FERTILITY IN LAYER BREEDERS FOLLOWING DIETARY FATTY ACID TREATMENTS

by

Olusola Samuel Olubowale

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Supervisor: Prof. J.P.C. Greyling
Co-supervisor: Mr. F.H. De Witt

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 for a degree to any university. I furthermore cede copyright of this dissertation in favour of the University of the Free State.

Olusola Samuel Olubowale

Bloemfontein

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ACRONYMS AND ABBREVIATIONS

AA	Arachidonic acid
ADF	Acid detergent fibre
AI	Artificial insemination
ALA	α-Linolenic acid
AME	Apparent metabolisable energy
AMEn	Apparent metabolisable energy corrected for nitrogen
ANOVA	Analysis of variance
AVP	Available phosphorus
BHT	Butylated hydroxytoluene
BPSE	Beltsville Poultry Semen Extender
Ca:AvP	Ratio of calcium to available phosphorus
CLA	Conjugated linoleic acid
CP	Crude protein
CVD	Cardiovascular disease
D	Dark
E	Embryo
DGLA	Dihomo-γ-linoleic acid
DHA	Docosahexaenoic acid
DM	Dry matter
DPA	Docosapentaenoic acid
DTA	Docosatetraenoic acid
ED	Early death
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAME	Fatty acid methyl esters
FCR	Feed conversion ratio
FE	Feed efficiency
FFA	Free fatty acids
FFDM	Fat free dry matter
GnRH	Gonadotrophin releasing hormone
HDL	High density lipoprotein

HO	High oleic acid
HSD	Honest significant difference
IPVL	Inner perivitelline layer
L	Light
LCT	Lower critical temperature
LD	Late death
LDL	Low density lipoprotein
LSD	Least significant difference
LT	Leukotriene
ME	Metabolisable energy
MD	Middle death
MUFA	Mono-unsaturated fatty acid
MUFA / SFA	Monounsaturated fatty acid to saturated fatty acid ratio
NDF	Neutral detergent fiber
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
PG	Prostaglandin
PUFA	Polyunsaturated fatty acid
PUFA / SFA	Polyunsaturated fatty acid to saturated fatty acid ratio
PV	Peroxide value
SFA	Saturated fatty acid
SMT	Sperm mobility test
SQI	Sperm quality index
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TX	Thromboxane
U/S	Unsaturated fatty acid to saturated fatty acid ratio
UCT	Upper critical temperature
UFA	Unsaturated fatty acid
UFA / SFA	Unsaturated fatty acid to saturated fatty acid ratio

CHAPTER 1
GENERAL INTRODUCTION

The importance of the poultry industry in terms of human nutrition and the agricultural economy in South Africa cannot be overemphasised. The consumption of chicken meat has increased in South Africa following the decline in per capita consumption of especially beef from the 1970's, a trend predicted to continue into the future. Generally, the animal production sector has consistently generated a higher income than the crop and horticulture agricultural sectors. The Department of Agriculture, Forestry and Fisheries (DAFF, 2012) estimated a gross income (R71 785 million) generated from animal production to be almost equal to the combined income from field crops (R35 789 million) and horticultural products (R37 020 million). According to the South African Poultry Association (SAPA), chicken meat and eggs remain the most important source of animal protein for South Africans, with an approximate consumption of 2 316 million tons (SAPA, 2012). The consumption of poultry products (meat & eggs) was approximately 30.9% more than the combined consumption of mutton, beef and pork during the same year (SAPA, 2012). The combined per capita consumption of poultry meat (36.12 kg per annum) and eggs (8.9 kg per annum) was reported to be exceedingly higher than that of beef (16.62 kg per annum), pork (4.60 kg per annum), mutton and goat (2.70 kg per annum). SAPA (2012) further reported that the total supply of 1 773 447 tons of poultry meat and 407 544 tons of eggs and egg products were a combination of supply by local producers and imported products. In sustaining the demand for eggs and chicken meat, there has consequently been a crucial need for the improvement of fertility in chicken parent stock of both the laying and broiler type.

Reddy (1995) suggested that the gradual decline in fertility of broiler breeders due to an increase in body weight, necessitated the implementation of artificial insemination (AI), as practised extensively in turkeys. This has suggested that reproductive intervention may be associated with the selection for growth, particularly in meat type lines of chickens (Reddy & Sajadi, 1990; Barbato, 1999). Such protocols, as well as the evaluation of breeding soundness in cockerels would however require techniques that involve the microscopic evaluation of semen. The current methods used in managing chicken flock fertility generally involve the selection of cockerels based on physical characteristics, such as comb size, body size and shank length. Wilson *et al*. (1979) reported these traits not to be accurate enough in predicting cockerel fertility, especially in broiler breeder cockerels. More recent studies on

reproduction have also advocated the need for semen quality evaluation of cockerels intended for breeding purposes. Many researchers (Donoghue, 1999; King *et al*., 2000; Parker & McDaniel, 2002) focussed on detailed scientific and sustainable methods for the evaluation of potential fertility in cockerels. These methods included protocols involving semen quality tests, using the evaluation of semen parameters such as volume, sperm motility, sperm viability and sperm concentration. Subsequently, the selection of cockerels based on sperm quality has been reported to translate into improved hatchability in the broiler breeder flocks (Eslick & McDaniel 1992; Parker & McDaniel 2002). However, one of the major factors affecting cockerel sperm quality has been diet composition (both chemically and physically). Supplementary dietary lipids have been shown to have an influence on cockerel semen quality (Cerolini *et al.,* 2006; Bongalhardo *et al.,* 2009), as well as fortifying eggs of hens with nutrients such as essential fatty acids (Grobas *et al.,* 2001; Mazalli *et al.,* 2004; Da Silva Filardi *et al*., 2005).

Dietary manipulation has generally been employed as a means of enhancing sperm quality, due to the strong relationship between the cockerel reproductive potential and overall flock fertility (Blesbois *et al.,* 1997, 2004; Cerolini *et al*., 2006). It is further evident that dietary fatty acids are deposited proportionately in the fatty acid methyl ester component of sperm (Blesbois *et al.,* 1997; Kelso *et al.,* 1997; Cerolini *et al.,* 2003). This means that regardless of the supplementary lipid sources consumed by cockerels, the proportion of the total relative abundance of saturated, mono-unsaturated- and polyunsaturated fatty acids in sperm do not change significantly, even although the percentage of specific omega-type fatty acids (omega-3; -6 and -9), within the category of total polyunsaturated fatty acids (PUFA's) are altered. Dietary lipid sources however affect cockerel sperm functionality differently, in relation to the specific fatty acids prevalent within the sperm phospholipids (Bongalhardo *et al.,* 2009). Omega-6 (n-6) type fatty acids (e.g. docosatetraenoic acid; DTA) are most prevalent in cockerel sperm (Cerolini *et al.,* 1997), as opposed to omega-3 (n-3) fatty acids that are predominant in mammalian sperm (Nissen *et al.,* 1981; Poulos *et al.,* 1986; Kelso *et al*., 1997). There however seems to be minor variation regarding the effect of n-3 and n-6 dietary sources on cockerel sperm quality (Kelso *et al*., 1997; Surai *et al.,* 2000; Cerolini *et al.,* 2003; Zanini *et al.,* 2003). Although the long chain fatty acids (docosahaexanoic acid; DHA) supplied by supplementary fish oil improved sperm motility in men (Nissen *et al*., 1981; Conquer & Tekpetey, 2003), bulls (Gholami *et al*., 2010) and cockerels (Cerolini *et al.,*

2006), its susceptibility to peroxidation has also been reported to affect sperm motility negatively (Ollero & Alvarez, 2003).

The main challenge regarding the usage of supplementary dietary lipids is concerning lipid oxidation. The unsaturated fatty acids (UFA's) readily undergo oxidation at the carbon atom adjacent to the double bond to form hydroperoxidase. In return, these give rise to free radicals and other short-chain end-products which eventually accelerate the process of oxidation (McDonalds *et al.,* 2011). Rancid odours resulting from dietary lipid oxidation occurs, normally when the peroxide threshold levels of 20 milli-equivalents peroxide/kg fat are reached in unsaturated fats of animal origin. While a higher threshold value of approximately 80 milliequivalents peroxide/kg fat has been quoted for vegetable oils (Leeson & Summers, 2005). The dietary supplementation of fish oil with varying levels of Vitamin E has been reported to reduce the lipid oxidation and improve cockerel sperm motility (Surai *et al.,* 1998; Zanini *et al.,* 1999; Cerolini *et al.,* 2006). Vitamin E supplementation may then be related to the reduced potency of feed anti-oxidants, especially in feed stored for prolonged intervals at high environmental temperatures (≥ 30°C) (Njobeh & Nsahlai, 2006).

The tendency of overall flock fertility to decline with age (Lapao *et al.,* 1999; Abudabos, 2010; Alsobayel & Albadry, 2012) is not only limited to the response of the cockerels alone (Cerolini *et al*., 2003). Insko *et al*. (1947) reported that the general fertility of hens deteriorate with age, resulting in a consequent lowering of hatchability of eggs in older flocks. Nonetheless, techniques in improving flock fertility have been more focussed on the cockerels *via* the control of the sex ratio (♂ : ♀) (Alsobayel & Albadry, 2012), as well as spiking at later ages (≥ 45 weeks of age) by means of the introduction of younger cockerels (Cassanovas, 2000). One important aspect in the dietary manipulation to improve semen quality is the added advantage of enhancing the general fertility of both cockerels and hens, as well as the deposition of essential nutrients (preferred fatty acids) in eggs. Although agreements regarding the effect of dietary lipids (particularly the polyunsaturated fatty acids) on the enrichment of chicken eggs are unanimous, there are contradictory results concerning the effect of these sources on the productive performance of the hen itself (Jiang *et al*., 1991; Grobas *et al.,* 1999a,b, 2001; Mazalli *et al.,* 2004; Da Silva Filardi *et al*., 2005). So for example other authors (Jiang *et al*., 1991; Mazalli *et al*., 2004; Da Silva Filardi *et al*., 2005) found that supplementary lipid sources had no effect ($P \geq 0.05$) on the production parameters (e.g. egg production and egg weight) during the peak production phase as well as the second

laying cycle. On the other hand, Grobas *et al.* (1999a; 2001) reported that the dietary supplementation of n-6 lipid sources had a positive ($P \leq 0.05$) effect on egg weight.

The chicken egg has long been recognised as a potent dietary element for human nutrition which could be used in supplying essential nutrients and also improving human health. Similarly, awareness on the role of fish oil enriched food products ('designer foods') in the supplying n-3 fatty acids needed in lowering the omega-6 to omega-3 ratio (n-6 : n-3) has generated interest in both human nutrition (Simopoulos, 1999; 2002) and human health (Lavie *et al.,* 2009). Especially as it implies that the risk of cardiovascular diseases are reduced. Also, the group of mono-unsaturated fatty acids (MUFA's) of omega-9 type (n-9) are being promoted for the nervous system myelinization in growing children (Uauy & Hoffman, 1991; Uauy *et al.,* 2007). The experimental diets evaluated in the present study included a control diet consisting of 50% linseed and 50% fish oil, pure fish oil, sunflower oil, high oleic acid sunflower oil and tallow; thereby supplying omega-3, omega-6, omega-9 and highly saturated fatty acids to the birds respectively.

The aims of this study were as follows:

- To investigate the influence of dietary lipid sources on the productive performance of cockerels.
- To evaluate the effects of dietary lipid sources on cockerel semen characteristics during both the early (≤ 50 weeks of age) and end-of-lay (≥ 60 weeks of age) productive phases.
- To determine the effects of dietary lipid sources on the fertility and hatchability in an end-of-lay flock.
- To investigate the influence of dietary lipid sources on the production performance of hens reared from early production to end-of-lay.

CHAPTER 2
LITERATURE REVIEW

2.1 Fats and oils in animal diets

The impact of nutrition on the physiology and anatomy of animals cannot be over-emphasized. Dietary lipids are increasingly being recognised as playing vital roles in the biological processes. The properties of lipids are influenced to a large extent by their constituent fatty acid composition. These fatty acids are then generally categorised as being either saturated or unsaturated, depending on the absence and/or presence of a carbon-carbon double bond. Unsaturated fatty acids are further subdivided into 2 groups, namely (i) mono-unsaturated fatty acids (MUFA's), which contain only one double bond and (ii) polyunsaturated fatty acids (PUFA's) which refers to those having more than one double bond (Bezard *et al.,* 1994). MUFA's normally contain omega-9 (n-9) fatty acids, whereas omega-3 (n-3) and omega-6 (n-6) fatty acids forms part of the PUFA's. Saturated fats are generally solid at room temperature and its incorporation as tallow in poultry diets have been popular from the mid-1990's. In addition to the biological functions of fats and oils, their inclusion in poultry feed has also ensured reduced dustiness and improved palatability thereof (Leeson & Summers, 2005).

2.1.1 Production of vegetable oils

The last century has witnessed an improvement in technological efficiency, with a concurrent commercial and domestic utilization of vegetable oils. In the agricultural sector, grains and their oil by-products, which are rich in n-6 fatty acids, have constituted a large proportion of the livestock feeds. The surge in availability and utilization of oils was thus brought about by practical innovations and interventions (Kirshenbauer, 1960). So for example, a continuous screw press (Expeller®) was invented by Anderson and the steam-vacuum deodorizer process were created by Wesson (Kirshenbauer, 1960). After World War I, solvent extraction of oilseeds became popular, leading to the large scale economical production of vegetable oils. Also in the human diets, hydrogenation for the purpose of solidifying the vegetable oils is constantly being utilised. This practise has however been found to lead to the formation of *trans*-fatty acids, as well as an increase in the concentration of linoleic acid (C18:2), with a subsequent decrease in the α-linolenic acid (C18:3) content of the oil (Emken, 1984).

2.1.2 Basic biochemical structure of fatty acids

Any fatty acid (FA) is characterised into two major groups, namely a carboxylic acid group at the one end and a methyl group having a carbon atom (named omega ω), at the other end. In the omega reference system, the number of carbon atoms was first described, together with the number of double bonds which separate (:) the carbon atoms. The closest double bond to the omega carbon usually features in the name of the unsaturated fatty acid (Stulnig, 2003). So for example, if the closest double bond is 6 carbon atoms distant, this fatty acid is then called omega (ω), or simply n-6. The categories, names and omega reference of certain of the more important fatty acids are presented in Table 2.1 (Stulnig, 2003).

Table 2.1 Categories and names of certain important fatty acids (Stulnig, 2003)

Category	Trivial name	Abbreviation	Omega references
Saturated FA's	Lauric acid		12:0
	Myristic acid		14:0
	Palmitic acid		16:0
	Stearic acid		18:0
MUFA's	Palmitoleic acid		16:1 ω-9
	Oleic acid	HO	18:1 ω-9
n-6 PUFA's	Linoleic acid*	LA	18:2 ω-6
	γ-Linolenic acid		18:3 ω-6
	Dihomo- γ -linolenic acid		20:3 ω-6
	Arachidonic acid	AA	20:4 ω-6
	Docosatetraenoic acid	DTA	22:4 ω-6
n-3 PUFA's	α-Linolenic acid*	ALA	18:3 ω-3
	Eicosapentaenoic acid	EPA	20:5 ω-3
	Docosahexaenoic acid	DHA	22:6 ω-3

* Essential fatty acid; FA's – fatty acids; MUFA's – mono-unsaturated fatty acids; PUFA's - polyunsaturated fatty acids.

2.1.3 Polyunsaturated fatty acids (PUFA's)

PUFA's are classified as either omega-3 (n-3) or omega-6 (n-6), depending on the fatty acids originating from the parent *α*-linolenic- and linoleic acid, respectively (Table 2.1). Both α-linolenic- and linoleic acid are termed essential fatty acids (EFA's), because of the incapacity

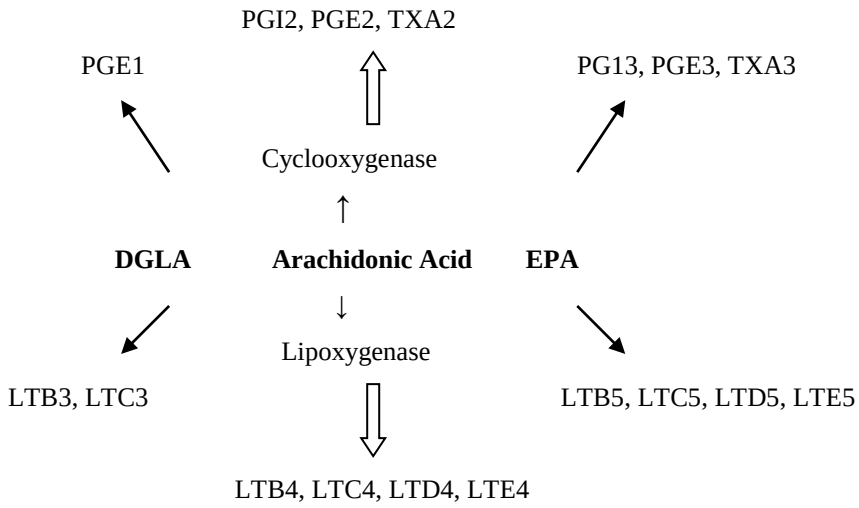
of the animal body to synthesize these specific fatty acids. Therefore, it is of critical importance that these FA's are supplied to animals by dietary means.

The main sources of linoleic acid include oils from maize, soybeans and animal fats, while α-linolenic acid (shorter chain n-3 type) is found in abundance in algae, canola, linseed and rapeseed oils. Marine food products such as fish oil are also good sources of longer chain n-3 derivatives, like docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Das, 2006).

2.1.3.1 Importance of essential fatty acids (EFA's)

Egg yolk is the main source of energy and essential fatty acids for the developing chick (Freeman & Vince, 1974). More than 80% of yolk fat being absorbed by the chick in the course of the 21 day incubation period (Noble & Cocchi, 1990; Cherian *et al*., 1997). Generally, EFA's fulfil important anatomical and physiological functions in the animal body, by constituting components of the cell membranes and influencing membrane fluidity and the behaviour of membrane bound enzymes and receptors (Das, 2006). The polyunsaturated fatty acids (PUFA's) help in hormonal regulations through the formation of important eicosanoids, such as prostaglandins (PGF), thromboxanes (TX), and leukotrienes (LT) for normal metabolism (Stulnig, 2003).

The eicosanoid derived from the omega-6 fatty acids, is generally arachidonic acid (AA), while eicosapentaenoic acid (EPA) is the main acid generated by the omega-3 fatty acids. The oxidative metabolism of AA produce eicosanoids, that favours pro-inflammatory, vasoconstriction and platelet aggregation activities (Calder, 2006). Conversely, EPA contributes to vasodilation and inhibits platelet aggregation. (Calder, 2006; Schmitz & Ecker, 2008). In Figure 2.1, the synthesis of eicosanoid from essential fatty acids is illustrated schematically. The activities of probiotics in the jejunal mucosa of gnotobiotic piglets have been specifically reported to be modulated by omega-3 (Bomba *et al.,* 2003). In another study Keys *et al*., (1957) reported that omega-6 fatty acids are important in lowering the blood serum cholesterol levels in animals. In addition, these EFA's often act as an antibiotic with ALA, by means of rapidly destroying *Staphylococcus aureus* (McDonald *et al.,* 1981).



DGLA = dihomo-γ -linoleic acid, EPA = eicosapentaenoic acid, LT = leukotriene, PG = prostaglandin; TX = thromboxane.

Figure 2.1 The eicosanoid synthesis from essential fatty acid derivatives (Simopoulus, 2002).

2.1.4 Historical background of fatty acids in the human diet

Significant changes have occurred in dietary food choices of man since the beginning of the agricultural revolution one thousand years ago. In spite of these changes, the human genes still bear remarkable similarities to that of their ancestors who lived during the Paleolithic period 40000 years ago (Eaton & Konner, 1985). These changes in eating habits induced changes in the lifestyle and a specific drift in disease manifestation. Studies on the evolution of diets (Eaton & Konner, 1985; Simopoulos, 1991; 1999) placed the emphasis on changes in diets, specifically the type and amount of essential fatty acids (EFA's) and the antioxidant content of foods prevalent in the modern society. A shocking observation was the relative high omega-6:omega-3 (n-6:n-3) ratios in modern western diets (12:1), compared to the low proportion (2.4:1) characteristic of the original hunter-gatherer diets of our ancestors (Simopoulos, 1995).

2.1.4.1 Modern foods *vs.* meat of game origin

The reduction in the omega-3 fatty acid content of animal carcasses has been blamed on the boost in agribusiness. Nowadays, domestic livestock such as cattle are predominantly fed grain diets which are rich in omega-6 fatty acids, resulting in their meat containing small quantities of α-linolenic acid (Crawford *et al*., 1969). On the other hand, wild animals and

birds whose diets are comprised mainly of natural plants have a higher deposit of ALA and leaner meat containing only 3% fat. Further, eicosapentaenoic acid (EPA) an important derivative of ALA, constitutes up to 4% of the total fat in wild animals (Ledger, 1968).

Production optimization has been the core focus of the modern agriculture, thus bringing about a decrease in the omega-3 fatty acid content of most natural foods. During the past 3 decades, commercially produced food products such as meat, eggs, green leafy vegetables, and fish have contained less quantities of these important PUFA's, compared to those food sources found in nature (Crawford, 1968; Sinclair *et al*., 1982; Cordain *et al*., 1998).

Table 2.2 Fatty acid levels (mg/g yolk) in egg yolk of chickens under different production systems (Simopoulos & Salem, 1989**)**

Fatty acid	Greek	Supermarket	Fishmeal	Flax
Saturated				
14:0	1.1	0.7	1.0	0.6
15:0	–	0.1	0.3	0.2
16:0	77.6	56.7	67.8	58.9
17:0	0.7	0.3	0.8	0.5
18:0	21.3	22.9	23.0	26.7
Total	100.7	80.7	92.9	86.9
Mono-unsaturated				
16:1ω7	21.7	4.7	5.1	4.4
18:1	120.5	110.0	102.8	94.2
20:1ω9	0.6	0.7	0.9	0.5
24:1ω9	–	–	0.1	–
Total	142.8	115.4	108.9	99.1
ω6 Polyunsaturated				
18:2ω6	16.0	26.1	67.8	42.4
18:3ω6	–	0.3	0.3	0.2
20:2ω6	0.2	0.4	0.6	0.4
20:3ω6	0.5	0.5	0.5	0.4
20:4ω6	5.4	5.0	4.4	2.6
22:4ω6	0.7	0.4	0.3	–
22:5ω6	0.3	1.2	0.2	–
Total	23.1	33.9	74.1	46.0
ω3 Polyunsaturated				
18:3ω3	6.9	0.5	4.1	21.3
20:3ω3	0.2	–	0.1	0.4
20:5ω3	1.2	–	0.2	0.5
22:5ω3	2.8	0.1	0.4	0.7
22:6ω3	6.6	1.1	6.5	5.1
P/S ratio	0.4	0.4	0.9	0.9
M/S ratio	1.4	1.4	1.2	1.1
ω6/ω3 ratio	1.3	19.9	6.6	1.6

P/S = polyunsaturated: saturated ratio; M/S = mono-unsaturated: saturated ratio

As a result of this destabilization of the fatty acid content and other nutritional factors, industrialized communities are now known to be affected by (i) an increase in the intake of omega-6, and trans-fatty acids, and a concomitant decrease in omega-3 intake; (ii) a decrease in the consumption of antioxidants, protein, and calcium, as well as other undesirable elements (Eaton & Konner, 1985; Simopoulos, 1999). In Table 2.2, the fatty acid levels (mg/g yolk) profile of hard-boiled eggs from different production enterprises are set out (Simopoulos & Salem, 1989). According to Table 2.2, "Greek eggs" represented eggs obtained from free-ranging chickens, while "Supermarket eggs" were standard copies of US Department of Agriculture eggs, found in US supermarkets. Fish meal eggs had their main source of fatty acids provided by fish meal and soybeans diets, while flax flour was the diet that provided the main source of fatty acid in flax eggs.

2.1.5 The importance of omega-6 : omega-3 ratio

As background two classes of essential fatty acids (EFA), namely n-6 in the form of linoleic and n-3 in the form of α-linolenic acids are not inter-convertible, as appropriate proportions must be provided in the diets. Both linoleic and α-linolenic acids are distinct in terms of their function, metabolic activities and are usually involved in opposing physiological functions.
Typically, a ratio of 4 : 1 (n-6 : n-3) is recommended for humans, however Western diets are grossly imbalanced with a ratio as high as 15-16 : 1 (Simopoulos, 2003a).

Fish is the main dependable and popular source of omega-3 fatty acids in human diets. It is therefore imperative to create more avenues by which these EFA's can be incorporated into human diets (Raper *et al.*, 1992; Lavie *et al.*, 2009) and chicken (Herber & Van Elswyk, 1996). One of the viable means of incorporating more omega-3 fatty acid in human diets, is the subsequent dietary manipulation in livestock, from which valuable food products are obtained.

The feeding of different sources and quantities of omega-3 PUFA's to dairy cows as well as broiler and layer type chickens have boosted its quantities, consequently reducing the n-6 : n-3 fatty acid ratio in milk, chicken meat (Coetzee & Hoffman, 2002; Cherian, 2007), and eggs (González-Esquerra & Leeson, 2001), respectively.

2.1.6 Effect of saturated and unsaturated fatty acids fed to layer chickens

As this project focuses on the period between peak (33 weeks of age) and end-of-lay (78 weeks of age) in laying hens, the digestion and utilization of the different type of fats is not expected to adversely affect the performance of chickens. Research has since shown that only young chickens (< 2 weeks old) are predisposed to a difficulty with the digestion and absorption of saturated fats (Garret & Young, 1975; Sibbald & Kramer, 1978; Chen & Chiang, 2005), which could be attributed to their digestive system not being fully developed and functional at that stage (Carew *et al.*, 1972).

More recently, Chen and Chiang (2005) reported that the ambient temperature, rather than the dietary polyunsaturated/saturated (P/S) fatty acid ratio is more important when considering weight gain, feed intake, and feed conversion ratio (FCR) in poultry. This is in agreement with the studies of Olomu and Baracos (1991) and Sanz *et al*. (2000). Contradictory observations have also been previously reported, with certain researchers reporting a better FCR when hens were fed unsaturated fat, compared to the hens reared on saturated fats (Zollitsch *et al.,* 1997; Crespo & Esteve-Garcia, 2002). Chien and Chiang (2005) then concluded that the modification in the dietary polyunsaturated/saturated (P/S) fatty acid profile of diets according to seasonal variation, may not affect poultry at all - as the experimental diets could not elicit any response in their metabolic heat production and growth performances. Furthermore, the use of other representative omega-3 (fish-, linseed- and canola oil) and omega-6 (sunflower oil) lipid sources did not affect the performance of the layers differently, when compared to tallow (Baucells *et al.*, 2000; Mazalli *et al*., 2004). Grobas *et al*. (2001) however reported higher egg weights for layers fed soybean oil, compared to tallow, linseed and olive oil. Scheideler and Froning (1996) reported an increased egg production for hens fed supplementary fish oil. Other parameters such as hen performance and shell quality were noted not to be affected by the type of oil i.e. saturated fatty acids (lard) or polyunsaturated fatty acids (canola-, cotton-, soybean- and sunflower oil) in diets of post-molt layers (Da Silva Filardi *et al.,* 2005).

Similar observations were made by Cachaldora *et al*. (2008) in an experiment where fish oil was added at 3 levels (0, 15, 30 g/kg) to a basal diet consisting of no fat. Production traits such as feed intake, laying rate, egg weight, yolk weight, Haugh units and shell thickness were found not to be affected by the dietary treatments - although yolk colour had reportedly

increased with supplemented basal fat diets, when compared to the non-supplemented basal diets.

Table 2.3 Performance characteristics of hens fed different fat sources during the second cycle of production (Da Silva Filardi *et al.*, 2005)

Fat source	Feed intake (g/b/d)	Egg production (%)	Egg weight (g)	Egg Output (g)	FCR (kg/kg)
Cotton oil	110.30	77.42	65.60	50.91	2.31
Soybean oil	110.12	78.52	65.42	51.36	2.26
Lard	110.30	78.62	68.20	53.61	2.17
Sunflower oil	111.65	76.35	68.34	52.13	2.27
Canola oil	108.52	75.27	68.03	51.23	2.24
F-value	0.79	0.57	1.43	0.39	0.38
SEM	2.49	3.81	2.47	3.48	0.17

2.1.6.1 Enrichment of chicken meat and eggs with n-3 fatty acids

Poultry products emerged in recent times as reliable sources of 'designer foods'. The manipulation of diets to increase the nutrient value of chicken meat and eggs for the improvement of human health is now being considered as a viable option in developed countries e.g. Canada (Leeson *et al*., 2007), Spain (Cachaldora *et al.*, 2008; Garcia-Rebollar *et al.*, 2008), Korea (Kim *et al.*,. 2007), and developing countries such as Brazil and Argentina (Da Silva Filardi *et al.*, 2005; Azcona *et al*., 2008). Amongst the categories of fatty acids, omega-3 essential fatty acids have expectedly received the most attention. Partly because of its health benefits (Simopolous, 2002), but also due to the capacity of dietary PUFA's to cause a greater change in the egg fatty acid profile than saturated and MUFA's whose effects are considered to be minimal (Noble *et al*., 1990; Baucells *et al*., 2000; Garcia-Rebollar *et al*., 2008). Similarly, the use of linoleic (omega-6) rich ingredients such as sunflower oil, which is a common practice in the poultry industry, has been associated with soft fatty tissue and greater susceptibility to lipid oxidation of the meat (Zollitsh *et al*., 1997; Sanz *et al*., 2000).

Although diets have been identified as a factor that influences the egg yolk lipid profile the most (Leskanich & Noble 1997), age of the bird, its strain and breed has also been reported to induce differences in the composition and fat profile of eggs (Edwards, 1964; Nielsen, 1998). In this regard, younger hens (less than 30 weeks of age) were reported to deposit fats of up to 50% lower in n-3 fatty acids into their egg yolks, compared to the older hens. It was also

found that the Hisex white layers had 30% more α-linolenic acid deposited in their eggs, than any other strains (Hy-line, Babcock, or Dekalb type layers) (Scheideler *et al.*, 1998).

Fish oil, linseed (flaxseed) oil and canola oil are currently popular natural sources rich in omega-3 that have routinely been incorporated into poultry feeds. Depending on the expectations of the desired products however, type of fat, source and its level of inclusion are the critical considerations that producers of omega-3 enriched poultry products have to contend with. It seems that when the precursor α-linolenic acid (C18:3n-3) is needed in higher quantities, linseed oil is the omega-3 source of choice to use in poultry diets. While fish oil remains the preferred ingredient when producers aim at producing longer chain omega-3 fatty acids (LCn-3) i.e. eicosapentaenoic acid (C20:5n-3), docosapentaenoic (C22:5n-3), and docosahexaenoic acid (C22:5n-3) enriched eggs. In Table 2.4, a summary regarding the effect of omega-3 types oil used in poultry diets with their respective fatty acid profiles is presented.

Table 2.4 Fatty acid profiles of natural sources of omega-3 lipid sources used in poultry diets ([†]NRC, 1993; [*]Herber & Van Elswyk, 1996).

Source	18:3n-3	20:5n-3	22:5n-3	22:6n-3	Σn-3	Σn-6	Σn-3: Σn-6
[†]Flaxseed oil	53.3	-	-	-	53.3	12.7	4.2
[†]Menhaden oil	0.3	11.0	1.9	9.1	25.1	1.5	16.73
[*]Marine algae	-	-	3.8	7.4	11.2	-	-
[*]Canola oil	12.0	-	-	-	12.0	20.2	0.59

A number of studies have shown that the quantity of α-linolenic acid deposition in the egg yolk could be increased by a higher dietary concentration of linseed oil. The fatty acid profile of the egg did not bring about a concomitant increase in the LCn-3 fatty acids, namely EPA, DPA, and DHA - indicating a limited conversion of ALA to its LCn-3 derivatives (Caston & Leeson 1990; Van Elswyk *et al.*, 1995; Scheideler & Froning, 1996; Basmacioglu-Malayoglu *et al.*, 2003; Garcia-Rebollar *et al.*, 2008). Fish oil has been identified as an alternative marine source (algae), rich in LCn-3, and the most popular in the poultry industry. The advantage that fish oil offers above linseed oil, is the direct deposition of LCn-3 into the egg following its incorporation into dietary treatments with the deposition of DHA being particularly profound (Gonzalez-Esquerra & Leeson, 2000c; Cachaldora *et al.*, 2006, Garcia-Rebollar *et al*., 2008). The n-3 deposition in the egg yolk is a gradual process, which could be completed within 14 days of feeding 1.5% fish oil to hens (Lin *et al*., 1995).

It is worthy to note that the major limitation to the high level inclusion of fish oil in the diet of layers has been the fishy flavour that tends to negatively impact on the egg products (Van Elswyk 1997; Surai & Sparks, 2001). This may restrict its level of usage in diets to produce an acceptable level of 300 mg n-3/100 g egg weight ratio, as proposed by the European Commission (EC, 2005). This limitation can however be overcome by an appropriate combination of fish oil and linseed oil in poultry diets to meet this EC standard, while at the same time not compromising its sensory quality (Garcia-Rebolla *et al.*, 2008). The addition of these combined n-3 sources has also been stated to affect neither the proportion of total fatty acids in yolk, nor the yolk fat content.

2.1.6.2 Omega-3 fatty acid in combination with other health improving fatty acids

Apart from the direct deposition of n-3 in eggs and meat of the chicken, omega-3 feed supplements have been used proportionately with other types of health beneficial fatty acids in poultry diets. So for example, conjugated linoleic acid (CLA) an isomer of linoleic fatty acid LA (C18:2), is known for its therapeutic nature (Belury, 2002), as well as the prevention of cancer (Eynard & Lopez, 2003; Lee *et al*., 2005). The feeding of CLA in combination with α-linolenic (n-3), linoleic (n-6) and high-oleic (n-9) was reported to improve eggshell quality and egg production, when compared to CLA diets that were not supplemented with these fatty acids (Kim *et al.*, 2007). These researchers further observed that the supplementation of CLA diets resulted in the increased deposition of the respective PUFA's in eggs - inducing a decrease in the saturation level of fatty acids within the eggs. A study by Aydin *et al*. (2001) also proved that oleic acid and certain PUFA's improved the hatchability of chicks by lowering the saturated : unsaturated fatty acid ratio in the yolk. Coetzee and Hoffman (2002) confirmed the importance of the nutritional impact of dietary omega-3 and omega-6 fatty acid profiles in the chicken. The researchers reported that no significant difference in the body weight and feed conversion ratio was recorded. The broilers that were fed a canola oil diet (omega-3), however had a significantly higher proportion of omega-3 fatty acids in their carcass, compared to the broilers that consumed a "famarol" oil diet (omega-6) throughout the trial. Similarly, a study on the popular South American "Campero" chicken delicacy revealed that the quality of this meat product could be fortified with omega-3 and omega-9 fatty acids by feeding the live chickens linseed oil and high oleic acid sunflower oil respectively (Azcona *et al.,* 2008). These researchers reported a significantly higher deposition of α-linolenic acid derivatives, particularly in the leg and breast meat of chicken. Azcona *et al.* (2008) also reported that high oleic (HO) acid sunflower oil had no effect

regarding the production performance of birds, which was similar to the results of Rodriguez *et al*. (2005).

The recent popularity of HO sunflower oil and HO seed in poultry diets has been related to the health benefits of this mono-unsaturated oil. Mono-unsaturated fatty acids (MUFA's) are reported to be effective in reducing the saturation of the intramuscular fats, without the simultaneous hazard of lipid oxidation, often experienced with PUFA's. It has the ability to combat cardiovascular disease by decreasing low-density lipoprotein cholesterol (Roche, 2001). Others researchers have claimed health benefits in the consumption of oleic acid, which includes a bactericidal effect, myelinization in the nervous system of growing children, and the reduction of the Her-2/neu oncogene, a pathology associated with mammary tumours (Uauy Dagach & Olivares, 2007).

Despite these reports on broiler meat, poultry eggs remains the most viable and easiest avenue for making essential n-3 PUFA's available for human consumption. This advantage is attributed to the more than 4 g of fatty acids contained in eggs, the high turnover of lipid and lipoprotein in the chicken's liver, as well as the short period of time (less than 3 weeks) required for n-3 fatty acid modified eggs to be produced (Leskanich & Noble, 1997; Cherian, 2009). A comprehensive review on the enrichment of eggs and chicken meat with omega-3 fatty acids, and how it affects production, performance and the perceived consumer acceptability of such products was published by Gonzalez-Esquerra and Leeson (2001).

Advanced biochemical technologies have been employed in producing omega-3 fortified food products for human consumption. In a recent experiment, Sharma *et al*. (2009) was able to produce a structured lipid containing a 1:1 ratio of omega-3 to omega-6 fatty acids. This was achieved through lipase-catalyzed acidolysis of groundnut oil, whereby the omega-6 fatty acids in the groundnut oil was proportionately replaced by concentrated omega-3 linseed oil.

2.1.7 Elongation and desaturation of n-3 and n-6 fatty acids

The essential n-3 and n-6 fatty acids, unlike some of the other unsaturated fatty acids (n-7and n-9) are generated by the animal through exogenous means only. During the process of digestion and absorption of dietary α-linolenic and linoleic fatty acids however, minute portions of these precursors (C18:3 and C18:2) are elongated and further desaturated to their

derivatives - C18:3 (n-3) to eicosapentaenoic (C20:5) or docosahexanoic (C22:5) acid and C18:2 (n-6) to arachidonic acid (C20:4). These two fatty acids (n-3 and n-6) are considered to be dietary essential to mammals, as deficiencies result in extensive health abnormalities (McDonald *et al.,* 2011). The conversion of the essential fatty acids to their respective derivatives is then mediated by certain processes, starting with the elongation by (Elovl)-2 and/or Elovl-5 elongases, and desaturation by delta-5 (Δ^5) and delta-6 (Δ^6) desaturases (Leonard *et al*., 2002; Jump, 2004). The subsequent eiocosanoids EPA, and AA metabolised through this alternate process depends to a large extent on the PUFA type of diet ingested (Simopoulos, 2003a; Stulnig, 2003).

In Table 2.5, a summary of the main symptoms observed due to a lack of EFA's is compiled (McDonald *et al.,* 2011). The prevalence of one essential fatty acid over another determines the respective eicosanoid activity i.e. pro-inflammatory or anti-inflammatory, vasoconstriction or vasodilation, promotion or inhibition of platelet aggregation, as discussed earlier. Apart from their active participation in digestion, the activities of enzymes Δ^5 and Δ^6 desaturases and elongases in the body are also known to be dependent on certain hormones (Brenner, 1981), age (Lopez Jimenez *et al.,* 1993), and diseases such as diabetes (Brenner, 1981) and obesity (Medeiros *et al.,* 1995).

Table 2.5 Symptoms associated with a deficiency in essential fatty acids in humans (McDonald *et al.* 2011).

Growth retardation
Increased permeability to water and increased water consumption
Increased susceptibility to bacterial infections
Sterility
Less stable bio-membranes
Capillary fragility
Kidney damage, haematuria and hypertension
Decreased visual acuity
Decreased myocardial contractility
Decreased ATP synthesis in liver and heart
Decreased nitrogen retention

The knowledge of correct eicosanoid generating ability of proportionate essential fatty acids has been extensively explored in the field of human medicine. Available reports in clinical trials show positive results in the prevention and treatment of pathological disorders e.g. cardiac diseases (Singh *et al*., 1997), psychological illnesses (Locke & Stoll, 2001), asthma (Broughton *et al*., 1997) and rheumatoid arthritis (James & Cleland, 1997). Much of this

success has been attributed to the partial replacement of arachidonic acid (a derivative of linoleic acid) by eicosapentaenoic and docosahexaenoic acid (derivatives of α-linolenic acid) in the membranes of the blood cell i.e. platelets, erythrocytes and germ-fighting monocytes, neutrophils and hepatocytes (Simopolous, 2003b).

Moreover, Simopolous (2002) suggested that the above studies may confirm that an increased omega-3 intake, with a simultaneous decrease in omega-6 intake, could help maximize the functions of therapy drugs taken by patients, as a result of the sparing effects and creating enabling a suitable biochemical environment, provided by the diet.

2.1.8 Fatty acid intake and human health

Diet composition, especially the type and amount of fat in foods has been of major concern in the human medical field. There is no doubt that a link exists between diets and prevalent diseases in a human society. Fat remains the most controversial of all nutrients, as it is associated with diseases such as obesity, heart disease and diabetes. Extensive research was conducted in the 1950's and the important role of omega-6 fatty acids in the prevention of cardiovascular diseases realised. This was then associated with the ability of omega-6 fatty acid in lowering blood serum cholesterol (Ahrens *et al.,* 1954; Keys *et al.,* 1957). Troisi *et al*. (1992) however cautioned that in spite of this good quality, most omega-6 fatty acids in human diets may actually cause an increase in the level of serum cholesterol by their transformation to *trans*-fatty acids through the hydrogenation process e.g. in the case of margarine.

2.1.8.1 Adverse effects of *trans*-fatty acids

The *trans* fatty acids are isomers of naturally occurring *cis* fatty acids. Apart from margarine, hydrogenated soybean oil, which is a major component of cooking oil, can contain up to 20% *trans* fatty acids (Leeson & Summers, 2005). Although information on the effect of *trans* fatty acids on the health of broilers and layers is very scarce, its adverse effects on human health have been extensively researched, as highlighted by Simopoulos (1995) in Table 2.6.

Table 2.6 Adverse effects of *trans*-fatty acids on certain body parameters in humans and rats (Simopoulos, 2002).

Increase
Low-density lipoprotein (LDL)
Platelet aggregation
Lipoprotein (a) (Lp-a)
Body weight
Cholesterol transfer protein (CTP)
Abnormal morphology of sperm (male rats)
Decrease / inhibit
Decrease or inhibit incorporation of other fatty acids into cell membranes
Decrease high-density lipoprotein (HDL)
Inhibit delta-6 desaturase (interfere with elongation and desaturation of EFA)
Decrease serum testosterone (male rats)
Cross the placenta and decrease birth weight (humans)

The Star newspaper in South Africa (17th March, 2012) reported in an article written by Dr. Beatrice Golomb, a professor at the University of California in which she discouraged the serving of margarine to schools and prisons. She was able to relate aggressive and irritable behaviour to the high consumption of margarine in a research survey conducted on 945 people. It was asserted that the *trans*-fatty acid generated during the margarine making processes, is known to cause high cholesterol levels, resistance to insulin, oxidation and inflammation. Heart problems may also have been responsible for the heightened aggression recorded in the respondents. This article undoubtedly caused a dent in the popularity of margarine as accessory delicacy in most households. Many margarine producers in South Africa e.g. Flora® are currently marketing their brands as a rich source of omega-3 and omega-6 essential (Plate 2.1) fatty acids (without any mention of *trans*-fatty acid) - this being conspicuously printed on their products. Further, *trans*-fatty acids alter the availability of important eicosanoids in the body of animals. These fatty acids appear to interfere with the desaturation and elongation of both the omega-6 and omega-3 polyunsaturated fatty acids. This would result in decreasing the quantity of arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) available to the normal metabolism (Simopoulos, 1995).

Plate 2.1 Margarine labelled high in omega-3 fatty acids and sold in South Africa

2.1.9 Fatty acid related diseases in poultry

Acute sudden death syndrome and chronic heart failure are two of the prevalent heart diseases affecting poultry (Nain, 2008). Broiler chickens in particular, are more predisposed to heart related diseases than the layer type chickens, because of their faster growth and higher metabolic rates (Julian, 2005). Cardiovascular related conditions have then been reported to be responsible for all mortalities and morbidities in commercial broiler flocks during rearing (Nain, 2008), as well as those dead-on-arrival chickens at the slaughter houses (Nijdam *et al.*, 2006). Poultry diets are characteristically high in dietary n-6 fatty acids, *via* oil sourced from linoleic acid rich feed components, such as maize and full fat soya (Cherian, 2007). This inclusion is known to be a major contributing factor to the prevalence of heart diseases in animals (Schmitz & Ecker, 2008; Simopoulos, 2008). The undesirable effects of n-6 on the circulatory system are not unrelated to the disproportionately high production of n-6 eicosanoid (arachidonic acid), with a concurrent low production level of n-3 derived eicosanoid.

Arachidonic acid was found to be present in high proportions in the heart lipids of broilers that died suddenly (Buckley *et al.*, 1987). Walton *et al.* (1999) also reported a lower

incidence of ascites in broiler chicken fed flaxseed, when compared to their counterparts fed diets containing a blend of animal fat and vegetable oil. Interestingly, the source of a particular essential fatty acid was suggested by Wang *et al.* (2004) to affect the immune system of laying hens in different ways. Their conclusion was based on the contradictory results obtained from different trials when sunflower- or animal oil was replaced by soybean- or linseed oil at the same inclusion level (5%). Wang *et al.* (2000) reported that while the former diet (sunflower-, animal oil) affected the serum and egg yolk total immunoglobulin G (IgG) concentration of the hen, the latter diet (soyabean-, linseed oil) did not.

2.1.9.1 Lipotoxicity in chickens

Lipotoxicity is a term usually used in humans and other mammals to describe the alteration of intracellular signalling of hormones leading to cellular malfunction and cell death, arising from the accumulation of triacyglycerol (TAG) and fatty acids in the non-adipocytes (Unger, 2002). This phenomenon has also been used to describe a pathological occurrence in poultry, caused by excessive fatty acid availability and altered fatty acid profiles in the body of animals, due to the disruption of intracellular signalling (Chen *et al.,* 2006). In order to establish a relationship between endocrine systems, functionality and the production performance in the laying hens, studies on the different relationships have been conducted. These studies included the ovarian morphology and the sensitivity of gonadal follicles to hormones, blood gonadotropins and sex steroid patterns, hypothalamic and pituitary responsiveness, and sex steroid outputs of ovarian follicles from lean and obese broiler breeder hens (Hocking *et al*., 1987, 1989; Bruggeman *et al*., 1988a,b; Onagbesan *et al*., 1999). Excessive feeding of chickens, mostly in a bid to maximize genetic selection for rapid growth, has been related to this dysfunctional manifestation (Richards, 2003). Broiler breeder chickens fed *ad libitum,* although attaining early sexual maturity, were reported to also show a dramatic drop in egg production and produce fewer eggs throughout their entire production period (Bornstein *et al*., 1984).

The effect of a disruption in the fatty acid balance on the hormonal system in the hen is probably felt most in the adipose tissue. Adipose tissue has then recently been identified as an endocrine organ, where the hormone leptin is secreted (Fruhbeck *et al*., 2001). Leptin as such is an important hormone, involved in many metabolic processes of digestion and absorption, as well as reproduction. The impairment or absence of leptin-induced gonadotropin secretion has led to increased feed intake, obesity and reproductive failure in mammals (Ahima &

Flier, 2000; Ben-Shlomo, 2002). Paczoska-Eliasiewicz *et al.* (2003) were able to relate the regression and reduction in progesterone and estradiol content in the ovaries to leptin treatment in starved Hy-line Brown layer hens. On the other hand, elevated levels of leptin were correlated with liver haemorrhage, which subsequently caused ovarian abnormalities in both White Leghorn hens (Walzem *et al.*, 1993) and broiler breeder hens (Chen *et al*., 2006). The schematic illustration of Chen *et al*. (2006) as presented in Figure 2.1 clearly shows the flow or progression levels leading to lipotoxicity and a dysfunction in the reproduction of hens.

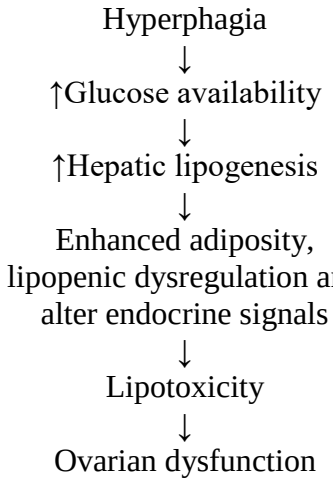


Figure 2.2 Schematic presentation of the progression of events leading to lipotoxicity and reproductive dysfunction in broiler breeder hens (Chen *et al*., 2006)

2.1.9.2 Steatosis

Steatosis, also known as fatty liver is a syndrome, associated with the dietary lipid profile, in which there is an accumulation of fat in the liver of an animal. Depending on the prevalent PUFA's the disease can either be termed microsteatosis or macrosteatosis (Selzner & Clavien, 2001; El-Badry *et al.,* 2007). Macrosteatosis although seen as large droplets of fat in a non-alcoholic fatty liver, is more acute (Araya *et al*., 2004). It is correlated to a combination of (i) a high intake of linoleic acid, (ii) the inadequate intake of α-linolenic acid or (iii) the defective desaturation and elongation of fatty acids - all of these factors which affect the metabolism of α-linoleic acid into its derivatives (Hautekeete *et al*., 1990; Simopolous, 2003b). The reason being that long chain omega-3 PUFA's are known to up-regulate the peroxisomal proliferator activated receptor-α [PPAR-α] (Levy *et al*., 2004), which then again enhances liver fatty acid oxidation (Kersten *et al*., 1999). With a concomitant increase in the transcription of fatty acid degradation genes, such as peroxisomal acyl-CoA oxidase (ACO)

and mitochondrial carnitine palmitoyl transferase-1 [CPT-1] (Kersten *et al*., 1999; Levy *et al*., 2004). This has practically been proven by a n-3 dietary supplementation in mice (El-Badry *et al*., 2007) and rats (Kurihara *et al*., 1994), to achieve an identical ratio of n-6 : n-3 fatty acids with a lean liver, and a significant reduction in the incidence of intrahepatic lipid accumulation and the percentage of large fat droplets i.e macrosteatosis.

2.1.9.3 Fatty liver haemorrhagic syndrome in the laying hen

Fatty liver haemorrhagic syndrome (FLHS) is a steastosis known to be a prevalent metabolic condition in commercial laying hens, particularly in high energy consuming, caged layers. This disease causes the distension of the hepatocytes with fat vacuoles resulting in the rupture of the liver and eventual death of the bird (Julian, 2005). Factors linked to this disorder are diverse, such as e.g. environmental, genetic, dietary and/or hormonal disorders (Squires & Leeson, 1988; Diaz *et al*., 1994; Julian, 2005).

Although rapeseed oil (erucic acid), and conjugated linoleic acid (CLA) have been associated with FLHS in layers (Cherian & Goeger, 2004; Julian, 2005), available reports regarding α-linolenic acid are contradictory. Bean and Leeson (2003) reported that feeding a diet containing 10% flaxseed for 25 weeks resulted in an increased case of liver haemorrhage in laying hens. However, any pathologic effect following flaxseed being included in the diet, was not observed. From the findings of Cherian and Hayat (2009), it would seem as if the long term feeding of 10% flaxseed brought about a reduction in liver lipids, as well as the number and magnitude of steatosis in laying hens.

2.1.10 Role of diets in the reproduction of hens

The relationship between diet and reproduction is said to be complex and goes as far back as the maternal diet, pre-conception and conception, early life, puberty and the adult stages. The diet of the dam during conception generally has an influence on the utero environment and subsequent impact on homeostasis regulation during adult life. Poor foetal nutrition is suggested to be a factor that is responsible for the adaptive manifestation of survival mechanisms in the later stages of life, through programming of the metabolism in a set pattern (Gluckman & Hanson, 2004). Rebel *et al*. (2006) defined this programming as the process whereby a stimulus or stress at a critical or sensitive period of development has a lasting or lifelong effect. Regarding the impact on reproductive performance, fatty acid related diseases were found to be generated mainly by the overfeeding of the broiler breeder

hens. The accumulation of triacyglycerol (TAG) in the adipose tissue was reported (Chen *et al.,* 2006) to alter the hormonal signalling, which subsequently causes lipotoxicity. Furthermore, the supplementation of the laying hen diet with fish oil has been reported to increase the incidence of cracked eggs, induce a reduction in fertility, and a reduction in the hatchability of fertile eggs (Herstad *et al.,* 2000).

2.1.10.1 Maternal nutrition and chick development

Maternal nutrition oriented disorders have been linked to elevated blood pressure in the adult guinea pig and rats (Persson & Jansson, 1992; Lillycrop *et al*., 2005) and affects the blood osmolality, blood pressure and body fat in adult sheep (Ross & Desai, 2005). During embryonic development, egg yolk is transported in the form of cholesteryl oleate from the yolk sac to the chick's liver, during incubation (Noble & Moore, 1964). In the chicken, maternal diets have been reported to influence the gene expression by its reflection in the proliferation of the intestinal cells of the chicks on day 3 and 14 (Rebel *et al*., 2006). In fact, studies show that the fatty acid composition of the chicken embryo during incubation and the hatched chick thereafter largely reflects the egg yolk fatty acid profile (Cherian & Sim, 1991). Cherian *et al.* (2009) further reported that maternal diets which were comprised of different levels of n-3 fatty acids were able to modulate and even alter the ventricle fatty acid composition, prostaglandin and thromboxane production, during the first 2 weeks after the hatching of the chick, differently. The first 2 weeks of life of newly hatched chicks have been identified as being very crucial, as the chick at this stage relies on passive immunity (antibodies from the hen), before its own immune system becomes functional (Smith *et al*., 1994; Rose, 1997).

Interestingly, Wang *et al*. (2004) did not observe any significant effects in the serum and total egg yolk immunoglobulin G (IgG) of laying hens fed 5% fat, with varying levels of α-linolenic acid. However, a higher concentration of IgG was reported in the hatchlings of hens that consumed lower ratios of LA : ALA (between 0.8 and 5.4 : 1), compared to those fed a higher ratio (12.4 : 1). In addition to yolk lipid composition, the maternal diet is also known to affect the survivability of fertile egg embryos and their subsequent hatchability (Wilson, 1997). As such, the type and proportion of fatty acid in the diets of the hen is of utmost importance. Embryonic mortalities have been recorded by Tullet (1990), when the ratio of C18:0 to C18:1 (n-9) exceeded 0.3. It has previously been reported that dietary cyclopropene fatty acids (sterculic and malvalic acids) reduce the hatchability due to their tendency to

increase the ratio of the stearic (C18:0) to oleic (C18:1) acid in egg yolk (Donaldson & Fites, 1970). This adverse effect was also investigated by Aydin and Cook (2009), where the negative influence of conjugated linoleic acid on hatchability was reversed by the inclusion of linoleic or oleic acids at a rate of 30 g/kg in CLA containing diets of layers. Aydin and Cook (2009) concluded that the ratio of UFA to SFA, rather than the ratio of C18:0 to C18:1(n-9), as the predominant factor related to embryonic mortalities within the CLA treatment.

Supplementation of eggs (*in ovo*) with nutrients influences the intestinal villus length of young chicks at hatch. It is noteworthy that this concept of programming is not limited only to the diet. Employing a protocol of circadian incubation, eggs could be exposed to certain temperatures (imprinting), that may influence their growth and feed conversion rate after hatching (Hamminga, 2012).

2.1.10.2 Fatty acid content and the performance of sperm

The assessment of fertilization in poultry involves a series of processes which include the characteristics of sperm, their behaviour in the female reproductive tract as seen in transportation and storage in the oviduct, as well as the binding and penetration into the ovum (Donoghue, 1999). Christensen *et al.* (1998), Blesbois *et al.* (2004), Cerolini *et al.* (2005), Zaniboni *et al.* (2006) and Kanyinji and Maeda (2010) are some of the researchers that investigated cockerel fertility, via dietary means e.g. fatty acids, Vitamin E and calcium in the diet. Their hypotheses were based on the knowledge of biological pathways of spermatogenesis and the anatomy and physiology of the sperm cell.

The lipid content of chicken sperm has been quantified and its membrane found to be characteristically high in n-6 PUFA's. According to studies, docosatetraenoic acid (DTA) (C22:4n-6) is the most abundant PUFA, accounting for between 17 and 30% of the total sperm fatty acids. They are followed by arachidonic acid C20:4n-6 (11 to 13%), while n-3 derived fatty acid ranking low and constituting only between 1 and 5% of sperm phospholipids (Cerolini *et al.*, 1997). This relative abundance of PUFA's is however not consistent with other species. Generally, turkey sperm contains a lower concentration of PUFA's than that of the chicken, while also containing a characteristically high concentration of C22:3n-9 (docosatrienoic acid) long chain PUFA's (Surai *et al.*, 1998a, Surai & Sparks, 2001; Blesbois *et al.*, 2004). However, docosahexaenoic acid, a derivative of n-3 PUFA's has

been shown to be the most abundant PUFA in mammalian sperm (Neill & Masters, 1972; Poulos *et al*., 1973; Lin *et al*., 1993). Dietary lipids are suggested to influence the fertility by affecting the sperm membrane structure, fluidity or sperm susceptibility to peroxidation and by altering its constituent n-6: n-3 ratio (Blesbois *et al.*, 1997). This assertion has been corroborated by Cerolini *et al.* (2005; 2006), reporting a transference of dietary n-3 fatty acid into the sperm membrane of the cockerel. It was also observed that the increase in the amount of sperm n-3 PUFA caused a simultaneous decrease in the n-6 PUFA content, although the prevalence (17%) of DTA was not affected (Cerolini, 2005). Bongalhardo *et al.* (2009) further demonstrated that dietary lipids affect the head and body of sperm cell differently.

2.1.10.3 Sperm quality and fertility in the cockerel

In the chicken, the n-6 derived PUFA, DTA has been regarded as the most important PUFA within poultry sperm and it has been suggested that it be used as an indicator of sperm quality, when assessing the reproductive efficiency in cockerels. This statement, being based on the seeming positive relationship that exists between the proportion of DTA found in the sperm, motility and fertility (Cerolini, 2005). A positive effect of DHA on sperm motility of cockerels (Cerolini *et al*., 2006), and men (Conquer & Tekpetey, 2003) has been reported. However, the negative effect on the total sperm concentration and production volume of chicken sperm specifically should not be overlooked (Cerolini *et al.*, 2005; 2006).

In addition, oils that are used as sources of n-3 fatty acid in diets, are known to be susceptible to peroxidation (Ollero & Alvarez, 2003; Cerolini *et al.*, 2006). Hudson and Wilson (2003) compared the lasting effect of poultry fat and menhaden (fish) oil and reported a similar number of fertile eggs produced during the first week following insemination. However, sperm enriched with n-3 from the menhaden oil resulted in a significantly higher number of fertile and hatched eggs during the second week post insemination, compared to the poultry fat treatment. Although only a small increase in the sperm n-3 content has been reported, the supplementation of diets with lipids rich in α-linolenic acid resulted in enhanced fertility during peak egg production (≤ 39 weeks of age) (Kelso *et al.*, 1997). When evaluating the fertility of 44 to 47 week old male broiler breeders, Blesbois *et al.* (1999) reported an increase of 4.4%, when the experimental diets were supplemented with menhaden oil at week 30 of age. The results of both studies appear to suggest that the effect of n-3 on sperm quality is transient and may be age dependent.

2.2 The characteristics of the Hy-Line ® variety Silver Brown layers

The layer strain to be used in the current experiment is the Hy-Line® Silver Brown layer. It is a prolific layer and popular chicken breed among commercial producers in South Africa. The production performance goals of Hy-Line® layers are summarized in Table 2.7 (Hy-Line Guide, 2008). These values were compiled from extensive commercial flock records gathered from all parts of the world. However, these performance figures are not a guarantee of the flock performance, as the productivity of a commercial flock of any layer strain will vary according to environmental and disease control scenarios (Hy-Line Guide, 2008).

Table 2.7 Production performance characteristics of Hy-Line Silver Brown hens (Hy-Line Guide, 2008)

Laying period (17 to 80 weeks of age)	
Percentage peak egg production	94-96%
Hen-day eggs:	
To 60 weeks of age	254
To 74 weeks of age	333
To 80 weeks of age	363
Liveability to 80 Weeks	95%
Days to 50% Production (From Hatch)	145 days
Egg weight at 32 weeks of age	59.5 g/Egg
Egg weight at 70 weeks of age	63.4 g/Egg
Total egg output:	
18 - 74 weeks of age	20.2 kg
18 - 80 weeks of age	22.1 kg
Body weight at 70 weeks of age	2.2 kg
Shell Colour	Uniform, Dark Brown
Shell Strength	Excellent
Haugh Units at 70 weeks of age	76
Average daily feed consumption (18-80 weeks of age)	116 grams/bird/day
FCR (kg / kg basis at 21-74 weeks of age)	2.19
Feather Colour	White with Brown tint
Temperament	Very calm, adapts well to any management

2.3 Chicken eggs

An egg is defined as a biological container in which the organic and inorganic materials required for the propagation of the species are contained. The packaging consists of the shell, various amino acids, lipids, minerals and vitamins that are required by the embryo, as it grows from a zygote to a chick. These are believed to be stored in the yolk and albumen (Etches, 1996).

2.3.1 The process of egg formation

Oviposition is the term used in describing the laying of an egg. Different organs of the body of the hen perform specific functions before oviposition can occur. The major components of the reproductive system involved in the formation and laying of an egg are the (i) ovary and (ii) oviduct. Organs such as the brain, liver and the skeletal system play a less prominent role (Robinson, 2002). The effective communication of these organs is coordinated by hormones and enhanced by lighting, which essentially ensures the efficient development of the hatching egg.

The brain as such, houses two important endocrine glands, namely the hypothalamus and the pituitary. The hypothalamus being the receptor of external light energy during photostimulation, is generally regarded as the 'main switch' stimulating the onset of egg production. The anterior pituitary (adenohypophysis) also located in the brain, secretes luteinizing hormone (LH) and follicle stimulating hormone (FSH) after being stimulated by the luteinizing hormone releasing hormone (LHRH) of the hypothalamus. Both LH and FSH are transported to the ovary within the hen, to stimulate the steroid hormones to be released from the follicular cells, where progesterone is specifically released from the largest follicle on the ovary (Robinson, 2002). The liver is the organ that produces lipoproteins and contributes to the yolk part of the egg. This synthesis is regulated by oestrogen, which if not properly controlled, may lead to the excessive production and storage of lipids by the liver, resulting in the disease known as fatty liver haemorrhaging syndrome (FLHS). This occurrence may also accompany the double yolk formation in broiler breeders, as a result of excessive (even *ad libitum*) feeding conditions. The presence of two or more yolks in close proximity in the oviduct generally disrupts egg and shell formation. Hocking *et al.* (1987) suggested a restricted feeding program as a means of controlling the hierarchy of the developing follicle in order to achieve enhanced egg production.

The skeletal system is important, especially to the laying hen (layers and breeders), as it functions as a storage facility and homeostatic balance of calcium for eggshell production and the maintenance of bone integrity and acceptable eggshell quality (Robinson, 2002). During the formation of an egg in the oviduct, the shell is usually formed at night, making it the most sensitive period for calcium mobilization. There is however a tendency of hens to increase their feed intake toward the end of the day in an attempt to provide adequate calcium during the night (Sauveur & Mongin, 1974).

Only the left oviduct is present and functional in most hens and egg formation starts with the deposition of the ovum, approximately 15 min after ovulation, followed by fertilization (if sperm are present), then the secretion of albumen, shell membrane, eggshell, and cuticle around the ovum. Four sections of the oviduct are essentially involved in the process of egg formation. These include the infundibulum, the magnum, the isthmus, and the shell gland or also known as the uterus (Etches, 1996). The infundibulum engulfs the ovulated ovum and creates an environment for sperm penetration of the ovum i.e. for fertilization to occur. It is comprised of a narrow chalaziferous region, where the chalaza is formed and which also serves as a sperm storage site. The egg at this early stage of formation spends up to 15 min in the infundibulum before moving on to the next section. The magnum is the section of the oviduct where the majority of albumen (egg protein) is formed. It is muscular in structure, creamy white in colour and represents the longest part of the oviduct. The egg traverses across this section in about 3 h (Hafez & Hafez, 2000). The isthmus is the next section following the magnum. It is smaller and longitudinal unlike the magnum, although it has ridged mucosa, similar to that of the magnum. This section of the oviduct is separated from the magnum by a narrow translucent region, devoid of any tubular glands. Both the outer shell membrane and the inner shell membrane are secreted as interwoven fibres during the 1½ h period that the egg stays in the isthmus (Etches, 1996). The last set of activities during egg formation in the oviduct occurs in the shell gland. The egg spends 18 to 22h in the shell gland (uterus), where it absorbs approximately 15 g of water, and exchanges numerous electrolytes such as Na^+, K^+, and Cl^- with the shell gland fluid. Ionized calcium (Ca^{2+}) is transferred from the blood for precipitation on the surface of the eggshell, in a process known as calcification. The quantity of water containing electrolytes absorbed by the egg however decreases with an increase in the rate of shell calcification (Taylor, 2003). The two major functions of the shell in relation to reproduction include the provision of calcium for the

developing embryo and structural medium for exchange of respiratory gases, while maintaining an appropriate water balance during incubation (Tullet, 1990).

2.3.2 Oviposition and laying clutches

Oviposition is a term used in describing the laying of an egg by the hen. The hen lays her eggs in sequence. Sequences refer to consequent days of laying that are separated by a pause period of 40 to 44 h (or more) in duration (Robinson, 2002). These ovulations may occur at varying intervals, from 2 eggs to as many as 360 eggs. Thus the ovulatory cycle of the hen is generally described in terms of the interval between successive ovulations (Etches, 1996). The hen continues to lay eggs, irrespective of mating with a cockerel. An egg becomes fertile, only when there are viable sperm cells present in her oviduct, either through natural mating or artificial insemination. Unlike in mammals with two functional ovaries, the hen has only one functional ovary, with the right ovary being degenerated.

2.3.3 Factors affecting egg size

There are differences in the size of avian eggs across species, as well as within breeds of the same species. The combination of eggshell conductance and the length of the incubation period are major factors contributing to the evolutional variation that occurs between species. So for instance, some hummingbirds may lay an egg of half a gram, while the egg of the ostrich can weigh as much as 1 kg. In the chicken, the weight of the fertile eggs range between 40 g and 75 g. A number of factors have then been associated with these differences - with the genetic potential and food availability playing prominent roles (Mortola & Awam, 2010). Other contributing factors to the variation in egg size within the same clutch include: (i) maternal size and age, (ii) seasonal variation and (iii) photoperiod programme. Although egg weight is positively correlated to the body weight of hens, which in turn produces larger offspring, (Batt & Prince, 1979; Ipek & Dikem, 2007) embryos of extremely large eggs are often susceptible to excess water retention, while the smaller eggs risk dehydration. Mortola and Al Awam (2010) reported that egg size has no major effect on incubation time and that only a slight variation may occur in eggshell conductance. By prolonging the time interval it was concluded that water balance was the main evolutionary determinant of maximal range in egg size.

Dietary manipulation has also been employed by many researchers to increase the weight of the chicken eggs. Increasing the nutritional levels of protein and fat and the amino acids

methionine and lysine resulted in increased egg weights. Basically, dietary manipulation of proteins and amino acids has a direct effect on the albumen part of the egg. Dietary fat (linoleic acid) also influences the yolk formation. However, although it appears that unanimous consensus has been reached on the positive effect of protein on egg weight, the same cannot be said regarding supplemental fat (energy). While Harms *et al*. (2000) and Wu *et al*. (2005) reported a higher egg weight, Leeson (2005) and Gunawardana *et al.* (2008) on the other hand, did not record any significant difference with an increased energy intake.

Yolk colour was also strictly influenced by the dietary fat (Gunawardana *et al*., 2008), as nutrients are known to be deposited in the egg through specific synthetic and transport processes. The composition of the egg does not tend to be subjected to significant changes, except where nutrients are in short supply in the body or affected by the hen's diet. Such nutrients generally include vitamins (e.g. riboflavin, biotin), proteins, and lipids (Tullet, 1990).

2.3.4 The egg as a protein source to humans

One important aspect of the chicken egg is in its use as a nutrient source to humans. The egg has been suggested and used as a functional food aimed at improving the nutritional status in the humans, wherever and whenever a deficiency is perceived. Over the past two decades the chicken egg in particular, has received considerable attention as the carrier of essential nutrients (Miles, 1998). Apart from the essential fatty acids mentioned (which have been reviewed in another section), other desirable nutrients have also been successfully incorporated into the egg. So for example the trace element selenium, a known anti-oxidant, which also enhances the immune system and inhibits the progression of HIV to AIDS among other benefits, has been incorporated into the egg (Surai & Dvorsky, 2001). Other reported nutrients include conjugated linoleic acid (CLA) (Cherian *et al.*, 2007), Vitamin E and even iodine (Rottger *et al*., 2008). Both conjugated linoleic acid (CLA) and Vitamin E have been reported to be effective anti-carcinogenic, hypocholesterolemic, anti-atherogenic and immunomodulatory agents (Traber, 1999; Belury, 2002).

2.4 The anatomy and physiology of poultry sperm

The sperm of the avian species have some unique features and characteristics that make it different from that of mammalian sperm. In contrast to the spindle shape of the sperm head of rams and bulls, the sperm head of the cockerels is cylindrical, and almost the same width

(approximately 0.5 µm in diameter) as the tail (Thurston & Hess, 1987). The length of the sperm tail is however considered to be excessive in relation to its head. The tail length being between 90 and 100 µm, is approximately eight times the length of the head, contrary to the much lower ratio which exists between the head and the tail of e.g. bull sperm (Salisbury & VanDemark, 1961). These anatomical features have been a major deterrent in the practice of artificial insemination in avian species, especially where the cryopreservation of semen is concerned.

Generally, the factor attributed to the problem of AI in poultry, is the inability of its semen to withstand long term storage, as is evident in the reduced fertility obtained in eggs, post AI. This has been linked to the structural and biochemical damage suffered by the sperm cells during the freezing process (Donoghue & Wishart, 2000). Lipids constitute a biochemical component of the sperm cell with an extended contribution to its metabolism and functionality. Thus many researchers have employed diverse methods, including dietary manipulation (Kanyinji & Maeda, 2010), as well as various semen extenders (Siudzinska, & Łukaszewicz, 2008) to enhance the long term storage survivability of chicken semen, with little success. In a study by Blesbois *et al*. (2008) although unexpected, the fertility of frozen-thawed semen was recorded to be higher than for fresh semen, following artificial insemination of the respective hens. It was however pointed out that a higher insemination dose and a greater frequency of AI favoured the frozen-thawed semen, compared to the fresh semen.

As a side note, it can be stated that although chicken sperm and that of turkey share similarities in terms of structure, the major difference lies in the *in vitro* oxygen required. While chicken sperm could retain its functions in both an anaerobic and aerobic environment, the sperm of the turkey requires high levels of oxygen, limiting the survivability of turkey sperm cells in an anaerobic environment (Sexton, 1974; Wishart, 1981). In Table 2.8 the differences in concentration (mM) of chicken and turkey seminal plasma when compared with blood plasma, is set out.

Table 2.8 The concentration (mM) of the major blood and seminal plasma components collected from the chicken and turkey (Lake & Wishart, 1984).

Component	Seminal plasma of chicken	Seminal plasma of turkeys	Blood plasma
Glucose	0.18	-	12
Cl^-	46	23	121
Na^+	145	140	160
K^+	13	20	6
Ca^{2+}	1.4	0.3	6
Glutamate	75	88	0.2
Lactate	3.7	2.4	5.5
Pyruvate	0.3	0.4	0.4
α-ketoglutarate	0.4	0.2	0.1
Carnitine	3.2	1.7	0.2
Acetyl carnitine	0.5 – 2.0	0.5 – 2.0	0.1
Protein (gl^{-1})			

2.5 Spermatogenesis

Senger (2005) described spermatogenesis as a process involving sequential mitotic and meiotic divisions and setting out the differentiation of spermatids into highly specialized spermatozoa. In simple terms, spermatogenesis involves the process by which sperm cells are produced in the seminiferous tubules of the testes of the male animal, and it consists of two phases, namely spermatocytogenesis and spermiogenesis (Gordon, 2005). Before spermatogenesis can occur however, there must be coordination of the responsible endocrine glands. So for example, the gonadotrophin releasing hormone (GnRH), secreted by the hypothalamus acts on the anterior pituitary gland, inducing the secretion of the follicle stimulating hormone (FSH) and the luteinizing hormone (LH). LH specifically acts on the Leydig cells which are dispersed between the spaces of the seminiferous tubules of the testis, for the secretion of the androgens - the most prominent one being testosterone (Etches, 1996).

The role of a spermatozoon is basically defined for the delivery of the male genetic material to the oocyte during fertilization (Senger, 2005). FSH controls the function of the Sertoli cells, which are meant to provide the micro-environment in which differentiation takes place and act as nursing cells for the developing sperm cell. The complete process of spermatogenesis varies in duration between animal species, but generally takes 17 days for completion to fully differentiated spermatozoa in adult cockerels (Etches, 1996). The flow chart in terms of the production of spermatozoa (Senger, 2005) is illustrated in Figure 2.3.

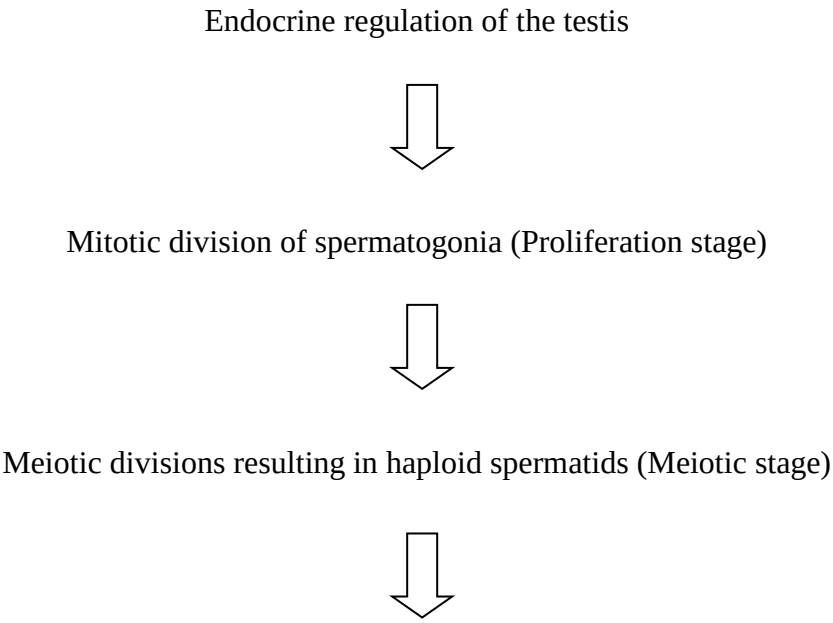


Figure 2.3 Production of spermatozoa (Senger, 2005)

2.6 Indicators of fertility in the cockerel

There are no strict evaluations for breeding soundness in the poultry industry. In a natural mating flock, the system commonly adopted by poultry producers involves modifying the male to female ratio, based on a number of physical attributes of the male. Generally, prior to onset of breeding (at 17 weeks of age), cockerels are selected according to comb and wattle size, colour, body size and shank length. Wilson *et al*. (1979) indicated however that these physical characteristics are not accurate enough in predicting cockerel fertility. Since then, many researchers (Donoghue, 1999; Parker & McDaniel, 2002) have advocated the need for a more scientific, detailed and sustainable method of the evaluation of the cockerel's fertility potential, before introduction into the breeding flock. The reports of these researchers emphasized the need for the evaluation of sperm as a more reliable selection method for cockerels intended for breeding in a commercial flock.

2.6.1 Semen quality assessment in the cockerel

Basically, the parameters usually assessed in the evaluation of semen are generally divided into macro and microscopic characteristics. As the name implies, macro analysis involves the visual appraisal of semen colour and the estimation of the ejaculate volume, while micro

analysis of the sperm include the evaluation of motility, viability, morphology and concentration.

2.6.1.1 Semen colour

The assessment of the colour of cockerel semen is the fastest and most convenient of all the parameters to record. As soon as semen is collected, visual appraisal can be conducted by the technician by means of inspection through the transparent semen collection tube. Generally, semen should appear pearly white, where a deviation from this occurs i.e. yellowish and white deposits, it suggests faecal contamination (Etches, 1996). Semen could also be densely opaque or watery, depending on its level of seminal dilution. The colour thus indicating high density or declining sperm numbers in the ejaculate (Sexton, 1980; Peters *et al.*, 2008). Occasionally, reddish brown semen may be encountered, indicating the presence of red blood cells. For the purpose of artificial insemination, it is best to work with pearly white semen, in order to maximize fertility (Etches, 1996).

2.6.1.2 Semen volume

The volume of the ejaculate is an important consideration, as it not only reflects the individual's reproductive potential, but is also used in calculating the insemination dose used in an AI protocol. Total sperm output (sperm production) of respective males, is generally the product of semen volume and sperm concentration. Different cockerels produce varying volumes of semen at different times (Anderson, 2001). A variation in volume within different species of poultry is known to exist, with a range of 0.1 – 0.9 ml, with a mean value of 0.35 ml - containing an approximate concentration of 5.7×10^9 ml^{-1} sperm for broiler breeders (Lake & Stewart, 1978). Peters *et al*. (2008) reported an ejaculate range of between 0.37 ml and 0.73 ml for strains of chicken reared under tropical, humid environments.

Factors that affect the ejaculate volume of cockerels then include species, breed, nutrition, age, frequency of ejaculation, and the technique of semen collection. Physical characteristics such as a large comb size have also been positively correlated to testicular size ($r = 0.69$, $P < 0.001$) (Tyler & Gous, 2008) and is associated with higher levels of androgens, increased mating activity and higher semen production (Nwachukwu *et al*., 2006; Galal, 2007).

The quantity and quality of feed available to the cockerel may affect its ejaculate volume, and the fertilizing capacity of the sperm. Cockerels that are underweight as a result of being

underfed produce lower volumes of semen (Renema *et al.*, 2007). Overweight cockerels on the other hand, also do not contribute positively to flock fertility. Thus, cockerels are usually placed on a specific feeding regime, in order to achieve a specific target body weight.

2.6.1.3 Sperm concentration

A determination of the total sperm output of an individual male is obtained as the product of the volume of the ejaculate and sperm concentration. Sperm concentration then referring to the number of sperm cells contained in an ejaculate. The number is either obtained through direct counting under the microscope, using diluted semen and a calibrated chamber of known volume e.g. hemocytometer or by a more advanced, quicker and precise automated method e.g. using the spectrophotometer (Etches, 1996). The sperm concentration of semen samples is ultimately important for the calculation of insemination doses available in an AI protocol. The number of inseminations (N) is thus calculated accordingly:

$$N = \frac{\text{Volume (ml) x concentration (cell ml}^{-1}\text{)}}{\text{100 million cells per insemination}}$$

The relationship between semen volume, concentration, and number of possible inseminations in different types of poultry has been well documented (Lake & Stewart, 1978). The sensitivity of the AI dose has been acknowledged in the research of Hudson and Wilson (2003), where the low fertility recorded between males fed dietary treatments was ascribed to the marginal dose of inseminated sperm utilized. Even though semen concentration is not regarded as being highly correlated to fertility, ejaculates containing less than 500 million sperm cells have been associated with low fertility rates (Bearden *et al*., 2004). Semen volume, sperm concentration and the number of inseminations per ejaculate for certain species of poultry are summarized in Table 2.9 (Lake & Stewart, 1978).

Table 2.9 The volume of semen, the concentration of sperm and the number of inseminations per ejaculate for species or types of poultry (Lake & Stewart, 1978)

Species or type	Volume (ml)		Concentration (10^9 cells ml^{-1})		Number of inseminations/ ejaculate ($100x10^6$)
	Mean	Range	Mean	Range	
Broiler breeder	0.35	0.1-0.9	5.7	3.0-8.0	20
Light weight egg layer	0.15	0.15-0.3	5.0	5.0-7.5	7.5
Medium weight egg layer	0.2	0.08-0.5	5.0	3.5-6.0	10
Light weight turkey	0.15	0.08-0.3	9.0	8.0-14.0	13.5
Heavy weight turkey	0.2	0.1-0.33	9.5	9.0-13.5	19
Guinea fowl	0.075	0.05-0.15	6.0	4.0-8.0	4.5
Pekin duck	0.23	0.1-1.0	4.0	0.02-6.0	9.2
Muscovy duck	1.1	1.0-1.5	1.8	-	20

2.6.1.4 Sperm motility

Sperm motility is the most common and at the same time, most subjective microscopic evaluation of semen quality. Motility can either be assessed as a whole, or individually. Mass sperm motility is usually conducted by diluting semen with the extender in a ratio of 1:3 or 1:4 and then grading the swirling mass of cells under the microscope, either on a 1 to 5 or 1 to 10 point scale (Etches, 1996). Blesbois *et al*. (2008) adopted a more concise scale of a 1 to 8 point scale, for the cryopreservation of chicken semen at a cryobank in France. Here it was used in predicting the fertilising potential of both fresh and frozen-thawed semen. Microscopic individual motility can also be conducted with a more diluted 1 : 100 semen sample. Cockerel sperm are generally most motile at their normal body temperature of ± 41 °C, but motility does decline with time after ejaculation (Froman & Feltman, 2010). Thus ejaculated semen intended for analyses are usually kept in a heated water bath at this temperature (± 41 °C). As stated earlier, sperm motility assessment under the light microscope is very subjective, and requires an operator with some degree of training. Thus a number of timely and objective methods have been developed through innovative technologies (Hafez & Hafez, 2000).

After two decades, the Computer Assisted Sperm Analysis (CASA) system has come in handy for a quick, objective and more comprehensive analysis of sperm motility. Other than motility, it can be used in evaluating sperm characteristics such as velocity and morphology (Verstegen *et al*., 2002). Certain motility parameters analysed with the aid of CASA in trials

involving chicken sperm, include the percentage of motile cells, average path velocity, progressive velocity, lateral head displacement, straightness, and linearity (Bongalhardo *et al.,* 2009).

2.6.1.5 Sperm morphology

Sperm structure analyses or sperm abnormalities are commonly conducted in the laboratory with the aid of eosin-nigrosin stain (Lake & Stewart, 1978; Bjorndahl *et al.*, 2003). As previously mentioned the spermatozoon of poultry is cylindrical, tapered at both ends and consists of an acrosome, a head, a midpiece and a tail (Etches, 1996). All these parts of the sperm cell are then prone to specific defects or abnormalities. Scientists are mostly concerned with the type and sum of abnormalities found within the live sperm cells. Such values have been used as indicators of the fertility in animals. Following staining, unlike the undamaged live sperm that appear white, sperm that are dead or injured as a result of compromised plasma membrane integrity tend to absorb the eosin stain, thereby appearing pinkish in colour under the microscope (Bakst & Cecil, 1997). Generally, live sperm are categorised as either live normal or live abnormal, with the abnormalities further being classified accordingly to abnormalities e.g. crooked acrosome, bulb head, broken neck, mid-piece swelling and tail looping or the tail being absent (Edens *et al.,* 1973; Lukaszewicz *et al*., 2008). Good quality semen is accepted as having up to 90% live and defect-free sperm cells (Bakst & Cecil, 1997). Lukaszewicz *et al*. (2008) further reported that different procedures for semen stain preparations described by other researchers also to have a significant effect on the sperm abnormality occurrence.

2.6.1.6 Advanced cockerel fertility tests

Other, but more technically advanced and objective protocols for semen evaluation include the following (i) sperm mobility test (SMT), (ii) sperm quality index (SQI) and (iii) sperm penetration into the perivitelline layer (IPVL) of the yolk. The SMT and SQI tests are assays involving simple mathematical calculations of two or more parameters evaluated in the sperm micro-analysis. The penetration of IPVL on the other hand constitutes a fertilization efficiency test conducted on a freshly laid egg, where both sperm from the cock and the egg from the hen are involved.

The sperm mobility test (SMT) is a sophisticated and objective assay that is currently being used in poultry. This technique was developed by Froman and McLean (1996) for the

analysis and evaluation of cockerel semen and evolved as a function of the product of the primary parameters of sperm quality i.e. the motile sperm concentration and more specifically the proportion of motile sperm moving in a straight line (velocity >30 m/s). Sperm are generally subjected to conditions similar to those encountered in the hen's reproductive tract, by swimming through a dense, inert Accudenz© solution (Froman *et al*., 2003). The Accudenz© solution is generally used in the laboratory together with the densimeter or the spectrophotometer, to evaluate cockerel (Froman & McLean, 1996) and turkey (Holsberger *et al.*, 1998; King *et al.*, 2000) semen. The mean sperm mobility index within a flock being first determined, and then semen samples collected from other cockerels in this flock ranked on a percentage basis, according to their standard deviation from the index (King *et al.*, 2000). Hudson and Wilson, (2003) employed this SMT method to evaluate semen quality, where the beneficial effect of dietary menhaden oil on the fertility of old broiler breeder males was evaluated.

The sperm quality index (SQI) is another less laborious and time consuming method by which the fertility of cockerel semen can be determined. The indexes generated by the OptiBreed sperm quality analyzer has then been correlated with sperm motility, concentration and viability of the cockerel (McDaniel *et al*., 1998). A photocell in the analyzer then has the capacity to monitor the frequency of light disruption caused by movement of sperm cells. The SQI method was successfully utilized by Parker and McDaniel (2002) to classify cockerels regarding fertility during an industrial trial.

One method, in which sperm function is evaluated *in vivo*, is the determination of sperm penetration into the inner perivitelline layer (IPVL) of the yolk in a freshly laid egg. The assay is generally conducted by fixing and staining the intact IPVL section with Schiff's reagent, as described by Bramwell *et al.* (1995). Unlike mammals where not more than a single sperm can penetrate the zona pellucida, multiple spermatozoa have been found to penetrate the germinal disc during fertilization in avian species. The sperm utilizes its acrosomal enzymes to digest holes into the protein fibres of the IPVL, which can easily be observed under the microscope (Bakst & Howarth, 1977). One evident advantage of using this non-invasive fertility technique, is that it is not only a reliable male fertility test, but also gives an insight into the reproductive performance of the hen. The age effect on fertility of turkey hens was also conducted by Fairchild and Christensen (2005) using this IPVL method.

2.6.2 Semen quality and hatchability of chicks

The quality of sperm has an effect on embryo survival during incubation and the eventual hatching of the chick. The consensus is that the detection of infertile eggs and occurrence of early deaths in embryos are major signs of male infertility from the donor flock (Parker & McDaniel, 2002; Wilson, 2004). The break-out analysis of eggs following candling between incubation day 7 and day 10 is generally motivated by the quest to detect any flock fertility problem emanating from poor semen quality. Further, to justify the need to conduct a thorough sperm quality evaluation on the male chicken prior to breeding, Parker and McDaniel (2002) divided 1800 twenty-two week old cockerels into 2 groups. Here, interestingly, the flock whose group of cockerels were selected, based on the top 80% SQI score at 26 weeks of age, recorded an up to 64% higher hatchability rate (in a 22 hatches pool), compared to a flock where cockerels were never subjected to a sperm analysis.

2.7 Flock fertility

The frequency and number of hatching chicks produced from incubated eggs have been used as criteria for the reproductive performance of a flock. This breeder flock problem is one of the main factors affecting hatchability, manifesting in a low percentage of chicks produced from the eggs set. Out of all the possible causes of infertile eggs highlighted by Wilson (2004), the one that may occur in an AI system is defective sperm in males and females producing abnormal eggs (germinal discs). There is however no practical approach of correcting hen abnormalities, hence the focus is directed at the cockerel. The cockerel's reproductive performance has a major impact on the reproductive efficiency of poultry operations (Bakst *et al*., 1994; Adenokun & Sonaiya, 2001), as well as that of toms in commercial turkey flocks (Donoghue, 1999). In a natural mating flock, the two main factors responsible for poor flock fertility are mainly (i) mating activity and (ii) sperm quality. As such, a method called spiking is commonly employed in commercial flocks, whenever there is a decline in fertility - usually between 40 and 45 weeks of age (Casanovas, 2000). This method involves the introduction of younger males (25 to 28 weeks of age) into a flock to re-ignite the interest of the older males (40 to 45 weeks of age) within the flock, regarding mating activities. However due to concerns regarding biosecurity, Casanovas (2000) also suggested a system of intra-spiking, where no males are imported into the farm. All males of the same age (40 to 45 weeks) are only exchanged between pens, and houses on the same chicken farm. This was reported to also yield higher fertility (3.4%) and hatchability (4.7%) rates, compared to spiking.

2.8 Artificial insemination in poultry

The first successful AI in birds was recorded in chickens more than a century ago, when Ivanov was able to produce fertile eggs from semen obtained from the *ductus deferens* of the cockerel (Burrows & Quinn, 1937). The current technique for AI in birds was first described by Burrows and Quinn (1937) and it has now been exclusively adopted as the means of reproduction, in especially commercial turkey production. Thus the flock fertility problem emanating from the tom's inability to mate effectively has been curtailed, as over 300 million turkey poults are being produced annually in the USA alone. The large sizes of normal commercial breeder flocks have been the major constraint in the implementation of AI in chickens. However, the continuous selection for growth, resulting in the increase in body weight and skeletal frame of cockerels, may render AI a viable option in the production of hatching eggs in future (Reddy, 1995). Among bird species, the artificial insemination technique of reproduction is not limited to poultry alone, but it is also being used as a tool in the conservation of endangered wild birds.

2.8.1 The abdominal massage technique for semen collection

The abdominal massage technique as first described by Burrows and Quinn (1937), is then the most extensively used method for the training and collection of semen from cockerels. Two technicians are generally required for this protocol, one technician being responsible for holding the wings and gently stroking the abdominal region toward the tail. The other technician then quickly pushes the tail forward with one hand, using the thumb and forefinger of the same hand to 'milk' the semen from the external papilla of the *ductus deferens*. The semen is generally collected in a calibrated tube in order to ascertain the ejaculate volume of a particular cockerel. Care is also taken to avoid faecal contamination of semen during the collection process. Cockerels are generally easily trained at puberty (from 17 weeks of age), and it takes approximately 30 seconds for ejaculation to occur, once the cockerel gets accustomed to the massage technique. In order to maximize semen quantity and quality it is important to avoid contamination coming from collecting equipment, blood and faeces (Burrows & Quinn, 1937).

The artificial vagina (AV) has been reported (Kasai & Izumo, 2001) as an alternative method of semen collection. This is normally said to be a more effective technique with ducks, in

terms of the ease of semen collection, semen quantity and quality, when compared to the abdominal massage technique. However, this assertion has yet to be proven in the chicken.

2.8.2 Sperm deposition in the vagina

Artificial insemination of hens as described by Burrows and Quinn (1937), involves the application of pressure to the hen's abdomen and the venting of the vaginal orifice, through the cloaca. This procedure is being referred to as everting, cracking or venting. Following venting, semen from the cockerel is deposited about 2 to 4 cm into the vaginal orifice, simultaneously with the release of pressure on the hen's abdomen. Artificial insemination procedures are preferably performed in the afternoon, when the oviduct of the hen is expected to be free of any hard shelled-eggs. The day of AI is usually recorded as Day 0 and fertilized eggs can be collected the following day.

2.8.3 The advantages of chicken AI

The application of AI in breeder flocks tends to offer a number of advantages. A placement ratio of 8 to 10 males per 100 females is generally maintained for good flock fertility. Aggression and injuries often occur when the ratio is higher. On the other hand, a lower ratio may result in a decline of mating frequency, hence poor flock fertility. Artificial insemination can help compensate for keeping a low male : female ratio, where only males whose semen have been evaluated are kept for breeding purposes (Donoghue, 1999).

The common practice of selective mating in a chicken flock can also be avoided if AI is incorporated in a breeder flock system. Also, battery cages can be used especially in situations where individual animal need to be identified. The ease of crossbreeding, and the use of older or injured males, which would have been culled in a natural flock are some of the other advantages AI tends to offer.

2.9 Behaviour of sperm in the sperm storage tubule

It is possible for a hen to continue laying fertile eggs up to 3 weeks after the deposition of semen in her vagina. The genital tract of the hen possesses unique crypt structures known as sperm nests, or sperm glands around the infundibulum and the uterovaginal junction.

The hen semen storage tubules (SST) are also located between the vagina and shell gland of the oviduct (Froman & Feltman, 2010). The testes of the cockerel being located inside its

body is probably one of the reasons why cockerel sperm remain motile at 41 °C and viable for such a long time in the hen's oviduct. Sperm are reported to be released from the SST episodically, with the assistance of smooth muscle contractions and/or ciliary activities, before accumulating in the mucosal folds of the infundibulum (Hafez & Hafez, 2000). It has also been suggested due to the reversible suppression of respiration and motility of the sperm. In addition to stabilization of the plasma membrane and maintenance of the acrosome, aid in the prolonged life of the sperm in the SST (Tabatabaei *et al.*, 2009).

2.10 Hatchability

The problem of low or poor hatchability is a global phenomenon. Surveys conducted on hatchability trends between 1985 and 2005 in the USA revealed that while advances in nutrition, genetic selection and management of turkey- and broiler flocks have been made, the same cannot be said of hatchability. Records showed that hatchability generally ranged between 79 to 82% for broiler eggs and 76 to 80% for turkey eggs over this period (Schaal & Cherian, 2007). Researchers also discovered an estimated US $500 million economic loss to be recorded in the year of 2005, due to unimproved hatching technologies.

2.10.1 Factors affecting the hatchability of eggs

A number of factors have been associated with the hatchability of poultry eggs. These hatchability problems are traced to the hatchery, egg handling, or the breeder flock. Incubation conditions, where deviations can occur, include amongst others temperature, humidity, egg orientation, turning frequency, and ventilation (Wilson, 2004). Kirk *et al.* (1980) whose research has been extensively referenced performed a comprehensive study on the interactive effects of these hatchability problem sources, using eggs from broiler breeders of the age between 26 and 60 weeks. The findings included the following:

- Egg collection from the nest at an hourly interval, rather than every 5 hours post oviposition, slightly reduced ($P < 0.10$) hatchability.
- There was an interactive effect of days (d) of egg storage and incubation temperature. Eggs stored for 2 days, hatched better when kept at 18 °C than those stored at 15 °C ($P < 0.05$). To the contrary, eggs after 8 days of storage hatched better when incubated at 15 °C, compared to those that were stored for the same number of days at 18 °C. This suggests a lower temperature for eggs stored for long periods gave better hatchability.
- It was also suggested that eggs from younger birds should preferably be stored, rather than eggs from an older flock.

- The relative humidity (RH) inside the incubator has a direct effect on the egg weight loss. It has been suggested that RH in the setter be decreased with an increase in flock age. The egg weight loss was observed to decrease with an increase in egg size, as the age of the flock advanced. Reduced hatchability was more pronounced in larger eggs (from older flocks) than small eggs (from the younger flock), when the RH was increased from 53 to 70%.
- Fumigation before egg storage did not result in an improved hatchability, even with eggs stored for 8 days.
- Physical conditions of the breeder stock are important considerations in hatchability. Loss of appetite and decreased egg production experienced in a flock between the age of 36 to 40 weeks, resulted in reduced hatchability.
- The age of the flock plays a major role in hatchability, as was evidenced in the lower hatchability recorded when a flock was 44 weeks of age and the egg weight was 65 g on average. It was generally stated that eggs of a moderate size hatched better, when compared to eggs from a very young flock (small body weight), as well as the eggs from very old flocks (heavy body weight).

2.10.2 Storage of eggs following AI

The handling of hatching eggs may be critical to the viability of the embryo, as the pre-incubational eggs exert an effect on the incubated eggs *via* the storage time, environmental conditions, hen-age and strain. Studies regarding the short-term storage effects on the hatching of eggs yielded contradictory reports. There is however agreement on the need to avoid the long term storage of eggs from an old flock (Kirk *et al*., 1980; Lapao *et al*., 1999). This accelerates the rate of deterioration of the already poor albumen quality. It was further reported by Lapao *et al.* (1999) that the decline in albumen height manifesting in eggs from old hens, caused a decline in hatchability, although the albumen pH was fairly constant for all flock ages after a 4 day period of egg storage.

2.10.3 Nutrition and egg hatchability

Hatchability problems emanating from breeders are usually not detected in time. It could take up to 4 weeks before such problems are traced to the flock, considering the 3 weeks of incubation and approximately 1 week of egg storage. Problems originating from the breeder flock are generally inter-linked and may be triggered by the environment, disease or nutrition. Nutritional deficiency and its severity in a chicken flock constitute nutritional factors from

which embryonic abnormalities may occur. The occurrence of infertile eggs following candling on embryonic day 7 of incubation is apparently the easiest way of detecting problems in the breeder flock. Eggs judged to be infertile appear clear at candling, and the broken-out analysis will show a small white-dot germinal disc, with no blood veins (Wilson, 2004).

2.11 Developmental stages of the chicken embryo

The development of poultry embryos has unique characteristics. Unlike mammals, the ovum is fertilized not by a single, but many spermatozoa. Second, the yolk does not cleave as successive cell divisions following fertilization. The third distinctive feature relates to the three phases involved in embryonic development. The initial phase is inside the oviduct, the second stage occurs when egg is laid and cools down to ambient temperature (embryonic diapause). The last phase takes place inside the incubator, during which time the embryonic development is re-activated (Etches, 1996). Large differences have however been found to exist between embryonic development of broiler and layer chickens. The broiler embryos seem to grow, accumulate protein and utilize the yolk faster than their layer counterparts. This appears to reflect the marked differences occurring during the later stages of life between these two chicken types, in terms of body weight, feed intake, protein degradation and the basal metabolic rate (Hocking *et al*., 1989).

CHAPTER 3
MATERIALS AND METHODS

3.1 Location and time-outlay of the study

This study was conducted at the poultry research facility on the Paradys experimental farm of the University of the Free State, Bloemfontein, South Africa. Guidelines regarding the animal care and welfare were followed as stipulated, and approved by the Animal Ethics Committee of the University of the Free State (Experiment No. 19/2011). The entire experimental period was 46 weeks (from 32 to 78 weeks of age), while the different experimental phases conducted during this project are summarised in Table 3.1.

Table 3.1 Experimental phases followed during this project

Phases	Period		Duration	Age of birds
	Onset	End	(weeks)	(weeks)
Egg production trial	6th Sept. 2011	24th July 2012	46	32 – 78
Egg quality characteristics	14th May 2012	21st May 2012	1	68
Semen evaluation (Trial 1)	15th Sept. 2011	15th Sept. 2011	12	35 - 46
Flock fertility	18th May 2012	10th August 2012	9	69 – 77
Artificial insemination	18th May 2012	13th July 2012	9	69 – 77
Egg collection & incubation	2nd June 2012	19th July 2012	7	71 - 77

As highlighted in Table 3.1, the feeding of the dietary treatments of both cockerels and hens started on the 6th of September, 2011. This date also marked the commencement of the egg production trial when the hens were 33 weeks of age. The other trials in this project involving the cockerels e.g. semen and flock fertility evaluations were conducted concurrently with the egg production trial.

3.2 Experimental birds

Seventeen week old Hy-Line Silver-Brown pullets (n=130 ♀) and cockerels (n=70 ♂) were purchased from a commercial pullet producer in the Parys area of the Free State Province, South Africa. On arrival, birds were individually housed in cages (1600 cm^2). The placement was done in such a way that visual contact between the cockerels and the hens was continuous. This was achieved by the alternate arrangement of the two sexes (Plate 3.1) in rows. A standard layer grower mash was fed from arrival of birds (week 17 of age), until the onset of the experimental dietary treatments (week 32 of age). During this time period, water and feed was provided *ad libitum* to all birds. At onset of week 32, all birds were allocated to

their respective dietary treatments, according to the corresponding colour coding on each bird's cage, used for identification purposes.

Plate 3.1 Experimental hen-house outlay

Prior to the allotment of experimental diets to the cockerels (n=70), all cockerels were regularly subjected to abdominal massage to train them for semen ejaculation, according to the technique first described by Burrows and Quinn (1937). Hence, each cockerel was massaged every second day, starting at 22 weeks of age. The 20 least sensitive cockerels with the lowest frequency of ejaculation were removed from the experiment just before the introduction of experimental diets at week 32 of age and did not partake in the semen evaluation trial, at 35 weeks of age. Thus ultimately, only 50 cockerels were chosen to partake in the study at week 32, based on their ease of response to the massage technique and their frequency of ejaculation. Thereafter the cockerels were randomly allotted to one of the five dietary treatments (n= 10 /treatment) for the duration of the entire project. Each bird was leg-tagged, according to the specific number on their respective cages. A period of three weeks was allowed following allocation of birds to the different dietary treatments, before the commencement of semen analyses, thereby making provision for dietary adaptation and allows the completion of spermatogenesis of approximately 17 days (Etches, 1996). Throughout the duration of Trial 1, the cockerels were abdominally massaged by the same two technicians twice weekly from 9:30. Only the semen sample from the second (2^{nd}) massage session was collected and used for analysis (described in section 3.6) in the

laboratory, while semen collected at the first (1st) massage session was discarded. Similarly, in order to allot the 125 hens required for the project, the five inferior hens were removed from the original purchased number of 130 hens. These five hens had been recorded to lay almost 100% cracked eggs on a weekly basis and were thus removed at week 32 of age, coinciding with the introduction of dietary treatments. The remaining 125 hens were then randomly allocated to each of the five dietary treatments (n=25/treatment) and received their respective experimental diets throughout the trial period (32 to 78 weeks of age). A one week feed adaptation period was allowed prior to the commencement of the hen production trial at 33 weeks of age.

3.3 General management of the experimental facility

3.3.1 Washing and disinfecting

Three weeks before the arrival of the chickens on the experimental farm, pre-arrival sanitation and precautionary activities were performed. The poultry house was physically cleaned by means of a "dry" and "wet water" cleaning procedure, with emphasis on the wooden slatted floors, battery cages and the removal of dust and dirt from the walls and roof. A chemical cleansing of the aforementioned infrastructure was undertaken after the initial "wet cleaning". When the house and equipment were deemed to be clean and dry, HYGEN© Q96 (active ingredient; Dimethylammonium chloride 91 g/l and Akyl Dimethyl Benzylammonium chloride 59 g/l) a disinfectant against micro-organisms and poultry diseases (such as Infectious Bursal disease and Newcastle disease), was sprayed. HYGEN© A30, an acid de-scalar (active ingredients; nitric- and phosphoric acid), was also diluted in water at a rate of 10 ml/l water and flushed through the nipple drinking system in all cages. All the feed buckets to be used in this project were thoroughly washed, rinsed and dried before being disinfected with HYGEN© Q96. All washing chemicals and disinfectants were supplied by a registered chemical company, (Kem-Klean Hygiene Systems, Cape Town, South Africa).

3.3.2 Housing of the birds

The poultry house was an elevated structure and all the birds (hens n = 125 and cockerels n = 50) were housed individually in metabolic battery cages of 1600 cm^2 / cage. The poultry house consisted of eight rows, with 25 individual bird cages within each of the eight rows (8 x 25). Each of these cages was fitted with an individual feeder tray and a nipple water drinker. All cages were also equipped with a wooden perch that enable birds to "exercise" as

well as feed and drink effortlessly. Every cage compartment was also numbered serially from 1 to 200, with a colour sticker corresponding to the assigned colour of the dietary treatment. Artificial lighting was adequately provided in the poultry house by means of fluorescent lights and regulated with a timer according to the breed requirements for a given reproductive developmental phase. On arrival of the birds (17 weeks of age), a 14 hour "light" and 10 hour "dark" (14L:10D) photoperiod was implemented. This photoperiod was gradually increased by 30 minutes/week, until a photoperiod schedule of 16L:8D was reached, at 26 week of age (Hy-Line guide, 2008). Sixteen hours of light (16L) was then maintained throughout the experimental period (32 to 78 weeks of age).

3.3.3 Routine management activities

Certain routine activities were performed regarding the care of experimental birds. Before the onset of the daily routine work, the indoor house temperature was recorded, using five minimum and maximum thermometers hung at strategic locations within the poultry house. These five individual minimum and maximum temperature readings were then used to calculate the mean daily minimum and maximum ambient temperature. Every morning at approximately 7:00, each bird cage was cleaned using a round hand brush and any possible excreta contamination carefully removed from remnants in the feeder. Next, the birds were individually fed. Depending on the specific day of the week (every second day), excreta was scraped from slatted wooden floor below the cages to ensure an easier "fall through" to the lower section of the building where the excreta accumulated. Eggs were removed daily from the cages at 9:30, marked for identification purposes, packed into pre-numbered plastic trays which were assigned to each experimental treatment. After recording of egg production, all eggs were also individually weighed for the calculation of other production performance parameters.

Plate 3.2 Experimental cockerels housed individually in metabolic cages

Plate 3.3 Individually housed experimental hen in a metabolic cage

3.4 Experimental diets

The five experimental diets (Table 3.3 and Table 3.4) were formulated using different supplementary lipid sources at a constant inclusion level (30 g/kg), to increase the dietary concentration of specific omega-type fatty acids. The number of double bonds (i.e. none, one, two or three) on the carbon atom were used as a reference in defining the dietary lipid saturation. Thus, an increase in the number of double bonds was associated with a higher level of fatty acid unsaturation and *vice versa*. Furthermore, the unsaturated fatty acids were grouped into three families, based on their oleic- (n-9), linoleic- (n-6) and α-linolenic acid (n-3) precursors. Thereby referring to the position of the double bonds nearest to the omega carbon atom, as illustrated by McDonald *et al*. (2011). The control diet was formulated using a blend (50 : 50) of linseed- and fish oil to increase the dietary levels of shorter chain omega-3 (n-3) fatty acids, such as α-linolenic acid. In the second experimental diet (fish oil treatment), refined, deodorised fish oil was used to increase the dietary concentration of longer chain n-3 fatty acids, such as eicosapentaenoic- (EPA), docosapentaenoic- (DPA) and docosahexaenoic acid (DHA). Sunflower oil was used to increase the dietary omega-6 (n-6) fatty acids such as linoleic acid, in the third treatment, while high oleic acid (HO) sunflower oil was used to increase the omega-9 (n-9) fatty acids (such as oleic acid), in the fourth treatment. Lastly, tallow was used as a supplementary lipid source in the saturated diet (SFA treatment) to increase the levels of the highly saturated fatty acids such as palmitic and stearic acid, and to lower the concentration of polyunsaturated fatty acids of the n-3 and n-6 type. These definite omega-type fatty acids were not exclusive to a specific diet, but the high concentration thereof within a respective diet was used in defining the treatment. Additionally, the total concentration (%) of mono-unsaturated fatty acids (MUFA's) and polyunsaturated fatty acids (PUFA's) were combined and defined as total unsaturated fatty acids (UFA's). The total dietary concentration of unsaturated and saturated fatty acids was further used as an indication of the specific dietary saturation, irrespective of the omega-type fatty acids. The control n-3 diet was higher in short chain (C18:3c9) n-3 fatty acids, compared to the n-3 treatment, where pure fish oil was used. This served as a good indicator regarding the effects of fatty acid chain length (irrespective of saturation), on dietary lipid oxidation and the consequent effects on the production and reproduction performance of the cockerels.

The objective was thus to formulate five *isoenergetic* and *isonitrogenous* diets, with a constant inclusion level (30 g/kg) of the various supplementary lipid sources, differing only

in their fatty acid profile. To ensure the formulation of *isoenergetic* diets, the apparent metabolizable energy (AME) content of each dietary lipid source was predetermined. The AME value of the respective lipid sources were calculated based on the free fatty acid (FFA) and the unsaturated to saturated fatty acid ratio (U/S), using the following prediction equation as described by Wiseman (1990).

$$AME\ (kcal/kg) = 239[A + (B \times FFA) + (C \times e^{(D \times U/S)})]$$

Where: FFA is the free fatty acids in g/kg
U/S is the unsaturated to saturated ratio

Constants to be used for birds older than 21 days of age were as follows:
A = 39.025
B = -0.006
C = -8.505
D = -0.403

The fatty acid methyl esters (FAME) of the experimental dietary lipid sources were determined by extracting the fat content thereof, using the method described by Folch *et al*. (1957). Extraction of the total lipid content of the respective dietary lipid sources was quantitatively performed using chloroform and methanol, in a ratio of 2:1. Butylatedhydroxytoluene was added at a concentration of 0.001% to the chloroform : methanol mixture, as an antioxidant. The extracts were dried under vacuum in a rotary evaporator and further dried overnight in a vacuum oven at 50^{o}C, with phosphorus pentoxide as a moisture absorbent. The total extractable fat content was determined by weighing and expressed as the percentage (%) fat (w/w) per 100 g lipid source.

Approximately 10 mg of the total lipids extracted during the Folch extraction process was transferred into a Teflon-lined screw-top test tube, by means of a disposable glass Pasteur pipette. Fatty acid methyl esters were prepared for gas chromatography by methylation of the extracted fat, using methanol–BF_3 (Slover & Lanza, 1979; Diaz *et al*., 2005). The fatty acid methyl esters were then quantified using a Varian GX 3400 flame ionization GC, with a fused silica capillary column - Chrompack CPSIL 88 (100 m length, 0.25 mm ID, 0.2 μm film

thickness). The column temperature was 40^{o} – 230°C (held for 2 minutes; 4°C/minute; held for 10 minutes). Fatty acid methyl esters in hexane (1 μL) were injected into the column, using a Varian 8200 CX auto sampler, with a split ratio of 100:1. The injection port and detector were both maintained at 250°C. Hydrogen, at 45 psi, functioned as the carrier gas, while nitrogen was employed as the make-up gas. The Varian Star Chromatography Software (VSCS) recorded the chromatogram. Fatty acid methyl ester samples were identified by comparing the relative retention times of the FAME peaks, from samples with those of standards obtained from SIGMA (189-19). Fatty acids were expressed as the relative percentage of individual fatty acids, as a percentage of the total fatty acids present in the sample. The following fatty acid combinations and ratios were calculated using the fatty acid data: (i) total SFA's, (ii) total MUFA's, (iii) total PUFA's, (iv) total UFA's, (v) total n-6 fatty acids, (vi) total n-3 fatty acids, (vii) ratio of PUFA's to SFA's, (viii) ratio of MUFA's to SFA's, (ix) ratio of UFA's to SFA's and (x) omega-6 to omega-3 (n-6 : n-3) ratio. Free fatty acid concentration of the lipid sources was determined according to the method number 940.28 for refined oils (AOAC, 2000). The results of these analyses (dietary fatty acid methyl esters) were comprehensively discussed in section 4.3.1 of Chapter 4.

Data of the FFA, U/S ratio, as well as the predicted AME values for the five lipid sources used during the experimental period are set out in Table 3.2. These predicted AME values were then used to formulate the five *isoenergetic* (12.6 MJ/kg DM) and *isonitrogenous* (170 g CP/kg DM) experimental diets as illustrated in Table 3.3 and Table 3.4, by means of the Format feed formulation program.

Table 3.2 Mean free fatty acid (g/kg), unsaturated to saturated ratio (g/g) and calculated apparent metabolisable energy (MJ/kg DM) of the different lipid sources used in the experimental diets

	Control[1]	Fish oil	Sunflower oil	HO[2] sunflower oil	Tallow
FFA[3]	0.15	0.39	0.16	0.49	1.78
U/S[4]	4.6	2.1	7.9	9.5	0.4
AME[5]	37.32	35.11	38.45	38.62	32.08

1 A blend of 50% fish- and 50% linseed oil
2 High oleic acid sunflower oil
3 Free fatty acids in g/kg
4 Unsaturated to saturated ratio as g/g
5 Apparent metabolisable energy in MJ/kg DM (Wiseman, 1990)

Due to a total dietary fat content of approximately 62 g fat/kg DM, as well as the duration of the experimental period (46 weeks), three batches of feed were mixed - in an attempt to limit the oxidation of the feed lipids and its subsequent effects on the animal production performances. The fat content of each batch of feed was extracted (Folch *et al*., 1957) and analysed (AOAC, 2000) for peroxide value (PV) (AOAC no. 965.33) and FFA concentration (AOAC no. 940.28), in an attempt to determine the lipid oxidative quality of the feed. In a further attempt to delay the rate of lipid oxidation within the respective diets, the premix used during the study was specially formulated with considerably higher vitamin E levels (100 000 IU/ton feed), as well as the addition of 150 g Sel-Plex™/ton feed as supplementary anti-oxidant.

Table 3.3 Mean physical composition (%) of experimental diets fed to cockerels and hens from 32 to 78 weeks of age (as is basis)

	Control	Fish oil (n-3)	Sunflower oil (n-6)	#HO Sunflower oil (n-9)	Tallow (SFA)
Yellow maize	528.27	535.31	524.33	524.33	550.25
Prime gluten (60%)	10.00	15.33	10.00	10.00	10.00
Wheat bran	80.00	75.00	85.00	85.00	54.00
Soybean oilcake (48%)	125.33	118.33	124.33	124.33	129.33
Sunflower oilcake (38%)	100.00	99.33	100.00	100.00	100.00
Limestone – coarse[1]	20.00	20.00	20.00	20.00	20.00
Limestone – fine[2]	89.33	89.33	89.33	89.33	89.00
Monocalcium phosphate	10.29	10.37	10.21	10.21	10.67
Salt	3.41	3.43	3.43	3.43	3.41
Linseed oil	15.00	0.00	0.00	0.00	0.00
Fish oil	15.00	30.00	0.00	0.00	0.00
Sunflower oil	0.00	0.00	30.00	0.00	0.00
HO[3] sunflower oil	0.00	0.00	0.00	30.00	0.00
Tallow	0.00	0.00	0.00	0.00	30.00
Choline powder[4]	0.15	0.19	0.15	0.15	0.17
L-Lysine	0.12	0.32	0.13	0.13	0.10
DL-Methionine[5]	0.09	0.06	0.09	0.09	0.08
Premix[6]	3.00	3.00	3.00	3.00	3.00

High oleic acid sunflower oil.
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 High oleic acid sunflower oil.
4 Minimum of 60% choline chloride.
5 DL-methionine powder, with purity of 99%.
6 Commercial mineral, vitamin and phytase enzyme premix containing 100 000 IU Vit. E/ton and 150 g Sel-Plex™/ton feed.

After calculating the predicted AME of the respective supplementary lipid sources (Table 3.2), the experimental diets (Table 3.3 and Table 3.4) were formulated according to the nutritional recommendations of a commercial feed supplier for laying hens, based on the mean egg production period. Subsequently, the specific nutrient composition for particular dietary ingredients, generated over a prolonged period of time was used. During diet formulation, the inclusion of full-fat soya, as well as poultry by-product meal was avoided, while the other feed ingredients such as sunflower oilcake (10%) were kept constant across treatments. This was done as a preventative measurement to ensure that the contribution of the fatty acids from specific feed ingredients did not interfere with those supplied by the respective supplementary lipid sources.

Table 3.4 Mean calculated chemical composition (g/kg DM) of diets fed to cockerels and hens during the experimental period (32 – 78 weeks of age)

	Control	Fish oil (n-3)	Sunflower oil (n-6)	#HO Sunflower oil (n-9)	Tallow (SFA)
AME[1] (MJ/kg DM)	12.38	12.38	12.39	12.39	12.39
Crude protein	170.61	170.50	170.53	170.53	170.63
Fat	62.64	62.70	62.66	62.66	62.64
Ash	160.72	160.22	160.85	160.85	159.80
Crude fibre	47.99	47.26	48.42	48.42	45.79
NDF[2]	130.39	128.39	132.05	132.05	121.90
ADF[3]	58.51	57.75	59.03	59.03	55.84
Calcium	40.12	40.10	40.11	40.11	40.08
Phosphorus	6.54	6.49	6.56	6.56	6.45
Magnesium	2.74	2.71	2.76	2.76	2.67
Potassium	6.56	6.36	6.58	6.58	6.43
Sodium	1.65	1.65	1.65	1.65	1.65
Chloride	2.79	2.85	2.80	2.80	2.78
AvP[4]	3.36	3.36	3.36	3.36	3.36
Ca:AvP[5]	131.53	131.47	131.55	131.55	131.33
Arginine	10.45	10.28	10.45	10.45	10.44
Isoleucine	6.31	6.29	6.30	6.30	6.35
Lysine	6.87	6.86	6.86	6.86	6.88
Methionine	3.09	3.09	3.09	3.09	3.09
Threonine	5.39	5.37	5.38	5.38	5.41
Tryptophan	1.75	1.71	1.75	1.75	1.74

[#] High oleic acids sunflower oil
[1] Apparent metabolisable energy in MJ AME/kg DM.
[2] Neutral detergent fibre.
[3] Acid detergent fibre.
[4] Available phosphorus.
[5] Ratio of calcium to available phosphorus.

3.4.1 Mixing of experimental diets

The preparation of the experimental diets by means of blending the respective lipid sources with the basal diet was done on the experimental farm. In an attempt to limit the negative effects of dietary lipid oxidation, only 300 kg feed per treatment was prepared at a time (single batch); ensuring that the feed would last for a maximum period of approximately 12-14 weeks, before it was necessary to mix the next batch of feed. For each treatment, 6 x 50 kg bags of the formulated basal diets from a commercial feed manufacturer were accurately weighed out (to the nearest 5 g), on an electronic scale. The total weight was then reduced, such that only 291 kg of the basal feed was left. Thereafter, 9 kg (3%) of the experimental oil was accurately weighed and blended into the adjusted basal feed and mixed thoroughly for 30 minutes, using an paddle-type feed mixer (Plate 3.4). After mixing to ensure thorough blending of the lipid source with the basal diet, the feed was re-bagged and labelled according to the specific dietary treatment. The tallow treatment was always the last experimental diet to be prepared, due to its unique characteristic of being solid at room temperature and the time needed to "melt" the fat into a mixable liquid form. Thus, before the commencement of activities, the tallow intended for mixing would be weighed (10% extra) and placed in a separate 20 l plastic bucket before heating in a "warm water bath". This was constructed using a plastic 220 l plastic container equipped with an electric element, generally used for heating water in a geyser. The water in the larger plastic container was kept at boiling point to ensure that the tallow inside the 20 l bucket would melt and be in a liquid form at mixing, when it needed to be blended with the basal diet. Tallow was therefore never exposed to an open flame, but melted by means of this warm water technique.

Plate 3.4 Blending of supplementary lipid source with the basal diet using a paddle-type feed mixer

3.4.2 Nutrition

The respective dietary treatments were allocated to the individual birds within the treatment by filling each bird's feed bucket and subsequently recording the combined 'feed and bucket' weight. To ensure *ad libitum* feed provision to birds, the feed allocation was approximately 120 g per hen and 110 g per cockerel per day, respectively - fed daily from their individual feed buckets. Subsequently, at the end of each week (7-day period), the remaining feed in the bucket was weighed, while the feed remnants were also scraped from the feeder trays of individual birds, weighed and recorded as 'feed residue'. The individual weekly feed consumption i.e. weekly feed intake (WFI) was thus calculated according to the following equation:

WFI = FBI – FB – Res

Where: WFI = weekly feed intake (g/bird)

FBI = Feed + bucket of the previous week (beginning) (g)

FB = Feed + bucket of the current week (end) (g)

Res = Feed residue of the current week (g)

3.5 Record keeping

Data of all the eggs produced during the respective collection weeks were individually recorded and summarized before being pooled for statistical analysis.

3.5.1 Egg production

Individual egg weights (g) were recorded by accurately weighing each egg to the nearest 0.01 gram using a Mettler PL 3000 scale, immediately after egg collection. The individual weighing of eggs and the visual inspection for intact eggshells was conducted concurrently. Hen-day egg production (%) was calculated according to Ahmad and Balander (2003), whereby the number of eggs produced was divided by the number of live birds in each treatment group. So for example, an egg was collected, visually appraised as sellable, cracked, or shell-less and then weighed. The number of sellable; cracked and shell-less eggs produced per week within each treatment was recorded on an individual bird basis. The combination of the aforementioned parameters was then used in the calculation of egg deformities (%). At the end of each week, the number of eggs produced by an individual hen, as well as the average weight of the eggs was calculated from the daily records. Hen-day production (%) was compiled on a weekly basis and thus expressed as weekly egg production (%) per individual hen.

Average egg weight = $\frac{\text{Total egg weight/week}}{\text{Number of eggs laid/week}}$

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

3.5.2 Egg output

Weekly egg output (g) was calculated for each hen according to Rose (1997), where the number of eggs produced was multiplied by the mean egg weight of the specific hen e.a.

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

3.5.3 Feed conversion ratio

The feed conversion ratio (FCR) of individual hens was calculated by dividing the weekly mean feed intake (g) by its corresponding mean egg output (g) and expressed as g/g (Rose, 1997).

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

3.5.4 Body weight

All birds (hens & cockerels) were weighed accurately to the nearest gram at the commencement of the trial (32 weeks of age) and at four week intervals thereafter, until the age of 76 weeks.

3.5.5 Egg component characteristics

At 68 weeks of age, before onset of the fertility study and artificial insemination (AI) of the hens, all eggs collected were used for the determination and calculation of certain internal egg quality characteristics such as: yolk weight (g), albumen weight (g), eggshell weight (g), yolk ratio (%), albumen ratio (%) and the percentage eggshell (%). Individual eggs were broken and the yolk and albumen separated using an egg yolk separator (Plate 3.5). After carefully removal of all adhering albumen and chalazae, the eggshell and yolks were weighed separately on a digital scale to the nearest 0.0001g (Plate 3.6). 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*., 1999b).

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

The albumen and yolk ratios were calculated as a percentage of the component weight (g), compared to the egg weight (g), using the formula as described by Kul and Seker (2004).

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

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

Plate 3.5 Separation of the egg yolk and albumen

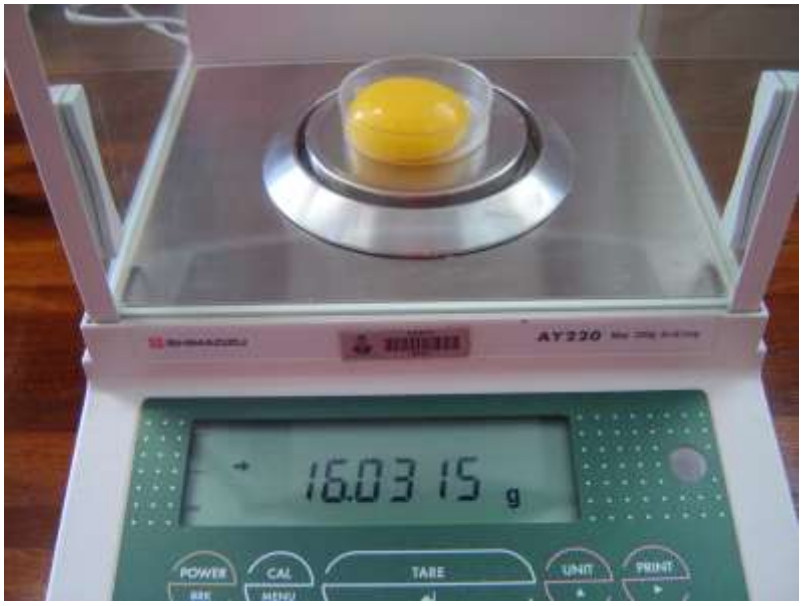


Plate 3.6 Weighing of the egg yolk

3.5.6 Analysis of yolk fatty acid methyl esters

Following the completion of the artificial insemination (AI) period (week 77), a total of 60 eggs (n=60) representing 12 eggs/treatment were analysed for the determination of fatty acid composition and proportion, as implemented for the diets. The same methods used for determination of FAME in the lipid sources and feed samples (discussed in section 3.4) were used for the determination of yolk FAME. Fatty acids identified were expressed as the relative percentage of individual fatty acids and as a percentage of the total fatty acids present in the sample (% FAME). Total extractable fat content was determined by weighing and expressed as the % fat (w/w) per 100 g egg yolk. The fat free dry matter (FFDM) content was determined by weighing the residue on a pre-weighed filter paper, used for fat extraction (Folch *et al*., 1957), after drying it according to procedure number 934.01 of the AOAC (AOAC, 2000). By determining the difference in weight, the FFDM was then expressed as % FFDM (w/w) per 100 g yolk. The moisture content of the yolk was determined by means of subtraction (100 - %lipid - %FFDM) and expressed as the % moisture (w/w) per 100 g yolk.

By using the concentration of individual fatty acids, the following fatty acid combination and ratios for the egg yolk were calculated, namely: (i) total SFA's, (ii) total MUFA's, (iii) total PUFA's, (iv) total unsaturated fatty acids (UFA's), (v) total n-6 fatty acids, (vi) total n-3 fatty acids, (vii) ratio of PUFA's to SFA's (PUFA/SFA), (viii) ratio of MUFA's to SFA's (MUFA/SFA), (ix) ratio of UFA's to SFA's (UFA/SFA) and the (ix) omega-6 to omega-3 (n-6/n-3) ratio. Furthermore, levels of n-9, n-6 and n-3 fatty acids were respectively expressed as mg n-9, mg n-6 or mg n-3 per g egg (shell included), by considering the egg weight and the % lipid within the egg yolk.

3.6 Semen collection and sperm analyses

All chemicals used during the experiment of semen evaluation (35 – 46 weeks of age) were manufactured by Sigma®. Semen collection and evaluation was performed twice per week (Thursdays and Fridays) during the 12 week semen evaluation trial period, as indicated in Table 3.1. With 25 cockerels (5 per treatment) being evaluated on Thursday, and the remaining 25 evaluated on Friday (individual bird evaluated once per week). The data collected from individual cockerels (total of 50 cockerels) on these two days, were pooled to represent the semen data for that particular week.

Semen collection and evaluation commenced at 9:30 on the day of analysis. Before the onset of semen evaluation, all equipment and laboratory tables were cleaned using a 10% alcohol solution, while the microscope slides and the eosin-nigrosin stain were pre-heated on a warm stage (40°C). A warm-water (42 °C) flask was used for semen incubation in the poultry house prior to microscopic evaluation of semen in the laboratory.

Semen samples were collected serially from five cockerels per batch before microscopic evaluation in such a manner that all five dietary treatments were represented within each batch. Each cock was dorso-abdominally massaged by gently stroking the back of the cockerel down its tail, while the second operator held a test tube (Plate 3.7), to collect the ejaculated semen (Burrows & Quinn, 1937). The ejaculate collected in a 15 ml calibrated tube (with lowest calibration of 0.5 ml), was then immediately placed into the vacuum flask with warm water (42°C). Semen with any visual faecal contamination was discarded before semen evaluation was done, while cockerels that failed to ejaculate were also recorded. The collected semen samples from a particular batch (five birds/batch) were removed from the poultry house and taken to the laboratory on the experimental farm for semen analyses. After the completion of the analyses for the first batch of five semen samples, the procedure was repeated until all 25 cockerels had been massaged on that specific day. A 35 minute time-frame was set between semen collection and the analysis of two successive batches, thereby ensuring that all five batches (25 cockerels) of semen analyses were completed before 12:30.

Plate 3.7 Semen collection using the abdominal massage technique

3.6.1 Macroscopic semen evaluation

Macroscopic semen analyses were conducted immediately on a batch of collected semen samples (n=5), following arrival at the farm laboratory – a 2 minute walk from the poultry house, where the birds were housed. The semen colour of the individual samples was visually appraised and recorded; whereafter the ejaculate volume was recorded directly from the graduated semen collection tube.

3.6.2 Microscopic semen evaluation

After the macroscopic evaluation of the semen samples was completed, 10 µl semen aliquots were taken for the microscopic sperm evaluation - starting with (i) sperm motility (%), (ii) sperm viability (%) and (iii) sperm concentration (ml). The total sperm output of each semen sample was calculated by multiplying the semen volume and sperm concentration (Hafez & Hafez, 2000). All glass microscope slides, test tubes, chemicals, and semen extenders to be used for semen analysis were kept warm (30°C), on a warm stage. The room temperature was regulated (30°C), in order to keep the semen evaluation environment constant.

Table 3.5 Composition of the Beltsville Poultry Semen Extender (Sexton, 1977)

Component	Level g/l	Primary function
Dipotassium phosphate	12.70	Buffer
Sodium glutamite	8.61	Chelator
Fructose substrate	5.00	Metabolic
Sodium acetate	4.30	Osmotic balance
Tes	1.95	Buffer
Potassium citrate	0.64	Osmotic balance
Monopotassium phosphate	0.65	Buffer

3.6.2.1 Sperm motility

After the initial macroscopic evaluation of individual semen samples (n=5/batch), microscopic sperm motility analyses followed immediately. The aliquot of 10 µl semen was sampled and mixed with 100 µl Beltsville Poultry Semen Extender BPSE (at a dilution rate of 1:10) in a clean 5 ml test tube. The BPSE was formulated in the laboratory according to the composition (Table 3.5), as specified by Sexton (1977). After thorough mixing, 10 µl of the

semen x BPSE mixture was gently pipetted onto a preheated slide, mounted under a light microscope (x40 magnification). A modified system of Blesbois *et al*. (2008) was used in grading the intensity of sperm movement and the degree of sperm agglutination on a scale of 0 to 8. At the same time, 100 sperm cells were counted with the aid of a cell counter and classified as the percentage of motile or non-motile sperm.

3.6.2.2 Sperm morphology assessment

The preparations of slides for both sperm viability and morphology were identical to that used for sperm motility, as previously described in paragraph 3.6.2.1. Again 10 µl of semen was sampled and mixed with a 100 µl eosin-nigrosin stain. Thereafter, 10 µl of the mixture was aspirated with the aid of a pipette and placed on a microscope slide, before making a smear. The slide was then left to dry and arranged in the slide rack for later evaluation. All sperm evaluations were performed by the same technician. After the completion of sperm motility evaluation of all the semen samples, the morphology slides were prepared and arranged on a slide rack to be evaluated. The evaluation was performed by placing a drop of immersion oil on the slide, followed by critical assessment of the sperm cells under the microscope (x1000 magnification) (Plate 3.8). Both sperm viability (percentage dead/live) and sperm morphology were determined and classified simultaneously by counting 200 sperm cells in different microscopic fields per slide, using a cell counter. The sperm viability analyses classified the sperm cells as either dead or alive. Live sperm cells appeared white, while the dead sperm appeared pinkish in colour, when viewed under the microscope. The colouration of the dead sperm cells was the result of the eiosin-nigrosin stain that penetrated through the plasma membrane (Bakst & Cecil, 1997).

Sperm morphology analyses were only conducted on the sperm cells judged to be alive. Sperm morphology was classified according to the classification system of Siudzinska and Lukaszewicz (2008), with certain modifications as either (i) normal or (ii) abnormal. The abnormal group were further divided into abnormalities of (ia) swollen heads, (ib) bent necks, (ic) defective mid-pieces, and (id) other abnormalities (i.e. looped heads, double heads, spermatids, etc.). Ultimately, the percentage live sperm for each semen sample was expressed, as a percentage of the 200 sperm cells, evaluated per slide. Sperm morphology on the other hand was expressed as a percentage of the total live sperm according to the following formulae (Lukaszewicz *et al.*, 2008):

Live sperm (%) = $\frac{\text{Number of live sperm}}{200} \times 100$

Sperm abnormalities (%) = $\frac{\text{Number of sperm abnormality}}{\text{Number of live sperm}} \times 100$

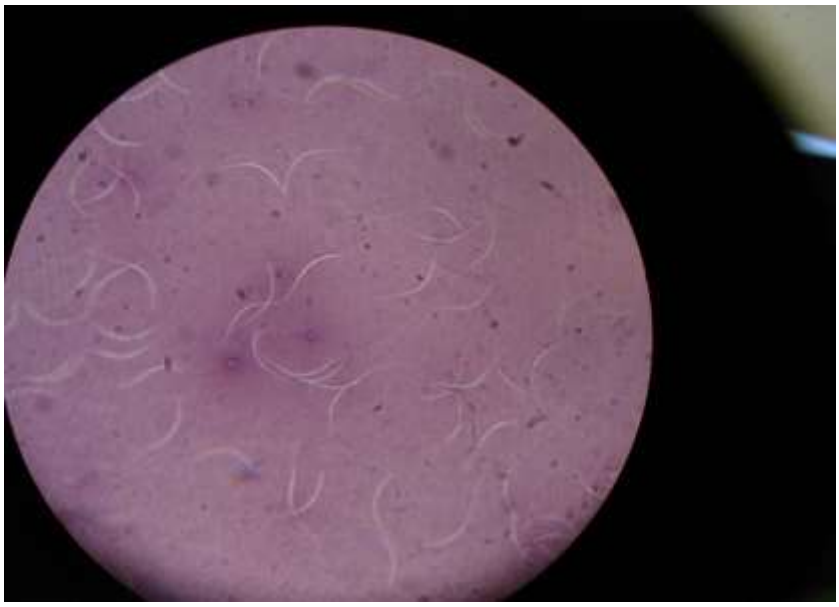


Plate 3.8 Microscopic view (x1000 magnification) of cockerel sperm

3.6.2.3 Sperm concentration determinations

As poultry semen is known regarding its high sperm concentration, the ejaculate was initially diluted with distilled water in a 1:100 ratio for evaluation (Anderson, 2001). In every semen batch evaluation, 10 µl of a semen sample was mixed with 990 µl distilled water (final dilution rate 1:100) in a 5 ml test-tube and placed in a test tube rack. After the completion of the evaluation of all sperm parameters for the day, all diluted semen intended for sperm concentration analyses were placed in the refrigerator at 4 ºC (to kill the sperm) and stored, until the following day when direct sperm counting was conducted by means of a haemocytometer. The cold storage was also done to prevent agglutination or disintegration of sperm cells, which could make counting difficult or even impossible.

Stored semen samples were removed from the refrigerator, whereafter 10 µl of each sample was pipetted into a clean Neubauer haemocytometer. All sperm cells in the five diagonal boxes of the haemocytometer were counted under a light microscope (x400 magnification).

To ultimately determine the sperm concentration of each semen sample, all sperm counted within these 5 fields were added and multiplied by a standard factor as well as the semen dilution factor, the product of which was expressed as 10^9 sperm/ml (Hafez & Hafez, 2000). The following equations were used for the calculation of the specific parameters:

Sperm concentration = Sperm count x multiplication factor x dilution factor

Sperm concentration = Haemocytometer count x 50 000 x 100

Total sperm output ($x10^9$ sperm/ejaculate) = Semen volume x sperm concentration

3.7 Artificial insemination (AI)

This phase of the project focused on flock fertility (combined effect of cockerels and hens) and was conducted between May and August, 2012 (Table 3.1). At the onset of the fertility study, all birds were 69 weeks of age, while artificial insemination of hens and egg incubation lasted for a 9 week period, until the age of 77 weeks. A two-week period was allowed before eggs intended for incubation (at week 71 of age) were collected from inseminated hens following the commencement of the trial at week 69 of age (Table 3.1). All 10 cockerels and 25 hens per treatment were used for the determination of fertility during this study. Semen collection, evaluation and AI of the hens were generally completed within a 150 minute period by the three technicians. Artificial insemination of the hens was always performed on Fridays, while egg collection for incubation purposes followed from the subsequent day (Saturday), until the next Thursday (eggs collected for a 6-day period). The collected eggs were stored for a maximum of three days at room temperature on the farm (mean maximum and minimum daily temperatures were 18°C and 5°C respectively, with an average daily temperature being 11.5°C during storage on the farm), before being set for incubation.

3.7.1 Artificial insemination procedure

The AI procedure commenced at 12:00 on a Friday, when the majority of hens had laid their egg for that day. The time of AI was thus chosen to ensure that the oviduct and uterus was "emptied-out" and without a developing egg within the uterus. The cocks in a specific dietary treatment were dorso-abdominally massaged and the ejaculates collected, as described earlier in paragraph 3.6. Individual ejaculates were quickly transferred to the water-bath (42°C), until semen collection from all cocks within the specific treatment was complete. Thereafter, the semen volume of each ejaculate was recorded and a slide for the microscopic evaluation of sperm viability individually prepared. The individual sperm motility of each semen sample

was determined microscopically as described in paragraph 3.6.2.1, before individually collected semen samples were pooled within a dietary treatment. After pooling the semen samples, the undiluted semen was utilised for the insemination of all 25 hens within the corresponding treatment group. An insemination dose of 0.06 ml was deposited at a depth of approximately 2 cm into the hen's vagina using an automatic pipette (Sadanand *et al.*, 2004), following the venting of the vaginal orifice (Plate 3.9). Although 0.05 ml semen has been suggested for routine AI (Etches, 1996; Aydin & Cook, 2009), it was decided to increase the insemination dose to 0.06 ml for the purpose of this study. Only after the successful insemination of a single treatment, did the collection and screening of semen for the next dietary treatment commence. All prepared slides for the sperm viability evaluation were analysed, as previously described in paragraph 3.6.2.2, immediately after the completion of AI. The AI procedure was reversed during the following weeks to ensure that the collection and insemination of the same dietary treatment differed to a certain degree in time from that of the previous week.

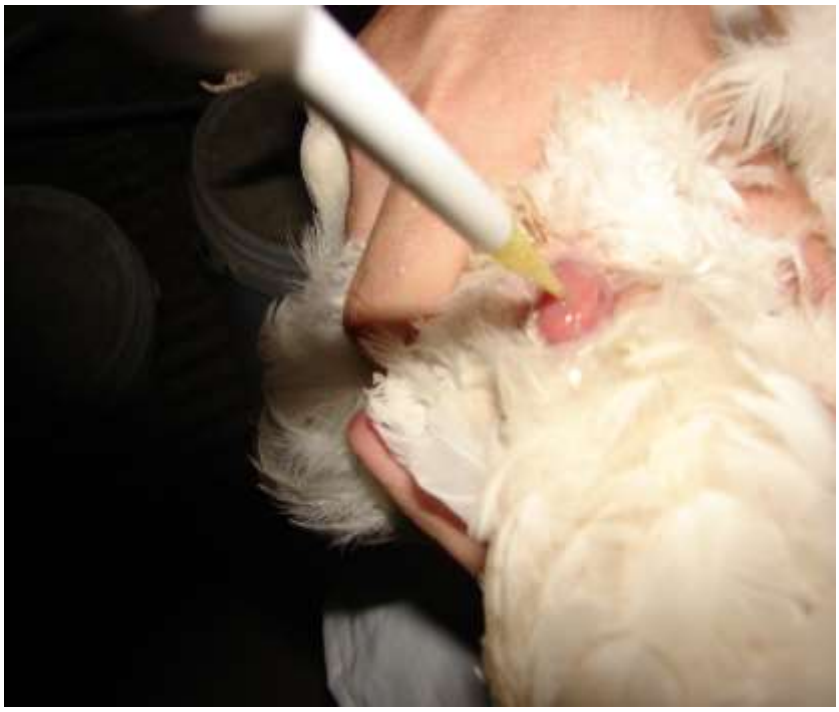


Plate 3.9 Artificial insemination of a hen using an automatic pipette

3.7.2 Egg collection, storage and incubation

Fertilised eggs from all hens (n = 125) were individually collected at 9:30, starting from the day following AI (i.e. Saturday). The eggs were individually marked with the date and hen

number before being weighed. Eggs collected on Saturday, Sunday and Monday following AI were set for incubation on that particular Monday afternoon, while eggs collected on the Tuesday, Wednesday and Thursday following AI the previous week, were set on that particular Thursday afternoon.

Eggs laid later than 9:30 in the day were left in the cage until the following day and recorded as such. All collected eggs were weighed and also classified as (i) sellable, (ii) cracked shell or (iii) shell-less. In each treatment group, only 12 eggs per day (n=12/treatment/day) in the sellable category (intact eggshells), which were free of any excreta or dust and not of extreme size (must be between 51 to 75 g) were selected for incubation (Lapao *et al.*, 1999; Mortola & Al Awam, 2010).

3.7.3 Incubation, fertility and hatchability

All presumed fertile eggs were not stored for more than three days, before being transported on the designated days (Mondays or Thursdays) from the experimental farm to the hatchery situated on the main campus of the UFS, for incubation purposes. On arrival at the hatchery, all eggs were individually weighed and set in the incubator on the same day at 37.5 °C and 60% relative humidity (RH) in a still air egg incubator (Mortola & Al Awam, 2010). Twelve eggs per treatment per day (n=12/treatment/day) for a period of 49 days were collected for incubation purposes. In total, 504 eggs per treatment (n=504/treatment) and 2520 eggs in total were thus incubated over a period of 70 days (49 + 21 days). Eggs were automatically turned inside the incubator every hour at an angle of approximately 35° to prevent the embryos from attaching to the shell membrane. The date of setting of the eggs in the incubator was recorded as embryonic day zero (E0). On embryonic day 18 (E18), all fertile eggs were individually placed (Plate 3.10) in a net bag (for the purpose of identification) and transferred into a smaller incubator that served as a "hatcher," until the expected day 21 of hatching.

After 22 days of incubation (21 days + 24 hours after the time of set) all hatched chicks were removed from the incubator and weighed individually before being marked with a neck tag (Plate 3.11). The eggs which had not hatched were removed with the chicks the following day (21 days of incubation + 24 hours after the time of set) and a break-out analysis conducted in order to determine the cause of non-hatching. Fully developed, but dead chicks with a partially broken shell were recorded as "pips".

Plate 3.10 Hatched chicks in respective colour bags and neck-tagged

Plate 3.12 Weighing of hatched chicks individually marked with a neck-tag

3.7.3.1 Candling of the eggs

Eggs were candled weekly on embryonic day 7 (E7) and day 14 (E14), to determine overall flock fertility (%) and embryonic mortalities, as set out by Aydin and Cook (2009). Candling was improvised by quickly taking eggs from the incubator into a dark enclosure, where the

light (250 lumen) from a handheld lamp was directed at the surface of each egg to identify the development of blood veins and to evaluate the size of the embryo. Eggs on day 7 (E7) that appeared 'clear' under the light were removed and classified as infertile. A break-out analysis subsequently revealed a small white germinal disc, with no blood veins. 'Clear' eggs having an enlarged germinal disc with no blood veins (blastoderm without an embryo), or no "dark eye" were classified as early deaths. The procedure of candling was repeated on day 14 (E14) and eggs without any visible appearance of bright red blood veins extending into the embryo were removed and broken for analysis of embryonic deaths. The break-out analysis and morphological identification regarding stage of embryonic deaths was adapted according to the technique of Wilson (2004).

Following every batch of chicks hatched, a residue analysis was conducted in order to determine the percentage of middle deaths (8 – 18 d), late deaths (19 – 21d), as well as "pips". The embryonic development classification system as described by Lapao *et al*. (1999) was used, with certain minor modifications in the present study, as follows:
The formulae (Lapao *et al.,* 1999) used for the calculation of (i) hatchability, and (ii) viability (hatchability of fertile eggs).

$$\text{Hatchability (\%)} = \frac{\text{Number of saleable chicks hatched}}{\text{Number of eggs set}} \times 100$$

$$\text{Viability (\%)} = \frac{\text{Number of saleable chicks hatched}}{\text{Number of fertile eggs}} \times 100$$

Chick weight as a percentage of egg weight was also determined in every treatment group.

3.8 Statistical analyses

3.8.1 Egg production study

Data gathered during the 46 collection week period were pooled for statistical analyses of parameter means during the long term exposure of experimental dietary treatments and analysed using a fully randomized one-way ANOVA design. The effect of dietary lipid sources on internal egg quality characteristics was also analysed using the same statistical model. The PROC ANOVA procedures of the SAS program (SAS, 2010) were used to test for significant ($P < 0.05$) differences between treatments. When significant differences were

recorded ($P < 0.05$), further multiple comparisons using Tukey's honest significant difference (HSD) test, were used to quantify treatment means.

3.8.2 Semen evaluation study

Data for the quantitative and qualitative analyses of semen parameters were analysed using a fully randomized one-way ANOVA design (SAS, 2010). Parameter means were compared using the least significant difference (LSD ± S.E.) test, at a significance level of $P \leq 0.05$, using the Duncan Multiple Range test as described by Cerolini *et al.* (2006). Pearson's correlation coefficients were also calculated between certain related semen parameters. Significance levels of $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ were used, based on the variability associated with the type of measurements.

3.8.3 Fertility study

The effects of dietary lipid source on overall flock fertility and chick hatchability were statistically analysed according to the least square means procedures, using the General Linear Models procedure (PROC GLM) (SAS, 2010). Eggs collected to partake in the incubation study from inseminated hens were individually considered and assigned a dummy binary of 0 or 1, depending on the negative or positive state under the following variables namely, (i) fertility, (ii) early death, (iii) mid-death, (iv) late death and (v) hatchability, as suggested by Harvey (1982) for the statistical analysis of discrete data, and as adopted by Lapao *et al*. (1999).

CHAPTER 4
EFFECTS OF DIETARY LIPID SOURCES ON COCKEREL SEMEN QUALITY AND FLOCK FERTILITY

4.1 Introduction

There exist no formal evaluations for breeding soundness in the poultry industry. In a commercial flock, the system commonly adopted by producers involves the modifying of the male to female ratio (♂ : ♀), based on a number of physical attributes of the male. Wilson *et al*. (1979) illustrated that the general selection of cockerels based on physical characteristics such as comb size, body size and shank length were not accurate enough in predicting cockerel fertility. The decline in flock fertility thus requires critical consideration in current breeding practices. So for example, artificial insemination has been suggested in broiler breeders (Reddy, 1995), as e.g. increases in body size tend to impact negatively on male fertility (Reddy & Sajadi, 1990; Barbato, 1999). Similarly, many researchers (Donoghue, 1999; King *et al.*, 2000; Parker & McDaniel, 2002) have advocated the need for a more scientific, detailed and sustainable method for the evaluation of the cockerel's potential fertility. These methods include protocols involving semen quality tests - through the evaluation of semen parameters such as volume, sperm motility, sperm viability, and sperm concentration. Subsequently, selection of cockerels based on sperm quality has also translated into improved hatchability in the broiler breeder flock (Eslick & McDaniel, 1992; Parker & McDaniel, 2002). However, one of the major factors affecting sperm quality, has been shown to be the diet composition. Dietary manipulation has been employed as a means of enhancing sperm quality, due to the strong relationship that exists between the male reproductive potential and flock fertility.

Dietary lipid sources generally affect cockerel sperm composition and functionality differently (Bongalhardo *et al.,* 2009), which could be a result of the specific proportions of the sperm fatty acids. The n-6 type of fatty acids (e.g. docosatetraenoic acid- DTA) being the most prevalent in cockerel sperm (Cerolini *et al.*, 1997), as opposed to n-3 type fatty acids that are predominant in mammalian sperm (Nissen *et al.,* 1981; Poulos *et al.,* 1986; Jiang *et al*., 1992; Kelso *et al*., 1997). Studies show that dietary fatty acids are deposited proportionately in the sperm (Blesbois *et al.*, 1997; Kelso *et al.*, 1997; Cerolini *et al.*, 2003). This means that regardless of the type of dietary oil consumed, the proportion of the total relative abundance of saturated (SFA), mono-unsaturated (MUFA's) and polyunsaturated

fatty acids (PUFA's) in the sperm do not change, even if the arrangements of the fatty acids within a group (PUFA's in particular) are altered.

Further, different sources of lipids with their characteristic fatty acid profiles have received considerable attention regarding sperm quality enhancement. Fish oil (n-3) for example, has been given preference over the conventional n-6 oils from plants such as soyabean, sunflower and corn regarding sperm motility (Cerolini *et al.,* 2006), and fertility (Blesbois *et al.,* 1997; Hudson & Wilson, 2003) in broiler breeders. Fish oil (n-3) also maintained fertility better in tom turkeys, when compared to sunflower (n-6) oil (Blesbois *et al.,* 2004). Although the relatively high concentration of long chain fatty acid docosaexahenoic acid (DHA) content in fish oil has been positively associated with sperm motility, it also predisposes fish oil to a faster rate of peroxidation, which may hamper its benefits (Ollero & Alvarez, 2003). Sperm motility and fertility in relation to the SFA, MUFA and PUFA composition of sperm phospholipids in the aging cockerel may thus make an all inclusive dietary lipid experiment imperative (Cerolini *et al.,* 1997).

Besides the influence on cockerels (sperm quality) and hens (egg size), fatty acids also contribute to chick embryonic development. The main energy for the developing chick embryo being supplied by fats in the egg yolk (Freeman & Vince, 1974). The alteration of the egg yolk fatty acid profile could therefore be detrimental to the growth and development of the embryo during the incubation period (Donaldson & Fites, 1970; Aydin & Cook, 2009).

The aims of the present study were to evaluate the effects of fish oil (n-3), sunflower oil (n-6), high oleic sunflower oil (n-9), tallow (SFA), and a control diet consisting of equal proportions (50: 50) of linseed and fish oil (n-3), on cockerel semen characteristics during peak production (week 32 – 46 of age) (Trial 1). Also, on the flock fertility and hatchability during the end-of-lay period (week 69 – 77 of age) stages (Trial 2).

4.2 Materials and Methods

4.2.1 Experimental birds and husbandry

Bird management and husbandry has been comprehensively discussed in paragraph 3.2 and 3.3 (Chapter 3). Fifty 17 week old HyLine Silver-Brown cockerels were housed individually in metabolic cages (1600 cm^2), within a natural ventilated building on the Paradys experimental farm of the University of the Free State, outside Bloemfontein. Each cage was

equipped with a single water nipple, feed tray and excreta tray. Birds were fed ad lib with a standard commercial layer mash up to the age of 32 weeks, when the experimental diets were introduced. From 22 weeks of age, all cockerels were trained for semen collection according to the "massage" technique first described by Burrows and Quinn (1937). At 32 weeks of age, 10 cockerels were randomly assigned to one of the following five dietary treatments: (i) the control diet (a blend of 50% fish and 50% linseed oil), (ii) pure fish oil (n-3), (iii) sunflower oil (n-6), (iv) high oleic (HO) acid sunflower oil (n-9) and (v) tallow (saturated fatty acids) supplemental diets. Access to water was on an *ad libitium* basis, while the supply of feed to the cockerels was limited to 110 g feed/bird/day from 32 weeks of age. A photoperiod schedule of 16 hours light and 8 hours darkness (16L:8D) was provided from week 32 of age, until the termination of the study at week 77 week of age (end of Trial 2). The daily minimum and maximum ambient temperatures (^{o}C) were recorded at five different locations within the hen-house and data were pooled to calculate the mean minimum and maximum hen-housed temperatures (^{o}C), of the first and second experimental periods, respectively.

4.2.1.1 Trial 1

After an adaptation period of 3 weeks to the experimental diets, semen collection and analyses commenced on week 35 of age and were conducted once a week on each individual cockerel, until 46 weeks of age. This time-frame of 12 weeks (from week 35 to 46 of age) was used for the first experiment (Trial 1) for the cockerels. During this time, individual feed consumption was determined weekly and used in the calculation of average daily feed intake (g/bird/day), while birds were individually weighed every four weeks to determine their body weight.

4.2.1.2 Trial 2

After the successful completion of Trial 1 (at 46 weeks of age), all birds remained on their respective experimental diets until the termination of the second trial (end of week 77 of age). The second trial commenced at 69 weeks of age and lasted for 9 weeks, until 77 weeks of age. The weekly collection of semen, as well as sperm analyses during the second trial (weeks 69 to 77 of age), were the same as that performed in Trial 1 (see paragraph 3.7; Chapter 3). The only difference between the first and second trial, was the artificial insemination of 25 hens (see paragraph 3.7; Chapter 3) in each of the corresponding dietary treatments after the evaluation of the semen quality characteristics. Weekly feed

consumption and monthly body weights of the cockerels were similarly recorded as in Trial 1. All chemicals used during the semen evaluation in Trial 1 and Trial 2 were supplied by Sigma®.

4.2.2 Experimental diets

The five different lipid sources, namely fish oil, sunflower oil, high oleic acid sunflower oil, tallow, and an equal (50 : 50) proportion of linseed and fish oil (representing the control diet), were used in formulating the five experimental diets. The objective was to formulate five *isoenergetic* and *isonitrogenous* diets, with a constant inclusion level (30 g/kg) of the various supplementary lipid sources - differing only in their fatty acid profile. To ensure the formulation of *isoenergetic* diets, the apparent metabolizable energy (AME) content of each dietary lipid source was predetermined. The AME values (Table 3.2) of the respective lipid sources were calculated based upon the free fatty acid (FFA) and the unsaturated to saturated fatty acid ratio (U/S), as described by Wiseman (1990). The physical (Table 3.3) and chemical (Table 3.4) composition of the experimental diets, as well as the preparation procedure are comprehensively described in paragraph 3.4 of Chapter 3.

4.2.3 Semen collection and sperm analyses

Procedures for semen collection and sperm analyses used in the present study have been described in paragraph 3.6 (Chapter 3). Semen collection and evaluations were done on two consecutive days (Thursday and Friday) per week and commenced at 09:30. The 50 experimental cockerels were divided into two groups (n = 25), where all 5 treatments were equally represented for each day (25 cockerels on Thursday and the other set of 25 cockerels on Friday) of analysis. This was done to ensure that collected semen samples were analysed within the shortest time possible, since analyses were conducted on an individual basis (not pooled). Semen was collected into 5 ml transparent graduated tubes (least graduation of 0.5 ml), using the abdominal massage technique (Burrows & Quinn, 1937) on the cockerels. The collection of semen was carried out in batches of 5 birds at a time, representing individual ejaculates from all five experimental treatments. Cockerels that failed to ejaculate or produced semen contaminated with faeces/uric acid were noted. All semen collected in a batch (n = 5) was kept warm (41 °C) in a water bath and analysed within 30 minutes after collection.

Semen volume was visually appraised directly from the graduated collection tube, while sperm motility was analysed on a warm microscope slide (x 400 magnification), by classifying 100 sperm cells as either motile or non-motile. Sperm concentration analyses were conducted by mixing 10 µl of the semen sample with 990 µl of distilled water (1 : 100), and counting the number of sperm with the aid of a haemocytometer (Hafez & Hafez, 2000). The morphology of the sperm was assessed using the eosin-nigrosin stain method and sperm were classified as either dead or alive. Two hundred (2 x 100) individual sperm cells were counted per slide, while the live sperm cells were further characterised into normal and abnormal sperm (Lukaszewicz *et al*., 2008). The results of each morphological category were then expressed as a percentage of the total sperm cells counted (200 cells).

4.2.4 Artificial insemination and egg incubation

At the age of 69 to 77 weeks, flock fertility was evaluated, by artificially inseminating hens with collected semen and the determination of chick hatchability. Semen from the cockerels in each treatment group (n = 10/treatment) was collected and individually assessed for ejaculate volume, sperm motility, and viability. Following sperm analyses, semen samples were pooled and used for the insemination of the hens (n = 25/treatment), within the corresponding dietary treatment of the cockerels. Hens were inseminated with 0.06 ml undiluted fresh semen, using a calibrated pipette (Plate 3.6; Chapter 3). To avoid low fertility in the initial weeks, the AI of the hens started two weeks (week 69 of age) prior to the commencement of the egg incubation trial at week 71 of age. The procedures regarding the artificial insemination of hens are fully described in paragraph 3.7, Chapter 3.

Eggs were collected throughout the week, starting from the day after insemination. Clean eggs (excreta free) with weights of 51 to 75 g were marked with the date of collection and the specific hen number. Eggs were stored for a maximum period of 3 days, before setting the "collection eggs" at 37.5 °C and 60% relative humidity (RH) in a still-air egg incubator (see paragraph 3.7.1 & 3.7.2). Twelve eggs per treatment per day (n = 12/treatment/day) for a period of 49 days were collected for incubation purposes. In total, 588 eggs per treatment (n = 588/treatment) and 2940 eggs in total (all treatments) were incubated over a period of 70 days. Eggs were automatically turned every hour at (40 °C) to prevent the embryos from adhering to the shell membranes. The date of setting the eggs in the incubator was recorded as embryonic day zero (E0).

Incubated eggs were candled at embryonic day 7 (E7) to identify infertile eggs, and the candling repeated on embryonic day 14 (E14) for the detection and removal of early deaths. On embryonic day 18 (E18), the remaining eggs in the incubator were transferred to individual net bags and set inside a separate still air hatcher at 36.5 ^{o}C, with a RH of at least 65%. Chicks that "completely hatched" from their shells after 21 days (+ 24 h) of incubation were individually weighed. A "hatching window" of 24 hours was allowed, thereafter all eggs that failed to hatch were removed from the incubator (Day 22 of incubation) to perform a break-out analysis in an attempt to categorize the reasons for non-hatching. The three categories used for classifying the "non-hatched" eggs were (i) middle deaths, (ii) late deaths and (iii) pips (fully developed chicks with a broken shell that failed to hatch completely).

4.2.5 Sperm fatty acid methyl esters

Before the termination of the study on week 78 (Chapter 3) semen from all individual cockerels per treatment were collected and pooled for the analyses of the sperm fatty acid methyl esters (FAME). The total lipid content of the samples were extracted according to the method as described by Folch *et al.* (1957) and comprehensively set out in paragraph 3.5.1 (Chapter3). The fatty acid composition, total fatty acid saturation, and the n-3, n-6, n-9 ratios of the fatty acids within cockerel sperm were calculated accordingly.

4.2.6 Statistical analyses

4.2.6.1 Semen evaluation study

Data for the quantitative and qualitative analyses of semen parameters were analysed, using a fully randomized one-way ANOVA design (SAS, 2010). Parameter means were compared using the least significant difference (LSD) test at a significance level of $P \leq 0.05$, according to the Duncan Multiple Range test. The Pearson correlation coefficients were also calculated for certain related semen parameters. A significance level of $P \leq 0.05$, $P \leq 0.01$ or $P \leq 0.001$ was used for the correlated parameters, based on the variation associated with the type of variable.

4.2.6.2 Fertility study

The effects of the dietary lipid sources on overall flock fertility and chick hatchability were statistically analysed according to the least square means procedures using the General Linear Models procedure (PROC GLM) (SAS, 2010). The eggs collected to partake in the incubation study from inseminated hens were individually considered and assigned a binary

dummy value of 0 or 1, depending on its negative or positive status under the following variables, namely (i) fertility, (ii) early deaths, (iii) mid-deaths, (iv) late deaths and (v) hatchability, as suggested by Harvey, (1982) for the statistical analysis of discrete data.

4.3 Results and Discussions

4.3.1 Fatty acid profile of the experimental diets

The effect of dietary lipid sources on the dietary fatty acid methyl esters (FAME) are presented in Table 4.1. From Table 4.1 it is evident that the relative prevalence of fatty acids can be associated with the specific lipid sources included in certain diets, and thus in describing that particular lipid source. The control treatment resulted in the highest ($P < 0.0001$) concentration (15.4%) of α-linolenic (C18:3n-3) acid. This was expected due to the inclusion of linseed oil. From the fish oil treatment, it was evident that this lipid source resulted in a significant increase ($P < 0.0001$) of eicosapentaenoic (C20:5n-3) and docosahexanoic (C22:6n-3) concentrations (8.2 and 2.6% respectively) - which are both long chain derivatives of α-linolenic acid (C18:3n-3). Also, high concentrations of saturated fatty acids C14:0 (5.2%) and C16:0 (18.2%) were recorded for the fish oil treatment. This can be partially explained by the fact that although fish oil is a polyunsaturated n-3 source, it remains a lipid source of animal (fish) origin. This was the main reason why there were limited differences, except in the case of stearic acid (C18:0), between the saturated fatty acid profile of fish oil and tallow. Likewise, the sunflower treatment recorded the highest ($P < 0.0001$) concentration (55.3%) of linoleic acid (C18:2n-6). The high oleic acid (HO) sunflower oil inclusion resulted in the highest concentration (54.9%) of oleic (C18:1n-9) acid, which is the predominant in the mono-unsaturated fatty acid group. Moreover, the tallow treatment recorded the highest concentrations of palmitic (C16:0) and stearic acid (C18:0), of respectively 20.0% and 17.1%.

Table 4.1 The mean effects of dietary lipid sources on the fatty acid methyl esters (*FAME) of the experimental diets

Parameter	Control	Fish oil (n–3)	Sunflower oil (n–6)	#HO Sunflower oil (n–9)	Tallow (SFA)	Significance *(P)*
Dietary fat (%)	5.08[a]	5.23[a]	5.17[a]	5.18[a]	5.17[a]	0.5122
***FAME (% of total fatty acids)**						
Saturated fatty acids						
C14:0 Myrisitic acid	2.39[b]	5.22[a]	0.06[c]	ND	2.01[b]	<0.0001
C16:0 Palmitic acid	13.03[b]	18.18[a]	9.14[c]	7.56[c]	19.99[a]	<0.0001
C17:0 Margaric acid	0.15[c]	0.25[b]	0.06[d]	0.04[d]	0.77[a]	<0.0001
C18:0 Stearic acid	3.36[b]	3.47[b]	4.56[b]	4.47[b]	17.11[a]	<0.0001
Mono-unsaturated fatty acids						
C16:1 Palmitoleic acid	2.81[b]	5.96[a]	0.08[d]	0.07[d]	1.39[c]	<0.0001
C18:1n-9 Oleic acid	23.18[d]	21.32[d]	27.82[c]	54.86[a]	30.24[b]	<0.0001
C18:1n-7 Vaccenic acid	1.63[b]	2.54[a]	0.66[d]	0.74[cd]	0.95[c]	<0.0001
C20:1n-11 Eicosenoic acid	0.65[b]	1.12[a]	0.20[c]	0.21[c]	0.18[c]	<0.0001
Polyunsaturated fatty acids (n-6)						
C18:2n-6 Linoleic acid	29.96[b]	27.09[c]	55.27[a]	29.87[c]	24.04[d]	<0.0001
C20:2n-6 Eicosadienoic acid	0.04[b]	0.08[a]	ND	ND	ND	<0.0001
C20:4n-6 Arachidonic acid	0.20[b]	0.39[a]	ND	ND	ND	<0.0001
C22:4n-6 Docosatetraenoic	0.41	0.66	ND	ND	ND	<0.0001
Polyunsaturated fatty acids (n-3)						
C18:3n-3 α-linolenic acid	15.38[a]	1.13[b]	0.84[b]	0.78[b]	0.95[b]	<0.0001
C20:5n-3 EPA[1]	4.26[b]	8.17[a]	ND	ND	ND	0.0002
C22:5n-3 DPA[2]	0.41[ab]	0.66[a]	ND	ND	ND	0.0021
C22:6n-3 DHA[3]	1.38[ab]	2.57[a]	ND	ND	ND	0.0008

[a,b,c,d] Means in rows with different superscripts differ significantly at $P < 0.05$; ND = not detected; # High oleic acid sunflower oil; [1] Eicosopentaenoic acid; [2] Docosapentaenoic acid;[3] Docosahexanoic acid.

The total saturated, mono-unsaturated, polyunsaturated, n-6, and n-3 fatty acids, as well as their specific ratios in the experimental diets following the inclusion are summarised in Table 4.2. Significant differences ($P < 0.0001$) were recorded for both the total concentration and ratios of fatty acids between the experimental dietary treatments. The control diet recorded the highest concentration (21.4%) of the total n-3 fatty acids, and had the second highest concentration of total PUFA's (51.8%). The fish oil (n-3) treatment, as expected, recorded the highest ($P < 0.001$) concentration of long chain n-3 derivatives, such as EPA, DPA, and DHA (8.2, 0.7 and 2.6%, respectively), as illustrated in Table 4.1. In addition, the fish oil (n-3) diet also contained a high proportion of saturated fatty acids. It was in this diet that the highest ($P < 0.0001$) concentration of myristic acid (5.2%) and palmitic acid (although numerically lower than tallow), was recorded. Consequently, the fish oil (n-3) diet was rated second highest in total saturated fats (28.2%), and also in n-3 (12.5%) concentration. Similar to being the second lowest ($P < 0.0001$) in PUFA: SFA (1.45: 1) and MUFA: SFA (1.13:1) ratios, as set out in Table 4.2. Further, it was evident that the sunflower (n-6) treatment recorded the highest total n-6 concentrations (55.3%). The ratio of PUFA:SFA, n-6:n-3 and n-6:n-9 ratios were also significantly ($P < 0.0001$) higher (3.8, 66.0 and 1.9, respectively) in the sunflower treatment, compared to the other treatments. The high oleic acid sunflower (n-9) treatment had the highest concentration of total mono-unsaturated fatty acids MUFA (55.9%) and UFA (although not statistically different from the sunflower (n-6) treatment). This resulted in the highest proportion of MUFA:SFA (4.16:1), MUFA:PUFA (1.82:1) and also n-9:n-3 (72.41:1) recorded in the high oleic acid sunflower treatment. The tallow treatment only resulted in the highest concentration (40.7%) of total SFA for all the groups and ratios of fatty acids recorded. These results are in agreement with that recorded by King (2012).

Table 4.2 The mean effects of dietary lipid source on the total fatty acid concentration and fatty acid ratios in the experimental diets

Parameter	Control	Fish oil (n–3)	Sunflower oil (n–6)	HO Sunflower oil (n–9)	Tallow (SFA)	Significance (P)
Total						
∑SFA	19.74^{c}	28.17^{b}	14.97^{d}	13.45^{d}	40.74^{a}	<0.0001
∑MUFA	28.49^{d}	31.55^{bc}	28.91^{cd}	55.90^{a}	34.27^{b}	<0.0001
∑n-6	30.35^{c}	27.75^{c}	55.27^{a}	29.87^{b}	24.04^{d}	<0.0001
∑n-3	21.42^{a}	12.53^{b}	0.84^{c}	0.78^{c}	0.95^{c}	<0.0001
∑PUFA	51.77^{a}	40.28^{b}	56.11^{a}	30.65^{c}	25.00^{c}	<0.0001
∑UFA	80.26^{b}	71.83^{c}	85.02^{a}	86.55^{a}	59.26^{d}	<0.0001
Ratio						
PUFA:SFA	2.64^{b}	1.45^{c}	3.76^{a}	2.28^{b}	0.61^{d}	<0.0001
MUFA:SFA	1.45^{c}	1.13^{d}	1.93^{b}	4.16^{a}	0.84^{e}	<0.0001
MUFA:PUFA	0.55^{d}	0.79^{c}	0.51^{d}	1.82^{a}	1.37^{b}	<0.0001
n-6:n-3	1.44^{d}	2.49^{d}	65.99^{a}	38.67^{b}	25.35^{c}	<0.0001
n-6:n-9	1.07^{b}	0.88^{c}	1.91^{a}	0.53^{e}	0.70^{d}	<0.0001
n-9:n-3	1.35^{c}	2.88^{c}	34.54^{b}	72.41^{a}	36.17^{b}	<0.0001

^{a,b,c,d,e} Means in rows with different superscripts differ significantly $P < 0.05$

[1] Means of 3 replicates

SFA = Saturated fatty acid

MUFA = Mono-unsaturated fatty acid

PUFA = Polyunsaturated fatty acid

UFA = Unsaturated fatty acid

4.3.2 Temperature

The average maximum and minimum hen-house temperatures as recorded during Trial 1 (week 33 to 46 of age), are set out in Figure 4.1. The gradual increasing trend in ambient temperature during this rearing period was characteristic of the transition from the spring to summer season (September to December) for the Bloemfontein area, where the trial was conducted. The mean maximum and minimum daily ambient temperatures were 29 °C and 13 °C respectively, with an average daily temperature of 21 °C being recorded for this period.

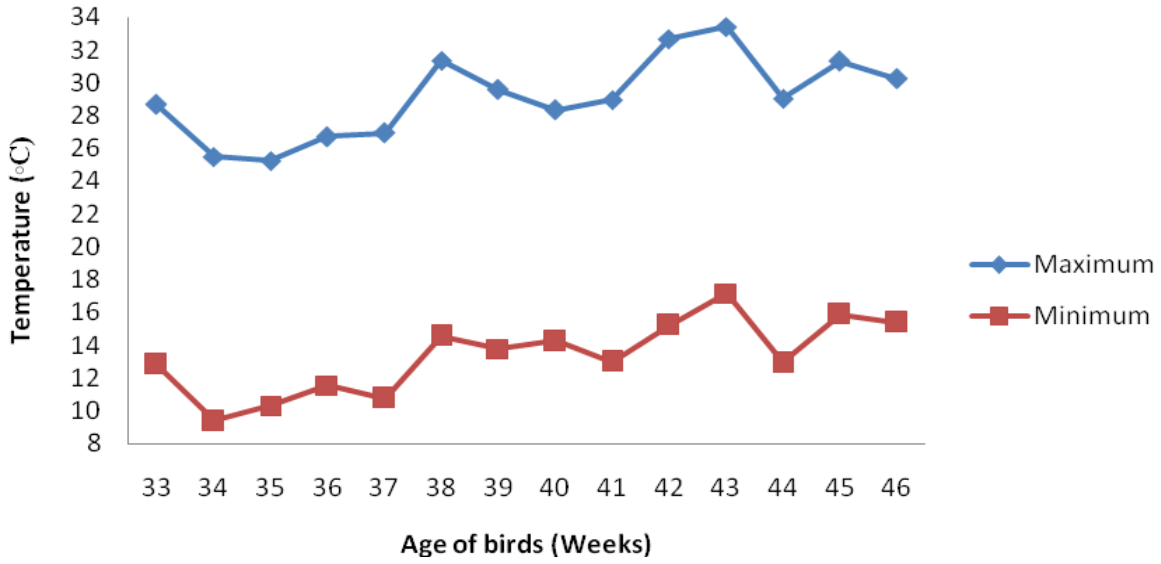


Figure 4.1 Average minimum and maximum hen-house temperatures from 33 to 46 weeks of age (Trial 1)

Ambient temperature generally plays an important role in controlling the metabolic energy of the bird, through the regulation of feed intake. A lower critical temperature (LCT) of 19 °C and upper critical temperature (UCT) of 27 °C has been suggested by Kleyn (2006), for optimal production in laying hens and falls within the range recorded in the current trial.

A slight deviation in feed intake could therefore be expected in the present study, in order for the birds to adjust to the prevailing temperatures (minimum 13 °C; maximum 29 °C), even though the mean daily temperature (21 °C) was within the comfort zone for layers.

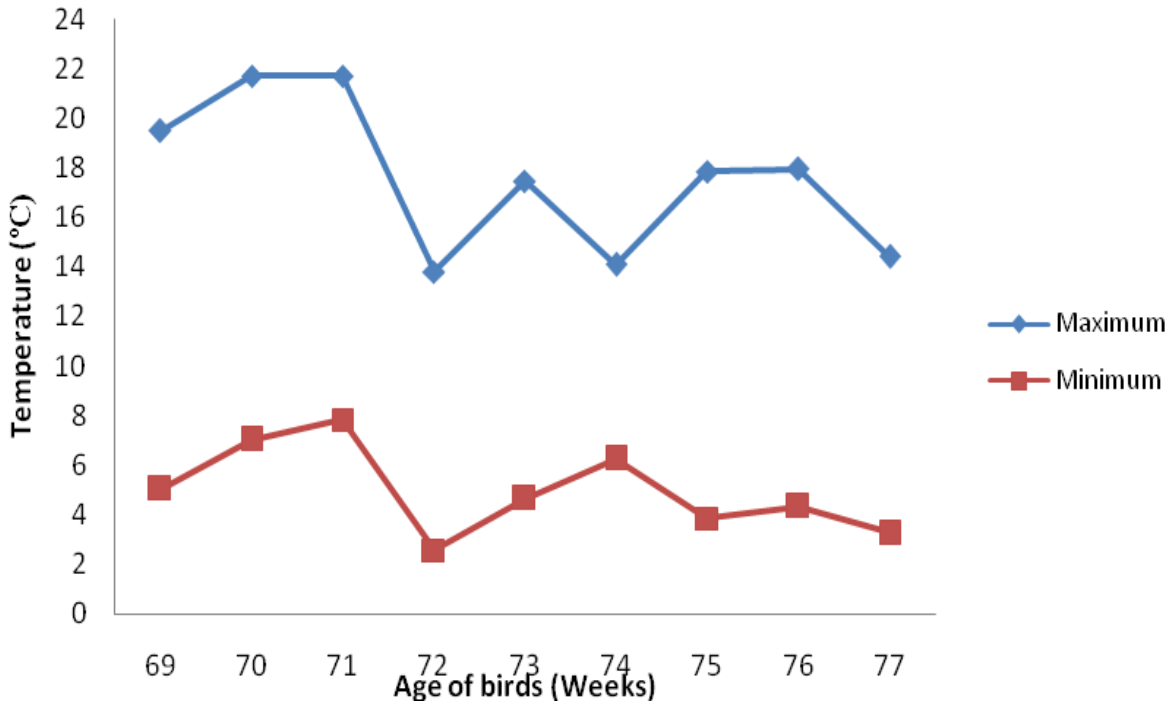


Figure 4.2 Average minimum and maximum hen-house temperatures from 69 to 77 weeks of age (Trial 2)

Figure 4.2 illustrates the environmental temperatures during Trial 2, when the birds were between 69 and 77 weeks of age. The dramatic decrease in ambient temperature occurring between the start (69 weeks of age) and the end (77 weeks of age) of the trial was characteristic of the end of autumn to the winter season (May to July) for the Bloemfontein area. The mean maximum and minimum daily temperatures recorded were 18 °C and 5 °C respectively, with an average daily temperature of 11.5 °C being recorded. Birds during the course of Trial 2 were expected to consume more feed (due to a lower ambient temperature) in order to ensure sufficient heat generation, compared to a lower feed consumption across treatments during Trial 1. A constant exposure of birds to temperatures higher than their thermo-neutral zones, normally predisposes them to a low feed intake. This deliberate decrease in feed consumption during hot weather by chickens, is then associated with the mechanism in reducing metabolic heat (Ayo *et al.*, 2010).

4.3.3 Effects of dietary lipid sources on the performance and semen quality of cockerels during Trial 1

In Figure 4.3, the effect of dietary lipid sources on the average daily feed intake of the cockerels during Trial 1 (33 – 46 weeks of age), is illustrated. Apparently, the period of feed

adaptation after the introduction of dietary treatments at week 32 was the longest (although these results were not statistically significant) for cockerels fed the fish oil (n-3) diet. These cockerels only consumed similar quantities of feed to cockerels in the other treatments, starting from week 40.

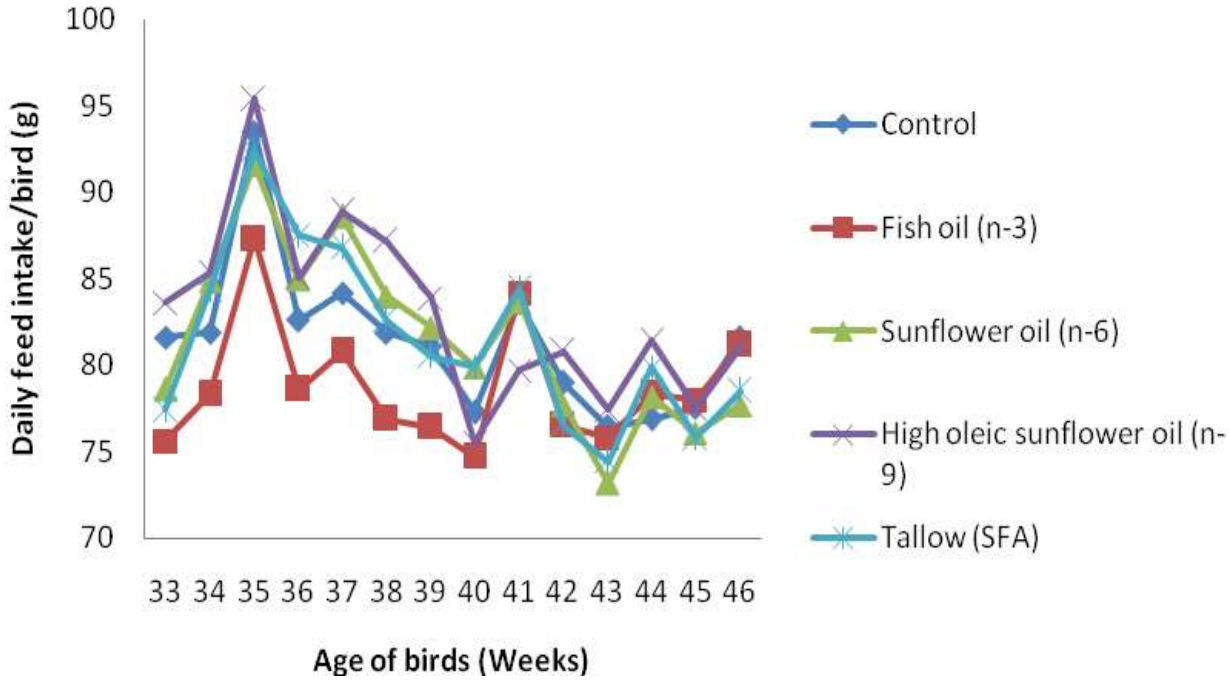


Figure 4.3 Mean (± s.e.) daily feed consumption of cockerels following introduction to treatments during Trial 1

Interestingly, this initial disparity in feed consumption was not recorded in cockerels maintained on the control treatment, which comprised of 1.5% fish- and 1.5% linseed oil. It could therefore be suggested that the higher number of long chain fatty acids supplied by the higher inclusion level (3%) of fish oil in the fish oil (n-3) treatment, was responsible for the manifested low feed intake of the cockerels until week 40 of age. Although no significant differences ($P \geq 0.05$) were recorded in the average daily feed intake of the cockerels. However, when the mean data were analysed on a weekly basis, the cumulative effect of the non-significant lower feed intake in the fish oil treatment group during weeks 33 - 40, resulted in a lower ($P < 0.05$) feed intake during the mean period (Table 4.3). However, as the trial progressed, cockerels seemed to adjust to this specific diet, with an appropriate improvement in feed intake.

The susceptibility of polyunsaturated fatty acids of animal origin (e.g. fish oil) to oxidation, is generally faster than that of plant origin. The characteristic rancid odour generally occurs in animal PUFA, when the peroxide threshold level of 20 meq/kg fat is surpassed, whereas the vegetable oils have a relatively higher threshold of 80 meq/kg fat (Leeson & Summers, 2005).

The effect of dietary lipid sources on the performance and semen quality characteristics of cockerels during the first trial (35 – 46 weeks of age) are summarised in Table 4.3. The average daily feed consumption and consequent body weight of the birds were significantly ($P < 0.05$) affected by the dietary lipid sources used. Cockerels maintained on the fish oil (n-3) treatment, recorded the lowest ($P < 0.05$) feed consumption (± 78.8 g/bird/day), when compared for the mean of the period of the other dietary treatments. As no other statistical significant differences in feed intake were recorded between the other experimental treatments, no tendency of dietary lipid source effect on feed intake could be registered. Similar to the feed intake results, the body weights of cockerels on the fish oil (n-3) treatment recorded the lowest values of all the dietary treatments (2499 g/bird). However, once again no tendency regarding the effect of dietary fatty acid saturation on body weights of the cockerels could be recorded.

The semen volume of the cockerels was significantly affected ($P < 0.01$) by the lipid source, which consequently led to a significant difference in the total sperm production ($P < 0.01$) of the cockerels, irrespective of the non-significant effect ($P = 0.5759$) of dietary fatty acid saturation on sperm concentration. From the results in Table 4.3, it was evident that cockerels in the tallow (SFA) treatment group recorded the highest ($P \leq 0.01$) semen volume (0.42 ml), whereas no significant ($P > 0.05$) differences in terms of semen volume were recorded between the other dietary treatments. The increased ($P < 0.05$) semen volume of cockerels on the tallow (SFA) treatment subsequently contributed to the highest ($P < 0.01$) sperm production (1.48×10^9sperm/ml) being recorded. Sperm motility of the cockerels in the control (54.8%), HO sunflower oil (53.7%) and tallow (55.1%) treatment groups were higher ($P < 0.05$) than that of the fish oil (48.1%) treatment. Sunflower oil treatment (50%) resulted in intermediate sperm motility.

Table 4.3 The effects (LSM ± S.E.M) of dietary lipid sources on the performance and semen quality characteristics of cockerels during Trial 1 (35 to 46 weeks of age)

Parameter	Control	Fish oil (n – 3)	Sunflower oil (n – 6)	*HO Sunflower oil (n – 9)	Tallow (SFA)	Significance *(P)*
Average daily feed intake (g/bird/d)	$81.36^{a} \pm 0.76$	$78.75^{b} \pm 0.59$	$81.53^{a} \pm 0.72$	$83.06^{a} \pm 1.02$	$81.49^{a} \pm 0.87$	0.0007
Body weight (g)	$2567^{a} \pm 17.4$	$2499^{b} \pm 17.3$	$2594^{a} \pm 30.3$	$2623^{a} \pm 16.4$	$2607^{a} \pm 27.4$	0.0005
Semen volume (ml)	$0.36^{b} \pm 0.015$	$0.35^{b} \pm 0.019$	$0.36^{b} \pm 0.016$	$0.36^{b} \pm 0.015$	$0.42^{a} \pm 0.020$	0.0061
Sperm concentration ($X10^{9}$ /ml)	3.33 ± 0.09	3.47 ± 0.09	3.53 ± 0.08	3.42 ± 0.08	3.42 ± 0.08	0.5759
Total sperm production ($X10^{9}$)	$1.20^{b} \pm 0.06$	$1.20^{b} \pm 0.07$	$1.29^{b} \pm 0.07$	$1.25^{b} \pm 0.06$	$1.48^{a} \pm 0.09$	0.0091
Sperm motility (%)	$54.8^{a} \pm 2.14$	$48.1^{b} \pm 2.10$	$50.0^{ab} \pm 2.26$	$53.7^{a} \pm 2.03$	$55.1^{a} \pm 2.10$	0.0365
Live sperm (%)	93.4 ± 0.49	91.7 ± 0.64	93.6 ± 0.45	92.6 ± 0.64	92.9 ± 0.45	0.2307
[1]Viable sperm (%)	86.3 ± 0.76	84.5 ± 1.09	87.5 ± 0.57	85.2 ± 0.79	86.1 ± 0.65	0.1549
[1]Sperm abnormalities (%)	7.07 ± 0.40	7.17 ± 0.52	6.13 ± 0.28	7.38 ± 0.33	6.85 ± 0.32	0.1463
Ejaculation rate (%)	$87.5^{a} \pm 0.03$	$79.2^{b} \pm 0.04$	$88.3^{a} \pm 0.03$	$94.2^{a} \pm 0.02$	$94.2^{a} \pm 0.02$	0.0008

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

[1] Calculated as a percentage (%) of the total sperm count

[*] High oleic acid

Further, the percentage of live sperm (%) was not significantly affected (P = 0.2307) by the dietary treatments. This trend was further evident in sperm viability (P = 0.1549), and sperm abnormalities (P = 0.1463). Moreover, the frequency at which cockerels ejaculated non-contaminated (faeces-free) semen seemed to be affected (P < 0.01) by treatment - with the fish oil (n-3) treatment performing the poorest (79.2%) in this regard

The dietary lipid source and perhaps the level of inclusion showed a limited and non-predictable response on the feed intake, body weight, and reproduction characteristics in the cockerels. The effect of lipid inclusion level was particularly evident in the reproductive differences between the control and the fish oil (n-3) fed cockerels, as both treatments contained fish oil, but at different levels. The possibility of peroxidation of the fish oil (n-3) diet used in this experiment could not be overruled, considering the significantly (P < 0.05) lower values recorded for most parameters measured. Firstly, the lower (P < 0.05) feed consumption of the fish oil (n–3) fed cockerels, compared to cockerels in the other dietary treatments, indicated the possibility of higher lipid oxidation and an unacceptability of this particular diet. Lipid oxidation generally manifests as rancidity and may cause a loss of feed quality and acceptability (Gillard, 1989), resulting in the reduced feed intake by the chickens (Lin *et al.,* 1989). Generally, unsaturated fatty acids, particularly those of animal origin are more prone to lipid peroxidation than the saturated fats (Leeson & Summers, 2005). The fish oil (n-3) treatment is of animal origin and higher in peroxidation rate than tallow (also animal source) and the high oleic acid sunflower (n-9) treatments at an unsaturation level (Table 4.2) - as well as having the highest concentration (Table 4.1) of long chain fatty acids (DHA, EPA), predisposes this diet to a faster rate of oxidation and rancidity. The level of fish oil inclusion could also be of importance, as the control treatment (1.5% fish oil/1.5% linseed oil) did not exhibit the similar superficial rancidity odour observed in the (3%) fish oil (n-3) treatment - despite being a higher (P < 0.05) unsaturated diet (Table 4.2). Storage of feeds in high ambient temperatures have also been reported by Batista *et al.* (1992) to increase the rancidity and induce a loss in diet quality, which was further associated to a increase in free fatty acids (FFA) and high (P < 0.001) peroxide values (Njobeh *et al.*, 2006).

One of the main effects reported of rancid feed on consumption has been the disruption of biological activities (Shermer & Calabotta, 1985), as observed in terms of the general performance of the fish oil (n-3) fed cockerels in the present study. Fish oil is generally a rich source of long chain fatty acids (DHA in particular), which is unfortunately also (due to its

relatively high number of double carbon bonds) a potential source of peroxidation. In Figure 4.3, it could be observed that cockerels fed the fish oil (n–3) diet took a longer time to adapt to this experimental diet (following the introduction of the birds to the respective experimental diets), at week 32 of age. This ultimately culminated in the fish oil (n-3) fed cockerels recording significantly lower ($P < 0.05$) feed intakes during the time interval (Table 4.3). It is not surprising therefore that the disparity regarding feed intake was also reflected in the body weight of the experimental cockerels (Engberg *et al.,* 1996). Fresh oil (n-3) with a peroxide value of less than 1 meq/kg fat, and/or the supplementation with Vitamin E (an anti-oxidant agent) was used by Tavarez *et al.* (2011) to improve the feed intake and body weight gain in chickens. Cabel *et al.* (1988) and Cabel and Waldroup (1989) also reported the need for an increased level of the synthetic antioxidant ethoxyquin for lipid storage, particularly when the environmental temperature was high.

As expected, there was a significant ($P < 0.01$) negative correlation between feed intake of the cockerels and the in-house temperature in all dietary groups e.g. the control ($r = -0.66$), fish oil ($r = -0.52$), sunflower oil ($r = -0.78$), HO sunflower oil ($r = -0.78$), and tallow ($r = -0.81$). Chickens tended to eat less during the warmer weather, in order to reduce the metabolic heat (Ayo *et al.,* 2010). Regardless of the experimental diets being *iso-energetic* (12.4 MJ/kg DM), as shown in Table 3.4, a marginal difference was exhibited in the manner in which cockerels responded to the ambient temperature during the trial. The pattern of feeding was strongly dictated by temperature in those cockerels fed the tallow (SFA) diet, inducing the cockerels to consume less feed during the warm days. Conversely, this trend was not particularly evident in cockerels maintained on the fish oil (n-3) diet.

Many investigations revealed that a relationship exists between dietary and sperm fatty acid profiles, as well as their composition and functionality. These studies show that dietary lipids are deposited proportionately in the sperm (Blesbois *et al.,* 1997; Kelso *et al.,* 1997; Cerolini *et al.,* 2003). This means that regardless of the type of dietary oil consumed, the proportion of the total relative abundance of saturated, mono-unsaturated and polyunsaturated fatty acid groups in the sperm phospholipids do not change - even if the arrangement of fatty acids within a group were altered. Cerolini *et al.* (2006) reported a reduction in the n-6 : n-3 ratio in response to a 100% and 74% increase in the long chain DHA and DPA, respectively in cockerel sperm, following dietary treatment with 1% fish oil, compared to those fed a n-6 rich diet. This change was further reported to be reflected in the sperm functionality, through

a better progressive forward sperm motility of the cockerels maintained on the 1% fish oil diet. This trend was also reported by Bongalhardo *et al.* (2009) in a dietary trial involving maize oil (n-6), fish oil (n-3) and linseed oil (n-3). It was further observed that a higher percentage of n-3 was deposited in the sperm of fish oil fed cockerels, even though the linseed oil diet contained the highest quantity of n-3 fatty acids. This could most probably be explained by the fact that the long chain EPA, DPA and DHA have an effect on sperm motility. This could then explain some of the results recorded in the current study.

Unsaturated lipid sources are generally more commonly used in poultry nutrition than the saturated lipids. This could then possibly be a major reason why current research focuses mainly on the PUFA's as a means of improving chicken semen quality, via dietary manipulation. One striking observation in the present study however was the positive effect of saturated fatty acids on the sperm production in the cockerels (Table 4.3) - in terms of volume and sperm output. The fish oil (n-3) fed cockerels also recorded a lower, although not significant ($P > 0.05$), semen volume, when compared to cockerels maintained on the other unsaturated fatty acid diets. The trend in semen volume, between cockerels that consumed unsaturated fats in the present study, was 0.35 ml for fish oil (n-3), 0.36 ml for sunflower (n-6) and for the HO sunflower (n-9) diets - similar to the 0.34 ml and 0.35 ml reported by Cerolini *et al.* (2006) for 22 to 54 weeks old broiler breeders fed n-3 and n-6 diets, respectively. The present results also bear similar tendencies with the study of Surai *et al.* (2000) with 26 week old Ross broiler breeder cockerels. Similar semen volume values (0.44 ml) were recorded for the maize oil (18:2n-6) and arasco oil (rich in 20:4n-6 and 18:1n-9) treatments, and a lower semen volume (0.43 ml) for the tuna oil (22:6n-3) treatment. Perhaps the slight variation in semen volume between the fish/tuna oil diet and the other unsaturated fatty acid diets in the current and that of cited studies could be ascribed to the relatively higher abundance and activity of the long chain fatty acids such as EPA and DHA, in the sperm cells of cockerels consuming fish oil. The inclusion of the tallow (SFA) diet as one of the experimental diets in the present study, illustrated that a significantly higher ($P < 0.05$) semen volume could be achieved by feeding a highly saturated diet to cockerels, when compared to the feeding of mono-unsaturated or polyunsaturated fatty acid diets. The tallow (SFA) treatment resulted in a higher semen volume (0.42 ml), when compared to the cockerels, maintained on an unsaturated fatty acid diet, in both the current study, as well as that reported by Cerolini *et al.* (2006). Terano *et al.* (1996) reported that in the bid to prevent arteriosclerosis, EPA and DHA help in regulating the proliferation of the smooth muscle cells

- by inhibiting DNA synthesis and replication and suppressing the progression of the synthesis phases in the cell cycle. This activity may also have occurred in the testes of the fish oil (n-3) fed cockerels, with a minimal impact on the proliferation of the Sertoli cells in the testes, and their ability to produce semen. By extension, it could also explain the negative effect of fish oil on sperm concentration, as reported by Cerolini *et al.* (2005; 2006).

Conversely, the tendency for saturated fatty acids to hasten muscle cell proliferation has been documented (Shiina, 1993; Terano *et al.*, 1996; Sudheendran *et al.*, 2010). Studies by researchers revealed that while fish and vegetable oils attenuated smooth cell proliferation, highly saturated lipid sources enhanced coronary smooth muscle cell proliferation (Shiina *et al.*, 1993; Terano *et al.*, 1996). Therefore, it is likely that tallow (SFA) exerted similar proliferation effects on the specialised sperm cells in the testes of cockerels maintained on this type of fat (SFA), during the present study. The prevalent fatty acids in each dietary treatment were believed not only to be deposited, but also to influence the testicular function of the cockerels. The effect may be manifested in semen volume, as early as 3 weeks after the introduction of the dietary treatments at week 32 of age - considering the 17 days duration of spermatogenesis in the cockerel (Senger, 2010). The proliferation effect of high saturated fatty acids concentrations was also recorded by Bermudez *et al.* (2008) in human artery smooth muscle cells, following the consumption of butter (highly saturated fatty acids). This was observed when triglyceride rich particles of these cells were incubated after eating butter (postprandial) for a period of 24 h. It is likely that the action of the tallow (SFA) saturated fat diet on the sperm cells was spontaneous, rather than delayed to the late phase of the trial. A determination of the testicular mass of the experimental cockerels in the present study may have justified this assumption. So for example, a study conducted by Surai *et al.* (2000) also clearly recorded an evidently higher semen volume, resulting from an increased testes mass, following dietary manipulation.

The sperm concentration was not significantly ($P > 0.05$) different between the dietary groups. Although Cerolini *et al.* (2005; 2006) reported sperm concentration to be negatively affected by the dietary n-3 inclusion, the present results agree more with Surai *et al.* (2000) who recorded no significant difference in sperm concentration between cockerels fed maize oil (n-6), arasco oil (20:4n-6 and 18:1n-9) or tuna oil (n-3). Bongalhardo *et al.* (2009) also reported no differences in sperm concentration between cockerels fed maize oil (n-6), fish oil (n-3), or linseed oil (n-3). However, the negative effects of long chain DHA on the total

sperm output reported by Cerolini *et al.* (2006) was confirmed ifsn the cockerels fed a fish oil (n-3) diet, in the current study. Certainly, the potential lipid oxidation characteristic of high inclusion levels of fish oil has been a main challenge and limitation to its use in chicken diets (Kelso *et al.,* 1996). Investigations revealed that increasing the concentration of fish oil would result in the concomitant disruption of the antioxidant system, by reducing the content of Vitamin E in the human and rat tissues. The earlier suspicion that lipid oxidation in fish oil (n-3) treatment may have affected the feed intake of cockerels negatively, was supported by a significantly lower ($P < 0.05$) sperm motility during the present study. Oxygen free radicals resulting from lipid oxidation have been discovered to affect the sperm motility in humans (McLeod, 1943) and chickens (Wales *et al.,* 1959). Similarly, Jones and Mann (1973; 1977b), reported that lipid peroxidation was responsible for a significantly reduced respiration rate and sperm motility in rams. They further incubated ram semen with peroxidized DHA, and recorded the release of the intracellular enzymes. With lactate dehydrogenase (LDH), glutamic oxalacetic transaminase (GOT), hyaluronidase acid, phosphatase and β-N-acetylglucosaminidase being transferred into the surrounding medium. This reportedly caused an irreversible hindrance to the fructolytic and respiratory activity of the sperm cells.

Another interesting finding of Jones and Mann (1977a) was the relationship between the susceptibility of damaged sperm cells to lipid peroxidation and their morphology. Thus, according to Jones and Mann (1977a), not only does the toxicity of lipid oxidation hinder sperm motility, it may also be harmful to the morphology of the sperm. In the present study, the sperm from the fish oil treated (n-3) cockerels recorded a low motility and viability, although the viability of sperm cells was not statistically different ($P = 0.1549$) between treatments. A positive role of DHA on sperm motility has been reported, but so also its susceptibility to sperm peroxidation (Ollero & Alvarez, 2003; Cerolini *et al.,* 2006). This may explain the observed improvement in sperm motility of cockerels fed the control diet in the current study. The control diet being a mix of 1.5% fish oil and 1.5% linseed oil, recorded a higher sperm motility, compared to the 3% sunflower oil (n–6) and the 3% fish oil (n–3) diets. This suggests that the inclusion level of DHA, especially from the fish oil source, is critical in determining either a positive contribution to sperm motility or a negative impact resulting from the lipid peroxidation. Lipid oxidative stress resulted in the same manner on sperm quality, as other common stress factors, such as fasting and extreme ambient temperatures. This negative impact could however be controlled with the supplementation of Vitamin E (Eid *et al.,* 2006). In another report, Lee *et al.* (1999) demonstrated a relationship

between genetically stressed cockerels and a low ejaculation rate following the abdominal massage technique. Likewise, cockerels of the fish oil (n–3) diet in the current trial showed stress symptoms via their low feed intake, as well as their poor semen ejaculation rate. Kabir *et al.* (2007) also reported low sperm motility values concomitantly with a declining feed intake in cockerels. In the current study, low sperm motility recorded was evident in the cockerels that consumed the higher (3%) fish oil (n–3) diet. This indicated that the sperm quality of the cockerels that consumed the fish oil (n–3) diet in the present study could have been compromised during the relatively low feed intake period (week 33 – 40 of age) - as illustrated in Figure 4.3. Blesbois *et al.* (2004) reported the fertility and embryo viability to indeed be lower during the feed adaptation period (week 28 – 31 of age) in male cockerels introduced to a diet containing 4.9% of an equal level of fish oil and extruded wheat, compared to those introduced to a standard soya oil (n-6) diet. This illustrated that the cascade of internal biochemical events culminating in sperm toxicity (due to the oxidation described earlier), may actually have begun externally with lipid peroxidation of the diet, stress, and low feed intake of the birds.

The high sperm motility recorded in the present study for the high oleic sunflower (n-9) fed cockerels, is in agreement with the improved motility reported by Zanini *et al.* (2003) who attributed their results to HO sunflower oil characteristic ability to enhance cellular membrane fluidity. Sperm motility of cockerels fed the tallow (SFA) diet also ranked high, although not statistically different from the sperm motility recorded in the control or the high oleic acid sunflower (n-9) fed cockerels. Relevant studies on saturated fats are not common, as PUFA's are generally used extensively in poultry diets. However, the fact is that the cockerels fed tallow (SFA) in the present study recorded a significantly higher ($P < 0.05$) semen volume, yet a similar sperm concentration to the other dietary treatments. It could be speculated that the tallow fed cockerels had a higher fraction of seminal plasma, compared to cockerels in the other dietary treatments. If this could be verified, the high sperm motility recorded in this group would not be surprising. The seminal plasma of avian species generally have the capacity to protect the sperm from lipid peroxidation (Fujihara & Koga, 1984; Cecil & Baskt, 1993). Surai *et al.* (1998) demonstrated the seminal plasma of cockerels to be able to inhibit spontaneous lipid peroxidation in a concentration dependent manner - even though blood plasma had the opposite effect. An explanation in previous work indicated that the superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity in semen plasma is deployed as a free radical trapping mechanism (Surai *et al*., 1998a). This means

then that the cockerels maintained on the tallow (SFA) diet would produce more seminal plasma, and by implication provide better protection of their sperm against oxidation. Tallow being a saturated fat (SFA), and therefore less prone to lipid peroxidation, when compared to other dietary treatments in this study, may also provide a sustainable environment for this beneficial trapping activity in the seminal plasma lipids of the chicken.

Interestingly, differences were recorded in the ejaculation rate of cockerels for all five dietary treatments, during the 12 week semen collection period and analyses. The ejaculation rate (%) being representative of the responsiveness of the 10 cockerels in each dietary treatment to abdominal massage, and subsequent ejaculation of semen, void of faecal contamination. The highest semen ejaculation rates (94.2%) were recorded by the cockerels in the HO sunflower (n–9) and tallow (SFA) treatment groups. In other words, these cockerels failed to (i) ejaculate or (ii) ejaculated faecal contaminated semen, only 7 out of the total 120 times following abdominal massage during Trial 1. The cockerels maintained on the control diet either failed to ejaculate or produced faeces-contaminated semen in 15 out of the 120 times, representing an ejaculation rate of 87.5%. Similarly, the cockerels fed a sunflower oil (n–6) diet recorded an ejaculation rate of 88.3% - meaning that the cockerels failed to ejaculate or produce contaminated semen in 14 out of the 120 times, following abdominal massage. The lowest ejaculation rate (79.2%) was recorded in the cockerels that consumed the pure fish oil (n-3) diet. This group of cockerels failed to ejaculate or they produced contaminated semen in 25 out of the 120 abdominal massages.

The ejaculation rates of cockerels in this experiment (Trial 1) may suggest that feed intake could be a potential source of stress to the cockerels. Resulting in cockerels recording either a high frequency of no semen ejaculation, or the occurrence of faeces contaminated semen. The present results are in agreement with that of Kabir *et al.* (2007), who showed a gradual decline in the rate of semen ejaculation with an increased feed restriction program for cockerels. Although the ejaculation rate recorded for cockerels in the fish oil (n–3) treatment (79.2%) was similar to the 74.6% and 73.2% reported by Hudson and Wilson (2003) for menhaden (fish) oil and poultry fat fed to cockerels in a fertility test - other management factors, barring the diet, may also be responsible for the relative lower cockerel ejaculation rates recorded. Lee *et al.* (1999) admitted that the lack of “massage experience” did affect the responsiveness of the young cockerels in the trial - when compared to older cockerels with more massage experience. In the present trial, efforts were made to ensure that all factors that

could influence the responsiveness of the cockerels to massage were eliminated, except for the dietary treatment. So for example, the semen collection procedure was performed on all cockerels by the same technician, and all birds were housed under similar conditions and in the same environment. Also cockerels were trained every other day for an 8 week period before the introduction of the experimental diets and the subsequent semen collection and recording of ejaculation rates. Adherence to strict management practices and semen collection procedures may therefore be responsible for the general high ejaculation rates (± 85%) recorded in the experimental cockerels. In a natural flock, stressful cockerels have been implicated in poor reproductive performance (Craig *et al.,* 1983; Shabalina, 1984).

Although not determined in the current study, stress generally has a profound impact on the endocrine system - the hypothalamus-pituitary-adrenal axis and thyroid gland in particular (Siegel, 1980). However, specific trials are required to understand the biochemical mechanisms of the dietary fatty acid induced stress on the cockerel's reproductive system. It has been revealed that differences do occur in the Vitamin E (anti-oxidant) content of the body tissues (Surai *et al.,* 1998b) and different brain regions (Surai *et al.,* 1998a) of a day old chick. Following this observation it was concluded that the highly unsaturated brain tissue (cerebrum) would be more susceptible to peroxidation under stress conditions (Surai, 1998).

The effect of dietary lipid source on the viable sperm proportion and sperm abnormalities are set out in Table 4.4. Dietary treatment had no effect ($P > 0.05$) on percentage of viable (live-normal) sperm, as well as the total sperm abnormalities. Data in Table 4.4 were calculated on the basis of the live sperm (with each parameter value representing a fraction of the percentage live sperm), while the total (200) sperm cells were used in calculating the percentage live sperm cells (Table 4.3). Dietary treatments affected ($P < 0.05$) the prevalence of all types of sperm abnormalities, except for the swollen head category. Swollen heads appeared to be the most occurring sperm abnormality in the cockerels maintained on all the dietary treatments - with the exception of those that consumed the fish oil (n–3) diet. The bent neck sperm abnormality was most prominent ($P < 0.05$) in the sperm of cockerels fed the high oleic (n–9) diet, while least dominant in the sunflower (n–6) fed cockerels. Cockerels that consumed the control, fish oil (n–3), and tallow (SFA) diets recorded mid-piece abnormalities that were significantly higher ($P < 0.05$) than those birds fed the sunflower (n–6) diet.

Table 4.4 The effects (LSM ± S.E.M) of dietary lipid source on the morphological characteristics (%) of cockerel sperm

Parameter (%)	Control	Fish oil (n – 3)	Sunflower oil (n – 6)	HO Sunflower oil (n – 9)	Tallow (SFA)	Significance (*P*)
†Viable sperm	92.3 ± 0.47	91.8 ± 0.68	93.4 ± 0.31	91.9 ± 0.40	92.5 ± 0.37	0.1161
†Sperm abnormalities						
Swollen head	2.41 ± 0.24	2.22 ± 0.21	2.38 ± 0.21	2.61 ± 0.22	2.35 ± 0.20	0.6089
Bent neck	1.64^{ab} ± 0.18	1.33^{b} ± 0.13	1.42^{b} ± 0.15	1.84^{a} ± 0.17	1.64^{ab} ± 0.16	0.0339
Mid-piece	1.40^{a} ± 0.12	1.21^{a} ± 0.12	0.90^{b} ± 0.09	1.17^{ab} ± 0.11	1.35^{a} ± 0.15	0.0223
Other	2.25^{b} ± 0.28	3.40^{a} ± 0.56	1.89^{b} ± 0.18	2.48^{b} ± 0.29	2.14^{b} ± 0.18	0.0150
Total abnormalities	7.70 ± 0.47	8.16 ± 0.68	6.59 ± 0.31	8.09 ± 0.41	7.47 ± 0.37	0.1161

^{a,b} Means in rows with different superscripts differ significantly at $P < 0.05$

† Calculated as a percentage of live sperm count

In the other undefined sperm abnormalities, cockerels maintained on fish oil (n–3) diet recorded a significantly higher ($P < 0.05$) value of 3.4%, compared to the other dietary treatments.

In Table 4.5 the correlation between the different semen parameters in cockerels fed diets with varying types of lipid sources during trial 1 (33 - 46 weeks of age), are set out. The total sperm output (cockerels in all treatments) exhibited a significantly high correlation with semen volume ($P < 0.001$) ($r = 0.87, 0.87, 0.90, 0.91, 0.93$) and sperm concentration ($P < 0.05$) ($r = 0.46, 0.58, 0.64, 0.61, 0.55$) for the control, fish oil (n–3), sunflower oil (n–6), HO sunflower oil (n–9) and tallow (SFA) diets, respectively. Semen volume in the tallow treatment recorded the highest ($P < 0.001$) correlation with sperm output ($r = 0.93$). Sperm output was then negatively correlated to sperm motility in the tallow fed cockerels, and showed a low, non-significant positive correlation in the cockerels of the other dietary groups. Semen volume was significantly correlated to sperm concentration in all the treatments, except the n-3 type diets (fish oil and control treatments). The highest correlation of $r = 0.30$ ($P < 0.01$) was recorded in the sunflower (n–6) diet, followed by $r = 0.28$ for the HO sunflower (n–9) treatment. Semen volume and sperm concentration in the tallow treatment group was also found to be significantly ($P < 0.05$) correlated ($r = 0.25$). A significant ($P < 0.01$), but low correlation was recorded between semen volume and sperm motility in the cockerels that consumed the HO sunflower oil (n–9) supplemented diet. Similar for the cockerels maintained on the n-3 diets ($P < 0.05$), while negative, with a non-significant correlation ($P > 0.05$) being recorded in the tallow fed cockerels. Sperm viability was not significantly correlated ($P > 0.05$) to sperm concentration in all dietary treatments - although negative correlation values were recorded in the cockerels maintained on the sunflower oil (n–6) and HO sunflower oil (n–9) treatments. Sperm viability was positive and significantly correlated ($P < 0.01$) to motility, only in cockerels fed the HO sunflower diet. Most of these correlations however recorded low values and no clear trend was observed, making the relationships difficult to explain.

Table 4.5 Correlations between semen parameters of cockerels (33 – 46 weeks of age) fed different supplemental dietary lipid sources

	Sperm output	Volume	Viability
Semen volume			
Control (n – 3)	0.87***		
Fish oil (n – 3)	0.87***		
Sunflower (n – 6)	0.90***		
High oleic (n – 9)	0.91***		
Tallow	0.93***		
Sperm concentration			
Control	0.58 **	0.17	0.10
Fish oil (n-3)	0.46**	0.04	0.12
Sunflower (n-6)	0.64**	0.30**	-0.15
High oleic (n-9)	0.61**	0.28**	-0.05
Tallow	0.55**	0.24*	0.13
Sperm motility			
Control	0.14	0.25*	-0.18
Fish oil (n-3)	0.18	0.22*	-0.04
Sunflower (n-6)	0.07	0.07	0.28**
High oleic (n-9)	0.17	0.28**	0.17
Tallow	-0.11	-0.07	0.07

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

A number of correlations for the sperm parameters determined in the present study are in agreement with those of Bongalhardo *et al*. (2009). The highly significant correlations of sperm output with semen volume and sperm concentration (Table 4.5) was not induced by the dietary treatments, but occurred as a result of these two parameters being the indices of sperm output. Similar to the present study, Bongalhardo *et al.* (2009) also found the total sperm output to be highly correlated with semen volume and sperm concentration, irrespective of the dietary lipid sources (maize-, fish- and flaxseed oil). Semen volume showed a more pronounced correlation with sperm output, compared to the sperm concentration in all the dietary treatments. However, where semen volume was only correlated with sperm concentration in the pre-trial diet of Bongalhardo *et al.* (2009), these parameters recorded significant correlations in all the treatments, except the two n-3 diets in the present study. Results regarding the correlation between sperm viability and motility are contradictory to that of Bongalhardo *et al*. (2009). Sperm viability was only significantly ($P < 0.01$) correlated ($r = 0.28$) to sperm motility in the sunflower (n-6) treatment group. The sperm viability data used in this correlation was only for that of the normal live sperm. It was observed that during the course of the study sperm motility was sometimes very high, even for samples in which high sperm abnormalities were later recorded. These samples could be identified following

critical assessment under the microscope during the sperm motility count. Further, the results of the present study regarding the absence of a significant correlation between sperm viability and concentration is in agreement with that of Bongalhrdo *et al.* (2009).

The results regarding the correlation coefficients between semen volume, body weight and feed intake are presented in Table 4.6. There were no significant correlations between body weight and semen volume in all the treatment groups. The overall correlation (r = 0.03) between these two parameters was also non-significant (P = 0.818). The correlation between body weight and feed intake however showed a significant relationship in the control (r = 0.49, P = < 0.001), high oleic sunflower (r = 0.39, P < 0.01), and tallow (r = 0.61, P < 0.0001) groups. The overall correlation between feed intake and body weight (r = 0.39) was also significant (P < 0.01), when correlations for treatments were pooled. Further, Table 4.6 demonstrated a weak (P > 0.05) correlation between feed intake and semen volume in all treatments, resulting in an overall poor, but significant correlation (r = 0.14, P < 0.05) between these two parameters.

Table 4.6 Correlations between body weight, feed intake and semen volume of cockerels (33 – 46 weeks of age) fed diets with different supplemental lipid sources

	Body weight	**P value***	**Feed intake**	**P value#**
Semen volume				
Control (n – 3)	0.25	0.0760	0.25	0.0747
Fish oil (n – 3)	0.12	0.3827	0.06	0.6573
Sunflower (n – 6)	-0.11	0.4310	-0.04	0.7684
High oleic (n – 9)	0.03	0.8183	0.09	0.5193
Tallow	0.13	0.3288	0.24	0.0748
Overall	0.03	0.818	0.14	0.0242
Feed intake				
Control	0.49	<0.0001		
Fish oil (n-3)	0.23	0.0724		
Sunflower (n-6)	0.17	0.1890		
High oleic (n-9)	0.39	0.0022		
Tallow	0.61	<0.0001		
Overall	0.39	0.0022		

P value* Significance level of body weight *vs.* feed intake and semen volume
P value# Significance level of feed intake *vs.* semen volume

4.3.4 Semen fatty acid methyl esters

The effect of dietary lipid sources on sperm fat free methyl esters (FAME) of cockerels (Table 4.7) and other sperm properties such as percentage fat, fat free dry matter (FFDM), moisture content (%) and the concentration of total fatty acids and the fatty acid ratios (Table 4.8) are summarised in the respective tables. Due to the small volume of the ejaculate (≤ 0.5 ml) for most of the individual cockerels and the low fat concentration of the cockerel sperm (± 0.6%), it was not physically possible to collect sufficient semen from cockerels for the analysis of FAME on an individual basis. Semen collected from individual birds within a dietary treatment were pooled and used for fat extraction and FAME analysis in duplicate. Data set out in Table 4.7 and Table 4.8 are therefore parameter means for the duplicated samples and were not statistically analyzed. Although it was not possible to perform the statistical analysis of sperm fatty acid data, it is evident that the dietary lipid source had an effect on the alteration of the sperm FAME, especially those fatty acids belonging to the PUFA n-3 and n-6 groups. Furthermore, fatty acids set out in cockerel sperm (Table 4.8) corresponded, to a limited extent, to the specific individual fatty acids (Table 4.1) and the types of fatty acids (Table 4.2) in the dietary treatments.

Generally, aging was reported to induce some changes in lipid concentration and fat phospholipid components in the cockerel spermatozoa fractions. When comparing sperm lipid changes of cockerels between 25 and 60 weeks of age, Kelso *et al.* (1996) reported an increase from 574 to 1122 µg per 10^9 spermatozoa in total fat. Similarly, changes were also reported to be accompanied by a slight increase in the sperm phospholipids, with its unsaturated fractions (phosphatidyl ethanolamine) being replaced with a more saturated fraction (phosphatidyl choline). However, studies have revealed clearer absolute and proportional changes in the sperm fatty acids concentrations following dietary lipid treatment (Blesbois *et al.*, 1997, 2004; Kelso *et al.*, 1997; Cerolini *et al*., 2006). From Table 4.7, it is clear that the concentration of sperm fatty acids are biologically regulated, considering the strict adjustment of the fatty acids within the sperm, as compared to the wider variation that exists in the dietary treatments (Table 4.1). With the exception of the tallow (SFA) fed cockerels, the concentration of stearic acid was evidently high in the sperm (17.7 – 18.1%), despite its low concentration in the unsaturated dietary treatments (3.4 - 4.5%) (Table 4.1). Similarly, the concentration of unsaturated fatty acids such as oleic (C18:1n-9) and linoleic

(C18:2n-6) acids were lower in the sperm (Table 4.7), compared to those in the other respective dietary treatments (Table 4.1).

The dietary linoleic acid is mostly converted to arachidonic and docosatetraenoic acid in the cockerel sperm. The amount of sperm α-linoleic acid (C18: 3n-3) was however higher than its dietary concentrations in all treatments, apart from the control diet, which had an excessively high concentration (15.4%, see Table 4.1) of this particular (C18:3n-3) fatty acid. The specificity of fatty acid deposition into sperm observed in the present study, agrees with previous reports of the dietary lipid effects (n-3 and n-6 in particular) on cockerel sperm (Blesbois *et al.*, 1997; Kelso *et al.*, 1997), as well that of toms (Blesbois *et al*., 2004).

Interestingly, the most dominant fatty acid (from 18% to 25.9%) in the sperm of all treatments was docosatetraenoic acid (DTA; C22:4). Although DTA was not detected in the sunflower (n-6), high oleic acid sunflower (n-9) and the tallow (SFA) diets, (and only in low quantities in the control (0.41%) and the fish oil (0.66%) diets) its high concentration in the cockerel sperm (18 - 25.9%) is worth mentioning. Docosatetraenoic acid (DTA) was however more prevalent in the sunflower (n-6), high oleic sunflower (n-9) and the tallow diets, compared to the two n-3 diets (control and fish oil treatments). The peculiarity of DTA in cockerel sperm necessitated its recommendation as a useful marker for the evaluation reproduction efficiency in cockerels (Cerolini *et al*., 2005). Also in the present study, the specific long chain n-3 fatty acids (docosapentanoic and docosahexanoic acids) were recorded to be particularly higher in the spermatozoa of cockerels fed the n-3 diets (control and fish oil), compared to the other treatments (Table 4.7). The relatively higher amount of DPA and DHA in these two n-3 treatments could be a result of the conversion of the precursor α-linolenic acid (C18:3) - as the dietary concentrations of both DPA (control = 0.41; fish oil = 0.66%) and DHA (control = 1.38; fish oil = 2.57%) were lower than the amount analysed in their respective spermatozoa (Table 4.7). Observations previously reported were for cockerels fed 6% linseed oil (Kelso *et al.*, 1997) and toms fed 5% supplemental fish oil diets (Blesbois *et al.*, 2004).

Table 4.7 Mean effect of dietary lipid sources on the fatty acid methyl esters (FAME[#]) of cockerel semen at week 78 of age

	Control	Fish oil (n-3)	Sunflower oil (n-6)	HO[1] Sunflower oil (n-9)	Tallow (SFA)
FAME[#] (% of total fatty acids)					
Saturated fatty acids					
C14:0 Myristic	0.39	0.42	0.35	0.39	0.45
C16:0 Palmitic	17.16	17.68	16.38	17.05	17.34
C18:0 Stearic acid	17.47	18.00	18.10	17.78	17.73
C20:0 Arachidic	0.45	0.46	0.43	0.48	0.44
C22:0 Behenic	0.48	0.43	0.46	0.48	0.51
C24:0 Lignoceric	4.10	5.28	2.01	2.98	2.67
Mono-unsaturated fatty acids (n-9)					
C16:1 Palmitoleic	0.45	0.51	0.37	0.38	0.49
C18:1 Oleic	14.23	13.67	11.84	13.35	12.65
C24:1 Nervonic	0.95	1.09	0.93	1.20	0.97
Polyunsaturated fatty acids (n-6)					
C18:2 Linoleic	3.05	2.13	4.05	2.62	2.61
C20:4 Arachidonic	8.30	7.18	10.84	10.69	10.40
C22:4 Docosatetraenoic	18.49	18.00	25.85	25.18	24.67
Polyunsaturated fatty acids (n-3)					
C18:3 α-Linolenic	2.38	2.43	2.12	2.51	2.30
C20:3 Eicosatrienoic	1.67	1.46	1.38	1.19	1.32
C20:5 Eicosopentaenoic	0.43	0.40	0.35	0.43	0.37
C22:5 Docosapentaenoic	3.05	3.83	0.16	0.21	0.28
C22:6 Docosahexanoic	2.97	3.95	0.28	0.35	0.45

[1] High oleic acid sunflower oil

Table 4.8 Mean effect of dietary lipid sources on the total fatty acid concentration (%) and fatty acid ratios of cockerel semen at week 78 of age

	Control	Fish oil (n-3)	Sunflower oil (n-6)	HO[1]Sunflower oil (n-9)	Tallow (SFA)
Sperm properties (%)					
Fat content	0.57	0.57	0.69	0.71	0.55
Fat free dry matter	5.75	5.64	7.01	6.81	5.28
Moisture	93.68	93.79	92.30	92.47	94.17
Total fatty acid concentration (%)					
ΣSaturated fatty acids	40.26	42.49	37.94	39.37	39.45
ΣMono-unsaturated fatty acids (n-9)	18.64	17.51	16.13	16.70	17.41
ΣPolyunsaturated fatty acids (n-6)	30.61	27.93	41.65	39.24	38.42
ΣPolyunsaturated fatty acids (n-3)	10.49	12.07	4.29	4.69	4.72
ΣPolyunsaturated fatty acids (n-6 & n-3)	41.10	40.00	45.94	43.93	43.14
ΣUnsaturated fatty acids	59.74	57.51	62.06	60.63	60.55
Fatty acid ratios					
PUFA:SFA	1.02	0.94	1.21	1.12	1.09
MUFA/SFA	0.46	0.41	0.43	0.42	0.44
UFA/SFA	1.48	1.35	1.64	1.54	1.53
MUFA/PUFA	0.45	0.44	0.35	0.38	0.40
n-6/n-3	2.92	2.31	9.72	8.37	8.13
n-9/n-6	0.61	0.63	0.39	0.43	0.45
n-9/n-3	1.78	1.45	3.76	3.56	3.68

[1] High oleic acid sunflower oil

Except in the tallow (SFA) fed cockerels, higher amounts of saturated fatty acids were recorded in the cockerel sperm (Table 4.8), compared to their respective dietary treatments (Table 4.2). An observation also reported in cockerels fed 5% maize oil (n-6) or fish oil (n-3) diets (Blesbois *et al*., 1997), as well as in turkey males fed a standard diet (n-6) or 1% fish oil (n-3) diet (Blesbois *et al.,* 2004). Saturated fatty acids are generally seen as "non-essential" and its endogenous synthesis in the cockerel's body could have therefore complemented the amount supplied by the exogenous dietary sources. In spite of the increase in the sperm saturated fatty acids, its proportion was low in relation to the concentration of unsaturated fatty acids (Table 4.8). The most evident proportional modifications of fatty acids occurred within the polyunsaturated fraction, particularly the n-6 : n-3 ratio. The conversion of the α-linolenic acid (C18:3n-3) to its longer derivatives within the sperm of the n-3 fed cockerels, seemed to have a simultaneous negative effect on the synthesis of the longer chain n-6 derivatives, such as AA and DTA from the precursor linoleic acid (C18:2n-6) - culminating in a lower n-6 : n-3 ratio analysed in the control and the fish oil fed cockerels. In short, results obtained in the present study agree with previous studies investigating the effects dietary lipid sources on poultry sperm fatty acid composition - especially the specificity of the sperm and the modification of its polyunsaturates (Blesbois *et al.,* 1997, 2004; Kelso *et al.,* 1997; Cerolini *et al*., 2003, 2006).

4.3.5 The effects of dietary lipid sources on the performance of cockerels at the end-of-lay period in Trial 2

Table 4.9 summarizes the results of Trial 2, which was conducted between the ages of 69 and 77 weeks - a productive phase, generally associated with a decline in breeder flock fertility and defined as the end-of-lay stage. The daily feed consumption of the cockerels was not significantly different ($P = 0.1907$), as set out in Table 4.9. The dietary lipid source also had no significant effect ($P = 0.1361$) on the percentage of live sperm between cockerels. However, other variables such as body weight ($P < 0.01$); semen volume ($P < 0.01$) and sperm motility ($P < 0.05$) were statistically influenced by the dietary lipid sources. Cockerels that consumed a fish oil (n-3) diet recorded a significantly lower ($P < 0.01$) body weight (2482 g), when compared to the cockerels in the other dietary treatments (whose body weights were similar). The variation in body weight could not have been a result of feed intake (no significant difference between treatments) during the period under consideration. This could be related to differences occurring in the dietary fatty acid profiles. Even then, the prevalence of the particular fatty acid needs to be considered. Regardless of the control and

fish oil (n-3) diets having similar fatty acid profiles (Table 4.1). The fact that the assigned cockerels consumed the same amount of feed (Table 4.9), the control fed cockerels however, still maintained similar body weights to the cockerels on the other experimental treatments (sunflower oil, HO sunflower oil and tallow). The high level of fish oil inclusion in the fish oil (n-3) diet (taste) may therefore have resulted in this lower body weight. This could possibly be ascribed to the increased concentration of EPA and DHA in the body tissue of the cockerels.

Both eicosopentaenoic and docosahexaonic acid have been noted for cell regress and anti-lipid accumulation abilities, which will ultimately also help in the production of leaner meat (Sudheendran *et al.*, 2010). The semen volume of the cockerels was also affected ($P < 0.05$) by the dietary treatments. The tallow (SFA) treatment resulted in the highest ($P < 0.01$) semen volume, compared to all the other dietary treatments. This observation regarding the higher semen volume recorded for the tallow treatment was similar to that in the first trial (35 – 46 weeks of age). Sperm motility was also affected ($P < 0.05$) by dietary treatments, with the cockerels in the tallow treatment attaining the lowest sperm motility (51.6%). The semen ejaculation rate for each cock was recorded and calculated throughout the course of the nine week trial period. Unlike Trial 1, only marginal differences in the ejaculation rate of the cockerels occurred between the dietary groups. So for example, only two ejaculation failures separated the highest from the lowest group in terms of ejaculation rate. The lowest semen ejaculation rate of 95.5% was recorded by the cockerels fed the control diet, while the highest rate of 97.8% was recorded in the cockerels fed the sunflower oil (n-6) diet. The cockerels allocated to the other three treatments were intermediate, recording a similar ejaculation rate of 96.7%. This means that the cockerels fed the three diets namely fish oil (n-3), high oleic acid (HO) sunflower oil (n-9) and tallow (SFA), failed to ejaculate or produced contaminated semen only in 3 out of the 90 massage procedures. The sunflower (n-6) fed cockerels failed only twice, while the control cockerels failed 4 out of the 90 times.

Table 4.9 The effects (LSM ± S.E.M) of dietary lipid source on the performance and semen quality of cockerels between 69 and 77 weeks of age

Parameter	Control	Fish oil (n – 3)	Sunflower (n – 6)	HO Sunflower (n – 9)	Tallow (SFA)	Significance (*P*)
Daily feed intake (g)	90.56 ± 1.02	90.55 ± 0.92	92.52 ± 0.52	92.46 ± 1.05	91.65 ± 1.26	0.1907
Body weight (g)	2600.2^{a} ± 23.1	2482.1^{b} ± 20.1	2575.6^{a} ± 31.4	2599.4^{a} ±30.7	2600.5^{a} ± 31.2	0.0027
Semen volume (mL)	0.39^{b} ± 0.015	0.36^{b} ± 0.019	0.39^{b} ± 0.012	0.39^{b} ± 0.013	0.44^{a} ± 0.021	0.0082
Sperm motility (%)	55.8^{ab} ± 2.20	56.7^{a} ± 2.03	57.9^{a} ± 2.06	58.7^{a} ± 1.76	51.6^{b} ± 1.86	0.0443
Live sperm (%)	87.9 ± 0.46	88.6 ± 0.42	89.5 ± 0.49	88.7 ± 0.47	88.4 ± 0.57	0.1361
Ejaculation rate (%)	95.5 ± 0.02	96.7 ± 0.02	97.8 ± 0.01	96.7 ± 0.02	96.7 ± 0.02	0.2701

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

The aim of the second trial was to evaluate the effects of the dietary lipid sources on the reproductive performance of older (69 – 77 weeks of age) cockerels. Thus, during a phase in their lifetime when cockerels are generally considered as having passed their optimal performance (loss of e.g. mating interest), in a natural flock mating system. Sperm phospholipids characteristics have been reported to change in response to fatty acid treatments in aging cockerels (Cerolini *et al.,* 1997; Kelso *et al.,* 1997). The proportion of saturated, mono-unsaturated and polyunsaturated fatty acids in the sperm phospholipid fraction however did not change in frequency as the cockerels aged, dietary treatments notwithstanding. The main PUFA in cockerel sperm recorded was C22:4n-6, and with the existence of n-6 prevalence over the n-3 PUFA's, unlike in mammalian sperm, where the n-3 type fatty acids was found to dominate the n-6 fatty acids (Nissen *et al.,* 1981). Thus, what researchers have been able to achieve through dietary manipulation, was the alteration (not substituting) of the n-6 : n-3 ratio in avian sperm - which has been demonstrated to be particularly pronounced in older birds, and positively correlated with fertility (Blesbois *et al.,* 1997, 2004). Results obtained in the present study agree with these researchers in this regard.

In the current trial (Trial 2), no symptoms of oxidative stress were recorded for the cockerels maintained on the fish oil (n-3) diet - as feed intake, ejaculation rate and semen quality were recorded not to be inferior to that obtained from cockerels on the other dietary treatments. This may be indicative of the better environmental weather conditions (Figure 4.1 and Figure 4.2) and aging of the cocks. Higher fertility rates have been reported by Blesbois *et al.* (2004) in older turkey toms, corresponding to periods when lower n-6 : n-3 ratios were recorded in the sperm of fish oil fed toms.

Although no significant difference was recorded between treatments in terms of semen volume, a significant difference ($P < 0.05$) did occur between the unsaturated- and the saturated fatty acid diets. The inflammatory effect of the saturated fatty acids on the smooth muscle cells, and perhaps on the Sertoli cells and testicular function (as described under section 4.3.3), may have been responsible for the higher semen volume in the tallow (SFA) treatment group, even at this later age of their production cycle (Surai *et al.*, 2000; Senger, 2010).

Sperm motility was the lowest ($P < 0.05$) for the tallow fed diet group. The concentration of C16:0 and C18:0 fatty acids were recorded to be negatively correlated with sperm motility (Cerolini *et al.,* 1997). Sperm content of these saturated fatty acids were also shown to be positively correlated with the age of the cockerels. Although the tallow (SFA) diet had the highest ($P < 0.0001$) concentration of saturated fatty acids (40.7% - see Table 4.2), the sperm FAME analysis showed that cockerels treated with tallow deposited a relatively lower amount of saturated fatty acid in their spermatozoa, compared to cockerels fed polyunsaturated fatty acids (Table 4.8). It is therefore difficult to associate the relatively lower sperm motility recorded in the tallow (SFA) fed cockerels with the level of sperm saturated fatty acid concentration. Conversely, the fish (n-3), sunflower (n-6) and HO sunflower oil (n-9) diets resulted in the best sperm motility during this mature reproductive phase, in the second study. Generally, a marked reduction was recorded in the C22n-6 sperm content of cockerels older than 60 weeks of age - even when fed the same standard layer diet (Cerolini *et al.,* 1997). The decrease in the C22n-6 was further confirmed by Blesbois *et al*. (2004) to be more prominent at 50 weeks of age for birds receiving an n-6, or an n-3 diet, compared to when birds were 34 weeks of age. The marginal decrease in the n-6 : n-3 ratio may have been responsible for the high sperm motility recorded in the cockerels fed the sunflower (n-6) diet. Blesbois *et al.* (2004) further associated the high fertility rate recorded during 50 – 60 weeks of age, with a decline in the n-6 : n-3 ratio, due to the more positive effects induced by the fish oil diet. The high sperm motility recorded for cockerels fed fish oil (n-3) may therefore be attributed to the better utilization of the fish oil diet by the older cockerels - as reflected by the improved feed intake. This assertion was further emphasized in the hatchability and fertility test conducted in the present study. Feed intake of the cockerels was higher in all treatments (Table 4.9); compared to a younger age (Table 4.3) when treatments were first introduced to the birds. Even the significantly lower ($P < 0.05$) feed intake recorded for the fish oil (n-3) fed cockerels in Trial 1 was observed to have been nullified in the trial.

All parameters measured in Trial 2, including the ejaculation rates, were superior to the same parameters measured in Trial 1, except for sperm viability in that a lower percentage live sperm was recorded in Trial 2, compared to Trial 1. The present study agrees with Kelso *et al.* (1996) regarding the lower percentage of live sperm in the older cockerels (86.1%), compared to younger cockerels (91.2%). These values are comparable to Trial 2 and Trial 1 in respective age phases.

This variation could only be explained by the better utilization of the diets by the aging birds, and/or seasonal variation. Trial 1 was conducted during the summer (high environmental temperature), whereas Trial 2 was conducted on the same birds during the winter (low environmental temperature). Van der Berghe *et al.* (1990) and Ruiz *et al.* (2000) reported that fats may be unstable at 30 ºC. High temperatures could also reduce the potency of feed antioxidants, thereby making the additives susceptible to oxidation. The potential hazard of a high environmental temperature (≥ 30 ºC) on stored broiler feed was further demonstrated by Njobeh *et al.* (2006), where a loss in anti-oxidation potency within one month, as well as increased free fatty acids concentrations ($P < 0.001$, $R^2 = 0.84$) and peroxide values ($P > 0.001$, $R^2 = 0.89$) after two months, were reported. Bird management, including the photoperiod schedule, feed allocation, massage technique, staff management and other semen evaluation procedures were identical for both trials. Apart from the tested dietary effects therefore, seasonal variation and aging of the cockerels were factors that could have also affected the cockerel sperm quality measured in the present study. Kelso *et al.* (1996), when comparing lipid changes in the spermatozoa of aging cockerels (25 *vs.* 60 weeks of age), reported a decrease in n-6 fatty acids and simultaneous increase in n-3 fatty acids in older cockerels, especially, when the arachidonic- (AA) and docosatetraenoic acid (DTA) concentration in spermatozoa were very high at 25 weeks of age (28.9 and 33.6% respectively). The concentrations of these fatty acids were reduced to 14% and 23.1% respectively when measured at 60 weeks of age. On the other hand, the levels of docosapentaenoic- (DPA) and docosahexanoic (DHA) acids were reported to be 6.19% and 5.8% respectively, in the sperm at 60 weeks of age - a remarkable improvement considering their non-detection in the younger cockerels (25 weeks of age).

Ayo *et al.* (2010) associated the voluntary decrease in feed intake during hot weather as a mechanism to reduce the metabolic heat in chickens. This is generally manifested with a consequent negative effect on the seminiferous cell differentiation, resulting in decreased semen quantity and quality (Edens, 1983; McDaniel *et al*., 1996). Also, the impairment of sperm motility of previously highly ranked cockerels by Karaca *et al.*, (2002) was related to the resultant low intracellular Ca^{2+} seminal plasma levels, which the researchers ascribed to heat stress.

4.3.6 Effects of dietary lipid sources on the fertility and hatchability of an old layer flock

The results presented in Table 4.10 summarise the fertility and embryonic mortalities of 69 – 77 week old layers (♂, ♀), which were fed diets enriched with different lipid sources. The egg weight (62.5 g) of the sunflower oil (n-6) treatment was higher ($P < 0.001$), compared to the other dietary treatments. Conversely, the lowest ($P < 0.001$) egg weights (59.3 g) were produced by the hens fed fish oil (n-3), while hens in the control, high oleic acid sunflower oil (n-9) or tallow (SFA) treatments produced intermediate egg sizes. Parameters measured during the early stages ($P < 0.05$) (fertility and early deaths) and the middle stage ($P < 0.01$) of incubation were affected by the dietary treatments. Nonetheless the same could not be said of the effects of diet during the latter stages ($P = 0.197$). The highest ($P < 0.05$) egg fertility (%) was recorded in the control and the HO sunflower oil (n-9) group - both 92.1%, followed by the sunflower oil (n-6) (89.6%) treatment. The lowest fertility (84.6%) occurred in the fish oil (n-3) treatment group. The fish oil (n-3) treatment however performed better regarding early embryonic mortalities, where the lowest ($P < 0.05$) percentage (2.8%) of early deaths was recorded. The highest early embryonic deaths were recorded in the sunflower oil (n-6) treatment and HO sunflower oil (n-9) groups, at 8.1 and 6.8% respectively.

The highest ($P < 0.01$) mid-incubation (8 – 18 days) mortalities occurred in the sunflower oil (n-6) (17.0%) and the control (16.3%) treatments. The fish oil (n-3) treatment still performed better than any of the other diets at this stage, yielding the lowest embryonic mortalities (9.3%), followed by the tallow (SFA) treatment (10.4%). As pointed out earlier, the number of embryonic deaths that occurred during the late incubation phases (19 – 21 days) did not differ ($P = 0.197$) significantly.

The hatchability, expressed as both a percentage of (i) fertile eggs and (ii) total eggs set were affected ($P < 0.005$) by the dietary treatments. The fish oil (n-3) treatment resulted in the lowest embryonic mortalities at all stages of embryonic development and recorded the highest hatchability percentage (75.9%) of the fertile eggs. The lowest hatchability (58.2%) of the fertile eggs was recorded for the sunflower (n-6) treatment, while intermediate hatchability results were recorded for the other dietary treatments.

Table 4.10 Effects (LSM ± S.E.M) of dietary lipid sources on the fertility and hatchability (%) of eggs obtained from layers between 69 and 77 weeks of age

	Control	Fish oil (n–3)	Sunflower (n–6)	HO sunflower (n–9)	Tallow (SFA)	Significance (*P*)
Egg weight (g)	$61.56^{b} \pm 0.25$	$59.25^{c} \pm 0.21$	$62.49^{a} \pm 0.22$	$61.06^{b} \pm 0.23$	$60.96^{b} \pm 0.24$	< 0.001
Fertile (%)	$92.14^{a} \pm 1.43$	$84.57^{c} \pm 1.90$	$89.63^{ab} \pm 1.64$	$92.09^{a} \pm 1.43$	$86.28^{bc} \pm 1.82$	0.008
Early deaths (%)	$4.78^{abc} \pm 1.13$	$2.81^{c} \pm 0.87$	$8.07^{a} \pm 1.46$	$6.78^{ab} \pm 1.33$	$4.78^{bc} \pm 1.13$	0.022
Mid-deaths (%)	$16.29^{a} \pm 1.96$	$9.27^{b} \pm 1.54$	$17.00^{a} \pm 2.02$	$13.84^{ab} \pm 1.83$	$10.36^{b} \pm 1.62$	0.004
Late deaths (%)	10.39 ± 1.62	6.81 ± 1.27	9.80 ± 1.60	7.63 ± 1.41	7.56 ± 1.40	0.197
Pips (%)	2.25 ± 0.79	1.97 ± 0.73	3.17 ± 0.94	3.67 ± 1.00	1.96 ± 0.73	0.499
Hatch of fertile eggs (%)	$62.32^{bc} \pm 2.70$	$75.97^{a} \pm 2.46$	$58.21^{c} \pm 2.81$	$65.50^{bc} \pm 2.63$	$70.95^{ab} \pm 2.62$	0.006
Hatch of eggs set (%)	$58.43^{ab} \pm 2.62$	$63.79^{a} \pm 2.54$	$51.14^{b} \pm 2.69$	$61.11^{a} \pm 2.60$	$61.06^{a} \pm 2.58$	0.005
Chick weight (g)	$39.77^{ab} \pm 0.25$	$37.63^{c} \pm 0.23$	$40.13^{a} \pm 0.24$	$39.23^{b} \pm 0.25$	$39.07^{b} \pm 0.25$	<0.001
Chick/egg (%)	64.98 ± 0.31	63.58 ± 0.29	64.48 ± 0.34	64.90 ± 0.31	64.43 ± 0.27	0.067

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

When considering chick hatchability (expressed of the total number of eggs set), the fish oil (n-3) and the sunflower oil (n-6) treatments still maintained their respective ranks as the groups with the highest (63.8%) and lowest (51.1%) hatchability. The HO sunflower oil (n-9) and the tallow (SFA) treatments recorded a similar hatchability (61.1%), compared to the fish oil (n-3) treatment. The hatchability of the control treatment was intermediate (58.4%).

Table 4.10 also demonstrates the chick characteristics in relation to the number of incubated eggs. Although differences ($P < 0.001$) occurred between chick weights at hatching, no differences ($P = 0.067$) in the ratio of chick weight to egg weight at setting were recorded. The trend in hatched live chicken weight between treatments seemed to be similar to that observed in the original weights of the incubated eggs. Hence, eggs from the sunflower oil (n-6) treatment resulted in the heaviest chicks at hatching (40.1 g), while the lightest chicks (37.6 g) hatched from eggs of the fish oil (n-3) treatment.

Although results regarding the fertility of eggs in the present study can be considered to be slightly lower than in younger (42 – 47 weeks of age) flocks fed corn oil (n-6) or fish oil (n-3) diets (Blesbois *et al.,* 1997), the hatchability of eggs was however generally low in all treatments. Investigations regarding egg fertility in a broiler flock (Kirk 1980; Fasenko *et al.,* 1992) and those of the turkey (Fairchild *et al.,* 2002) have established a consistent increase of fertility and hatchability up to a peak, before declining as the flock ages. Pedroso *et al.* (2005) reported an increase from 85.3% to 96.8% in fertility as Cobb breeders progressed from the pullet (25 – 27 weeks of age) to the breeder (32 – 37 weeks of age) stage. Indeed, Insko *et al.* (1947) reported a tendency of fertility to decline with age, and this was supported by Cerolini *et al.* (1997). Demonstrating a negative correlation between age and flock fertility, with fertility being monitored at 24, 39, 54, and 72 weeks of age in the Cobb broiler breeder strain. Kelso *et al.* (1997) reported a consistent decline in the fertility of broiler breeder cockerels after peaking at week 39. However, the 65.4 % and 69.5% fertility rates for 72 week old cockerels maintained on soybean oil (n-6) and linseed oil (n-3) in the trial of Kelso *et al.* (1997), were lower than the 89.6% and 92.1% recorded in the current study for cockerels maintained on sunflower oil (n-6) and the control (n-3) treatments, respectively. The high inclusion level (3%) of fish oil may not have been responsible for the low fertility (84.6%), recorded in the present study. Blesbois (1997) reported that cockerels maintained on a 5% fish oil diet to be more ($P < 0.0001$) fertile (96.0%) than their counterparts maintained on a 5% maize oil (n – 6) diet (91.6%). The fertility of the fish oil (n-3) treatment in this current

study however showed a strong similarity to the fertility rate (83.7%) reported by Herstad *et al.* (2000) in broiler breeders (from 6 to 32 week of age), treated with a 3% fish oil diet, supplemented and with 40 mg synthetic Vitamin E.

The marked differences occurring in the fertility rates of the two n-3 diets (control and fish oil) recorded, was unexpected and difficult to explain. Firstly, there seemed to be an overall improvement (compared to Trial 1; summarised in Table 4.3) in the sperm quality (motility in particular) and performance of the fish oil fed cockerels. Secondly, sperm fatty acid methyl esters (FAME) analyses presented in Table 4.8 showed a higher concentration of long chain fatty acids, such as DHA and EPA, which previous researchers reported to be positively related to sperm motility (Cerolini *et al*., 2006), or translating to higher fertility rates (Blesbois *et al.,* 1997).

Flock fertility is generally regarded as a function of the male chicken. Thus attempts have been made to relate sperm motility to the fertility of the flock. The n-3 fatty acids (from fish oil sources in particular) have the potential of contributing positively to the cockerel's reproductive performance at an old age (Blesbois *et al.,* 2004). This assertion was confirmed in the sperm motility of cockerels maintained on the two types of n-3 diets (control and fish oil) in the present study (Table 4.9). It however seems that the enhancement did not translate to egg fertility in the fish oil (n-3) group following AI. Sperm motility is generally seen as a good indicator of fertility due to the high positive correlation between these two parameters (Cerolini *et al.,* 1994). Furthermore, Donoghue (1999) used sperm motility to determine the success rates of sperm-egg binding, a critical stage in the fertilization process. Hence, the group of cockerels whose sperm mobility was classified as good, exhibited a significantly higher fertility rate, compared to the cockerels whose semen was classified as poor. The present trial showed a partly similar relationship between sperm motility and fertility for cockerels treated with the experimental diets. The cockerels maintained on the mono-unsaturated (high oleic sunflower) and polyunsaturated diets (control, fish oil, sunflower) recorded a higher ($P < 0.05$) sperm motility, compared to those on the saturated tallow treatment (Table 4.9). A trend that was consistent with the fertility of the flock, with the exception of the fish oil (n-3) fed cockerels (Table 4.10). Factors other than sperm motility and fatty acid concentration could therefore be responsible for the unexpectedly low fertility recorded in the fish oil (n-3) treatment, during the late reproductive phase.

Contrary to other reports (Hudson & Wilson, 2003; Alsobayel & Albadry, 2012) egg fertility in the present study was not particularly related with early embryonic deaths (Table 4.10). It would appear that the treatments with the highest fertility were also yielded the highest early embryonic mortalities - except for the control treatment, where high fertility and low early embryonic mortalities were recorded. Following the negative correlation recorded between egg fertility and early embryonic mortalities, Fairchild *et al.* (2002) suggested that factors aimed at improving flock fertility (dietary fatty acid in the present study) could be helpful in reducing early embryonic mortalities. Perhaps a more meaningful result would have been recorded in the current study if the numbers of infertile eggs and early deaths were combined within a treatment.

In addition to the occurrence of infertile eggs, early embryonic mortality was also associated with sperm quality (Huyghebaert *et al.*, 1984; Bramwell & Howarth, 1997). Bramwell and Howarth (1997) specifically associated early embryonic deaths in chickens and increased sperm penetration of the germinal disc, with high sperm concentration. It was however difficult to establish the relationship between sperm quality and early embryonic mortality in the present study, as the penetration of sperm into the inner perivitelline layer of the egg yolk was not determined under the microscope.

The general low hatchability of the set eggs means that factors other than that directly affecting flock fertility must be considered. Attention must therefore be shifted from the effect of sperm quality to adopted procedures of incubation, as well as the effect of dietary lipid sources on hen production performances (particularly egg size) and embryo survivability. The saturated fatty acid concentration was not increased to a hazardous level (< 41%) in the yolk of any treatment in the current study (Table 5.4). The uncharacteristically high (57%) saturated fatty acid concentration in the yolk of hens fed 5 g/kg conjugated linoleic acid (CLA) diet was concluded to have resulted in the 0% hatchability of fertile eggs after day 7 of incubation (Aydin & Cook, 2009). From Table 4.10, it can be seen that the highest category for embryonic mortalities were in the “mid-death” category followed by the “late death” category. Presumably the number of times eggs were removed from the incubator for the purpose of candling and transfer to the hatcher resulted in increased embryonic mortalities.

In the present study, candling was conducted twice (embryonic day 7 and 14), while eggs were also taken out of the incubator on day 18 in order to have each egg separated in individual net bags for ease of identification of the hatched chicks (after 21 days). On the other hand, in many experiments such as reported by Blesbois, *et al.* (1997), Ruiz and Lunam (2002) and Hudson and Wilson (2003), candling was conducted only once. Embryonic mortalities were generally evaluated after the hatching of the eggs (day 21 – 22). Some literature also reported that no candling was done until, after hatching of the eggs at day 21 (Pedroso *et al.*, 2005). The frequency of candling and exposure of eggs to environmental conditions outside the incubator may have adverse effects on the eventual hatchability - as reflected in the high number of embryonic mortalities that occurred after the 2nd candling (middle & late deaths) in the present study. However irrespective of the general high number of middle and late mortalities recorded across treatments, it was also observed that embryonic mortalities were significantly higher ($P < 0.05$) in the early and middle stages of incubation, for the sunflower (n-6) treatment group. This culminated in the lowest egg hatchability for this particular dietary treatment. Table 4.10 illustrates the average egg weight (62.5 g) of hens maintained on the sunflower (n-6) treatment to be significantly higher than that of the other treatments. As a matter of fact, the stage (late productive phase) at which the present trial was conducted could have had an effect on the more pronounced egg size of the sunflower oil (n-6) treatment. The size of the eggs has been implicated in the reduced hatchability rates of the incubated eggs (Mandlekar, 1981; Asuquo & Okon; 1993). Further, Abiola *et al.* (2008) reported a hatchability of 97% for medium size eggs (51 – 57 g), while 83% was reported for large eggs (57.4 – 69.6 g), in an Anak broiler breeder flock. The results obtained complemented the best hatchability recorded in medium size eggs (45 – 56g), reported earlier by Asuquo and Okon (1993). Egg weights obtained could have played a role in the variation in terms of hatchability, especially when the significant difference in egg weight appeared to follow an opposite hatchability trend (Table 4.10). So for example, the sunflower (n-6) diet produced the heaviest eggs ($P < 0.05$) while those hens on the fish oil (n-3) treatment recorded the smallest egg sizes. The hatchability in terms of both total eggs set and fertile eggs was the highest ($P < 0.05$) in the fish oil (n- 3) group, and the lowest for the sunflower oil (n-6) treatment group. Mortola and Al Awam (2010) proposed that the challenges of over-hydration occurring in large eggs may hamper the survival rates of the embryos during the incubation process.

The implication of egg size at set was quite evident in chick weight at hatching on day 21. Chick weight patterns were similar to the differences elaborated in egg weight between treatments. The present results confirm the reports of various researchers (Batt & Prince, 1979; Michel *et al.,* 2003; Dzialowski & Sotherland, 2004; Ipek & Diken, 2007; Abiola *et al.,* 2008) in terms of the positive relationship between egg size and the corresponding weight of the hatching chick. This was evident ($P < 0.001$) in the current study, as the highest chick weight (40.1 g) was recorded in the sunflower (n-6) treatment, while at the same time the fish oil (n-3) treatment produced the lightest chick weight (37.6 g) (Table 4.10). Embryos from different egg sizes followed different growth trajectories, which also conforms to differences in yolk assimilation rates towards the end of incubation, eventually resulting in different hatchling weights (Mortola & Al Awam, 2010). The ratio of the egg to chick (egg : chick) weight (percentage wise) was not affected ($P > 0.05$) by the experimental diets, and seemed to be very similar or constant, irrespective of the dietary treatment.

4.4 Conclusions

Results from this study indicated that the dietary lipid sources affected the fertility in the chicken, during the different growth phases (≤ 46 and ≥ 69 weeks of age). Inclusion levels of either 1.5% fish oil/1.5% linseed oil (n-3) or 3% high oleic sunflower (n-9) could be used in maintaining sperm motility, both at the peak and during the end of the production life cycle of cockerels. Also, a high fertility, as well as an acceptable level of hatchability could be achieved in older birds (≥ 69 weeks of age), by feeding either of these n-3 or n-9 supplementary lipid sources. The polyunsaturated fraction of the sperm was most affected by the dietary fatty acids.

Supplementary n-3 using fish oil (3%) was better utilized by older cockerels. This was reflected by improved sperm motility, when compared to younger cockerels. Further, higher hatchability of fertile eggs was an advantage of feeding 3% fish oil diet to an old flock. The n-6 lipid source (sunflower oil) did not have any adverse effect on sperm quality (motility) during both the peak and latter stages. This lipid source (n-6) could however cause reduced hatchability in an old flock.

Saturated fatty acid lipid (tallow) apparently boosts the total sperm output (through increased semen volume), throughout the productive life of the cockerels. Sperm motility and fertility could however be compromised in older cockerels. Moreover, this study also highlighted the

need for a holistic approach in determining the effects of dietary lipid sources on cockerel sperm quality. Thus, factors such prevailing weather conditions (temperature), as well as the ejaculation of contamination-free semen of cockerels during different seasons are important physiological considerations that also warrant attention.

CHAPTER 5
EFFECT OF DIETARY LIPID SOURCES ON PRODUCTION PERFORMANCE AND EGG QUALITY CHARACTERISITICS OF LAYERS

5.1 Introduction

In addition to the conventional uses of supplementary lipids in layer feeds, the focus has also turned to the human health benefit of eggs being produced by the incorporation of different dietary fatty acids. These attempts in the enrichment of eggs through dietary means, could also have an impact on the performance of the laying hen. There have been contradictory results regarding the influence of lipid sources (used in supplying saturated, mono-unsaturated and polyunsaturated fatty acids) on the performance of layers. Jiang *et al*. (1991), Mazalli *et al.* (2004) and Da Silva Filardi *et al*. (2005) reported no effect of supplementary fats on production parameters (egg production and egg weight) during the peak production phases, as well as the second laying cycle. However, Grobas *et al.* (1999a,b; 2001) reported an effect of unsaturated lipids on egg weight. Nonetheless, agreement has been consistent by researchers, regarding the deposition of dietary mono-unsaturated and polyunsaturated (n-3; n-6) fatty acids into the egg yolk of hens using different fatty acid rich dietary sources (Grobas *et al.,* 2001; Da Silva Filardi *et al.,* 2005; Cachaldora *et al.,* 2006; Antruejo *et al.,* 2011). These findings could have profound human health implications, considering the general awareness of an appropriate ratio of omega-6 : omega-3 fatty (n-6 : n-3) acid dietary intake (Simopoulos *et al.,* 1995; Simopoulos, 2002). Furthermore, mono-unsaturated (n-9) fatty acids have been recommended for the nervous system myelinization in growing children (Uauy Dagach & Hoffman, 1991; Uauy Dagach & Olivares, 2007).

The aim of this study was thus to investigate the effects of saturated, mono-unsaturated, and polyunsaturated (n-3, n-6) lipid sources on the production parameters during part of peak egg production and the entire end-of-lay phases (46 weeks) in layers - as well as determining the quality of eggs produced, following such dietary manipulation.

5.2 Material and Methods

5.2.1 Birds and housing

The housing and husbandry of the layers (17 weeks old) was comprehensively discussed in section 3.2 (Chapter 3). One hundred and twenty-five (125) hens were randomly allocated to five dietary treatments at 32 weeks of age, with each treatment consisting of 25 replicates (n

= 25 replicates/treatment). Each bird received 115 – 120 g of feed per day, to ensure a feeding level of satiation, while water was provided *ad libitum* using nipple drinkers. Egg collection and assessment was conducted before 09:00 on a daily basis, following a one week feed adaptation period. The minimum and maximum temperatures were recorded at five different locations within the hen-house at 08:00 in the morning for the entire experimental period. Production data was recorded for a total duration of 46 weeks, starting during the peak production phase (33 weeks of age), until a late phase in production (78 weeks of age). Further, the data of all the eggs produced during the successive weeks were individually recorded and summarized, before being pooled for statistical analyses. Individual body weights of the hens were also recorded monthly, starting from 32 weeks until 76 weeks of age.

All eggs laid during week 67 and 68 were then collected for quality evaluation. The Folch *et al.* (1957) procedure described for the diets in paragraph 3.4 was also used for the fatty acid methyl esters (FAME) analyses on a representative portion of these egg yolks, during the period (68 weeks) - immediately before the commencement of the fertility trial, as described in section 4.3.4.

5.2.2 Diets

The experimental diets with their respective lipid sources, as well as its preparation of the diet have been described in detail in section 3.4 (Chapter 3). The mean physical composition (Table 3.3), calculated chemical composition (Table 3.4), as well as the dietary fatty acid methyl esters (Table 4.1) and fatty acid ratios (Table 4.2) of dietary treatments are also summarized accordingly. Lipid sources were included at a constant 30 g/kg inclusion level, in order to produce five *isoenergetic* (12.4 MJ AME/kg DM) and *isonitrogenous* (171 g CP/kg DM) treatments - differing only in their degree of lipid saturation and fatty acid profiles. Briefly, the control diet was formulated using a blend (50 : 50) of linseed and fish oil to increase the dietary levels of omega-3 (n-3) fatty acids - particularly the precursor, α-linolenic acid. In the second experimental diet (fish oil n-3 treatment), refined, deodorised fish oil was used to increase the dietary concentration of the longer chain n-3 fatty acids, such as eicosapentaenoic- (EPA), docosapentaenoic- (DPA) and docosahexaenoic acid (DHA). Sunflower oil was used to increase the dietary omega-6 (n-6) fatty acid precursor, linoleic acid in the third treatment. High oleic acid (HO) sunflower oil was used to increase the omega-9 (n-9) fatty acids - particularly the oleic acid in the fourth treatment. Lastly, tallow

was used as a supplementary lipid source in the saturated diet (SFA treatment) to increase the level of highly saturated fatty acids, such as palmitic and stearic acid and to lower the concentration of polyunsaturated fatty acids of the n-3 and n-6 type. These definite omega-type fatty acids were not exclusive to a specific diet, but the high concentration thereof within a respective diet was used for defining the treatment.

5.2.3 Feed intake

Hens were individually fed (± 115 – 120 g/bird/day) between 07:30 and 08:30 daily, from a feed bucket assigned to each bird. Feed intake was first determined at week 33 of age, after one week of adaptation, and for successive weeks thereafter, until the end of the production trial at week 78 of age. Feed refusals and the buckets of each bird were weighed once a week and data were used for the calculation of the weekly feed intake (g/bird/week), as discussed in paragraph 3.4.2 (Chapter 3). The average daily feed intake (g/bird/day) was thereafter determined from the calculated weekly feed intake (g/bird/week). The feed conversion ratio (FCR) of the individual hens was calculated (Rose, 1997) by dividing the weekly mean feed intake (g) by the corresponding mean egg output (g) and expressed as gram/gram (g/g). The mean egg output then represented the product of egg number and egg weight for an individual hen per week.

5.2.4 Egg production

Egg weight (g) was recorded by accurately weighing the individual eggs to the closest 0.01 gram using a Mettler PL 3000 scale, immediately after egg collection each morning. The individual weighing of the eggs and the visual inspection of the intact egg shells was conducted concurrently. Hen-day egg production (%) was calculated according to Ahmad and Balander (2003), whereby the number of eggs produced was divided by the number of live birds for each treatment. So for example, an egg was collected, visually appraised as sellable, cracked, or shell-less and then weighed. The number of sellable cracked and shell-less eggs produced per week within each treatment was recorded for each individual bird. The combination of the aforementioned parameters was then used in the calculation of egg deformities (%). At the end of each week, the number of eggs produced by an individual hen, as well as the average weight of the eggs was calculated from the daily records. Hen-day egg production (%) was summarised on a weekly basis and then expressed as weekly egg production (%) per individual hen.

5.2.5 Egg component characteristics

At 68 weeks of age, 12 sellable eggs (n = 12/ treatment) of each treatment were randomly chosen from each treatment for 7 consecutive days (in total 84 eggs/treatment) - in order to determine certain egg component characteristics e.g. yolk weight (g), albumen weight (g), eggshell weight (g), yolk ratio (%), albumen ratio (%) and the percentage eggshell (%). Individual eggs were broken to enable the separation of the components (yolk and albumen), using an egg yolk separator (Plate 3.5). After careful removal of all adhering albumen and chalazae, the eggshell and yolk were weighed separately, to the nearest 0.0001 gram (Plate 3.6). Albumen weight (g) was calculated as the difference between total egg weight (g) and the weight of the eggshell (g) plus the yolk weight (g) (Grobas *et al*., 1999b).

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

The albumen and yolk ratios were calculated as a percentage of the component weight (g), compared to the egg weight (g). The formulas as described by Kul and Seker (2004) were used for the calculation thereof:

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

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

A total of 420 eggs (n = 420), representing 84 eggs/treatment was thus evaluated.

5.2.6 Egg fatty acid methyl esters

Before the termination of the study on week 78 (Chapter 3), 12 sellable eggs per treatment were randomly chosen for the analyses of the egg yolk fatty acid methyl esters. The total lipid content of the yolk samples were extracted according to the method, as described by Folch *et al*. (1957) and comprehensively set out in paragraph 3.5.1 (Chapter3). The fatty acid composition, total fatty acid saturation, and the n-3, n-6, n-9 ratios of the fatty acids within each egg yolk was determined accordingly.

5.2.7 Statistical analyses

To determine the effects of long term exposure to the different dietary lipid sources on production performance and egg quality characteristics, data generated during the observation period of 46 weeks were pooled for statistical analysis of the parameter means, using a fully randomized one-way ANOVA design. The PROC ANOVA procedures of the SAS program (SAS, 2010) were used to test for significant ($P < 0.05$) differences between treatments.

When significant differences were found ($P < 0.05$), a further multiple comparison, using Tukey's honest significant difference (HSD) test, was used to compare the treatment means.

5.3 Results and Discussions

5.3.1 Temperature

The average maximum and minimum hen-house temperatures recorded during the course of experimental period (46 weeks) are set out in Figure 5.1. The entire duration of the trial covered two extreme seasons (summer and winter), in the central South African region. The clear gradual increase in temperature from week 33 to 51 of age, before a decreasing trend in environmental temperature up to the end of trial (Week 78), signified the transition from autumn to the peak of summer, and then from summer to the mid-winter within the region (Free State). The highest mean environmental temperatures (37 °C maximum and 20 °C minimum) were recorded in mid-January, when the birds were 50 weeks of age. Similarly, the coldest ambient temperature (14 °C maximum and 3 °C minimum) occurred during mid-June, when the birds were 72 weeks of age. The average maximum and minimum temperatures for the entire 46 week period were calculated to be 28 °C and 13 °C respectively, giving a mean temperature of 20 °C. Hens in the current study were expected to consume less feed during the early stages of the trial, compared to the late phase, considering the persistent high ambient temperature until week 58 of age. Exposure to a constant high ambient temperature generally causes a reduction in feed intake and consequent lower egg production in hens (Usayran *et al.*, 2001).

Although all these parameters were not tested, it is known that ambient temperature may influence the performance of chickens in different ways. Etches *et al.* (1995) and Nalini *et al.* (2008) indicated chickens under heat stress to elicit behavioural adjustments, as well as the impairment of the physiological, hormonal, and molecular mechanisms in the chicken. Olarenwaju *et al.* (2010), reported a significantly higher concentration of plasma glucose and cortisol (stress hormone) in heavy broiler chickens, reared under a high ambient temperature environment (26.7 °C), compared to those reared in medium (21.1 °C) and low (15.6 °C) temperature conditions. Exposure of broiler chickens to ambient temperatures exceeding their comfort zones have been shown to adversely affect body weight gain, feed conversion ratio and meat yield (Washburn, 1985; Howlinder & Rose, 1989), while excessive high temperatures also lowered egg production in layers (Usayran *et al.*, 2001). As the present study spanned across 46 consecutive weeks, it was expected that the temperature impact

would have been compensated for by the changes in season - which remained constant across treatments. Therefore, it was decided to pool the data to give a good representation of layer performance throughout the productive life.

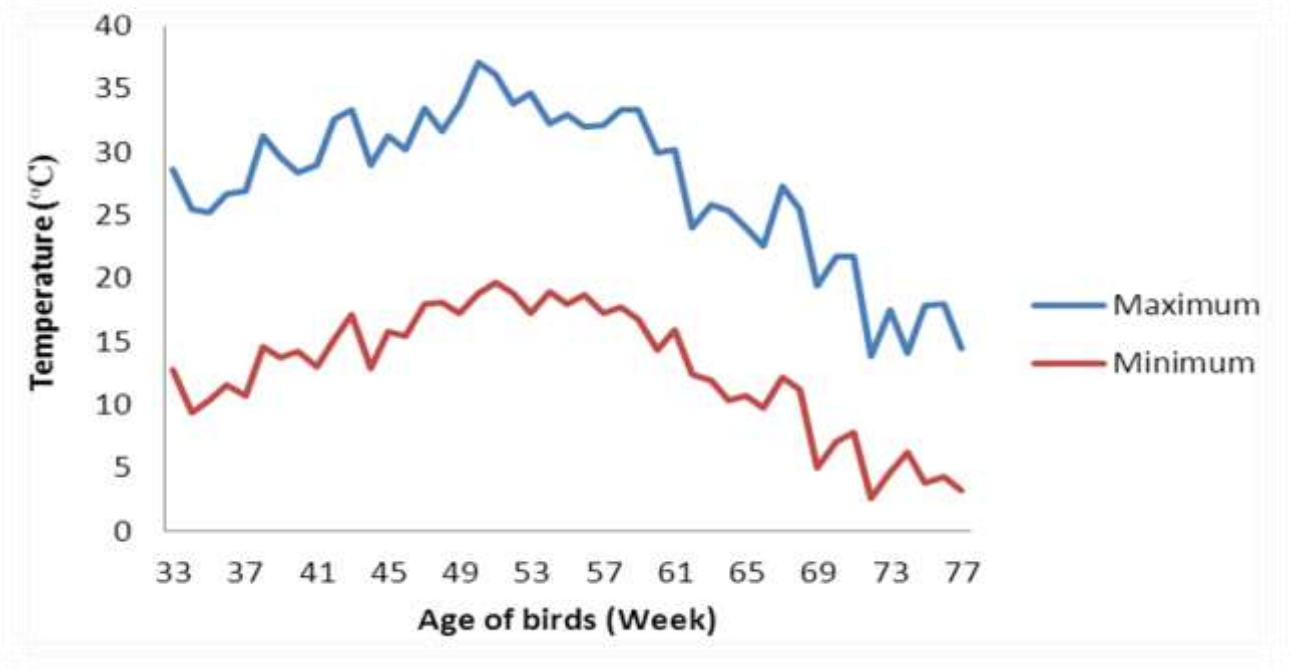


Figure 5.1 Average minimum and maximum hen-house temperatures from 33 to 77 weeks of age

5.3.2 Production performance

The effect of dietary lipid sources on the production performance parameters are summarized in Table 5.1. All measured parameters except hen-day egg production, were significantly influenced ($P < 0.05$) during the 46 week exposure period by lipid source treatment. Dietary lipid sources affected the feed intake ($P < 0.001$), sellable eggs ($P < 0.01$), egg weight ($P < 0.001$), egg output ($P < 0.001$), feed conversion ratio ($P < 0.001$) and body weight ($P < 0.001$). The apparently high coefficient of variation (CV) recorded for the hen-day egg production (14.9%) and the sellable eggs (18.8%) parameters could be ascribed to different causes. Perhaps the deliberate inclusion of all eggs produced during the trial (46 weeks), without any statistical adjustment to eliminate individual variation (on account of abnormally low weekly egg production), due to factors such as bird's health, could have resulted in this high CV for hen-day egg production.

Table 5.1 The mean (± S.D) effects of dietary lipid source on laying hen performance during a 46 week (33 to 78 weeks of age) production period

Parameter	Control	Fish oil (n – 3)	Sunflower oil (n – 6)	*HO Sunflower oil (n – 9)	Tallow (SFA)	Significance (*P*)	CV[1] (%)
Feed intake (g/bird/day)	102.97[b] ± 9.57	102.68[b] ± 9.38	104.55[a] ± 8.36	102.34[b] ± 10.33	101.07[c] ± 9.15	<0.001	7.45
Hen-day egg production[#] (%)	85.56	85.17	84.40	85.13	84.72	0.178	14.91
Sellable eggs[#] (%)	93.30[a]	92.09[ab]	93.16[a]	92.14[ab]	90.81[b]	0.008	18.81
Egg weight (g)	58.18[c] ± 3.91	57.08[d] ± 3.25	59.29[a] ± 4.03	58.5[bc] ± 3.62	58.61[b] ± 3.58	<0.001	6.02
Egg output (g)	347.06[ab] ± 55.38	338.40[c] ± 57.34	352.59[a] ± 49.37	349.03[ab] ± 55.96	346.20[b] ± 56.02	<0.001	14.16
Feed conversion ratio	2.13[b] ± 0.48	2.19[a] ± 0.52	2.10[b] ± 0.31	2.10[b] ± 0.40	2.09[b] ± 0.36	<0.001	19.07
Body weight (g)	1822[c] ± 147	1779[d] ± 112	1834[bc] ± 127	1853[ab] ± 152	1856[a] ± 136	0.001	6.97

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

[*]HO = high oleic acid sunflower

[1] Coefficient of variance (%)

[#] Mean values for hen-day egg production (%) and sellable eggs (%)

Consequently, the high CV (%) of the hen-day egg production parameter affected the egg output CV (14.2%), as well. This then ultimately resulted in a high CV (19.1%) being recorded for the feed conversion ratio between treatments. On the other hand, the high coefficient of variation (CV) recorded for the sellable eggs (%) parameter may not be unrelated to cumulative human effects during egg appraisal (checking of cracked and shell-less eggs), necessary for data collection over the 46 weeks trial period (King, 2012).

The objective of this trial was ultimately to monitor the performance of laying hens in the long term (46 weeks). Results presented therefore include the overall performance during part of the peak and the entire end-of-lay production phase. The highest ($P < 0.001$) mean feed intake (104.55 g/bird/day) was recorded in the sunflower oil (n-6) hens while the tallow treatment resulted in the lowest (101.07 g/bird/day) feed consumption. The present results during the 46 week period are not in agreement with previous dietary fats studies that reported no significant differences in all productions parameters during the peak egg production stage (Jiang *et al.*, 1991, 1992; King, 2012), or end-of-lay phase of production of the hens (Baucells *et al.*, 2000; Mazalli *et al.*, 2004; Da Silva Filardi *et al*., 2005).

On the other hand, Celebi and Macit (2009) attributed differences in feed intake of hens fed tallow and linseed oil, to the effect of the level of dietary fatty acid saturation on the digestibility and absorbability of the nutrients (Grobas *et al.*, 2001). Apart from this possibility, the duration of feed storage could also be a potential reason for variation observed in the current study, as a bird's feed intake is largely determined by its energy needs. Since diets in the present study were formulated to be *isoenergetic* (12.4 MJ AME/kg DM), the extended period of feed replacement between new batches of feed mixed could have resulted in minor differences in energy content of the diets.

Although the absence of significant effects ($P = 0.178$) of the dietary lipid sources on egg production were recorded in the present study, it is in agreement with certain studies (Baucells *et al.*, 2000; Mazalli *et al.*, 2004; Da Silva Filardi *et al*., 2005). The current study also agrees with the range of egg production (83 – 85%) reported by Mazalli *et al.* (2004) in which 46 weeks old laying hens were treated with similar dietary lipids sources and levels as in the present study for 20 weeks - until the end of 66 weeks of age.

Furthermore, the tallow (SFA) treatment resulted in the lowest ($P < 0.01$) percentage of sellable eggs (90.8%) being produced - a direct effect of the high (9.05%) percentage of cracked eggs produced by the tallow (SFA) fed hens. The occurrence of cracked eggs was the most common type of deformity encountered in all the treatments, with the highest frequency being recorded in the tallow treatment and the lowest in the control and sunflower oil (n-6) treatment hens (6.4 and 6.3% respectively). King (2012) also indicated that the diet in which the lipid saturation was increased with 3% tallow, resulted in the lowest ($P < 0.015$) percentage of sellable eggs (89.2%) - using a similar dietary treatment as in the current study. The percentages of other types of deformities such as shell-less, and soft shells were negligible, and the occurrence as low as 0.14% being recorded in the tallow fed treatment, in the current study.

The heaviest ($P < 0.001$) eggs were produced by hens fed sunflower oil (n-6) diet (59.29 g), while the lightest eggs (57.08 g) were produced by the fish oil (n-3) treatments. Grobas *et al.* (2001) reported similar findings, where hens that were fed a soybean oil (n-6) diet laid heavier eggs, when compared to other fat supplemented diets (tallow, olive oil, linseed oil) - despite no significant differences in feed intake being recorded between treatments. Further, the light egg weights recorded in the two n-3 treatments during the present study are in agreement with Scheideler and Froning (1996) for the control diet, as well as Herber and Van Elswyk (1996) for the fish oil treatments. Herber and Van Elswyk (1996) further implicated the effects of n-3 fatty acids on lipid metabolism, with circulating oestradiol being responsible for the lower yolk and consequent egg weights. A similar observation was made during this study following the low ($P < 0.001$) egg (58.42 g) and yolk (16.53 g) weights for the fish oil (n-3) treatment, in particular (Table 5.2).

Table 5.2 Mean (± SD) effect of dietary fatty acid profile on certain egg component characteristics during the end-of-lay period (68 weeks of age)

Parameter	Control	Fish oil (n – 3)	Sunflower oil (n – 6)	#HO Sunflower oil (n – 9)	Tallow (SFA)	Significance (P)	CV[1] (%)
Egg weight (g)	60.74^{ab} ± 4.12	58.42^{c} ± 4.04	61.88^{a} ± 3.72	60.47^{ab}± 4.26	60.1^{b}± 4.49	<0.001	6.73
Shell weight (g)	7.15^{b} ± 0.57	7.08^{b} ± 0.54	7.46^{a} ± 0.75	7.27^{ab} ± 0.66	7.06^{b} ± 0.66	<0.001	8.84
Yolk weight (g)	17.64^{a} ± 1.77	16.53^{b} ± 1.73	17.56^{a} ± 1.89	17.05^{ab} ± 1.99	17.23^{a} ± 1.90	<0.001	10.56
Albumen weight (g)	36.04^{ab} ± 3.04	34.90^{c} ± 2.55	36.88^{a} ± 2.91	36.16^{ab} ± 2.93	35.72^{bc} ± 2.55	<0.001	8.00
Shell ratio (%)	11.79^{b} ± 0.85	12.13^{a} ± 0.84	12.06^{ab} ± 0.92	12.04^{ab} ± 0.98	11.76^{b} ± 0.98	0.004	7.66
Yolk ratio (%)	28.86 ± 2.30	28.22 ± 1.78	28.38 ± 2.67	28.17 ± 2.25	28.72 ± 2.05	0.101	8.06
Albumen ratio (%)	59.21 ± 2.45	59.66 ± 1.79	59.57 ± 2.79	59.80 ± 2.52	59.55 ± 2.43	0.422	4.00
Yolk:Albumen (%)	49.22 ± 5.78	47.42 ± 4.39	47.94 ± 6.66	47.34 ± 5.80	48.43 ± 5.48	0.067	11.56

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

[#]HO = high oleic acid sunflower

[1] Coefficient of variance (%)

It would seem as if the reduction implication of egg weight was more pronounced in the fish oil, than in the control treatment (which was comprised of 1.5% of fish- + 1.5% linseed oil). The lower egg output ($P < 0.001$) of the fish oil (n-3) treatment, was as a consequence of the lower egg weight, rather than the hen-day egg production rate. This trend was also recorded in the sunflower (n-6) treatment, which also recorded the highest egg output per bird. The feed conversion ratio was affected ($P < 0.001$) by the dietary lipid source, in a similar pattern as the egg weight and -output. Birds in the fish oil (n-3) treatment group showed a poor ($P < 0.001$) feed conversion rate (2.19), compared to the other diets. This result was due to the fish oil (n-3) fed hens having a lower ($P < 0.001$) egg output, compared to the other treatments. Body weight of the hens was also influenced ($P < 0.001$) by the dietary lipid sources, but was however not directly related to the dietary lipid saturation. The saturated (tallow) treatment resulted in the heaviest body weights (1856 g), while the fish oil (n-3) treatment resulted in the lowest body weights (1779 g) being recorded. Caston *et al.* (1994), Scheideler and Froning (1996) and Yannakopoulos *et al.* (1998) reported a decrease in body weight and consequently egg weight, when feeding linseed oil to laying hens.

5.3.3 Egg components

The effects of dietary lipid sources on internal egg components and their relative proportions are set out in Table 5.2. All component parameters presented in Table 5.2 were affected ($P < 0.001$) by the dietary lipid sources, except in the case for ratios such as eggshell ratio ($P < 0.05$) and the yolk : albumen ratio ($P = 0.067$). The egg weights recorded maintained the same pattern as reported in paragraph 5.3.2, although there was a general increase in egg weights across treatments during the latter period of egg component evaluation (week 68 of age). Obviously, egg weights were expected to be higher at 68 weeks of age, compared to the mean egg weight between 33 and 78 weeks as presented in Table 5.1 - due to ageing of the birds and an increase in body weight of hens. While the total lipid, phospholipid and cholesterol content at 21 weeks of age has been reported to be similar to another group of hens of 56 weeks of age, the weights of the whole egg and yolk of the older hens were higher ($P < 0.05$) than the younger group of birds (Nielson, 1998). The sunflower oil (n-6) treatment fed hens recorded the heaviest ($P < 0.001$) egg weights (61.88 g), while the lightest egg weights (58.42g) were produced by the fish oil (n-3) fed hens, in the present study. The implication of this pattern being that the eggs from the sunflower oil (n-6) treatment also recorded high weights in all components - although having similar yolk weights to the control, HO sunflower oil (n-9) and tallow (SFA) treatments. The fish oil (n-3) hens produced

eggs with the lowest ($P < 0.001$) albumen weight (34.90 g) and yolk weight (16.53 g). Shell weight was however similar to the control, HO sunflower (n-9) and tallow (SFA) diet produced eggs. The heavy eggs recorded in the sunflower oil treatment was expected, as Grobas *et al.* (2001) also reported higher egg weights with a similar n-6 diet (soya oil), compared to hens fed a tallow, olive- or linseed oil diet. This consequently resulted in significantly higher albumen weights in the soya oil treatment, compared to the other supplemented fats, with slight increases also recorded in the other egg components.

Generally, dietary supplemented fats have been reported to improve both yolk and albumen weights (Safaa *et al*., 2008). In the present study, fish oil (n-3) was the only treatment where low ($P < 0.05$) yolk weight (16.53 g) was recorded, whereas the other n-3 lipid source (control diet) produced similar yolk weights to the other treatments (sunflower-, HO sunflower oil and tallow). Van Elswyk *et al.* (1995) suggested that the hypolipodemic characteristics of lipid sources such as fish oil could reduce liver lipogenesis and the transport of lipids from the blood to the ova - thereby resulting in reduced egg and yolk weights. However, not all studies were able to record these negative effects of fish oil on the yolk weights (Cachaldora *et al*., 2006; Garcia-Rebollar *et al.,* 2008).

The control diet used in current study therefore confirms the reports of Aymond and Van Elswyk (1995), Pheko *et al.* (1998), and King, (2012) who reported no effect of dietary linseed oil on yolk weight. Also, results of the current study are in agreement with previous experiments where the negative effects of fish oil on yolk weight were reported (Marshall and Van Elswyk 1994; Herber & Van Elswyk, 1996; Van Elswyk 1997; King, 2012). Moreover, certain researchers have also reported the negative effects of other n-3 sources (chia seed) on yolk weights (Ayerza & Coates 1999; Antruejo *et al*., 2011).

Further, albumen weights recorded differed ($P < 0.05$) between the sunflower oil (36.88 g) and the fish oil (34.90 g) dietary treatments. This may not be a direct effect of treatment on the hen's lipid metabolism, as is the case with yolk weight. It may have resulted from the same factors determining the higher ($P < 0.001$) and lower egg weights recorded for the sunflower (n-6) and fish oil (n-3) dietary treatments, respectively. Yolk to albumen ratio (yolk : albumen) was not affected by dietary lipid source in the current study, which is in agreement with Grobas *et al.* (2001).

5.3.4 Yolk fatty acid methyl esters

The effect of dietary lipid source on the yolk properties such as percentage yolk fat, fat free dry matter (FFDM), moisture content (%), fatty acid methyl esters (FAME) on egg yolk (Table 5.3), concentration of total fatty acids and the fatty acid ratios (Table 5.4) - as well as the total n-9, n-3 and n-6 contents per yolk weight (Table 5.5), are summarised in the respective tables. The fatty acids presented in the yolk FAME analysis (Table 5.3) corresponded to the specific individual fatty acids (Table 4.1) and total concentration of fatty acids (Table 4.2), as determined for the five dietary treatments. No significant effect of dietary lipid sources on any of the egg yolk fat ($P = 0.28$), free fat dry matter ($P = 0.12$) or moisture ($P = 0.53$) parameters were recorded. The fatty acids concentrations of prominent fatty acids were however significantly ($P < 0.001$) different, according to their prevalence in the respective dietary treatments, except in the tallow (SFA) fed hens. The control diet (1.5% linseed & 1.5% fish oil) resulted in the highest ($P < 0.001$) deposition of α-linolenic acid (2.6%). The control diet was also recorded the second highest concentration of total n-3 (9.2%), polyunsaturated fatty acids (23.1%), as well as the second lowest n-6 : n-3 ratio (1.52 : 1), as indicated in Table 5.4.

The fish oil (n-3) diet recorded the highest concentration ($P < 0.001$) of long chain n-3 polyunsaturated fatty acids (PUFA's), such as eicosopentaenoic acid (EPA) (10.0%), docosapentaenoic (DPA) (0.9%), and the docosahexanoic acid (DHA) (8.5%). Also, the total n-3 fatty acids and the n-6 : n-3 ratio were respectively determined to be highest (10.7%) as well as the lowest (1.14 : 1) in the fish oil (n-3) dietary treatment group. This ultimately resulted in the highest ($P < 0.001$) quantity (g) of n-3 per yolk weight (0.42 g/yolk), as well as its highest concentration (mg) per gram of whole egg (7.89 mg/g whole egg) for this particular (fish oil) treatment.

Table 5.3 The mean (± SD) effect of dietary lipid source on the egg yolk properties and fatty acid methyl esters (FAME) at 78 weeks of age

	Control	Fish oil n – 3)	Sunflower oil (n – 6)	#HO Sunflower oil (n – 9)	Tallow (SFA)	Significance (*P)*
Yolk properties (%)						
Yolk fat	31.49	30.34	30.26	31.44	30.99	0.28
Yolk free fat dry matter	19.03	20.12	20.49	20.22	20.74	0.12
Yolk moisture	49.49	49.54	49.26	48.34	48.28	0.53
FAME# (% of total fatty acids)						
Saturated fatty acids						
C14:0 Myristic	0.35^{bc}	0.50^{a}	0.23^{d}	0.28^{cd}	0.41^{b}	<0.0001
C16:0 Palmitic	26.70^{b}	30.29^{a}	26.71^{b}	25.69^{b}	26.70^{b}	<0.0001
C17:0 Margaric	0.16^{c}	0.21^{b}	0.16^{c}	0.13^{c}	0.31^{a}	<0.0001
C18:0 Stearic	9.62^{b}	9.37^{b}	11.57^{a}	8.76^{b}	9.75^{b}	0.0012
Mono-unsaturated						
C16:1 Palmitoleic	2.65^{a}	2.72^{a}	1.55^{b}	2.42^{a}	2.94^{a}	<0.0001
C18:1n-9 Oleic	35.30^{b}	31.76^{c}	32.77^{bc}	44.28^{a}	42.14^{a}	<0.0001
C18:1n-7 Vaccenic	1.87^{ab}	1.97^{a}	1.23^{c}	1.68^{b}	2.02^{a}	<0.0001
C20:1n-11 Eicosenoic	0.22	0.23	0.18	0.20	0.21	0.195
Polyunsaturated fatty acids n-6						
C18:2n-6 Linoleic	12.6^{b}	10.99^{d}	20.41^{a}	12.42^{bc}	11.34^{cd}	<0.0001
C20:2n-6 Eicosadienoic	0.10^{c}	0.08^{c}	0.27^{a}	0.13^{b}	0.10^{bc}	<0.0001
C20:4n-6 Arachidonic	1.10^{c}	0.99^{c}	3.77^{a}	2.88^{ab}	2.59^{b}	<0.0001
Polyunsaturated fatty acids n-3						
C18:3n-3 α-Linolenic	2.55^{a}	0.21^{b}	0.13^{b}	0.12^{b}	0.16^{b}	<0.0001
C20:3n-3 Eicosatrienoic	0.11^{b}	0.14^{ab}	0.21^{a}	0.17^{ab}	0.19^{ab}	0.026
C20:5n-3 Eicosopentaenoic	0.54^{b}	0.96^{a}	ND	ND	ND	<0.0001
C22:5n-3 Docosapentaenoic	0.53^{b}	0.91^{a}	ND	ND	ND	<0.0001
C22:6n-3 Docosahexanoic	5.52^{b}	8.49^{a}	0.69^{c}	0.68^{c}	0.83^{c}	<0.0001

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

#HO = high oleic acid sunflower

Table 5.4 Mean (± SD) effect of dietary lipid source on the total fatty acid concentration (%) and fatty acid ratios of egg yolk

	Control	Fish oil (n – 3)	Sunflower oil (n – 6)	HO Sunflower oil (n – 9)	Tallow (SFA)	Significance (*P*)
Total fat concentration (%)						
Σ Satuarated fatty acids	36.89^{bc}	40.47^{a}	38.67^{ab}	34.90^{c}	37.32^{bc}	<0.0001
Σ Mono-unsaturated fatty acids (n-9)	40.06^{b}	36.76^{bc}	35.74^{c}	48.65^{a}	47.41^{a}	<0.0001
Σ Polyunsaturated fatty acids (n-6)	13.81^{c}	12.07^{d}	24.56^{a}	15.49^{b}	14.05^{bc}	<0.0001
Σ Polyunsaturated fatty acids (n-3)	9.24^{b}	10.71^{a}	1.03^{c}	0.967^{c}	1.22^{c}	<0.0001
Σ Polyunsaturated fatty acids	23.05^{b}	22.77^{b}	25.59^{a}	16.46^{c}	15.27^{c}	<0.0001
Ratios						
UFA : SFA[1]	1.72^{ab}	1.47^{c}	1.59^{bc}	1.88^{a}	1.69^{ab}	<0.0001
PUFA : SFA[2]	0.63^{a}	0.56^{b}	0.66^{a}	0.47^{c}	0.41^{d}	<0.0001
MUFA : SFA[3]	1.10^{b}	0.91^{c}	0.93^{c}	1.41^{a}	1.28^{a}	<0.0001
MUFA : PUFA[4]	1.75^{b}	1.62^{b}	1.40^{b}	3.02^{a}	3.17^{a}	<0.0001
n-6 : n-3[5]	1.52^{d}	1.14^{d}	24.39^{a}	17.21^{b}	12.27^{c}	<0.0001
n-9 : n-6[6]	2.91^{a}	3.05^{a}	1.46^{b}	3.20^{a}	3.43^{a}	<0.0001
n-9 : n-3[7]	4.45^{b}	3.47^{b}	35.79^{a}	57.51^{a}	43.84^{a}	<0.0001

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

[1] The ratio of unsaturated to saturated fatty acids

[2] The ratio of polyunsaturated to saturated fatty acids

[3] The ratio of mono-unsaturated to saturated fatty acids

[4] The ratio of mono-unsaturated to polyunsaturated fatty acids

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

[6] The ratio omega-9 to omega-6 fatty acids

[7] The ratio of omega-9 to omega-3 fatty acids

Interestingly, the highest ($P < 0.001$) total SFA, as a result of the high palmitic acid concentration, also manifested in the fish oil (n-3) diet yolk. The sunflower (n-6) diet recorded the highest concentration ($P < 0.001$) of linoleic acid (20.4%), as well as its derivatives – eicosadienoic (0.3%) and arachidonic (3.8%) fatty acid. This resulted in the highest yolk ($P < 0.001$), Σn-6, PUFA and PUFA : SFA (24.6%, 25.6% and 0.66 :1 respectively) concentrations - as well as n-6 : n-3 ratio (24.39 : 1), quantity (g) of n-6 per yolk weight (g) (0.97g/yolk) and concentration (mg) per gram of whole egg (18.18 mg/g whole egg). Further, the high oleic (HO) sunflower (n-9) treatment was particularly more effective ($P < 0.001$) than the other dietary treatments in terms of the deposition of the main mono-unsaturated fatty acids – oleic acid into the egg yolk (44.3%). The HO sunflower oil (n-9) induced yolk, further resulted in the highest MUFA (48.7%), UFA : SFA (1.88 : 1), MUFA : SFA (1.41 : 1), MUFA : PUFA (3.02 : 1), as well as n-9 to n-3 ratio (57.51 : 1) and n-9 concentrations (g) per yolk weight (g) (1.98 g/yolk) and its quantity (mg) per gram of whole egg (38.37 mg/g whole egg). The results obtained for the tallow (SFA) treatment did not follow the same pattern as observed in the unsaturated diets, regarding the deposition of its prominent fatty acids in the egg yolk. The egg yolk with the highest ($P < 0.0001$) palmitic acid concentration (30.3%) was obtained from the fish oil (n-3) dietary treatment, while the highest concentration of stearic acid (11.6%) was recorded in the sunflower oil (n-6) treatment - despite the tallow diet being the most concentrated regarding these two prominent saturated fatty acids (Table 4.1). It was evident in the present study, that the egg yolk FAME can be considerably altered through dietary means. It was however noteworthy, that unlike the unsaturated lipid sources – mono-unsaturates (high oleic sunflower oil n-9), and polyunsaturates (sunflower oil n-6, fish oil n-3 and linseed oil n-3), which resulted in a concomitant increase of their respective egg yolk fatty acids. The saturated fatty acids could not be significantly elevated by the inclusion of a highly saturated lipid diet, such as tallow. These results have been supported by various researchers (Noble *et al.,* 1990; Da Silva Filardi *et al*., 2005). Additionally, rather than increasing the concentration of saturated fatty acids, the use of tallow (SFA) was observed to increase both the concentration and proportion of egg yolk MUFA's, which ultimately agrees with Grobas *et al.* (2001) and Cachaldora *et al.* (2008).

It is evident from the present study that unlike the saturated lipids, the mono-unsaturated and the polyunsaturated lipid sources yielded consistent changes in their yolk fatty acid content, respectively. This is in agreement with previous investigations regarding the nutritional

improvement of meat (Rodriguez *et al.,* 2005; Azcona *et al.,* 2008) and eggs (Leeson *et al.,* 2007; Garcia-Rebollar *et al.,* 2008; Saafa *et al.,* 2008; Celebi & Macit, 2009) by means of dietary manipulation. Previous experiments have also indicated that dietary inclusion as low as 15 g/kg could effectively increase the total n-3 yolk fatty acid content (Herber & Van Elswyk, 1996; Gonzalez-Esquerra & Leeson, 2000a; Schreiner *et al.,* 2004; Safaa *et al.,* 2008). The present study agrees with these researchers. It could clearly be seen that the control (1.5% linseed & 1.5% fish oil) and the 3% fish oil diets, both increased the n-3 yolk content significantly (Table 5.5).

Table 5.5 The effect of dietary lipid sources on the total mono-unsaturated (n-9), and polyunsaturated (n-3 and n-6) fatty acid contents per yolk weight (g) and milligram per whole egg (shell inclusive)

Parameter	Control	Fish oil (n – 3)	Sunflower oil (n – 6)	#HO Sunflower oil (n – 9)	Tallow (SFA)	Significance (P)
Yolk fat (g)	4.26	3.95	3.94	4.07	4.11	0.119
g/yolk*						
n -9	1.71[b]	1.45[c]	1.41[c]	1.98[a]	1.95[a]	<0.0001
n-6	0.59[b]	0.48[c]	0.97[a]	0.63[b]	0.58[b]	<0.0001
n-3	0.39[a]	0.42[a]	0.04[b]	0.04[b]	0.05[b]	<0.0001
mg/whole egg[Δ]						
n-9	30.90[b]	26.89[b]	26.48[b]	38.37[a]	35.77[a]	<0.0001
n-6	10.63[c]	8.85[d]	18.18[a]	12.22[b]	10.59[c]	<0.0001
n-3	7.09[b]	7.89[a]	0.76[b]	0.76[b]	0.92[b]	<0.0001

[a,b,c,d] Row means with different superscripts differ significantly at $P < 0.05$
[#]HO = High oleic acid sunflower oil
[*]gram omega/gram of yolk
[Δ]mg omega/gram of whole egg

Grobas *et al*. (2001) reported similar increases in the n-9 (MUFA), as well as the n-6 and n-3 (PUFA) concentrations of different chicken strains, using olive- (n-9), soya- (n-6) and linseed oil (n-3), respectively. The fish oil treatment seemed to show a higher n-3 yolk deposition rate than the control treatment (10.7% *vs*. 9.2%), resulting in a better n-6 : n-3 ratio (1.14 : 1 *vs.* 1.52 : 1), despite the obvious higher n-3 concentration in the control diet (Table 4.1). This could mean a better utilization of the fish oil by the experimental hens. Further, a linear and quadratic increase in yolk concentration of long chain fatty acids (EPA, DHA) resulted from inclusion levels (0, 15 and 30 g/kg) of fish oil to different basal diets (Cachaldora *et al.,* 2008).

It was however further reported that an interactive effect of the added fish oil was more pronounced in the basal diets with no fat or the 50 g/kg lard, than those of the 50 g/kg linseed

or soybean diets. This could perhaps explain the higher deposition of EPA and DHA in the yolk of the 30 g/kg fish oil (n-3), compared to the 30 g/kg (50 : 50) fish oil : linseed fed hens, in the present study.

Marine sources of n-3 origin are particularly popular for human health benefits by direct incorporation of the long chain DHA into the eggs (Simopolous, 1999; 2002). This did not however reduce the importance of plant sources of n-3 such as e.g. linseed and canola oils due to their better lipid oxidative stability (Cherian & Sim, 1991; 1992). The rate of n-3 deposition has also been reported by the authors to vary between these two sources, with linseed being superior (8.8%) to canola oil (2.4%). These α-linolenic acid rich sources could also be used in increasing the long chain fatty acids (e.g. DHA) of egg yolk. The conversion to these longer fatty acid derivatives being carried out by certain endogenous processes - starting with the elongation of (Elovl)-2 and/or Elovl-5 elongases, then desaturation by delta-5 (Δ^5) and the delta-6 (Δ^6) desaturases (Leonard *et al*., 2002; Jump, 2004). Although the functionality of these enzymes were not tested during the current study, it was clearly observed - considering the resulting high (5.5%) content of DHA recorded (Table 5.3) from a relatively low dietary concentration (1.4%) of the same fatty acids in the control diet (Table 4.1). Furthermore, Cherian *et al.* (1996) and Carrilo-Dominguez *et al.* (2005) attributed the detection of this long chain fatty acid in the egg yolk to the capability of chickens to elongate the precursor α-linolenic acid to its metabolites (EPA, DPA and DHA) during the formation of yolk lipids in their liver. Additionally, linseed has been used at graded levels in combination with fish oil to improve the sensory acceptability, while maintaining the quality of fish oil enriched eggs (Garcia-Rebollar *et al.*, 2008). Although not investigated, the disadvantage of increased dietary fish oil levels is the fishy odour it tends to impact on the egg. There are disparities in the available results regarding the nutritional benefits of increasing levels of fish oil. Adams *et al*. (1989) reported that a 6% fish oil dietary treatment resulted in lower n-3 yolk concentrations, compared to a 3% fish oil treatment. Further, Van Elswyk *et al*. (1995) and Van Elswyk (1997) found no concomitant increase in n-3 egg yolk content between the 1.5% and 3% fish oil diets. Conversely, a consistent incorporation of DHA was reported by Gonzalez-Esquerra and Leeson (2000b) with a 0 to 6% graded level of dietary menhaden oil.

In the current study, a slight improvement was recorded in the incorporation of the total n-3 (10.71%) and the total n-3 per gram of yolk (0.42 g/yolk) of the fish oil treatment, compared

to the control treatment. It was noteworthy to take into account the higher palmitic concentration (and resultant saturated fat) also detected in the eggs produced from the fish oil diet - which may not be common for n-3 fatty acids of plant origin. According to Antruejo *et al.* (2011), palmitic acid concentrations were to be found higher in n-6 rich (maize) diets, compared to those n-3 rich diets from chia and linseed. Another possibility was the quality of the supplied fish oil - a similar observation reported by King (2012). The saturated palmitic acid (C16:0) concentration of pure fish oil could vary between 13.4% and 20.2% (Basmacioglu-Malayogly, 2009), which could explain its high concentration (18.2%) in the fish oil (n-3) diet (Table 4.1). Saturated fat, particularly palmitic acid has been related to cardiovascular diseases according to the American Heart Association. The two n-3 sources used in the present study (linseed oil and fish oil), resulted in a considerable lowering in n-6: n-3 yolk ratios (less than 2 : 1), compared to the sunflower (24 : 1), high oleic sunflower (17 : 1) and tallow (12 : 1) diets. This means that both sources were the only dietary treatments (out of the 5 tested) that achieved an acceptable minimum ratio of 10 : 1, as recommended by the FAO. The balance of n-6 : n-3 is generally very critical because of the competition that exists between linoleic (n-6) and α-linolenic (n-3) acid for the same Δ-6 desaturate enzymes responsible for the conversion of these precursors for the longer chain metabolites (linoleic acid to arachidonic acid, and α-linolenic acid to EPA, DPA and DHA). An excessively high n-6 : n-3 ratio, as determined in the sunflower oil (n-6) treatment (24.39 : 1), could compromise the health of consumers by inhibiting the conversion and availability of these important long chain n-3 fatty acids, irrespective of the n-3 concentration. The low ($P < 0.001$) MUFA concentrations recorded in the sunflower (n-6) fed hens, seem to be a result of its high ($P < 0.001$) concentrations of PUFA's, as previously asserted by different studies (Ayerza & Coates, 2000, Ayerza *et al.*, 2002; Antruejo *et al.*, 2011). Mazalli *et al.* (2004) and Da Silva Filardi *et al.* (2005) also recorded a corresponding increase in yolk oleic acid (n-9) in the eggs obtained from an n-9 (canola) rich source.

The importance of mono-unsaturated fatty acid consumption has been emphasized for growing children (Uauy Dagach & Hoffman, 1991; Uauy Dagach & Olivares, 2007). The n-9 lipid source in the present study (high oleic sunflower) did not only increased the concentration of mono-unsaturated fatty acids in the egg yolk, it also elevated the proportion of unsaturated fatty acids to saturated fatty acids (1.88 : 1) significantly (Table 5.4). This is could be regarded as an important factor in human nutrition, considering the renewed awareness on the high consumption of saturated fats, as captioned in the "great fat debate"

(Lichtenstein, 2011; Mozaffaria, 2011; Zelman, 2011). It was observed in the present study that tallow (SFA) did not increase the stearic acid concentration of egg yolk as reported by Celebi and Macit (2009). It rather positively affected the mono-unsaturate properties of the yolk, as previously reported by Grobas *et al.* (2001) and Cachaldora *et al.* (2008). Although stearic acid was high ($P < 0.0001$) in the tallow treatment (17.1%), it was drastically reduced in the egg yolk (9.8%) and did not differ to other treatments - except for the sunflower oil (n-6) (11.6%), despite being relatively lower in these diets (Table 4.1 and Table 5.3). The PUFA to SFA ratio also increased ($P < 0.05$) in the egg yolk according to Da-Silva Filardi *et al.* (2005), with an inclusion of 3% lard (saturated pig fat) to the poultry diet, compared to the yolk of eggs obtained from cotton oil, sunflower oil, or canola oil fed hens. These current results were complemented by Baucells *et al*. (2000) in that the total SFA concentrations in egg yolk ranged between 30 and 38%, irrespective of the dietary lipid source supplemented.

5.4 Conclusions

Egg weight was the main production parameter influenced ($P < 0.05$) in the long term by dietary lipid sources. Diets high in n-3 (omega-3) fatty acids may cause a reduction in egg weight, while heavier eggs could be obtained by using lipid sources high in n-6 (omega-6) fatty acids. This could implicate a specific effect of n-3 fatty acids on body weight and chicken lipid metabolism. Furthermore, it can be concluded that the tallow treatment resulted in a reduced ($P < 0.05$) number of sellable eggs. Current results also show that unsaturated dietary lipid sources could alter the prevalence of fatty acid methyl esters (FAME) in the egg yolk in a similar dietary way, with more pronounced effects in the polyunsaturated fatty acids. This means that dietary supplementation of these lipid sources (PUFA's) could increase the fatty acid concentrations in the eggs. The inclusion of tallow (SFA) however, did not result in a distinct corresponding stearic or palmitic acid elevation, but rather in an increase in the mono-unsaturates, similar to the n-9 source (high oleic sunflower), which made it equally important in increasing the unsaturation to saturation (UFA : SFA) ratios in eggs. From a human nutrition perspective, both the control and fish oil (n-3) treatments resulted in an ideal n-6: n-3 ratio (< 2 : 1). The eggs from these treatments could therefore be suitably labelled as omega-3 enriched. On the other hand, yolk n-9 : n-6 ratio was not particularly related with the dietary lipid source.

CHAPTER 6
GENERAL CONCLUSION AND RECOMMENDATIONS

6.1 General conclusions

Generally, dietary lipid sources affected the productive and reproductive performances of both cockerels and hens. Results from the present study indicated that the supplemented lipid sources affected the sperm quality of the cockerels, during the different growth phases (≤ 46 and ≥ 69 weeks of age). Inclusion levels of either 1.5% fish oil + 1.5% linseed oil (n-3) or 3% high oleic acid (HO) sunflower (n-9) may be used for maintaining sperm motility, both during the peak and at the end of the production life cycle of the cockerels. Also, high fertility, as well as an acceptable level of hatchability was achieved in older birds (≥ 69 weeks of age), by feeding either of these n-3 or n-9 supplementary dietary lipid sources.

Supplementary n-3 fatty acids, using e.g. fish oil (3%), were better utilized by older (≥ 69 weeks of age) cockerels. This was reflected in an improved sperm motility (56.7%; $P < 0.05$), when compared to the younger (≤ 46 weeks of age) cockerels (48.1%; $P < 0.05$). However, the differences in the prevailing ambient temperatures during the two age phases (Trial 1 and Trial 2) may also have had a more pronounced effect on the fish oil treatment - from the perspective of lipid oxidation susceptibility. Higher hatchability of the fertile eggs (76.0%; $P < 0.05$) was one advantage experienced in feeding a 3% fish oil diet to an old flock of hens and cockerels. The n-6 lipid source in the present study (sunflower oil) did not record any adverse effect on sperm quality (motility) during both the peak and later production phase. This lipid source (n-6) may however have caused reduced hatchability (51.1%, $P < 0.05$) in the old hen flock, due to the tendency of increased egg weights. Saturated fatty acid lipid (tallow) supplementation apparently boosted the total sperm output (through increased semen volume), throughout the productive life of the cockerels. This could be advantageous in a flock incorporating a relatively lower male to female ratio.

It was not clear whether the poor productive performance and sperm quality of cockerels fed the fish oil treatment during the Trial 1 was due to a lower feed consumption (78.75g/bird/day, $P < 0.05$), or hormonal disruption caused by suspected lipid oxidation - judging by the evidently inferior semen quality recorded (sperm motility and sperm viability). It should be noted however, that performances (with the exception of sperm viability) of cockerels in all treatments were generally better during Trial 2 - a period when the birds were

older and cooler environmental temperatures prevailed. The polyunsaturated fatty acid (PUFA) sperm profile was most affected by the lipid sources, and either the control diet or that of 3% fish oil would decrease the n-6 : n-3 ratio in the cockerel sperm.

The high cost of high oleic acid sunflower oil (n-9) may be a limitation, when considering its use in a layer breeders production system, regardless of its positive effects on sperm quality, as well as fertility (92.1%; $P < 0.05$) in the old flock.

Moreover, the present study highlighted the need for a holistic approach in determining the effects of dietary lipid sources on cockerel sperm quality. With factors such as the effect of weather on feed intake, together with the ejaculation of contamination free semen by cockerels are important factors contributing to the physiological state of the cockerel, which could eventually be reflected in the sperm quality. The present study is a first in South Africa to investigate the effect of saturated, mono-unsaturated, polyunsaturated n-6, and two polyunsaturated n-3 dietary lipid sources in a single trial. This is particularly important in terms of the reliability of the results - as the subjectivity of measurements during semen evaluation were reduced considerably, by ensuring e.g. the uniformity of bird condition and semen evaluation procedures. The relevance of semen quality evaluation in a chicken breeder system was also highlighted in the present study. Further, instead of the usual spiking (which may be an avenue for disease or heightened aggression), artificial insemination may also be employed in the event of a lack of mating interest in the old flock - with the high fertility level of the old cockerels recorded in the current trial. However, the practicability of AI on commercial chicken breeder farms remains doubtful, due to the labour intensiveness thereof.

The study conducted on the hens demonstrated that egg weight was the main production parameter affected ($P < 0.05$) in the long term (33 to 78 weeks of age) by the dietary lipid sources. This could be a specific effect of dietary fatty acids on the body weight and the chicken lipid metabolism. Furthermore, it was concluded that the tallow treatment resulted in a reduced (90.8%; $P < 0.05$) number of sellable eggs. Also, the inclusion of tallow did not result in a distinct corresponding stearic or palmitic acid increase, but it rather increased the mono-unsaturates, similar to the n-9 source (high oleic sunflower), as opposed to the actions of the dietary unsaturated fatty acids (control, fish oil, sunflower oil and high oleic sunflower oil). Both the control and the fish oil treatments produced eggs which could then be correctly labelled as “omega-3 enriched eggs”, due to their nutritionally acceptable n-6 : n-3 ratios

(1.52 and 1.14 respectively). Although omega-9 concentrations of egg yolk could be increased by either a mono-unsaturated (high oleic sunflower oil) or a highly saturated (tallow) lipid source, the consequent effect on the n-6 fatty acids was however not very distinct, confirming the unique relationship that exists between the n-3 and the n-6 fatty acids.

Monitoring of the hens over a long period (46 weeks) also accommodated the variation that could emanate from the utilization of dietary lipids between the peak and the end-of-lay stages of production in the hen.

6.2 Recommendations

The following recommendations could be made, based on the results generated from the current study in the evaluation of the effect of dietary lipid source supplementation on layer breeder performance.

- For future research, studies are required to understand the hormonal interaction of dietary lipid source supplementation, preferably those supplied by fish oil, on the reproductive performance of the cockerels.

- The uncharacteristic high semen volume produced by the saturated lipid source (tallow) used during the present study warrants further research.

- As the sex ratio is maintained at approximately 1♂: 10 ♀, it may be necessary that a thorough semen evaluation is performed, prior to the introduction of cockerels into a breeder flock.

- The type of dietary lipid supplementation employed should be based on the aim of production - meaning that lipid treatments have different effects on the quantity and quality of eggs for the egg producer, as well as on the ultimate fertility and the hatchability.

- Health beneficial eggs can be produced either by a combination of linseed oil and fish oil, (as used in the present study), or the sole use of dietary fish oil treatments. Although not evaluated, it is presumed that the sensory quality of the fish oil treated egg may be compromised.

ABSTRACT

Fertility in layer breeders following dietary fatty acid treatments

by

Olusola Samuel Olubowale

Supervisor: Prof. J.P.C. Greyling
Co-supervisor: Mr. F.H. De Witt
Department: Animal, Wildlife and Grassland Sciences
University: University of the Free State
Degree: M.Sc (Agric)

A study was conducted to evaluate the effects of dietary lipid sources on the productive and reproductive performances of cockerels and hens between 32 and 78 weeks of age. Five different dietary lipid sources namely fish oil, sunflower oil, high oleic (HO) acid sunflower oil, tallow and an equal (50 : 50) proportion of linseed and fish oil (representing the control diet), were used in formulating the five experimental diets. The objective was to formulate five *isoenergetic* and *isonitrogenous* diets, with a constant inclusion level (30 g/kg) of the various supplementary lipid sources, differing only in their fatty acid profile. The experiment on the cockerels (n = 10/treatment) was conducted in two phases- from 35 to 46 weeks (Trial 1) and 69 to 77 weeks of age (Trial 2), running concurrently with the hen (n = 25/treatment) production performance (32 to 78 weeks of age) trial. The following parameters were measured in the cockerels: daily feed intake, body weight, semen volume, sperm concentration, sperm motility, sperm viability and semen ejaculation rate. While lower ($P < 0.05$) daily feed intake (78.75 g/bird/day), body weight (2499 g), sperm motility (48.1%) and ejaculation rate (79.2%) was recorded in the dietary fish oil (n-3) treatment, a higher ($P < 0.05$) semen volume (0.42 mL) was recorded in the tallow (SFA) fed cockerels during Trial 1. During Trial 2, no significant differences ($P > 0.05$) were recorded in most of the parameters measured, except in the body weight of cockerels on the fish oil (n-3) treatment, which was the lowest ($P < 0.05$). Semen volume of the tallow (SFA) fed cockerels remained high ($P < 0.05$), with the lowest ($P < 0.05$) sperm motility (51.6%) however being recorded. The hatchability test conducted during Trial 2 revealed a lower fertility (84.6%), but higher ($P <$

0.05) hatchability of fertile eggs (76%) in the fish oil (n-3) treatment. The lowest hatchability rates of both eggs set (51.1%) and fertile eggs (58.2%) were recorded in the sunflower (n-6) treatment. All production parameters, except hen-day-egg production were affected ($P < 0.05$) by the dietary lipid supplementation over a mean period of 46 weeks (33 to 78 weeks of age). The heaviest ($P < 0.001$) eggs were produced in the sunflower (n-6) treatment group (59.3 g), while the fish oil (n-3) fed hens produced the lightest eggs (57.1 g). This resulted in a higher ($P < 0.001$) yolk and albumen weights being recorded in the sunflower oil (n-6) treatment. The body weights of the hens (1779 g) fed fish oil (n-3) were also low ($P < 0.001$), compared to the other treatments. A higher number of sellable eggs, resulting from differences in percentage of cracked eggs, were also recorded in the control (93.3%) and sunflower (93.2%) groups, and compared to those obtained with the tallow (90.8%) treatment. The concentrations of yolk fatty acids and their relative total proportions were influenced by dietary lipid sources, except where oleic fatty acid (n-9) was increased ($P < 0.001$) in the tallow (SFA) treatment. Consequently, the omega-6 to omega-3 (n-6 : n-3) ratio was lowered according to their dietary sources; 1.52 : 1, 1.14 : 1, 24.39 : 1, 17.21 : 1 and 12.27 : 1 for the control, fish oil (n-3), sunflower (n-6), high oleic sunflower (n-9), and tallow (SFA) treatments, respectively.

The present study therefore showed that dietary lipid sources have an effect on the productive and reproductive performance of the cockerels and hens. However, environmental factors such as ambient temperature could influence the utilization of dietary fish oil, as observed in the performance and semen quality of the cockerels.

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