soft-shelled, malformed eggs) that was not appropriate to sell to consumers from the total egg production and expressed as a percentage. The following equation was used to calculate sellable egg production:

$$\text{Sellable egg production} = \frac{\text{Sum of total eggs produced – cracked and mis formed eggs}}{\text{Number of collection days}} \times 100$$

3.2.8.3 Egg output and feed efficiency

Individually marked feed buckets were allocated to each bird and the combined feed and bucket weight were recorded on a weekly basis. The daily feed allocation of 120 g per hen per day was used to ensure that the nutrient requirements of the birds were met. Feed intake of

individual birds were determined on a weekly basis by weighing back the feed bucket and feed refusals. The individual weekly feed intake was calculated according to the following equation:

WFI= IFBW – EFB – Res

Where:
WFI = weekly feed intake (g/bird)
IFBW= Initial feed bucket weight at beginning of a week (g)
EFB= Feed bucket weight at end of a week (g)
Res= Residual feed in feeder trough at end of a week (g)

Weekly egg output (g) was calculated for each hen during the respective data collection weeks of 24, 28, 32, 36 and 40 of age. The number of eggs produced were multiplied by mean egg weight of the specific individual hen expressed as (Rose, 1997):

Egg output (g) = Number of eggs x mean egg weight (g)

To calculate the feed efficiency (FE) of individual bird's, the weekly mean egg output (g) was divided by its equivalent mean feed intake (g/bird), according to the formulae of Clark *et al.* (2019):

$$\text{FE (g/g)} = \frac{\text{Weekly mean egg output (g)}}{\text{Weekly mean feed intake (g)}}$$

3.2.8.4 Body weight

Hens were individually weighed (closest 5 g) at week 24, 28, 32, 36 and 40 of age as graphically illustrated in Figure 3.2.

Figure 3.2 Recording of individual hen body weight during week 24, 28, 32 and 40 of age.

3.2.8.5 Statistical analysis

A fully randomized one-way ANOVA design was used for statistical analysis of the production parameter means. To test for significant differences ($P < 0.05$) between treatment means, SAS software (SAS Enterprise Guide, 2021) was used to perform the PROC ANOVA procedure. Tukey's honest significant difference test (HSD) was used to quantify differences between treatment means if recorded.

3.3 Results and discussions

3.3.1 Temperature and humidity

To ensure the bird's well-being, optimum productivity, and efficient feed utilization, environmental characteristics such as air temperature, relative humidity, and air quality are critical (Wang *et al.*, 2019b). For adult laying hens, the house temperatures can range between 15°C and 28°C, with a relative humidity between 40% and 80% (Freitas *et al.*, 2017). While an ambient house temperature of 21°C to 26°C is ideal for egg production, there seems to be a reduction in egg production if environmental temperatures increase above 26°C (Freitas *et al.*, 2017). In addition, Holik (2015) stated that for every one-degree Celsius increase above 23ºC, the hens feed intake is reduced, leading to a decrease in egg numbers, egg size and shell quality. Since birds used in this study were housed in a naturally ventilated layer house without any electrical fans, hens from different treatments were evenly distributed throughout the house in an attempt to minimize effects from temperature differences on their production performance. The mean weekly temperatures of the experimental facility are illustrated in Figure 3.3. The gradual increase in temperature over time is normal for the corresponding time of year (June to November) in Bloemfontein where the study took place.

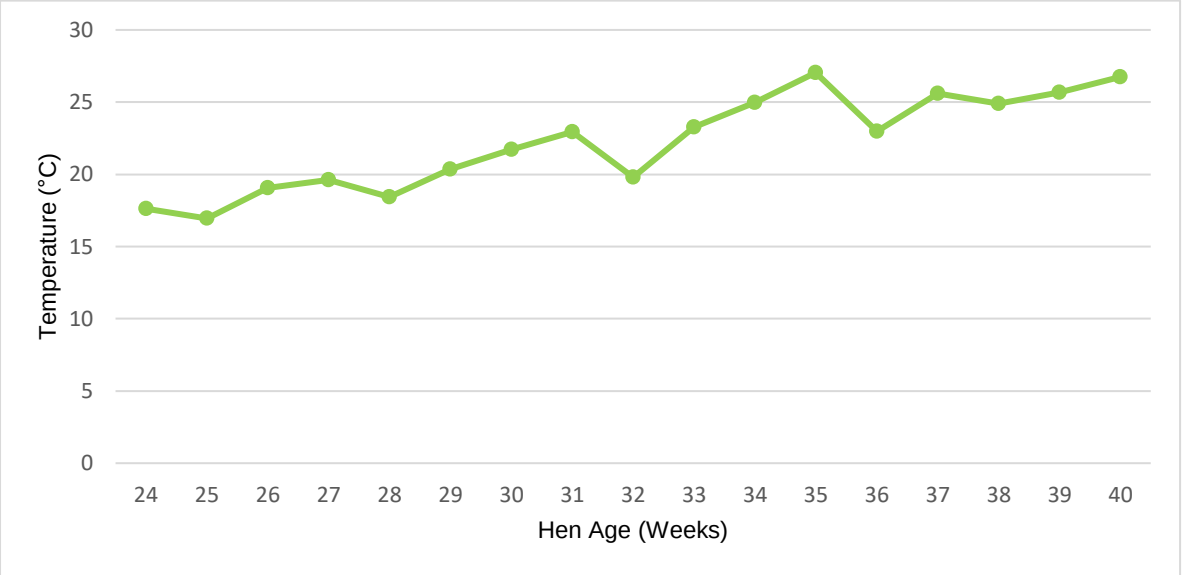

Figure 3.3 Mean weekly temperature (ºC) during the trial period.

The mean weekly relative humidity (%) that was recorded during the experimental period is shown in Figure 3.4. The gradual decline in RH is normal for the time of year in Bloemfontein during June to September, while a gradual increase is recorded from October to November.

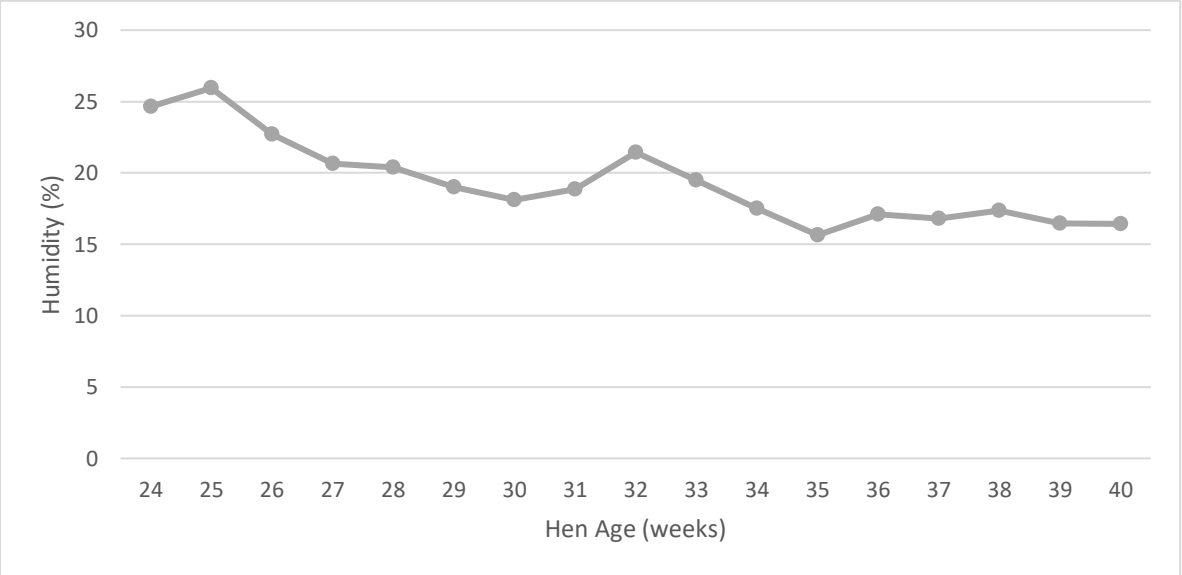

Figure 3.4 Average weekly relative humidity (%).

Temperature and RH play an essential role in the production of laying hens because heat stress could decrease feed intake and production performance, resulting in a lower profitability. Heat stress also affects the post-absorptive metabolism of nutrients like proteins, lipids, and carbohydrates, even with reduced feed intake (Okpara *et al.*, 2016; Kang *et al.*,

2020). Commercial laying hens are genetically selected for high egg output and lower body weight in order to increase the FCR, therefore egg production performance may also decline in colder weather. Environmental and heat-transference statistics suggest that laying hens expend four times more energy to maintain their body temperature in cold weather (Hu & Cheng, 2021).

Figure 3.5 depicts the average daily feed intake of the birds within their respective dietary treatments between the ages of 22 and 40 weeks of age. When comparing the effect of environmental temperatures on feed intake of birds during the peak-lay experimental period (22 to 40 weeks of age), it could be concluded that the effect of environmental temperature was consistent between dietary treatments during the experimental period. Despite the variation between the weekly temperatures, it is evident from production results summarised in Table 3.6 and Figure 3.5 that it had no effect on feed intake and egg production parameters between dietary treatments during the experimental period. It is interesting to note that the lower feed intake during week 35 of hen age could be because of the higher ambient temperature during that week as indicated in figure 3.3.

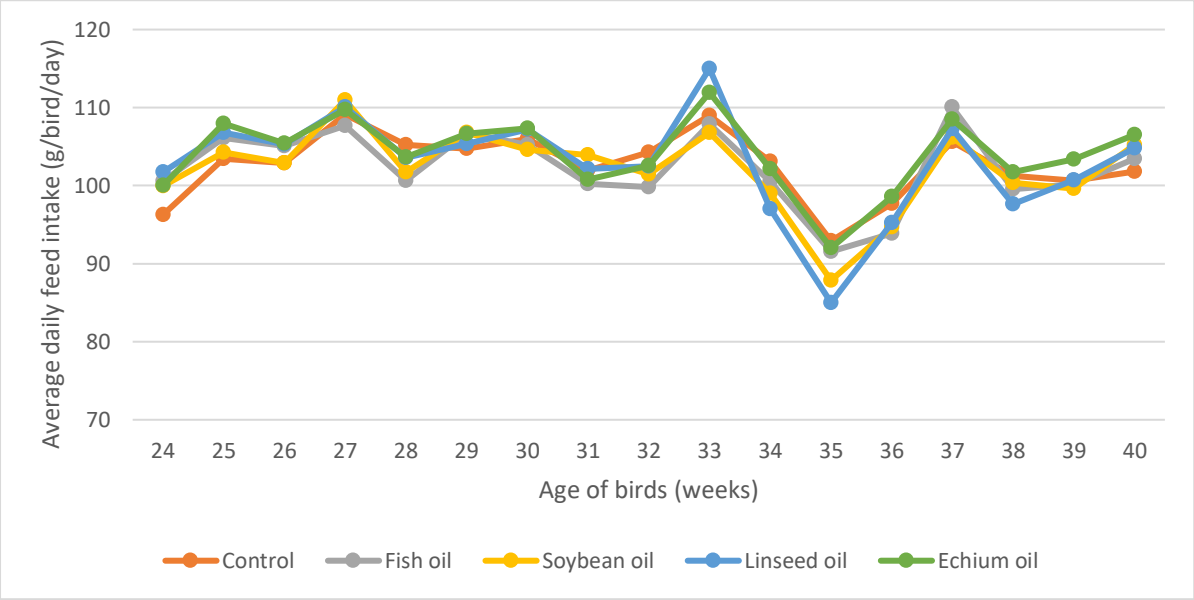

Figure 3.5 Average daily feed intake (g/bird/day) of experimental treatments (24 to 40 weeks of age)

3.3.2 Chemical analysis of dietary treatments

Feed samples were used for chemical analysis as shown in Table 3.3. The chemical analysis of the control dietary treatment differed in some instances from the mean formulated chemical composition, as illustrated in Table 3.2 (Paragraph 3.2.5).

Table 3.3 Mean dry matter chemical analysis (g/ kg DM) of the different experimental diets

	Control	Fish oil	Soybean oil	Linseed oil	Echium oil
Dry matter (g/kg)	908.77	909.05	909.60	911.23	908.29
Ash (g/kg)	146.77	143.39	141.25	138.99	145.96
Gross energy (MJ/Kg)	16.08	16.51	16.22	16.29	16.35
AME[1]	11.90	12.22	12.00	12.05	12.10
Crude fat (g/kg)	32.64	45.56	44.97	44.93	44.07
Crude protein (g/kg)	162.90	162.78	163.66	167.54	160.27
NDF[2] (g/kg)	215.57	222.64	222.52	212.94	218.31
ADF[3] (g/kg)	78.86	88.57	80.77	81.20	76.64
Ca[4] (g/kg)	54.31	51.66	51.19	51.18	53.98
Mg[5] (g/kg)	3.58	3.54	3.49	3.47	3.36
K[6](g/kg)	3.88	3.93	4.01	3.98	4.46
Na[7] (g/kg)	1.75	1.85	1.97	2.20	1.96
TP[8] (g/kg)	6.45	7.32	7.01	8.23	7.01
Ca:TP[9]	8.42	7.05	7.30	6.21	7.71

[1]Apparent metabolizable energy, calculated using the factor of 0.74 (Sakomura, 2004).
[2]Neutral detergent fibre
[3]Acid detergent fibre
[4]Calcium
[5]Magnesium
[6]Potassium
[7]Sodium
[8] Total phosphorus
[9] Ratio of calcium to total phosphorus.

The dry matter content, ash, crude fat, and crude protein content of the current study's basal diet chemical analysis only have minor deviations from that of the formulated feed. The current study's neutral detergent fibre (NDF) and acid detergent fibre (ADF) were higher than the formulated feed. This could be due to the high starch content of the maize-based diet, and the lab where the chemical analysis was done used the fibertec method from Van Soest & Robertson (1979), while the formulated diet's analysis was based on the Ankom fibre analyzer.

Since a digestibility study was not performed in the current study and the free fatty acids of the individual oils were not obtained, a calculated energy utilisation efficiency was used to calculate the AME using the gross energy values from Table 3.3. Sakomura (2004) determined that the energy utilising efficiency of a caged laying hen at maintenance at 31 °C is 0.74. Using this factor, the AME of the current study's feed analysis would be as follows: 16.08 MJ/Kg x 0.74= 11.90. A possible reason for the lower AME of the current analysis could be because of the higher mineral content, especially Ca. This higher Ca content could be due to the type of limestone used in the feed formulation, since the solubility of these sources differ.

Table 3.4 presents mean effect of dietary omega-3 lipid sources on the fatty acid methyl esters and Table 3.5 presents the mean effect on fatty acid concentration (%) and fatty acid ratios of layer diets used during the experimental period. The FAs presented in Table 3.4 are not the only detected FAs and the dietary concentrations and ratios of n-3 FAs in Table 3.5 are calculated using the complete fatty acid profile, rather than only using the FAs from Table 3.4.

The dietary fatty acid profile for each treatment is as expected when compared to the fatty acid profiles of the lipid sources as indicated in literature (Table 2.1). The echium oil treatment, as expected contains the highest concentration of stearidonic acid (4.04%) with the fish oil treatment (0.60%) being the only other dietary treatment where SDA was also detected. The n-6 LA is as expected the highest for the soybean oil treatment (49.47%) followed by the control treatment (47.05%). With the fish oil treatment being the only dietary treatment containing EPA (3.94%), DPA (0.26%) and DHA (2.79%), it also had the highest content of the SFA palmitic acid, which could be part of the reason why the fish oil treatment had the highest Total SFA (Table 3.5) concentration. The highest dietary total n-3 FA concentration were recorded for the linseed oil treatment (18.30%) followed by the echium oil (15.93%). Therefore the n-6/n-3 ratio of the LO treatment (2.12 : 1) followed by the EO treatment (2.65 : 1) were the lowest.

Table 3.4 The mean effect of dietary omega-3 lipid sources on the fatty acid methyl esters of layer diets used during the experimental period

	Control	Fish oil	Soybean oil	Linseed oil	Echium oil
FAME (FA as % of total FA)					
Saturated Fatty Acids					
Myristic (C14:0)	0.09	2.06	0.08	0.06	0.04
Palmitic (C16:0)	14.44	16.71	13.05	11.62	12.06
Margaric (C17:0)	0.06	0.15	0.06	0.05	0.06
Stearic acid (C18:0)	2.89	3.07	3.45	3.11	3.00
Monounsaturated Fatty Acids					
Palmitoleic (C16:1c9)	0.21	2.48	0.16	0.15	0.16
Oleic (C18:1c9)	29.17	23.61	26.68	25.44	24.17
Vaccenic (C18:1c7)	2.62	2.99	2.46	2.23	2.12
Eicosenoic (C20:1c11)	-	-	0.23	-	-
Polyunsaturated Fatty Acids (n-6)					
Linoleic (C18:2c9,12)	47.05	36.05	49.47	38.38	38.52
Eicosadienoic (C20:2c11,14)	0.01	0.05	-	0.01	-
Arachidonic (C20:4c5,8,11,14)	-	0.27	-	-	-
Polyunsaturated Fatty Acids (n-3)					
α-Linolenic (C18:3c9,12,15)	2.70	3.26	3.59	18.30	11.81
Stearidonic (C18:4c6,9,12,15)	-	0.60	-	-	4.04
Eicosopentaenoic (C20:5c5,8,11,14,17)	-	3.94	-	-	-
Docosapentaenoic (C22:5c7,10,13,16,19)	-	0.26	-	-	-
Docosahexanoic (C22:6c4,7,10,13,16,19)	-	2.79	-	-	-

Table 3.5 The mean effect of dietary omega-3 lipid sources on fatty acid concentration (%) and fatty acid ratios of layer diets used during the experimental period

	Control	Fish oil	Soybean oil	Linseed oil	Echium oil
Total fatty acid concentrations (%)					
Total Saturated Fatty Acids	17.99	22.64	17.11	15.26	15.57
Total Monounsaturated Fatty Acids	32.10	29.81	29.62	27.91	26.53
Total Polyunsaturated Fatty Acids	49.91	47.56	53.26	56.82	57.90
Total Unsaturated fatty acids	82.01	77.36	82.89	84.74	84.43
Total Omega- 6 Fatty Acids	47.21	36.53	49.68	38.52	41.97
Total Omega-3 Fatty Acids	2.70	11.02	3.59	18.30	15.93
Total long chain n-3 (EPA, DPA & DHA)	-	6.99	-	-	-
Fatty acid ratios					
PUFA/SFA[1]	2.78	2.10	3.11	3.72	3.72
n-6/n-3[2]	17.63	3.32	13.92	2.12	2.65

[1]The ratio of polyunsaturated to saturated fatty acids.

[2] The ratio of omega-6 to omega-3 fatty acids.

3.3.3 Production performance

The effects of different dietary n-3 PUFA lipid sources on the production performance parameters of hens during the peak laying period are shown in Table 3.6. The different lipid sources, varying in their n-3 PUFA chain length, did not affect the feed intake (*P*= 0.6826), feed efficiency (*P*= 0.7452), egg output (*P*= 0.6320), egg weight (*P*= 0.0586), or sellable egg production (*P*= 0.9748). Despite no significant differences in terms of feed intake of the hens, the soybean oil treatment resulted in the highest (*P*< 0.05) mean hen body weight (1849 g) during peak production, while that of the control treatment was the lowest (1729 g). In addition, it is evident from Table 3.6 that the total egg production of the soybean oil treatment (94.3%) was lower (*P*= 0.0460) compared to the linseed oil treatment (96.7%). While egg weight did not differ significantly (*P*= 0.0586), it is notable that the soybean oil treatment had the highest egg weight (55.8 g), and the control treatment had the lowest egg weight (53.9 g) when tested at *P*< 0.10 instead of *P*< 0.05. Due to the heavier egg weights of the soybean oil treatment, the mean egg output (364.22 g) of the soybean oil treatment was also numerically (*P*> 0.05) higher than that of the other treatments. Findings of the present study are in agreement with that of Basmacioglu *et al.* (2003); Omar *et al.* (2014); Omidi *et al.* (2015); Neijat *et al.* (2016); Ekine *et al.* (2020) who recorded no significant differences in daily feed intake, feed efficiency (Omidi *et al.*, 2015) and egg weight (Zou & Wu, 2005; Omar *et al.*, 2014; Omidi *et al.*, 2015; Ekine *et al.*, 2020) when using different dietary supplemented lipid sources.

Although the egg weights in the present study did not differ significantly at *P*< 0.05, results of this study are partly supportive to the findings of Dong *et al.* (2018) who also found that supplemental soybean oil as well as coconut oil result in higher (*P*< 0.05) egg weights compared to fish oil. Dong *et al.* (2018) investigated the possible effects of dietary linoleic acid on egg weight, but since coconut oil and fish oil had similar levels of linoleic acid, 21.7 mg/g and 20.5 mg/g respectively with 68.3 mg/g in the soybean oil treatment, they concluded that the high content of DHA (139.7 mg/g) and EPA (88.8 mg/g) in fish oil could be a factor in poor performance and decreased in egg weight. According to Herber & Van elswyk (1996), feeding isonitrogenous and isoenergetic diets containing marine algae (2.4% and 4.8% inclusion) enhanced the amount of DHA in egg yolks. However, the inclusion of 4.8% microalgae (MA) in the diet had a negative impact on egg production and egg weight, this could be due to the high level of MA being more vulnerable to oxidation. Lipid sources becomes more vulnerable to oxidation as the concentration of long chain PUFAs increase, therefore determining the effect of DHA enrichment on egg yolk oxidative stability is necessary (Herber & Van Elswyk, 1996; Ao *et al.*, 2015). Dey *et al.* (2021) concluded that egg weight (g) was higher (*P*< 0.05) with dietary treatments containing 1.5% (61.58 g) and 2.5% (60.55 g) dietary soybean oil compared to the control diet treatment (58.47 g), when fed to laying hens over a 15-week

Table 3.6 The mean (± S.E.) effect of different omega-3 lipid sources on the production performance of Hy-Line Silver-Brown layers during peak production (24 weeks - 40 weeks)

	Control	Fish oil	Soybean oil	Linseed oil	Echium oil	P	CV[1] (%)
Daily feed intake (g/bird/day)	101.07±1.17	99.66±1.34	100.88±1.25	101.58±1.27	102.29±1.37	0.6826	6.94
Feed efficiency (g/g)	0.48±0.01	0.48±0.01	0.49±0.01	0.48±0.01	0.48±0.01	0.7452	6.43
Egg output (g)	356.27±4.11	357.63±4.92	364.22±4.95	363.43±5.36	363.70±4.14	0.6320	7.16
Egg weight (g)	53.93±0.50	54.34±0.45	55.81±0.58	54.35±0.54	55.28±0.47	0.0586	5.09
Total egg production (%)	94.88^{ab} ±0.64	96.03^{ab} ±0.60	94.25^{b} ±0.70	96.69^{a} ±0.51	95.06^{ab} ±0.61	0.0460	3.53
Sellable egg production (%)	90.98±2.18	91.11±2.51	90.88±1.25	91.16±1.68	89.57±1.65	0.9748	11.50
Mean body weight (g)	1729^{b}±15.99	1789^{ab}±18.77	1849^{a}±18.31	1803^{a} ±18.11	1807^{a}±21.22	0.0004	5.66

^{a,b} Row means with different superscripts differ significantly at $P< 0.05$.

[1] Coefficient of variance (%)

period. Dey *et al.* (2021) ascribed their findings to the high concentration of linoleic acid in soybean oil. Results of the current study are in contrast with that of Batkowska *et al.* (2020), who reported that eggs from the control group (52.70 g) were heavier ($P< 0.05$) than treatments with a dietary lipid inclusion of 2.5% soybean oil (51.16 g) and linseed oil (50.55 g), respectively.

In Table 3.6 it is shown that even though the SO treatment had the lowest ($P< 0.05$) total egg production, the egg output of the SO treatment was not negatively impacted because of the heavier egg weights. In contrast with the current results, Küçükersan *et al.* (2010) found that the egg production of the soybean (82.7%) and fish oil (82.1%) treatments to be the highest ($P< 0.05$) compared to sunflower (74.1%) and hazelnut oil (77.5%), supplemented at a concentration of 3%. Furthermore, results of the present study (Table 3.6) also agree with that of Küçükersan *et al.* (2010) who found that the soybean oil treatment recorded the highest ($P< 0.05$) egg weight (64.3 g) compared to sunflower (63.7 g), fish (62.7 g) and hazelnut (62.6 g) oil treatments. When investigating the effects of four different oil sources (soybean, fish, linseed and rapeseed oil) at two inclusion levels (1.5% and 3%) Ceylan *et al.* (2011) reported no effect ($P> 0.05$) on egg production, egg weight, daily feed intake, FCR or body weight after 12 weeks on the experimental diets. Furthermore, Elkin *et al.* (2021) found no differences ($P> 0.05$) in egg production and performance parameters when feeding laying hens four diets containing 4% stearidonic acid enriched soybean oil along with either 2% high oleic acid sunflower oil; 20% high linoleic acid safflower oil or 2% microalgae oil.

When comparing extruded linseed (EL), ground linseed (GL) and a fish oil source (inclusion: 10%, 10% and 3.4% respectively) added to a control diet (no supplementary lipid source) over a 61-day period, it was found that the fish oil treatment had higher ($P< 0.01$) egg production (98%) than the EL (95%), GL (95%) and the control (89%) treatments (Zotte *et al.*, 2015). Gao *et al.* (2021) investigated the effect of different concentrations of soybean oil, lard and a mixture of these two oils (1.5% and 3% of each) on production performance, and reported that the 1.5% inclusion of soybean oil have the highest ($P< 0.05$) egg production (89.3%) while the 3% lard (81.3%) and mixed oils (77.9%) resulted in the lowest egg production. According to Dey *et al.* (2021), the inclusion of 1.5% soybean oil resulted in numerically better egg production (94.1%) compared to a 2.5% soybean oil (90.2%) inclusion, concluding that lower levels of dietary linoleic acid improve performance and egg quality. Results from the current study followed a similar trend to that of Dey *et al.* (2021), where the linseed oil treatment with lower linoleic acid concentration had better egg production (96.7%) than the soybean oil (94.3%) treatment. Furthermore, in the current study as well as other studies (Grobas *et al.*, 1999 and Dong *et al.*, 2018) it is noticeable that a higher dietary linoleic acid content could

deliver heavier egg weights. In contrast other authors (Pérez-Bonilla *et al.*, 2011; Costa *et al.*, 2015; Irawan *et al.*, 2022) found that linoleic acid had no effect (*P*> 0.05) on egg weight or size.

In the current study it was observed that the echium oil treatment, recorded the numerical (*P*> 0.05) second highest egg weight (55.28 g) but the lowest sellable egg production (89.6%). The higher coefficient of variation (11.5%) of the sellable eggs as reported in Table 3.6 could be ascribed to variation within a treatment regarding the number of cracked and/or shell-less eggs produced during peak production. Since this a calculated trait, it was not expected to have a normal distribution, because various animal and/or environmental factors could influence it. There is very little literature available regarding the use of echium oil in laying hen diets - with some studies conducted on broilers (Kitessa & Young, 2008; Al-Khalaifah *et al.*, 2020) and some on SDA enriched soybean oil (Elkin *et al.*, 2015, 2021).

Najib & Al-Yousif (2014) conducted a study to assess how the performance and egg size of the Saudi local birds were affected by different weight groups at maturity (line of the bird) and different quantities of supplemental fat. These birds were divided into six treatments at the age of 22 weeks, including two weight groups (medium and light weight) and using three different isoenergetic diets with different fat levels (0, 2, and 4% maize oil). Najib & Al-Yousif (2014) reported a highly significant (*P*< 0.001) interaction between the fat level and the breed (weight) of the bird on egg weight. Medium weight birds responded better to the greater level of linoleic acid in the diet with an average egg weight of 51 g, compared to the egg weights of lower level of dietary linoleic acid (48.11 g) and the control (47.29 g) (Najib & Al-Yousif, 2014).

Pérez-Bonilla *et al.* (2012) found that hens with a higher body weight will lay heavier eggs. In a study consisting of 288 Lohmann White layers divided into three groups according to body weight (Light: 1400 – 1500 g; Medium: 1500 – 1600 g and Heavy: 1600 < g), Lacin *et al.* (2008) reported that egg weight was heavier (*P*< 0.01) for the heavy body weight group (66.3 g) than for the medium (65.0 g) and light body weight groups (64.6 g). Daikwo *et al.* (2011) also reported a positive (*P*< 0.05) correlation between body weight and egg weight as well as body weight and clutch size. Wolc *et al.* (2010) found the correlation between body weight and egg weight to be positive but low (R^2 = 0.25).

In the current study (Table 3.6) the feed intake of the control group (101.1 g/bird/day) was slightly higher than that of the soybean oil treatment (100.9 g/bird/day) and the fish oil treatment (99.7 g/bird/day), which might be ascribed to the lower GE and dietary fat content (Table 3.3) of the control treatment. Furthermore, the slightly higher feed intake of hens in the

control treatment could therefore not be the reason for the lower (P= 0.0004) body weights recorded in this treatment. Abdulla *et al.* (2017) found that broilers fed a diet with 6% added soybean oil were heavier (P< 0.05) than those fed a 6% linseed oil diet after six weeks, and ascribed the results to higher fat deposition of the SO treatment. Wealleans *et al.* (2021) have presented a range of metabolizable energy values for older poultry with and without the moisture, insoluble impurities and unsaponifiable matter (MIU) content of the lipid source. The AME (MJ/kg) range without MIU were as follows: FO: 33.4 –37 MJ/kg; SO: 37.2 – 38.4 MJ/kg and LO: 33 – 38.8 MJ/kg and when including MIU were FO: 32.8 – 36.6; SO: 36.6 – 38.2 and LO: 32.82 – 38.4 MJ/kg (Wealleans *et al.*, 2021). While oils generally follow published profiles, there is great disparity in the FA composition among different types of oils. This range in energy value is more than what nutritionists typically presume, and energy-diluting factors are prevalent (Wealleans *et al.*, 2021). Even though oils of the different treatments were added on top of the control diet during the present study, therefore diets were not formulated to be isoenergetic, the analysed chemical composition of the dietary treatments (Table 3.3) showed that the energy (MJ/kg) would also not be the cause for significant difference in body weight. Additional explanations for variation between the findings of the present study (Table 3.6) regarding body weight and total egg production, and that of cited literature could be ascribed to the genetic make-up of the animals, age of birds, hormonal metabolism, experiment duration, lipid sources, lipid inclusion levels, and dietary AME values.

3.3.4 Conclusions

Results of the current study indicated that most production performance parameters are generally unaffected by the inclusion of different lipid sources high in n-3 FA of either short (C18:3) or long (C20:5; C22:6) chain length. The differences (P< 0.05) recorded in total egg production (%) and body weight (g) could most probably be ascribed to the variation in dietary FA profile and/or the oxidative status of the different lipid sources, or unknown factors that require further investigation. There is no clear indication that the n-3 type lipid sources are the cause of the differences (P< 0.05) in the abovementioned parameters. Overall, it can be concluded that the type of dietary n-3 FAs won't have a negative impact on egg production during the peak laying period, given that the nutrient composition of the diets complies with the requirements of the birds during a period of high egg output.

Chapter 4

The effect of different omega-3 lipid sources on internal and external egg quality characteristics during peak lay

Abstract

This study investigated the effect of supplementary dietary omega-3 lipid sources on the internal and external quality and the fatty acid profile of eggs. Four lipid sources, each with a different omega-3 fatty acid chain length, were added to the basal diet at a rate of 1.5% during the experimental treatments. Fish- (EPA, C20:5 & DHA, C22:6), soybean- (ALA, C18:3), linseed- (ALA, C18:3), and echium (SDA, 18:4) oil were the lipid sources used. A total of 150 Hy-Line Silver-Brown hens (aged 21 weeks) were divided among the five experimental diets (n = 30 per treatment). In addition to measuring egg quality parameters of 20 eggs per treatment (n = 100) and weighing each egg produced during weeks 24, 28, 32, 36, and 40, twelve eggs per treatment were selected randomly at 33 weeks of age to analyse the egg yolk fatty acid methyl esters (FAME). Data were pooled to calculate the peak laying period's average internal and external egg quality values; a one-way ANOVA was used to analyse the data. The dietary treatments did not affect any of the shell quality parameters. The yolk weight and diameter were the highest ($P< 0.05$) for the soybean oil (14.43 g and 39.82 mm) and lowest for the control treatment (13.73 g and 38.65 mm). The yolk colour of the control was lighter ($P< 0.05$) than yolks from n-3 lipid treatments. The α-linolenic acid concentration of the linseed oil (3.62%) treatment was the highest ($P< 0.05$), followed by the echium oil treatment (2.88%). Yolks from the fish oil treatment, compared to the echium oil treatment, had the highest EPA (0.16% vs. 0.04%) and DHA (1.85% vs. 1.20%), but echium oil had the highest DPA (0.19% vs. 0.23% %). Linseed and echium oil treatments resulted in the highest ($P< 0.05$) levels of total n-3 PUFA (4.80% & 4.38%), as well as the lowest n-6/n-3 ratios (2.81 : 1 & 3.52 : 1) in egg yolk.

Keywords: Stearidonic acid, echium oil, egg quality, linseed oil, polyunsaturated fatty acids

4.1 Introduction

Egg weight and eggshell quality are two of the key factors egg producers take into account, whereas consumers are more concerned with shelf-life, external appearance, and sensory traits like yolk colour (Roberts, 2004; Ahmadi & Rahimi, 2011; Faitarone *et al.*, 2016; Preisinger, 2018). Eggshell quality affects the integrity and safety of egg content, cracked eggs result in a downgrade of about 8 to 10% of eggs produced which causes significant economic losses for egg producers (Akbari Moghaddam Kakhki *et al.*, 2020; Benavides-Reyes *et al.*,

2021). Eggshell mineralisation and quality are affected by various variables including hen age, health, genetics, housing environmental and nutritional factors (Harikrishnan & Mohan, 2018). El-Husseiny *et al.* (2008) reported that the inclusion of balanced levels of vegetable oil and animal fat sources in laying hen diets affect different aspects including feed intake and feed conversion ratio, as well as egg quality and egg size.

Due to their high concentration of saturated fat (approximately 3 g/100 g egg) and cholesterol (between 200 and 300 mg/100 g egg), egg consumption resulted in much debate amongst health organisations and nutritional experts. Due to dietary cholesterol intake and the possible link to cardiovascular disorders (CVD), the public had previously been advised against consuming more eggs (Alagawany *et al.*, 2018). However, Eilat-Adar *et al.* (2013) reported that cholesterol from eggs has a limited effect on blood cholesterol levels and CVD, despite the fact that overall consumption has declined due to the unwarranted, growing dread of high cholesterol levels in eggs. Furthermore, Aguillón-Páez *et al.* (2020) reported that replacing dietary saturated fatty acids (SFA) with polyunsaturated fatty acids (PUFA) will result in a positive effect on the cardiovascular system. Omega-3 PUFA enriched eggs meet the increased demand of health conscious consumers for animal protein products high in omega-3 PUFAs (Ahmad *et al.*, 2012; Alagawany *et al.*, 2019). Because the conversion of α-linolenic acid to EPA and DHA is somewhat limited, the added health benefits from the longer chain n-3 fatty acids may be insignificant, which is why stearidonic acid, that is more easily converted, could potentially offer better health benefits (Baker *et al.*, 2016). Because fish oil could cause eggs and meat to have pungent flavours, flaxseed and echium oils are preferred in poultry diets instead of fish oil because they effectively transfer PUFAs from diet to meat and eggs (Sarica, 2017).

Basmacioglu *et al.* (2003) found that dietary supplemental fish oil and flax seed did not affect egg-, yolk- or albumen weight, yolk-, albumen- or shell ratio, shell weight or shell strength and thickness in a negative manner. Bean & Leeson (2003) also found no difference ($P > 0.05$) in egg weight, shell weight, albumen height, and shell thickness when hens were fed a 10% flax seed diet compared to a control (0% flax seed). However, these authors reported that the yolk weight decreased ($P < 0.05$) when hens were fed flax seed. In another opinion, Gürbüz *et al.* (2012) reported that an increase in dietary linseed concentration (from 0% to 10% inclusion) resulted in a decreased eggshell weight and thickness. In contrast, Senkoylu *et al.* (2005) found no effect on shell weight, shell thickness, albumen height and cracked egg percentages by feeding full fat soybeans from 0 to 22% dietary inclusion levels. However, Kim *et al.* (2019) reported that laying hens fed a control diet with no lipid inclusion produced eggs with greater

(*P*< 0.05) eggshell thickness than hens fed a 4% soybean oil diet, while egg yolk colour was higher (*P*< 0.05) in the control treatment compared to the 4% soybean oil diet.
Zhao *et al.* (2021) found the total yolk n-6/n-3 ratio to be successfully altered when comparing a 1.5% fish oil treatment (1.89 : 1) with the control treatment (19.2 :1). Batkowska *et al.* (2021) reported that the yolk ALA content was significantly higher (*P*< 0.05) for a 2.5% linseed oil treatment (1.59%) than for the non-supplemented control (0.58%), with the ALA from the 2.5% soybean oil treatment (0.94%) not differing significantly from the control. The results of Omidi *et al.* (2015) showed that the total yolk n-3 content was significantly (*P*< 0.01) affected by a 3% lipid inclusion of fish oil (3.66%) and soybean oil (0.55%) when compared to the non-supplemented control (0.36%). Although the n-6/n-3 ratio of the fish oil treatment (2.55 : 1) differed significantly from the control (33.52 : 1), the soybean oil treatment (34.94 : 1) did not differ significantly due to a significant increase of n-6 PUFAs. The findings of Kitessa & Young (2008) showed that a dietary inclusion of 3% echium oil, a source of SDA, was better (*P*< 0.01) than 3% rapeseed oil inclusion in improving the total n-3 content of the thigh (9.89% vs. 3.35%) and breast (8.43% vs. 3.12%) intramuscular fat.

Currently, there are no studies available that examines the possible effect of echium oil, rich in n-3 PUFA stearidonic acid, on egg quality parameters and deposition of long chain n-3 PUFAs into the egg yolk. Therefore, this chapter will explore the effects of different sources of omega-3 poly unsaturated fatty acids on these quality parameters.

4.2 Materials and methods

4.2.1 Experimental birds and husbandry

The same birds as previously discussed in paragraph 3.2.2 were used for egg collection for the determination of internal and external egg quality characteristics. These hens received the experimental dietary treatments (n=30 birds/treatment) from 21 weeks of age as previously explained in paragraph 3.2.2. Feed supply was 120 g/bird/day, while water was supplied *ad libitum* via individual drinker nipples. A photoperiod schedule of 16 hours daylight and 8 hours dark (16L:8D) was maintained during the trial and the number of eggs laid was recorded daily at 8:00 AM.

4.2.2 Characteristics of egg components

A total of 20 eggs per treatment per day (n = 100) collected during specific weeks (weeks 24, 28, 32, 36 and 40 of age) were used for the determination and calculation of certain internal egg quality characteristics such as: albumen weight (g), yolk weight (g), yolk colour, albumen

ratio (%), yolk ratio (%), yolk- and albumen index, haugh units, as well as eggshell quality traits such as shell thickness and the percentage eggshell (%).

4.2.3 Egg and shell quality

A total of 20 eggs per treatment per day (n=100 eggs/collection week) were selected at random on a daily basis during specific weeks (weeks 24, 28, 32, 36 and 40 of age) to measure external and internal egg quality traits. After individual egg weight (0.01 g) was recorded, each egg was broken on an egg break-out stand (Figure 4.1) with a flat, transparent glass surface and a mirror angled at 45º were used to accurately measure the yolk and albumen (Nahashon *et al.*, 2007; Lokapirnasari *et al.*, 2019).

Figure 4.1 The egg break-out stand being levelled

The procedure described by Crosara *et al.* (2019) were used to wash the eggshell under slow running water to remove adhering albumen. The eggshells were then dried at room temperature before weighing (0.01 g) individually and measuring of shell thickness. An AMES micrometre (accurate to 0.001 mm) was used to measure individual eggshells in triplicate at the blunt end, equator and sharp end of each shell (Figure 4.2) (Ahmad & Balander, 2003; de Witt *et al.*, 2009; Vezzoli *et al.*, 2016). In total, nine shell thickness readings per individual eggshell were recorded for calculation of the mean eggshell thickness measurement.

Figure 4.2 Using AMES micrometre to record shell thickness

The following calculations were used to calculate additional eggshell quality parameters:

Egg surface area (ESA) = $3.9782W^{0.7056}$ (Carter, 1975)

Where:

W = egg weight (g)

Shell weight per unit surface area (SWUSA) (mg/cm^2) = $\frac{SW}{ESA}$ (Wells, 1967)

Where:

SW = shell weight (g DM)

ESA = egg surface area

Percentage eggshell (%) = $\frac{SW}{EW}$ x 100 (Orban & Roland, 1990)

Where:

SW = shell weight (g DM)

EW = egg weight (g)

Eggshell Ca content (g) = SW (g) x 0.373 (Simons, 1986)

Where: SW = shell weight (g DM)

4.2.4 Egg yolk and albumen measurements

Using a digital vernier calliper (Figure 4.3), the diameter of the egg yolk was accurately measured (0.01 mm) at two different locations by using the cross-over technique described by Berardinelli *et al.* (2008). The two yolk diameter readings obtained by using this technique allowed for improved accuracy when calculating an average yolk diameter. The albumen was also measured (0.01 mm) according to the cross-over technique (lengthwise and across) for the calculation of the mean albumen diameter. A digital AMES tripod micrometre (Figure 4.4), accurate to 0.001 mm, was used to measure the height of yolk and albumen. Yolk height was measured at the highest point in the centre without separating the yolk from the albumen (Bagh *et al.*, 2016). The thick albumen was measured approximately 5 mm from the yolk and not on the chalazae, while the thin albumen was measured approximately 5 mm from the edge of the thick albumen. These measurements were obtained by using the same technique as described by Keener *et al.* (2006). The measurements were then used in further calculations of internal egg quality parameters.

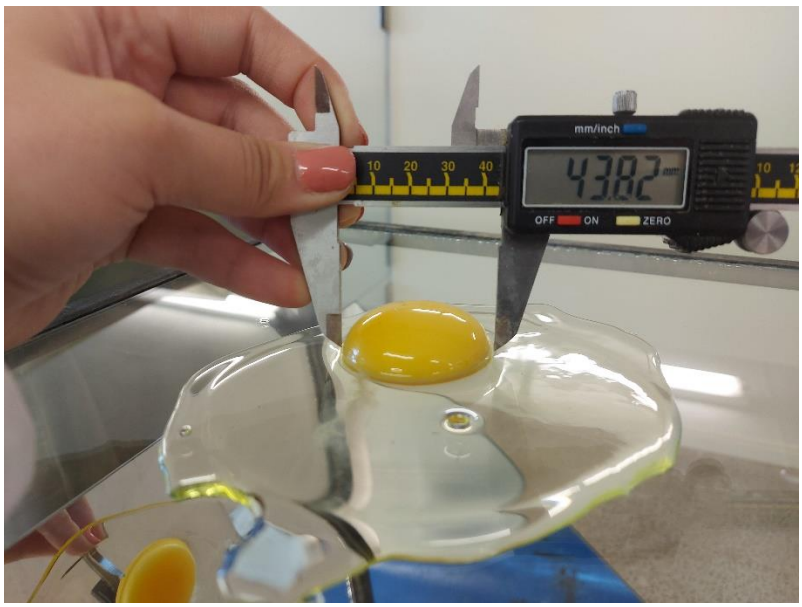

Figure 4.3 Using a vernier calliper to measure egg yolk diameter

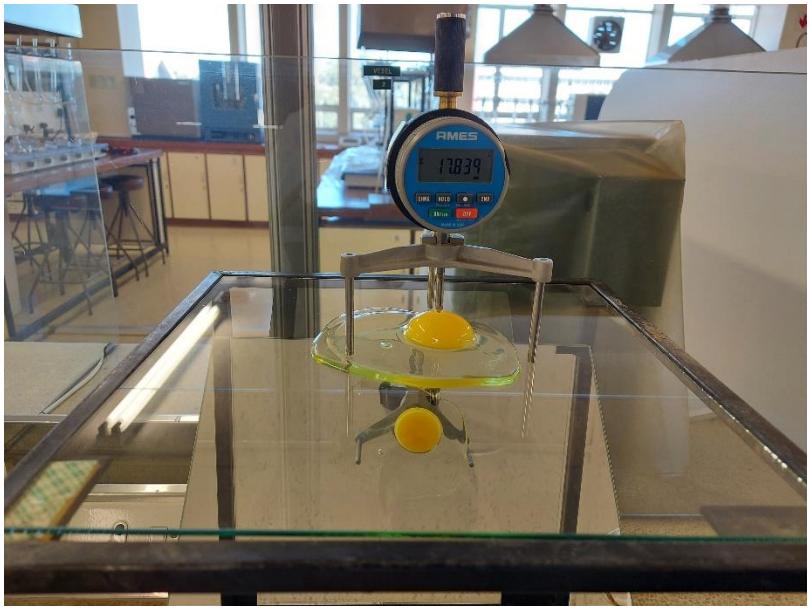

Figure 4.4 Using the AMES tripod micrometre to measure yolk height

4.2.5 Egg yolk colour

A DSM Roche® colour fan was used to determine egg yolk colour, following the technique described by Vuilleumier (1969). The same egg used for measuring yolk width and height was used for evaluating yolk colour, by holding a white page underneath the surface of the glass plate, thereby removing adjacent colours from the background, and comparing the yolk colour with the blades of the colour fan (Figure 4.5). The same person was used to determine yolk colour throughout the experimental period to reduce operator variability.

Figure 4.5 Using a DSM Roche® colour fan to determine yolk colour

4.2.6 Yolk and albumen characteristics

After all the yolk and albumen measurements were recorded on the break-out stand, the egg contents were slowly placed in an egg separator to remove the albumen from the yolk as shown in Figure 4.6. The yolk was then placed on a damp paper towel and carefully rolled to remove any adhering albumen and chalazae (Figure 4.7) before placing the yolk on a petri dish for weighing (0.01 g) (Figure 4.8).

Figure 4.6 Using an egg separator to separate the yolk from the albumen

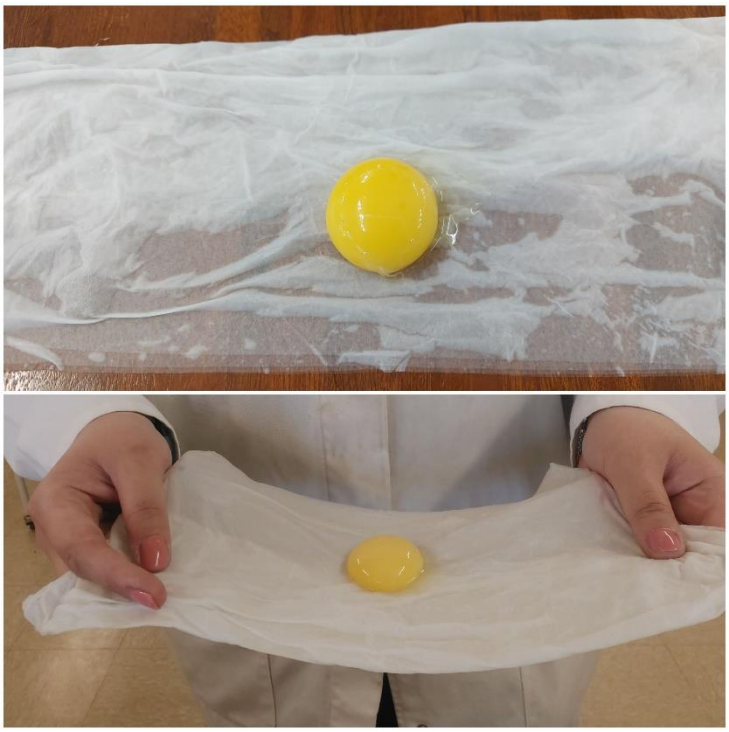

Figure 4.7 The yolk is carefully placed on a damp towel and slowly rolled to remove adhering albumen

Figure 4.8 Egg yolk being weighed after albumen was removed

Albumen weight (g) was calculated as the difference between the total egg weight (g) and the weight of the eggshell (g) plus the yolk weight (g) (Grobas *et al.*, 1999; Tůmová & Gous, 2012) by using the following equation:

Albumen weight (g) = Egg weight – (Yolk weight + shell weight)

The yolk and albumen ratios were calculated as a percentage of the specific component weight (g) in relationship to the egg weight (g), using the following formulae of Kul & Seker, (2004):

Albumen ratio (%) = (Albumen weight/ Egg weight) x 100
Yolk ratio (%) = (Yolk weight/ Egg weight) x 100

To determine the albumen index the albumen height (mm) was divided by the albumen diameter (mm) (measurements which was recorded as in paragraph 4.2.4) and was calculated by using the following equation (Kul & Seker, 2004):

$$\text{Albumen index (\%)} = 100 \times \frac{\text{Albumen height}}{\text{Albumen diameter}}$$

Similarly, the yolk index was calculated by using the yolk height (mm) and yolk diameter (mm) in the following equation (Kul & Seker, 2004; Wang *et al.*, 2019):

$$\text{Yolk index (\%)} = 100 \times \frac{\text{Yolk height}}{\text{Yolk diameter}}$$

4.2.7 Haugh unit

Albumen height determined on the egg break-out stand (as described in paragraph 4.2.4) was used for the calculation of individual the Haugh units of eggs, as described by Eisen *et al.* (1962). The following equation was used to calculate the Haugh unit:

$$\text{Haugh unit} = 100 \log \{H - [\sqrt{G}\,(30W^{0.37} - 100)/100] + 1.9\}$$

Where:
H = albumen height in mm
W = egg weight
G = Gravitational constant = 32.2

4.2.8 Yolk fatty acid analysis

A total number of 60 eggs (n=12 eggs/treatment) were collected during week 33 of age for the determination of fat free dry matter, percentage yolk fat, yolk moisture content as well as yolk fatty acid composition and fatty acid ratios.

Using the procedures firstly described by Folch *et al.* (1957), chloroform and methanol were used for the quantitative extraction of the total lipid content from individual egg yolks. Butylated hydroxytoluene, an antioxidant, at a concentration of 0,001%, was added to the chloroform-methanol mixture that was used in a ratio of 2:1. The fat extracts were then dried under vacuum by using a rotary evaporator. The extracts were left overnight in a vacuum oven at 50°C with a moisture absorbent, phosphorus pentoxide. The gravimetrical determination of total extracted fat from egg yolk is expressed in percentage fat (w/w) per 100 g egg yolk. By weighing the residue after drying on a pre-weighed filter paper that was used for the Folch extraction, the fat free dry matter (FFDM) content was verified. This difference in weight can be expressed as a percentage FFDM (w/w) per 100 g material. The material's moisture content is expressed as percentage moisture (w/w) per 100 g material and is determined by doing the following subtraction:

Percentage moisture per 100 g egg yolk = 100 - lipid (%) -FFDM (%)

A disposable Pasteur pipette was used to transfer a lipid aliquot (20 mg) of the egg yolk into a screw top test tube that is Teflon-lined. Using 0.5 N NaOH in methanol and 14% boron trifluoride in methanol (Park & Goins, 1994), fatty acids were trans esterified to form methyl esters. A Varian 430 flame ionisation GC with a fused silica capillary column, Chrompack CPSIL 88 (100m length, 0,25 mm ID, 0.2 µm film thicknesses) was used to quantify FAMEs from the feed fat and egg yolk. Starting with an isothermic period of 40°C for 2 minutes, the analysis was performed. The temperature was then increased at 4°C/minute to a maximum of 230°C. After that an isothermic period of 230°C for 10 minutes followed. A Varian CP 8400 Autosampler was then used to inject FAMEs n-hexane (1 µL) into the column. Both the injection port and detector were maintained as 250°C. Functioning as the carrier gas, was hydrogen at 45 psi and as the makeup gas nitrogen was used. The chromatograms were recorded by Galaxy Chromatography Software.

By comparing the retention times of FAME peaks from samples with the standards acquired from Supelco (Supelco 37 Component Fame Mix 47885-U, Sigma-Adric Aston Manor, Pretoria, South Africa), FAME samples were identified. The internal standard used was nonadecanoic acid (C19:0) (SIGMA N553377-IG) to improve quantitative FAME estimation. Acquired from Merck Chemicals (Pty Ltd, Halfway House, Johannesburg, South Africa), all the other reagents and solvents were of analytical grade. The FA composition was depicted as each individual FA to the proportion (%) of all the FAs present in the sample. The FAs in the egg yolk that were specifically from the n-3 and n-6 families were then quantitatively expressed as mg FA per 100 g egg yolk. A calculation of the following fatty acid combinations was done, namely: omega-3 (n-3) FA, omega-6 (n-6) FA, total saturated fatty acids (SFA), total monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), PUFA/SFA ratio (P/S), n-6/n-3 ratio.

4.2.9 Statistical analysis procedure

External and internal egg quality data that was collected within the specific collection weeks (week 24, 28, 32, 36 and 40 of age and week 33 for FA analysis) were pooled for statistical analysis. Similar to the egg production (paragraph 3.2.10.5), the fully randomised one-way ANOVA design was used to determine the effect of dietary n-3 type lipid sources on external and internal egg quality traits. The PROC ANOVA procedure (SAS, 2021) was used to test for significant differences ($P< 0.05$) between treatments. The Tukey's honest significant difference test (HSD) was used to identify significant differences between treatment means.

4.3 Results and discussions

4.3.1 Shell quality

The effect of different omega-3 fatty acid sources on egg weight, eggshell thickness and eggshell quality traits are shown in Table 4.1. The different lipid sources, varying in n-3 PUFA chain length, did not affect ($P > 0.05$) the egg weight, shell weight, shell thickness or any of the shell quality variables. Although not significantly different ($P < 0.05$), it seems from Table 4.1 that the egg weight ($P = 0.0586$), egg surface area ($P = 0.0571$) and calculated eggshell calcium content ($P = 0.0517$) would record differences between the dietary lipid sources if tested at $P < 0.1$.

Results of the present study agrees with that of Dey *et al.* (2021), who also reported no effect ($P > 0.05$) on eggshell quality parameters using dietary oil inclusion levels of 1.5% and 2.5% for soybean oil. Furthermore, Güçlü *et al.* (2008) reported that by comparing eight different dietary oil sources at a 4% inclusion level each, no effect ($P > 0.05$) on eggshell thickness of laying quail were recorded. The results of the present study are supportive to that of many authors (Basmacioglu *et al.*, 2003; Bean & Leeson, 2003; Mousavi *et al.*, 2017; Dong *et al.*, 2018; Moura *et al.*, 2019; Josling *et al.*, 2019) who found no effect ($P > 0.05$) on eggshell quality parameters when various dietary lipid sources at different inclusion levels were added to layer diets. Despite the fact that Panaite *et al.* (2021) reported no effect ($P > 0.05$) of 6% linseed meal and 2% carotenoid inclusion level on eggshell weight and shell breaking strength, they recorded an increase ($P < 0.05$) in eggshell thickness when compared to a control treatment with no added linseed meal. The findings of Attia *et al.* (2022) showed an increase ($P < 0.05$) in eggshell thickness, SWUSA and percentage eggshell when dietary treatments with 3% maize oil in combination with 2% fish oil and 5% linseed oil were fed. These results indicate that additional dietary oils do not have a negative effect on shell quality nor eggshell calcium metabolism (Attia *et al.*, 2022).

Table 4.1 The mean (± S.E) effects of different omega-3 fatty acid sources on egg shell quality characteristics during peak production (24 weeks - 40 weeks)

	Control	Fish oil	Soybean oil	Linseed oil	Echium oil	P	[1]CV (%)
Egg weight (g)	53.93±0.50	54.34±0.45	55.81±0.58	54.35±0.54	55.28±0.47	0.0586	5.09
Shell weight (g)	5.746±0.06	5.73±0.05	5.89±0.08	5.84±0.06	5.95±0.05	0.052	5.71
Shell Thickness							
Blunt end (µm)	0.34±0.00	0.35±0.00	0.34±0.01	0.34±0.01	0.34±0.0.10	0.4414	5.69
Sharp end (µm)	0.36±0.00	0.37±0.00	0.36±0.00	0.37±0.00	0.36±0.00	0.9395	5.18
Equator (µm)	0.36±0.00	0.36±0.00	0.37±0.00	0.36±0.00	0.36±0.00	0.7550	4.45
Average shell thickness (µm)	0.36±0.00	0.36±0.00	0.36±0.00	0.36±0.00	0.36±0.00	0.4547	4.44
Quality variables							
Egg surface area (cm^2)	66.29±0.43	66.66±0.38	67.92±0.49	66.66±0.47	67.47±0.41	0.0571	3.59
[2]SWUSA (mg/cm^2)	0.09±0.00	0.08±0.00	0.09±0.00	0.09±0.00	0.09±0.00	0.2411	4.59
Percentage eggshell (%)	10.53±0.10	10.38±0.07	10.40±0.12	10.57±0.08	10.60±0.08	0.3331	4.91
Calcium content (g)	2.11±0.02	2.10±0.02	2.16±0.03	2.15±0.02	2.19±0.02	0.0517	5.71

[1] Coefficient of variance (%).

[2]Shell weight per unit surface area.

4.3.2 Internal egg quality

The effect of different omega-3 lipid sources on internal egg quality characteristics during peak production are shown in Table 4.2. Dietary n-3 lipid source had no effects ($P > 0.05$) on egg weight, shell weight, yolk height, yolk ratio, yolk index, albumen weight, albumen diameter, thick albumen height, thin albumen height, albumen ratio, albumen index or haugh units. The only differences ($P < 0.05$) between dietary treatments were recorded for yolk weight ($P = 0.005$) yolk diameter ($P < 0.001$) and yolk colour ($P < 0.001$). Yolk weight of the soybean oil treatment (14.43 g) was heavier ($P < 0.05$) compared to that of the control (13.73 g) and fish oil (13.83 g) treatments but did not differ ($P > 0.05$) from that of the echium oil (14.20 g) or linseed oil (14.07 g) treatments. Similarly, the yolk diameter of the soybean oil treatment (39.82 mm) was wider ($P < 0.001$) compared to the linseed (39.15 mm), fish oil (38.81 mm) and control (38.65 mm) treatments, but did not differ ($P > 0.05$) from the echium oil (39.28 mm) treatment. The mean yolk colour of the control treatment (7.99) was lighter ($P < 0.001$) than the fish oil (8.40), soybean oil (8.39), echium oil (8.39) and linseed oil (8.38) treatments when compared on the DSM Roche® colour fan (Figure 4.5).

Internal egg quality traits of the present study (Table 4.2) differs from authors (Basmacioglu *et al.*, 2003; Cherian *et al.*, 2007; Cachaldora *et al.*, 2008; Pérez-Bonilla *et al.*, 2011; Petrović *et al.*, 2012; Kralik *et al.*, 2018; Perić *et al.*, 2019; Aguillón-Páez *et al.*, 2020) who reported no effect ($P > 0.05$) of dietary lipid sources and inclusion levels on the egg yolk weight. In addition, Bean & Leeson (2003) recorded lower yolk weights ($P < 0.05$) in laying hens that consumed diets containing 10% flaxseed as compared to the control treatment, despite no differences ($P > 0.05$) in egg production, egg weight, shell weight, albumen height, or shell thickness Also, egg yolk weight results of the present study differ from that of Dey *et al.* (2021) who recorded differences ($P > 0.05$) on boiled yolk weight when comparing yolks from a non-supplemented control treatment to that of 1.5% and 2.5% soybean oil supplementation. Supplementing laying hen diets with microencapsulated fish oil at inclusion levels of 0, 20, 40 and 60 g/kg resulted in a decrease ($P < 0.05$) in egg yolk weights (17.0, 16.1, 16.2 and 15.7 g respectively) (Lawlor *et al.*, 2010), which is partly supported by the present study (Table 4.2) where the fish oil treatment recorded the second lowest ($P < 0.05$) yolk weight (13.83 g). Gao *et al.* (2021) also reported that an 8% fish oil dietary inclusion caused a significant decrease in egg yolk weight. Egg yolk weight results for the control treatment (Table 4.2) does not support the findings of Zotte *et al.* (2015) who reported that the control diet resulted in heavier ($P < 0.05$) egg yolk weights when compared to treatments supplemented (between 3.4% to 10.0%) with commercial fish oil, ground linseed or extruded linseed oil. Furthermore, present

Table 4.2 The mean (± S.E) effects of different omega-3 fatty acid sources on internal egg quality characteristics during peak production (24 weeks- 40 weeks)

	Control	Fish oil	Soybean oil	Linseed oil	Echium oil	P	[1]CV (%)
Egg weight (g)	53.93±0.50	54.34±0.45	55.81±0.58	54.35±0.54	55.28±0.47	0.059	5.09
Shell weight (g)	5.746±0.06	5.73±0.05	5.89±0.08	5.84±0.06	5.95±0.05	0.052	5.71
Albumen weight (g)	35.22±0.47	34.89±0.35	35.67±0.47	35.67±0.47	35.35±0.38	0.478	6.66
Albumen diameter (mm)	103.89±0.85	104.97±0.73	105.21±0.83	105.33±0.75	102.94±0.95	0.194	4.33
Thick albumen height (mm)	9.18±0.14	9.25±0.20	8.86±0.16	8.75±0.18	9.06±0.20	0.138	9.67
Thin albumen height (mm)	1.77±0.04	1.69±0.04	1.71±0.05	1.69±0.04	1.81±0.04	0.144	12.97
Albumen index (%)	5.39±0.11	5.34±0.14	5.13±0.12	5.07±0.10	5.41±0.09	0.100	11.54
Albumen ratio (%)	64.36±0.26	64.08±0.26	63.68±0.29	63.47±0.25	63.68±0.22	0.108	2.22
Yolk weight (g)	13.73^{b}±0.12	13.83^{b}±0.15	14.43^{a}±0.18	14.07^{ab}±0.13	14.20^{ab}±0.13	0.005	5.59
Yolk diameter (mm)	38.65^{c}±0.12	38.81^{bc}±0.14	39.82^{a}±0.21	39.15^{bc}±0.15	39.28^{ab}±0.12	<0.0001	2.16
Yolk height (mm)	18.34±0.09	18.39±0.08	18.49±0.13	18.29±0.11	18.44±0.08	0.640	2.92
Yolk index (%)	47.56±0.23	47.45±0.22	46.78±0.29	46.86±0.31	47.05±0.19	0.111	2.92
Yolk ratio (%)	25.14±0.24	25.39±0.22	25.78±0.25	25.83±0.21	25.59±0.19	0.163	4.77
Yolk colour	7.99^{b}±0.003	8.40^{a}±0.01	8.39^{a}±0.01	8.38^{a}±0.01	8.39^{a}±0.01	<0.0001	0.55
Haugh units	74.03±0.71	73.95±0.93	71.91±0.76	71.96±0.78	73.40±0.51	0.106	5.63

[1] Coefficient of variance (%).

[a,b,c,] Row means with different superscripts differ significantly at $P< 0.05$.

results regarding egg yolk weights for the control and soybean oil treatments (Table 4.2) also differ from those of Batkowska *et al.* (2020) who indicated that the control treatment (no added lipids) resulted in heavier ($P< 0.05$) yolk weights (13.69 g) than treatments containing 2.5% soybean oil (13.21 g) and 2.5% linseed oil (13.31 g). Koppenol *et al.* (2014) reported that linoleic acid increases the production of lipoproteins absorbed by the developing yolk, therefore increasing yolk weight and consequently also egg weight. From Table 4.2 it is notable that the numerical highest ($P= 0.059$) egg weight (55.81 g) recorded for the soybean oil treatment resulted in the highest ($P= 0.005$) yolk weight (14.43 g), thereby supporting the findings of Koppenol *et al.* (2014). In addition, these findings regarding yolk weight of the soybean oil treatment are supportive to literature (Table 2.1) indicating that soybean oil have a high linoleic acid (LA) concentration in comparison with linseed and echium oil which have similar LA concentrations.

Furthermore, egg yolk colour results of the present study are partly in agreement with many authors (Soliman & El-Afifi, 2020; Dey *et al.*, 2021; Attia *et al.*, 2022) who found that the addition of different levels of dietary supplemental oils such as linseed, palm, soybean, maize and fish had no effect on yolk colour. Cachaldora *et al.* (2008) found that in comparison to non-lipid supplemented control diets, yolk colour increased with the addition of soybean oil, linseed oil, or lard, but decreased linearly with the level (0%, 1.5% and 3% inclusion) of fish oil supplementation. In addition, Dong *et al.* (2018) reported the yolk colour of the coconut oil treatment (6.6) to be higher than the soybean oil treatment (6.4) where the fish oil treatment (6.5) did not differ ($P> 0.05$) from either treatment. Current results (Table 4.2) indicate that only the control treatment (no oil supplementation) resulted in a lower ($P< 0.05$) yolk colour (7.99), while none of the oil supplemented treatments differ ($P> 0.05$) from one another in terms of yolk colour. Faitarone *et al.* (2016) concluded that vegetable oil inclusion in layer diets, as colour metrically evaluated, did not have a significant effect on yolk colour. The results of Pérez-Bonilla *et al.* (2011) suggest that yolk colour increased ($P< 0.001$) when hens were given diets supplemented with lard compared to those supplemented with soybean oil and/or acidulated vegetable soap-stocks. Najib & Al-Yousif (2014) reported that a 4% dietary maize oil supplemented diet resulted in paler ($P\leq 0.001$) egg yolks than a non-supplemented control (5.30 vs. 5.85) diet. These authors (Najib & Al-Yousif, 2014) indicated that in order to create isocaloric dietary treatments, the 4% maize oil diet had more maize replaced with oil than the control dietary treatment, which in turn could contribute to the paler yolks. Faitarone *et al.* (2016) indicated that the fat solubility properties of carotenoids and xanthophylls will influence the intestinal absorption of these pigments which is enhanced when supplementary lipids are included in the diets. Although these authors do not highlight the importance of individual FA in the depositing of these pigments into the egg yolk, it seems that data from the

present study (Table 4.2) supports this rationale whereby the four omega-3 enriched diets resulted in darker (P< 0.001) egg yolks than that of the non-supplemented control treatment.

4.3.3 Yolk fatty acid methyl esters

The effects dietary supplemental n-3 lipid sources on egg yolk properties during week 33 of hen age are shown in Table 4.3. From Table 4.3 it is evident that dietary n-3 lipid sources did not have a significant effect on yolk fat percentage (P= 0.829) but did influence yolk fat free dry matter (P =0.003) and yolk moisture (P= 0.007) concentration. The soybean oil treatment that recorded the highest (P< 0.05) mean egg yolk weight (14.43 g) during the peak production period (Table 4.2), also recorded the highest (P= 0.003) yolk fat free dry matter (20.97%) and lowest (P= 0.007) yolk moisture content (46.29%).

Table 4.3 The mean (± S.E) effects of different dietary omega-3 lipid sources on egg yolk properties at 33 weeks of age

	Yolk fat (%)	Yolk fat free dry matter (%)	Yolk moisture (%)
Control	32.43±0.21	19.29^{b}±0.29	48.28^{a}±0.39
Fish oil	32.61±0.23	19.80^{ab}±0.27	47.60^{ab}±0.36
Soybean oil	32.73±0.26	20.97^{a}±0.35	46.29^{b}±0.40
Linseed oil	32.78±0.29	19.84^{ab}±0.34	47.38^{ab}±0.37
Echium oil	32.76±0.19	19.51^{b}±0.26	47.73^{ab}±0.31
P	0.829	0.003	0.007

^{a,b,c,d} Column means with different superscripts differ significantly at P< 0.05

The effects of dietary supplemental n-3 lipid sources on fatty acid methyl esters (FAME) during week 33 of hen age are presented in Table 4.4. It should be emphasised that the specific FAME presented in Table 4.4 only includes the particular individual FAs that were also recorded for the diets (Table 3.4) and it is not the only FA recorded within the egg yolk. As anticipated, it is clear from differences that egg yolk FAME were influenced (P< 0.001) in line with the different dietary n-3 PUFA treatments. The control treatment (no added lipid source) resulted in egg yolk with the highest (P< 0.001) concentration of palmitic acid (C16:0; 26.92%) as well as the palmitoleic (C16:1c9; 4.45%), oleic (C18:1c9; 43.43%) and vaccenic acid (C18:1c7; 4.64%). The fish oil treatment produced the highest (P< 0.001)

Table 4.4 The mean (± S.E) effects of different dietary omega-3 lipid sources on fatty acid methyl esters of egg yolk during week 33 of hen age

	Control	Fish oil	Soybean oil	Linseed oil	Echium oil	P
FAME (% of total fatty acids)						
Saturated fatty acids						
Myristic (C14:0)	0.34^{b}±0.02	0.43^{a}±0.02	0.29^{c}±0.01	0.26^{c}±0.01	0.28^{c}±0.01	<0.0001
Palmitic (C16:0)	26.92^{a}±0.28	26.27^{ab}±0.34	25.55^{b}±0.24	23.98^{c}±0.24	26.22^{ab}±0.40	<0.0001
Margaric (C17:0)	0.06^{d}±0.00	0.13^{a}±0.01	0.09^{bc}±0.00	0.08^{cd}±0.00	0.11^{b}±0.01	<0.0001
Stearic acid (C18:0)	6.15^{b}±0.20	6.69^{b}±0.17	6.63^{b}±0.20	6.55^{b}±0.18	7.70^{a}±0.22	<0.0001
Monounsaturated fatty acids						
Palmitoleic (C16:1c9)	4.45^{a}±0.23	4.06^{ab}±0.14	3.08^{c}±0.18	3.46^{bc}±0.15	2.89^{c}±0.12	<0.0001
Oleic (C18:1c9)	43.43^{a}±0.49	42.76^{ab}±0.43	41.45^{bc}±0.32	43.20^{a}±0.42	40.39^{c}±0.35	<0.0001
Vaccenic (C18:1c7)	4.64^{a}±0.09	4.50^{a}±0.06	4.07^{b}±0.04	4.09^{b}±0.06	3.59^{c}±0.04	<0.0001
Eicosenoic (C20:1c11)	-	-	0.03^{a}±0.00	-	-	<0.0001
Polyunsaturated fatty acids (n-6)						
Linoleic (C18:2c9,12)	12.45^{c}±0.25	11.81^{c}±0.33	16.19^{a}±0.30	12.80^{c}±0.25	14.34^{b}±0.20	<0.0001
Eicosadienoic (C20:2c11,14)	0.05^{b}±0.00	0.02^{c}±0.00	0.07^{a}±0.01	0.03^{c}±0.00	0.03^{c}±0.00	<0.0001
Arachidonic (C20:4c5,8,11,14)	0.81^{bc}±0.08	0.46^{d}±0.03	1.05^{a}±0.07	0.61^{cd}±0.04	0.90^{ab}±0.04	<0.0001
Polyunsaturated fatty acids (n-3)						
α-Linolenic (C18:3c9,12,15)	0.59^{c}±0.03	0.71^{c}±0.02	0.85^{c}±0.03	3.62^{a}±0.12	2.88^{b}±0.07	<0.0001
Stearidonic (C18:4c6,9,12,15)	-	-	-	-	0.02^{a}±0.00	<0.0001
Eicosapentaenoic (C20:5c5,8,11,14,17)	-	0.16^{a}±0.01	-	0.03^{b}±0.00	0.04^{b}±0.00	<0.0001
Docosapentaenoic (C22:5c7,10,13,16,19)	0.01^{c}±0.01	0.19^{ab}±0.02	0.04^{c}±0.01	0.13^{b}±0.01	0.23^{a}±0.02	<0.0001
Docosahexanoic (C22:6c4,7,10,13,16,19)	0.20^{c}±0.03	1.85^{a}±0.15	0.40^{c}±0.03	1.00^{b}±0.07	1.20^{b}±0.08	<0.0001

^{a,b,c,d} Row means with different superscripts differ significantly at $P< 0.05$.

concentrations of margaric acid (C17:0; 0.13%), eicosapentaenoic acid (EPA; C20:5c5; 0.16%) and docosahexaenoic acid (DPA; C22:6c4; 1.85%) in egg yolk, while it also recorded the second highest concentration of docosapentaenoic acid (DHA; C22:5c7; 0.19%). The soybean oil treatment resulted in the highest (P< 0.001) concentration of linoleic (16.19%), eicosadienoic (0.07%) and arachidonic acid (1.05%). The linseed oil treatment produced the highest (P< 0.001) concentration of α-linolenic acid (3.62%). The egg yolks from the echium oil treatment had the highest (P< 0.001) content of stearic acid (7.70%), SDA (0.02%) and DPA (0.23%). While the echium oil treatment resulted in egg yolks that have similar levels of palmitic acid (26.22%) in comparison to yolks from the control (26.92%), fish oil (26.27%) and soybean oil (25.55%) treatments, the stearic acid (7.70%) content of egg yolk from the echium oil treatment was the highest (P< 0.001).

Given the dietary concentration (Table 3.4) of α-linolenic acid (ALA) of the control diet (2.70%), fish oil (3.26%), soybean oil (3.59%), linseed oil (18.30%) and the echium oil treatment (11.81%), it is interesting to note how the dietary ALA relates with the egg yolk ALA concentration - 0.59%, 0.71%, 0.85%, 3.62% and 2.88% for the respective dietary treatments (Table 4.4). While EPA (3.94%), DPA (0.26%) and DHA (2.79%) were only detected in the fish oil diets (Table 3.3), the concentrations of these long chain n-3 PUFA were notable in the egg yolks of the linseed and echium oil treatments – suggesting conversion from shorter chain n-3 PUFA in these specific cases.

Ngo Njembe *et al.* (2021) reported that laying hens are proficient in converting ALA to DHA, by reporting that a diet containing 20% flaxseed resulted in an ALA enrichment of up to 500 mg/egg and an increase in the DHA content of up to 80 mg/egg. Yannakopoulos *et al.* (2005) stated that laying hens consuming an n-3 PUFA enriched diet will change ALA into DHA and deposit both types of n-3 PUFA in the yolk by displacing less SFA during this process. The results of this study (Table 4.4) is supportive to the findings of Yannakopoulos *et al.* (2005); Ngo Njembe *et al.* (2021) whereby the linseed oil treatment with the highest dietary concentration of ALA (18.30%) resulted in the highest (P< 0.001) egg yolk concentration of ALA (3.62%) and the third highest in EPA (0.03%), DPA (0.13%) and DHA (1.00%). Kitessa *et al.* (2012) reported that by comparing vegetable oils that either contains ALA or stearidonic acid (SDA), it seems that SDA is seen as a better precursor for converting into long-chain n-3 PUFA such as EPA, DPA and DHA. Current results (Table 4.4) support the findings of Kitessa *et al.* (2012), whereby the echium oil treatment had the highest dietary concentration of SDA (4.04%) and resulted in the highest (P< 0.001) yolk concentrations of DPA (0.23%), second highest EPA (0.04%) and DHA (1.20%) when compared to the fish oil treatment which recorded the highest EPA (0.16%) and DHA (1.85%) concentrations in the egg yolk. Since the

linseed and echium oil diets didn't contain DHA, it is likely that the increase in the concentrations of yolk long chain omega-3 PUFA is a result from the desaturation and elongation of ALA in the liver during follicle development (Ayerza & Coates, 2001).

Table 4.5 presents the total FA concentrations and ratios of the egg yolk during week 33 of age. Additionally, rather than solely using the selected individual FAME as shown in Table 4.4, the egg yolk fatty acid concentrations and ratios shown in Table 4.5 were calculated using the complete fatty acid profile (FAP) of egg yolk for comprehensiveness. Furthermore, from Table 4.5 it is evident that the control treatment recorded the lowest (P< 0.001) concentration of total yolk n-3 FA (0.80%) as well as the highest (P< 0.001) ratio (16.83 : 1) of n-6/n-3 PUFA. Because of the FA composition of the yolk from the fish oil treatment it resulted in having the highest concentration of total long chain n-3 PUFA (EPA + DPA + DHA; 2.20%) as indicated in Table 4.5. The soybean oil treatment has the highest concentration of total n-6 PUFA (17.36%) and the second highest n-6/n-3 ratio (13.56 :1) as a result of its specific yolk FA composition. As shown in Table 4.5, the linseed oil treatment produced yolks with the lowest n-6/n-3 ratio (2.82 : 1), the highest mg n-3/ gram edible egg (4.46 mg/g egg) and double bond index (96.73) in the egg yolk as well as the highest concentration of total n-3 PUFA (4.80%). The yolks from the echium oil treatment did not differ (P> 0.05) from the linseed oil treatment with regards to total n-3 PUFA concentration (4.38%), n-6/n-3 ratio (3.52 : 1) and mg n-3/ gram edible egg (4.09 mg/g egg). From Table 4.5 it is interesting to note that the echium oil treatment resulted in egg yolks with the highest (P< 0.001) concentration of total SFA (34.32%), while the dietary FA concentrations (Table 3.5) of the fish oil diet resulted in the highest total SFA (22.64%) concentration, while the echium oil diet had the second lowest total SFA (15.57%) concentration. Given the considerable concentrations of SFA such as palmitic and stearic acid in egg yolk, it is expected that the total SFA concentration of egg yolk to be higher than that of the total concentrations of n-6 and n-3 PUFA.

Results (Table 4.5) regarding the mean total saturated fatty acid (SFA) concentration of egg yolk are in agreement with Poureslami *et al.* (2011) who reported that the SFA of egg yolk range between 28% and 38%. Guclu *et al.* (2008) also found the SFA of quail egg yolks to range between 30% and 38% when having a 4% lipid inclusion of different oils, with the fish oil treatment having the highest (P< 0.05) SFA concentration (37.07%) and the olive oil treatment the lowest (31.43%). It is further interesting to note that although the total concentration of SFA in egg yolk are similar (Table 4.5), it is clear that the echium oil treatment resulted in the lowest (P< 0.001) total concentration of MUFA (45.90%) and the highest (P< 0.001) concentration of total PUFA (19.78%).

Table 4.5 The mean (± S.E) effects of different dietary omega-3 lipid sources on total fatty acid concentrations and fatty acid ratios of egg yolk during week 33 of hen age

	Control	Fish oil	Soybean oil	Linseed oil	Echium oil	P
Total fatty acid concentration (%)						
Total saturated fatty acids	33.48^{ab}±0.35	33.56^{ab}±0.48	32.57^{b}±0.27	30.86^{c}±0.26	34.32^{a}±0.51	<0.0001
Total monounsaturated fatty acids	52.36^{a}±0.50	51.23^{a}±0.66	48.78^{b}±0.33	50.89^{a}±0.46	45.90^{c}±0.50	<0.0001
Total polyunsaturated fatty acids	14.15^{c}±0.33	15.21^{c}±0.36	18.65^{ab}±0.35	18.25^{b}±0.38	19.78^{a}±0.25	<0.0001
Total omega- 6 fatty acids	13.35^{c}±0.29	12.30^{c}±0.34	17.36^{a}±0.32	13.45^{c}±0.28	15.40^{b}±0.21	<0.0001
Total n-3 fatty acids	0.80^{d}±0.04	2.91^{b}±0.18	1.29^{c}±0.05	4.80^{a}±0.14	4.38^{a}±0.06	<0.0001
Total unsaturated fatty acids	66.51^{bc}±0.35	66.44^{bc}±0.48	67.43^{b}±0.27	69.14^{a}±0.26	65.68^{c}±0.51	<0.0001
Total long chain n-3 - EPA + DPA + DHA	0.21^{c}±0.03	2.20^{a}±0.18	0.43^{c}±0.04	1.16^{b}±0.07	1.47^{b}±0.09	<0.0001
Fatty acid ratios						
PUFA/SFA[1]	0.42^{b}±0.01	0.45^{b}±0.01	0.57^{a}±0.01	0.59^{a}±0.01	0.58^{a}±0.01	<0.0001
n-6/n-3 ratio[2]	16.83^{a}±0.36	4.45^{c}±0.34	13.56^{b}±0.33	2.82^{d}±0.07	3.52^{cd}±0.04	<0.0001
mg n-3 / g egg yolk	8.03^{d}±0.36	29.09^{b}±1.79	12.86^{c}±0.46	47.95^{a}±1.40	43.79^{a}±0.59	<0.0001
mg n-3/g edible egg (whole edible egg)	0.74^{d}±0.03	2.72^{b}±0.17	1.22^{c}±0.04	4.46^{a}±0.17	4.09^{a}±0.08	<0.0001

^{a,b,c,d} Row means with different superscripts differ significantly at $P< 0.05$.

[1]The ratio of polyunsaturated to saturated fatty acids.

[2] The ratio of omega-6 to omega-3 fatty acids.

Given the daily energy requirements of 2500 kcal for men and 2000 kcal for women, a daily intake of ALA of 2.8 g/day and 2.2 g/day is advised for adult men and women, respectively, while Ngo Njembe *et al.* (2021) indicate that the minimum daily intake for DHA and EPA + DHA should be 250 mg/day and 500 mg/day, respectively. Currently, claims for n-3 PUFA enriched products can only be made in Canada, the United States of America (USA), and the European Union (EU) (Vahmani *et al.*, 2015). Legislation in Canada indicate that each 100 g serving of an n-3 PUFA enrich food source must contain at least 300 mg of total n-3 fatty acids (Dugan *et al.*, 2015; Huang *et al.*, 2018; Subhani *et al.*, 2020). Foods in the USA can be described as "sources" or "rich" in ALA if they contain between 160 and 320 mg, but no claims can be made for EPA or DHA (Vahmani *et al.*, 2015). Foods in the EU may be labelled as sources of n-3 PUFA if they contain 300 mg of ALA or 40 mg of EPA and DHA combinedly per 100 g of product, or as rich in n-3 PUFA if they contain 600 mg of ALA or 80 mg of EPA and DHA combined per 100 g (Vahmani *et al.*, 2015). According to the South African Department of Health (DOH, 2014), food items may be labelled respectively as a (i) "source of n-3", (ii) "high in n-3" or (iii) "very high in n-3" if they contain at least 75 mg, 150 mg or 300 mg of n-3 PUFAs in total per single serving (one egg). The results of the current study (Table 4.5) show that the mg n-3/g edible egg was the highest ($P< 0.001$) in the linseed oil (4.46 mg/g) and echium oil (4.09 mg/g) treatments followed by the fish oil (2.72 mg/g) then the soybean oil (1.22 mg/g) and the control (0.74 mg/g) treatment. Given these results (Table 4.5) one egg with an average weight of 56 g would produce 250 mg n-3 for the linseed oil, 229 mg n-3 for the echium oil, 152 mg n-3 for the fish oil, 68 mg n-3 for the soybean oil and only 41 mg n-3 for the control treatment. King *et al.* (2012) reported that when diets supplemented with lipid sources such as fish oil, sunflower oil, high oleic acid sunflower oil and/or tallow at a dietary inclusion level of 30 g/kg, the fish oil treatment resulted in eggs with the highest n-3 PUFA concentration of 7.78 mg n-3/g egg. The findings of Aguillón-Páez *et al.* (2020) demonstrate that without impacting performance parameters negatively, dietary inclusion levels of 13.5% flaxseed resulted in elevated levels of n-3 PUFA (6.92%) in eggs compared to the non-supplemented control (1.6%) diet.

Simopoulos (2004) indicate that by considering the egg's nutritional quality in terms of the n-6/n-3 ratio, the ideal ratio ranges between 1 : 1 and 4 : 1 for improved human health. The results of the current study indicate that a dietary inclusion of 1.5% linseed and echium oil were equally effective in decreasing the n-6/n-3 ratio of the egg yolk (2.82 : 1 and 3.52 : 1 respectively) in comparison to the recommendations of Simopoulos (2004). Present results are supportive to that of various authors (King *et al.*, 2012; Huang *et al.*, 2018; Aguillón-Páez *et al.*, 2020; Feng *et al.*, 2020) in terms of the efficiency of using dietary n-3 PUFA lipid sources such as fish and linseed oils to reduce the n-6/n-3 ratio in the egg yolk to improve the health

properties for human consumption. The inconsistencies in data regarding the ideal inclusion levels as well as the potential role of echium oil and/or other lipid sources that could serve as fatty acid modifiers need clarification, especially with the ever-increasing demands of health-conscious consumers.

4.4 Conclusions

Although current results indicate that egg quality characteristics such as egg weight, yolk weight, yolk diameter and yolk colour are influenced by dietary n-3 PUFA lipid sources, no clear trend could be established in terms of the role of specific dietary fatty acids on other internal egg quality traits. Results suggest that by using different n-3 lipid sources for dietary supplementation, it is possible to alter the fatty acid methyl esters (FAME) of egg yolk in a more favourable manner. Additionally, it is evident that the linseed and echium oil treatments resulted in the highest levels of total n-3 PUFA (4.80% & 4.38%), as well as the lowest n-6/n-3 ratios (2.81 : 1 & 3.52 : 1) in egg yolk. Furthermore, despite fish oil recording the highest concentration of long chain n-3 PUFA (EPA+DPA+DHA) in egg yolk, it was not sufficient to decrease the n-6/n-3 ratio in egg yolk to the same extend than linseed oil. The challenges associated with high concentrations of PUFA n-3 in egg yolks may include sensory appeal to consumers as well as lipid oxidation. Even though these two aspects were not examined in this study, it should be further investigated to determine the interaction between n-3 PUFA lipid source and inclusion level on oxidation stability. Lastly, the limited response of depositing stearidonic acid from the echium oil treatment into egg yolk should be better quantified in terms of the metabolic route of this type of n-3 PUFA during yolk fat synthesis.

Chapter 5
General Conclusions and Recommendations

It is known that omega-3 polyunsaturated fatty acids (n-3 PUFAs) have numerous health benefits for humans. However, to ensure that these health benefits are realised, it is essential to ensure that the optimal omega-6 to omega-3 (n-6/n-3) ratio of human diets is less than 4 : 1. Modern western lifestyles resulted in diets containing an n-6/n-3 ratio of between 16 to 20 : 1 – resulting in an increase in non-communicable diseases due to the inflammatorily action of high concentrations of n-6 PUFAs. The high n-6/n-3 ratio could be ascribed to modern-day diets that are processed with plant oils such as sunflower oil that are high in n-6 PUFAs, as well as the lack of dietary consumption of fish and marine products that are rich in long-chain (LC) n-3 PUFAs such as like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The fatty acid profile (FAP) of egg yolk can be manipulated through dietary lipid sources to have a higher concentration of n-3 PUFAs. This enrichment of eggs provides a functional food source rich in essential fatty acids (EFAs), amino acids, minerals and vitamins. While the manipulation of fatty acid methyl esters (FAME) of egg yolks has been widely researched, the available literature is scarce regarding the use of lipid sources high in stearidonic acid (SDA), which is seen as an n-3 PUFA of C18:4 type on the FA enrichment of egg yolk.

According to the findings of the current study, most of the production parameters were not affected ($P > 0.05$) using various dietary n-3 lipid sources. The only parameters that were affected ($P < 0.05$) were mean body weight and total egg production, whereby the mean body weight of hens in the control treatment (1729 g) was the lowest ($P < 0.05$), while those of the soybean oil (1849 g), linseed oil (1809 g) and echium oil (1807 g) were higher. There seems to be no clear link between daily feed intake and body weight of the hens, but it is interesting to note that the lowest non-significant ($P > 0.05$) feed intake (99.66 g/bird/day) of hens in the fish oil treatment were associated with the second lowest body weight (1789 g) of hens while having the highest dietary gross energy (16.51 MJ/kg) and crude fat (45.56 g/kg) content. It is recognised that different fatty acids' associative dynamic action reduces the metabolic heat produced during digestion, thus increasing the energy efficiency of a lipid source. However, it could be postulated that the lower feed intake of the fish oil treatment is somewhat linked to the higher energy and dietary crude fat concentration instead of the specific associative dynamics of the fatty acids. In addition, another possible reason for the lowest daily feed intake recorded on the fish oil treatment could be linked with the organoleptic properties of fish oil and the fact that birds might be hesitant to consume diets containing rancid oils. Since none of the lipid sources were tested for oxidative stability before dietary inclusion, this suggestion

should be addressed with the needed caution. Total egg production was the highest ($P< 0.05$) for the linseed oil treatment (96.69%) and the lowest for the soybean oil treatment (94.25%). However, the lower egg production of the soybean oil treatment did not negatively impact egg output (g) since this treatment resulted in the numerical highest ($P>0.05$) egg output (364.22 g). Despite these random responses in terms of egg production, it seems that no clear link could be established between dietary n-3 lipid source and total egg production. The production study's results indicate that dietary supplementation with n-3 lipid sources had no adverse effect on egg production parameters, while the responses between the four types of n-3 lipid supplements seem to be similar for most traits.

Furthermore, the results of the present study also show that different n-3 lipid sources do not negatively impact external or internal egg quality parameters. None of the shell quality parameters was affected ($P>0.05$) by the n-3 lipid source. The results indicate that shell thickness and calcium metabolism are not influenced ($P>0.05$) by the additional 1.5% inclusion of lipid sources into the basal control diet during peak production (24 to 40 weeks of hen age). Egg yolk weight (14.43 g) and diameter (39.82 mm) from the soybean oil treatment were higher ($P< 0.05$) than the other n-3 lipid source treatments. The control treatment (with no added lipids) and the fish oil treatment (highest dietary lipid concentration) resulted in the lowest yolk weights (13.73 g & 13.83 g, respectively), suggesting that the use of dietary supplemental lipid sources does not necessarily influence egg yolk weight *per se*. The heavier ($P< 0.05$) yolk weight of the soybean oil treatment could be ascribed to the higher n-6 linoleic acid (LA) concentration of soybean oil, which is linked with higher yolk weights in prior studies. The egg yolk colour of the control treatment (7.99) was less yellow ($P< 0.001$) in comparison with those yolks from the n-3 supplemented sources (8.38 - 8.40). These egg yolk results may either be explained by the fact that (i) n-3 PUFA lipid sources influence the intestinal absorption of carotenoids and xanthophylls, which aid in transferring these pigments into the egg yolk and consequently darker yolk colours, or (ii) the fact that 15 g/kg lipid inclusion level into the four trial diets resulted in a higher absorption efficiency of the fat-soluble carotenoids.

Lastly, current results indicate that a low dietary level (1.5%) of supplementary n-3 lipid sources successfully altered not only the entire fatty acid methyl ester (FAME) profile of egg yolk but precisely that of the n-3 PUFA concentration in particular. The concentration of α-linolenic acid was the highest ($P< 0.001$) in the egg yolk of the linseed oil treatment (3.62%) and second highest in the echium oil treatment (2.88%). The echium oil treatment had the highest content of docosapentaenoic acid (DPA) (0.23%), while the egg yolk from the fish oil treatment had the highest ($P< 0.001$) concentration of EPA (0.16%) and DHA (1.85%) followed by the echium oil treatment (0.04% and 1.20% respectively). These results show that

incorporating a SDA source in layer diets may produce an egg that can be an alternative food for fish and seafood products while delivering enough DHA and DPA. The dietary linseed oil treatment resulted in egg yolks with the lowest (P< 0.0001) n-6/n-3 ratio (2.82 : 1), followed by the echium oil treatment (3.52 : 1). This is a clear indication that the echium oil treatment (Stearidonic acid, 18:4, n-3) was efficient in improving the n-6/n-3 ratio of the egg yolk to be of an optimal ratio for human consumption. Furthermore, by calculating the total concentration of n-3 egg, it was evident that the linseed (4.46 mg/g edible egg) and echium oil (4.09 mg n-3/g edible egg) was much higher (P< 0.0001) than those n-3 concentrations of the fish and soybean oil treatments (2.72 & 1.22 mg/g edible egg respectively). These results suggest that even with a relatively low dietary inclusion level (15 g/kg) of n-3 lipid sources, it is possible to produce n-3 enriched eggs, which could serve as a wholesome addition to the diet of people who prefer lacto-ovo-vegetarian lifestyles and/or people who are allergic to fish and fish products. Fatty acid-enriched eggs with a higher n-3 PUFA concentration and a lower ratio of n-6/n-3 PUFAs, could be seen as an excellent alternative to regular eggs for people living with non-communicable diseases.

To conclude, supplemental lipid sources with different n-3 FAP did not have a negative (P> 0.05) influence on the production performance as well as internal and/or external egg quality parameters during the peak production period of laying hens (24 – 40 weeks of age). The n-3 PUFA content of egg yolks were successfully altered in agreement with the different dietary n-3 lipid sources. The linseed-, echium- and fish oil treatments produced eggs containing sufficient n-3 PUFAs to be labelled as "high in n-3 FA" according to the South African Department of Health guidelines.

Despite the beneficial effect of more PUFAs in the diets, it remains crucial that the diets should be mixed adequately and stored in such a manner to minimise lipid peroxidation, despite the fact the current study did not explore the possible effects of oxidation on production performance and/or yolk FAME. Therefore, further research needs to be done to determine how lipid oxidation could affect the different types of n-3 FAs deposited into the egg yolk. Another aspect that needs further investigation is the deposition of dietary n-3 PUFAs in the body tissue of laying hens and how it affects the overall body and liver fat content of laying hens which might result in animal health and welfare aspects. In addition, the consumer sensory acceptability of n-3 enriched eggs will aid in understanding the reception of n-3 enriched eggs within modern markets. Lastly, further investigation in terms of the financial sustainability of producing n-3 PUFA enriched eggs, as well as the general consumers' acceptance of these eggs, is urgently needed – especially under local South African conditions whereby the cost become a considerable influencer in terms of purchasing decisions.

ABSTRACT

This study's objective was to investigate the effect of dietary omega-3 lipid sources on the production performance, external and internal egg quality of laying hens during the peak production period (21 - 40 weeks of age). The five dietary treatments consisted of 1.5% inclusion level of four lipid sources (varying in omega-3 fatty acid chain length) mixed "on top" into a basal diet which was also used as the control treatment. The lipid sources used were fish- (EPA, C20:5 & DHA, C22:6), soybean- (ALA, C18:3), linseed- (ALA, C18:3) and echium oil (SDA, 18:4). One hundred and fifty Hy-Line Silver-Brown hens (21 weeks of age) were allocated to the five experimental diets (n=30 birds/treatment).

During weeks 24, 28, 32, 36 & 40, all eggs produced were recorded and weighed individually, while feed intake and body weight of birds were determined. Twenty eggs/treatment/day (n = 100 eggs/treatment/week) were randomly selected for internal- and external egg quality parameters during the designated collection periods. Twelve eggs per treatment (n = 12 per treatment) were randomly selected during week 33 of age for the analysis of egg yolk fatty acid methyl esters (FAMEs). Statistical analysis of the data was performed using the SAS (2021) fully randomised one-way ANOVA procedure ($P< 0.05$).

According to the findings, omega-3 lipid sources had no effect on feed intake, feed efficiency, or egg output. The body weights of the birds receiving the control treatment (1729 g) were lower ($P< 0.05$) than that of the birds that received the dietary omega-3 lipid sources. Sellable eggs did not differ across treatment means even though hen-day egg production was lower for the soybean oil treatment than linseed oil (94.3% vs. 96.7%). Regarding the influence of omega-3 lipid supply on laying hens' peak production performance, no discernible trends were found. None of the shell quality parameters were affected by the dietary treatments.

Most of the internal yolk quality parameters were unaffected by the n-3 lipid sources, except for yolk weight, diameter and colour ($P< 0.05$). The soybean oil treatment resulted in having a higher ($P< 0.05$) yolk weight and diameter (14.43 g and 39.82 mm) than the control treatment (13.73 g and 38.65 mm). The control treatment's yolk colour was paler ($P< 0.001$) than the yolks from the n-3 lipid treatments. The linseed oil treatment resulted in yolks with the highest percentage of α-linolenic acid (3.62%; $P< 0.001$), followed by the echium oil treatment (2.88%). Yolks from the fish oil treatment were higher in EPA (0.16% vs 0.04%) and DHA (1.85% vs 1.20%) than yolks from the echium oil, whereas echium oil yolks had higher levels of DPA (0.19% vs 0.23%). Linseed and echium oil treatments resulted in the highest ($P< 0.05$)

levels of total n-3 PUFA (4.80% & 4.38%), as well as the lowest n-6/n-3 ratios (2.81 : 1 & 3.52 : 1) in egg yolk.

It can finally be concluded that layer hen diets supplemented with a low level of n-3 lipid sources will in general not have a negative impact on egg production and internal or external egg quality traits. Echium oil seems to be a viable lipid source to raise the n-3 PUFAs in the egg yolk and to lower the n-6/n-3 FA ratio. Finally, it is necessary to evaluate the market potential for fatty acid-enriched eggs in South Africa as well as consumer preference.

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