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# THE PHYSIOLOGICAL RESPONSES OF GUINEA FOWL, MUSCOVY DUCKS AND JAPANESE QUAIL TO HIGH–FAT DIETS

JANINE DONALDSON

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD).

Johannesburg, 2016

## DECLARATION

I declare that the work contained in this thesis is my own, with all assistance acknowledged. It is being submitted for the degree of Doctor of Philosophy (PhD) in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

…….

(Janine Donaldson)

…… day of …… 2016

## ABSTRACT

Animal fats and vegetable oils are often added to poultry diets to improve growth performance or to modify the lipid profiles and overall lipid content of the edible bird tissues to be consumed by humans. Effects of these high-fat diets (HFD) on the health status of birds during production are rarely investigated and most research involving current poultry production practices is performed in chickens and extrapolated to alternative poultry species. The current research investigated the effects of HFD feeding on the overall health status and tissue lipid profiles of some alternative poultry species which are becoming popular as table birds. Briefly, Guinea fowl (*Numida meleagris*), Muscovy ducks (*Cairina moschata*) and Japanese quail (*Coturnix coturnix japonica*) were fed a HFD rich in saturated fatty acids (Guinea fowl and Muscovy ducks) or HFDs of varying fatty acid profiles (Japanese quail) or a standard diet for a period of between four and twelve weeks. Oral glucose tolerance test parameters, erythrocyte osmotic fragility indices and serum metabolic health markers were used to assess the overall health status of the birds, following the HFD feeding. Liver lipid content, caecal microflora, organ masses, intestine lengths, the mass, length and relative density of the femurs, as well as the liver, breast muscle and thigh muscle lipid profiles were also assessed in the majority of the birds. The HFDs were well-tolerated by the birds, with no obvious adverse effects observed with respect to the health status of the birds. Dietary fatty acids were successfully transferred to the edible tissues of the Japanese quail and despite the very high level of lipid supplementation, the overall lipid content of the edible tissues remained within normal ranges. Thus if necessary, in terms of the modification of the edible tissue lipid profiles of poultry birds, HFDs of this nature can be used during poultry production without any adverse health implications for the birds. The birds should remain healthy during the feeding period, avoiding any additional production costs related to the maintenance of the birds' health status. In terms of potentially increasing the overall lipid content of the edible bird tissues as a means of increasing the total amount of lipids ingested by humans, the significantly increased level of lipid inclusion used in the present study was unsuccessful in doing so. Thus, it seems that regardless of the increased lipid inclusion, the overall lipid content of the edible bird tissues is somewhat regulated within a certain range. Future studies should focus on the mechanisms responsible for the resilience of these birds to the HFD feeding and the mechanisms involved in the regulation of the overall content of lipid deposition within the edible bird tissues.

## ACKNOWLEDGEMENTS

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To my friends (particularly the girls from Wits, Alessandra, Sam and Vernice) who suffered through this process with me and listened to my whining, I am very grateful for your understanding and reassurance, as well as our Friday G & T sessions which helped take my mind off things for a while. To my husband and my family, your constant encouragement, love and support has brought me through the darkest of times and enabled me to see the light at the end of a very long and dark tunnel, I will be eternally grateful.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AMP | Adenosine monophosphate |
| AMPK | Adenosine monophosphate-activated protein kinase |
| AST | Aspartate transaminase |
| ATP | Adenosine triphosphate |
| AUC | Area under the curve |
| CPT-1 | Carnitine palmitoyltransferase |
| HFD | High-fat diet |
| IL-6 | Interleukin 6 |
| OGTT | Oral glucose tolerance test |
| PBS | Phosphate-buffered saline |
| PPARα | Peroxisome proliferator-activated receptor alpha |
| STD | Standard diet |
| SREBP-1c | Sterol-responsive element binding protein-1c |

## PREFACE

This thesis has been written and submitted in the following manner: Chapter 1 is a review of the literature concerning the use of added dietary lipids as a source of energy in diets formulated for poultry, as well as the use of alternative poultry species, including Guinea fowl, Muscovy ducks and Japanese quail, as alternative sources of protein for human consumption. The metabolism of lipids in avian species is also discussed. The possible HFD-induced metabolic disturbances including changes in glucose tolerance, erythrocyte osmotic fragility, bone health, gut microbiota balance and fat deposition within birds are also included in the chapter. The organoleptic consequences resulting from altered lipid deposition in the meat of poultry birds are also discussed, together with the implications for humans following consumption of the poultry meat. The overall objective and specific aims for each of the two main studies which form part of this thesis are also stated in Chapter 1. Chapters 2 and 3 include the introduction, methods and results sections for study 1 and study 2, respectively, as well as a discussion of the results obtained in each study. The overall discussion of the results obtained from both studies, as well as conclusions for the thesis as a whole is presented in Chapter 4.

Study 1 was performed using Guinea fowl and Muscovy ducks which were fed a HFD based on palm oil and lard (high in saturated fatty acids) for either four, eight or twelve weeks. A diet composed mainly of saturated fatty acids was used for study 1 as the consumption of saturated fatty acids has been shown to increase the risk of developing components of the metabolic syndrome and thus more likely to result in observable metabolic changes within the birds. The possible HFD-induced changes in glucose tolerance, erythrocyte osmotic fragility, serum metabolic/general health profile, liver lipid content and liver histology, as well as the gut microbiota balance were investigated. In order to extend the spectrum of alternative poultry species investigated, Japanese quail (another poultry species which is becoming increasingly popular as a table bird) were used for study 2. Different HFDs were used in study 2, with varying fatty acid profiles, in order to get a better representation of the different types of animal and vegetable fat sources which are traditionally used in the formulation of poultry feed. The HFDs were composed of coconut oil, lard, palm oil, soyabean oil and sunflower oil. The same parameters assessed in study 1 were also assessed in study 2. Additionally, bone density (femur) and resulting fatty acid profiles of the edible bird tissues (liver, breast and thigh muscles) were assessed following consumption of the different HFDs in the Japanese

quail. These aspects are important in terms of current poultry production practices and overall production costs, as well as the resulting poultry meat products provided to consumers.

## WRITTEN OUTPUTS EMANATING FROM THE WORK CONTAINED IN THIS THESIS

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Donaldson J, Pillay K, Madziva MT, Erlwanger KH (2015). The effect of different high-fat diets on erythrocyte osmotic fragility, growth performance and serum lipid concentrations in male, Japanese quail (*Coturnix coturnix japonica*). *Journal of Animal Physiology and Animal Nutrition* **99**: 281-289.

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Donaldson J, Madziva MT, Erlwanger KH. The progressive effects of a high-fat diet on glucose tolerance, serum biochemistry, liver histology and liver and fat pad mass of Guinea fowl (*Numida meleagris*) and Muscovy duck (*Cairina moschata*).

## CONFERENCE PRESENTATIONS

40th Congress of the Physiological Society of Southern Africa, Cape Town, South Africa, 2012; Poster presentation: "The effects of a high-fat diet on Guinea fowl (*Numida meleagris*) erythrocytes" J. Donaldson, R. Dangarembizi, B. Mtetwa, M. T. Madziva and K. H. Erlwanger.

4th Cross Faculty Symposium, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa, 2013; Oral presentation: "The effects of a high-fat diet on the osmotic fragility of Guinea fowl and Muscovy duck erythrocytes" J. Donaldson.

43rd Congress of the Physiological Society of Southern Africa, Parys, South Africa, 2015; Poster presentation: "The effects of a high-fat diet rich in saturated fatty acids on glucose tolerance and the liver of Guinea fowl (*Numida meleagris*) and Muscovy duck (*Cairina moschata*)" J. Donaldson, M. Madziva, K. H. Erlwanger.

7th Cross Faculty Symposium, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa, 2016; Oral presentation: "The effects of different high-fat diets on the health status and tissue lipid profiles of male, Japanese quail (*Coturnix coturnix japonica*)" J. Donaldson.

## STATEMENT OF CONTRIBUTION TO DATA COLLECTION AND ANALYSIS

I, Janine Donaldson, hereby declare that I was responsible for the majority of the data collection for both study 1 and study 2, as presented in Chapters 2 and 3. The processing of the liver samples collected from the birds, in both study 1 and study 2 for histological analysis, was performed by a laboratory technician (Margaret Badenhorst) within our research group. The proximate content (fat content and crude protein content) and energy analysis of the diets used in study 2, as well as the analysis of the total lipid content and fatty acid profiles of the liver and muscle tissue samples from study 2 were performed at a South African National Accreditation System -accredited analytical laboratory [Agricultural Research Council's Irene Analytical Services Laboratories, South Africa]. The studies were designed in conjunction with Associate professor KH Erlwanger and Dr MT Madziva. I performed all the statistical analyses on the data collected and I wrote the manuscripts for the papers emanating from this thesis. The manuscripts were reviewed by Associate professor KH Erlwanger and Dr MT Madziva.

# CHAPTER ONE: LITERATURE REVIEW

## 1.1 Poultry production

The poultry production industry in South Africa is one of the largest contributors to the South African agriculture sector (Davids et al., 2015) and offers a number of economic and social advantages (Meissner et al., 2013a). According to the most recent report compiled by the South African Poultry Association (SAPA) in 2012, the South African poultry industry contributed approximately 22 % of the total income for the agricultural sector, with a gross income of over R37 billion (SAPA, 2012). In addition to its contribution to gross domestic profit, the South African poultry industry is also important in terms of job creation and providing food security (SAPA, 2012; Meissner et al., 2013a). Of the total number of workers employed in the agricultural sector in South Africa, about 10 % of them are employed in the poultry production sector (Mkhabela and Nyhodo, 2011; SAPA, 2012), providing direct employment within the industry to approximately 77 000 people. The poultry industry also plays a significant role in providing food security for the South African population in that it provides the cheapest form of animal protein for human consumption (Davids et al., 2015). In fact, in the year 2011, of the total amount of meat products consumed in South Africa, 55 % was chicken meat (Davids et al., 2015).

Broiler chicken meat constitutes the majority of poultry meat consumed by people worldwide, making up approximately 70 % of total poultry consumption (Al-Nasser, 2006). Poultry meat is a favourite animal protein source amongst consumers. Besides the affordability of poultry meat (Al-Nasser, 2006; Petracci et al., 2013), its perceived health benefits also contribute to its popularity. Poultry meat has a high protein and low fat content, as well as a balanced omega-6: omega-3 polyunsaturated fatty acid ratio and low cholesterol content (Vukasovic, 2010; Daniel et al., 2011; Petracci et al., 2013). According to statistics obtained by the Food and Agriculture Organization of the United Nations, poultry meat is one of the highest consumed animal proteins globally (13.6 kg per capita, per year) and is only superceded by pork (15.8 kg per capita, per year) (FAOSTAT, 2014; Font-i-Furnols and Guerrero, 2014). Global data obtained by the European Commission Organisation for Economic Co-operation and Development (OECD) indicates that although the predicted increase in gross consumption of meat worldwide is largely due to increases in poultry and pork meat consumption, by 2022 poultry meat is expected to be the most consumed meat in the world (European-Commission, 2012; Henchion et al., 2014).

As a result of the ever-increasing world population which was estimated to be approximately 7.2 billion people as of the end of 2014 (World Bank Group, 2015), conventional animal protein sources worldwide are not sufficient in meeting the increasing human protein requirements (Ayyub et al., 2014). With a projected escalation in the world population to about 9 billion people by the year 2050 (Hawkesworth et al., 2010), an estimated increase of between 60-70% in food will be needed to feed the global population (Muchenje and Mukumbo, 2015). The highest incidence of food insecurity occurs in Sub-Saharan Africa, with approximately 51.6 % of South African households being food insecure (Labadarios et al., 2009; Muchenje and Mukumbo, 2015; Musemwa et al., 2015). Thus there is a need to increase the viability of natural resources in Africa to guarantee their use by forthcoming generations (Muchenje and Mukumbo, 2015).

During the last few decades there has been an overall initiative by researchers and poultry farmers alike to increase the production of chicken meat, however not much attention has been paid to the potential increase in meat production from alternative poultry species (Biswas et al., 2015). More recently, in order to try and alleviate the consumer pressure on the domestic chicken as a protein source, the use of alternative or non-traditional poultry species is being investigated. These non-traditional species include, amongst others, guinea fowl, ducks and quail (Biswas et al., 2015). Utilization of these non-traditional poultry species in the coming years as alternative sources of protein for human consumption could have a significant contribution to meeting global protein demands and alleviating the current food security status of many developing countries (Geldenhuys et al., 2013; Biswas et al., 2015). The alternative poultry species considered in this thesis were the Guinea fowl (*Numida meleagris*), Muscovy duck (*Cairina moschata*) and Japanese quail (*Coturnix coturnix japonica*). These avian species have the potential to be produced on a larger scale, with the aim of providing an alternative protein source to the population of South Africa, over and above the domestic chicken.

### 1.1.1 The Guinea fowl (*Numida meleagris*)

Guinea fowl are part of the family *Numida*. The helmeted Guinea fowl (*Numida meleagris*) is the most well-known of the Guinea fowl species (Madzimure et al., 2011). Guinea fowl are gaining popularity as an alternative poultry source worldwide and although they are native to Africa,

Guinea fowl production/rearing is widespread across the world, including Europe, North America and Australia (Moreki and Seabo, 2012). In South Africa, the helmeted Guinea fowl is relatively widely distributed (Geldenhuys et al., 2013). Guinea fowl production is specifically important within third-world, developing countries, as a relatively cost-effective approach to alleviating poverty and improving the sustainability of rural economies (Mwale et al., 2008; Madzimure et al., 2011). Guinea fowl can be reared successfully with little effort under semi-intensive conditions (Yildirim, 2012) and are thus ideal for small-holder farmers, worldwide.

In addition to the relatively effortless rearing of the Guinea fowl, the birds also have relatively short reproduction cycles, which together with their resistance to various common poultry diseases and parasites, is advantageous in terms of poultry production (Mwale et al., 2008; Madzimure et al., 2011). Guinea fowl meat is popular with consumers due to its wild, gamey flavour as well as its low fat (4 % of total body mass) and high protein content (23 % of total body mass) (Mareko et al., 2006; Madzimure et al., 2011) compared to that of broiler meat (15 % of total body mass composed of fat) (Jiang et al., 2012). Despite the Guinea fowl's small skeletal frame, the carcass yields a large proportion of meat (Mareko et al., 2006; Elhashmi et al., 2012). The fact that Guinea fowl are seasonal breeders (Moreki, 2009) may be viewed as a disadvantage with regards to the production of Guinea fowl as an alternative protein source. However, they have been shown to start laying their eggs at the beginning of the warm season and continue laying for as long as up to eight or nine months (Moreki and Seabo, 2012). Additionally, the use of artificial lighting has been shown to extend the egg-laying period (Moreki and Seabo, 2012).

Another alternative poultry species which is already quite popular in certain parts of the world is the Muscovy duck. Despite the high fat content of the meat from the Muscovy ducks, it is regularly consumed as a protein source by humans.

### 1.1.2 The Muscovy duck (*Cairina moschata*)

The Muscovy duck originated from Central and South America and is part of the family *Anatidae* (Huang et al., 2012; Yakubu, 2013). In Africa, ducks, including the Muscovy duck, form the second

largest population of poultry birds next to chicken (Téguia et al., 2008). Muscovy ducks are widely used to produce meat and serve as an alternative to conventional poultry meat (Adzitey, 2012). Duck meat production has increased dramatically over the past few decades and, in 2010, world duck meat production reached 3.9 million tonnes, with China responsible for approximately 69 % of total production (Huang et al., 2012).

In terms of poultry production, the Muscovy duck is advantageous in that it is a relatively hardy bird and is able to adapt easily to a variety of harsh environmental conditions, specifically very hot climates (Adzitey, 2012; Yakubu, 2013). As a result of its hardiness and resistance to a number of common poultry diseases, the Muscovy duck is well suited for small-scale rural farmers in Africa and other developing countries. The rearing of Muscovy ducks as an alternative protein source for human consumption could therefore add to food security worldwide (Yakubu, 2013). The consumption of duck meat as an alternative protein source is popular as a result of its unique flavour, as well as its favourable composition of amino acids and fatty acids (Pingel, 2011).

Muscovy duck meat contains significant proportions of aspartic acid and glutamic acid, as well as the essential amino acids, lysine and methionine, in both the breast and thigh muscles (Aronal et al., 2012). This trend is comparable to that observed in chickens; however the concentration of methionine is significantly higher in both the breast and thigh muscles of Muscovy ducks compared to that of chickens (Hamm, 1981; Aronal et al., 2012). With respect to protein quality, Muscovy duck meat is relatively on par with chicken meat. Muscovy duck thigh muscles have a significantly higher essential amino acid index compared to the thigh muscles of chicken; however the essential amino acid index of Muscovy duck breast muscles is slightly lower than that observed in the breast muscles of chicken (Hamm, 1981; Aronal et al., 2012). Duck meat is also rich in various vitamins and minerals, such as niacin, selenium and iron (Adzitey, 2012). Despite a favourable fatty acid profile, duck meat has a higher overall fat content (compared to that of chicken) which is usually not well accepted by consumers (Jiang et al., 2012).

The Japanese quail is known to be a much leaner bird than the Muscovy duck. Thus it may be a healthier choice as an alternative protein source for the health-conscious consumer.

### 1.1.3 The Japanese quail (*Coturnix coturnix japonica*)

The Japanese quail (*Coturnix coturnix japonica*) represents a plausible alternative to the domestic chicken, in terms of animal protein supply for human nutrition. Like the domestic chicken, the Japanese quail also belongs to the order *Galliformes* and the family *Phasianidae* (Minvielle, 2004). The Japanese quail is becoming an increasingly popular source of poultry meat and eggs worldwide, in parts of Asia, the Middle East, Europe, America and Africa (Bulus et al., 2013; El-Daly et al., 2014). Japanese quail production is advantageous compared to that of other poultry species due to a number of unique qualities which Japanese quail possess. Firstly, the Japanese quail is a relatively small bird, thus requiring minimal space for housing. In fact, between eight to ten adult quails can be reared in the same space required for one adult chicken (Haruna et al., 1997). Consequently, as a result of their small body size, Japanese quail have lower feed requirements than chickens, thus reducing overall production costs (Owen and Dike, 2013). Japanese quail have a high growth rate, attain sexual maturity relatively early and have a short generation interval, thus allowing for the production of many generations of quail throughout the year (Afrose et al., 2011; Owen and Dike, 2013). In terms of meat quality, quail meat, like chicken meat, is a source of protein of high biological value, with a beneficial amino acid profile (Genchev et al., 2008). Quail meat is tender with a unique, ‘gamey’ flavour (Dauda et al., 2014) and has a low calorific content, making it a suitable choice for health-conscious consumers (Owen and Dike, 2013).

Since alternative poultry species such as the Guinea fowl, Muscovy duck and Japanese quail are being explored as substitutes for the domestic chicken for human protein consumption, it is important to investigate the metabolic effects of current poultry production practices with respect to poultry feeds and poultry nutrition on each specific species individually. One such current practice includes feeding poultry birds a HFD. The metabolic effects of these HFDs, as well as the effects on the overall health status and well-being of the birds during production need to be investigated. With this information, poultry feeds for alternative poultry species can then be tailored towards species-specific requirements.

## 1.2 Poultry feed formulation

Poultry feed represents the primary cost involved in poultry production, accounting for between 60 to 70 % of total production costs (Baéza et al., 2015; Begli et al., 2016). Poultry feed ingredients may vary according to species-specific requirements; however the basic principles of poultry nutrition are generally applicable to all poultry birds produced as meat and egg-laying birds (Ravindran, 2013). Poultry feeds are typically formulated using a number of different ingredients including animal and vegetable fats, cereal grains, cereal by-products, plant protein sources, animal protein sources, crystalline amino acids, vitamins, minerals and feed additives, amongst others (Jegede et al., 2011; Jegede et al., 2012; Oladokun and Johnson, 2012; Ravindran, 2013). Table 1.1 shows a list of common poultry feed ingredients used worldwide. Together these feed ingredients provide the nutrients and energy required for maintenance of the overall health of the birds, as well as to ensure optimal growth and reproduction (Kratzer et al., 1994). The largest proportion of poultry diets consists of energy sources such as maize, followed by plant and animal protein sources such as soyabean meal and fishmeal, respectively (Ravindran, 2013). The use of various carbohydrate and protein sources in the formulation of poultry feed will first be briefly discussed, followed by a more in depth discussion of the animal and vegetable fats used. Although vitamins, minerals and fibre also form an important part of poultry diets, this thesis is focussed on the macronutrient content of poultry diets.

**Table 1.1** Common ingredients used to formulate poultry feeds.

| Ingredients used to formulate poultry feeds |
|---|
| **1. Energy sources:**<br>- Cereals (mainly maize), cereal by-products (serve as a source of fibre)<br>- Vegetable oils and animal fats |
| **2. Plant protein sources:**<br>- Soyabean meal |
| **3. Animal protein sources:**<br>- Fish meal<br>- Meat meal<br>- Bone meal |
| **4. Mineral supplements:**<br>- Calcium supplements<br>- Phosphorus supplements<br>- Trace mineral premixes<br>- Salt<br>- Sodium bicarbonate |
| **5. Miscellaneous:**<br>- Vitamin premixes<br>- Crystalline amino acids (methionine, lysine, threonine)<br>- Non-nutritive feed additives (antibiotics, enzymes etc.) |
| |

### 1.2.1 Carbohydrates in the formulation of poultry feed

Cereal grains including corn, wheat, barley and maize constitute the dietary carbohydrates which are commonly used in the formulation of poultry diets (Kratzer et al., 1994; Ravindran, 2013). Most of the carbohydrates within these cereal grains occur as starch which is highly digestible for poultry (Moran, 1985). Thus, the cereal grains serve as sources of readily available energy for the birds.

Maize accounts for roughly 65 % of the total metabolisable energy content and 20 % of the total protein content in poultry diets (Cowieson, 2005). The protein content of maize is somewhat low at approximately 80 $g.kg^{-1}$, which is lower than that found in wheat and barley (110 $g.kg^{-1}$). The energy content however, is somewhat higher in maize versus wheat and barley, at approximately 14 $MJ.kg^{-1}$ versus 12 $MJ.kg^{-1}$, respectively (Cowieson, 2005). In addition to its' high energy content, maize also contains very small concentrations of anti-nutritional factors such as phytin, trypsin inhibitors and lectin (Eeckhout and De Paepe, 1994; Cowieson, 2005). Thus, in comparison to wheat and barley, maize is considered to be of relatively high nutritional value for use in poultry diets. The energy levels of poultry diets are key factors which influence the amount of feed consumed by the birds during production, as birds eat predominantly to satisfy their energy needs (Ravindran, 2013). Thus it is important that poultry diets are formulated with appropriate amounts of energy in order to maintain the overall health of the birds. Not all of the gross energy present within the poultry feed is actually utilised by the birds. The amount of energy available from the feed for use by the bird is referred to as the metabolisable energy content (Palić et al., 2012). The metabolisable energy content of a diet is directly related to the absorption and overall availability of the various dietary nutrients which is directly dependent on their digestibility (Palić et al., 2012).

Subsequent to the energy source in poultry diets, the protein source constitutes the next largest component of poultry diets (Ravindran, 2013; Beski et al., 2015).

### 1.2.2 Proteins in the formulation of poultry feed

Both plant and animal protein sources are used in the formulation of poultry diets; however the major proportion of dietary protein is usually supplied by plant protein sources (Ravindran, 2013;

Beski et al., 2015). The preferred plant protein source used to formulate poultry diets is soyabean meal (Kim et al., 2012a; Ravindran, 2013). Soyabean meal has a crude protein content of between 40-48 % (Ravindran, 2013). In comparison to other oilseed meals, soyabean meal contains a good balance of essential amino acids, as well as a much higher metabolisable energy content (Ravindran, 2013; Beski et al., 2015).

In addition to soyabean meal, other oil seed meals, such as canola and sunflower oilseed meal, are sometimes used as alternative plant protein sources in the formulation of poultry diets. However, dissimilar to soyabean meal, most other plant protein sources need to be supplemented with animal protein sources and crystalline amino acids in order to ensure that the plant-based diets meet the amino acid requirements for poultry meat and egg production, which vary according to the productive state of the bird (Kratzer et al., 1994; Ravindran and Bryden, 1999; Beski et al., 2015). In fact, animal protein sources are primarily used to balance the amino acid content of plant-based diets, rather than as the primary protein source since they are quite pricey (Ravindran and Bryden, 1999; Beski et al., 2015).

The primary animal protein sources which are used in the formulation of poultry diets are fish meal and meat/bone meal. These animal protein sources are attained from animal products following the slaughter of the animals and are then processed for use in animal/poultry feed (Ravindran, 2013). Fish meal consists of the dried, crushed carcasses of fish. Fishmeal contains high concentrations of good quality protein, minerals, B-vitamins and essential fatty acids (Ravindran, 2013). In contrast to the beneficial amino acid and fatty acid composition of fishmeal, the use of fishmeal in the formulation of poultry feeds has been associated with reduced sensory meat quality (Bou et al., 2004; Bou et al., 2009). The development of ‘fishy’, ‘off’ odours and flavours in the meat of birds consuming the fishmeal is due to the presence of breakdown products from the oxidation of omega-3 polyunsaturated fatty acids present, as well as nitrogenated compounds usually formed during fish spoilage (Hargis and Van Elswyk, 1993; Bou et al., 2009). Thus, there has been a move towards replacing fishmeal as an animal protein source in the formulation of poultry feed with alternative protein sources.

Meat/bone meal also contains large amounts of good quality protein together with significant amounts of calcium and phosphorus (Bozkurt et al., 2004; Caires et al., 2010). Meat/bone meal is obtained primarily from the bones, ligaments and tendons of mammals, as well as some skeletal muscle, lungs, liver tissue and gastrointestinal tract (Ravindran, 2013). Meat/bone meal has a lower nutritional value compared to that of fishmeal and is thus used less often compared to fishmeal as a dietary protein supplement to plant-based poultry diets (Ravindran, 2013).

Dietary protein serves as a supply of amino acids for the overall maintenance of growth, as well as various other bodily functions, including the growth of muscle and various metabolic reactions (Kratzer et al., 1994; Ravindran, 2013). Poultry do not have specific protein/amino acid requirements; they merely require a sufficient supply of both essential and non-essential amino acids to meet the birds' physiological needs (Ravindran, 2013). Methionine, lysine, threonine, leucine, isoleucine, tryptophan, valine, histidine, phenylalanine and arginine are all considered to be essential amino acids for poultry (Ravindran, 2013). Protein/amino acid requirements vary according to the production state of the bird (i.e. young, growing birds require increased amounts of dietary protein compared to older birds), as well as among different breeds or strains of poultry (Kratzer et al., 1994). Thus, poultry diets need to be tailored for specific types of poultry birds and growth stages.

In addition to the protein content of poultry diets, the amount and type of dietary fats which are commonly added to poultry diets need to be carefully considered.

### 1.2.3 Fats in the formulation of poultry feed

Poultry feeds are commonly formulated with added animal or vegetable fats as a source of concentrated energy which has been shown to result in improved feed conversion and increased growth rates (Kratzer et al., 1994; Nobakht, 2012; Sahito et al., 2012). Besides serving as an added source of energy, the fat added to poultry diets also serves as a binding agent during feed preparation, resulting in decreased dustiness of the feed and thus reduced dust feed loss (Mahesar et

al., 2011; Sahito et al., 2012; Ibrahim et al., 2014). In addition to reducing dust feed loss, the addition of dietary fat to poultry diets has also been shown to result in increased diet palatability.

Increased diet palatability following the addition of dietary fat is thought to involve the sensation of taste. The oral perception of fat was thought to be primarily dependent on the texture of the ingested fat, as well as on olfaction (Cartoni et al., 2010). Recent studies in mammals however, have identified candidate fat taste receptors and confirmed that taste does play a role in the detection of and preference for certain fatty acids (Cartoni et al., 2010). It is not certain whether the same is true for avian species as no homolog avian taste receptor gene has been identified as yet. However predicted taste receptor genes have been found in the chicken genome database, thus it is a possibility (Roura et al., 2013). Not only does the added dietary fat in poultry diets reduce dust feed loss and improve the palatability of the diet, it also plays an important role in increasing the absorption of fat soluble vitamins and various dietary nutrients.

Increased absorption of fat-soluble vitamins and improved digestion and absorption of all nutrients have been observed following the addition of fat to poultry diets (Baião and Lara, 2005; Mahesar et al., 2011; Sahito et al., 2012; Ibrahim et al., 2014). Dietary fats play an important role in the absorption of fat-soluble vitamins, vitamins A, D, E and K. Fat-soluble vitamins are highly lipophilic substances which are readily incorporated into lipid/oil droplets and are thus processed in the same manner in which dietary lipids are processed within the gastrointestinal tract (Borel, 2003). The intraluminal events which influence the absorption of major lipid classes are thus also important in the intestinal absorption of fat-soluble vitamins (Borel, 2003). These intraluminal events include emulsification, incorporation into mixed micelles, diffusion across the unstirred water layer and then transport across the intestinal enterocyte membrane (Borel, 2003). Once absorbed, the fat-soluble vitamins are then incorporated into portomicrons for transportation (Drevon, 1991). Thus, a deficiency in dietary fat would impair the absorption of fat-soluble vitamins. In addition to improving the absorption of fat-soluble vitamins, the addition of fat to poultry diets has also been shown to improve the overall digestion and absorption of all ingested nutrients.

The reduced rate of passage of digesta within the gastrointestinal tract of birds, following dietary supplementation with fat, is said to be responsible for the improved digestion and absorption of ingested nutrients (Palmquist, 2002; Baião and Lara, 2005; Firman et al., 2008; Monfaredi et al., 2011). The addition of dietary fat to poultry feeds results in a more viscous feed texture, which would in turn cause the feed to move through the gastrointestinal tract at a much slower rate, slowing gastrointestinal transit time. The digestion, absorption and subsequent utilization of nutrients are dependent on the rate of passage of the ingested feed through the gastrointestinal tract (Rochell et al., 2012). The rate of feed passage governs the amount of time available for interaction between ingested nutrients and digestive enzymes, absorptive surfaces and gut microbiota (Rochell et al., 2012). Thus, the higher the level of inclusion of fat in poultry diets, the more viscous the ingested feed and the slower the rate of feed passage through the avian gastrointestinal tract. Thus, there is more time available for hydrolysis of ingested nutrients by digestive enzymes, which would result in improved intestinal absorption of nutrients.

Since the addition of fats to poultry feed is associated with positive outcomes, we need to identify the biochemical and physiological processes responsible for the absorption and metabolism of fat in avian species. Additionally we also need to identify the biochemical and physiological processes involved in the deposition of fat within the body tissues of the birds.

#### 1.2.3.1 Biochemical basis of lipid metabolism

The majority of lipids exist in the form of triglycerides/triacylglycerides, which are composed of a single glycerol molecule esterified to three fatty acids (Meeker, 2009) (See Figure 1.1). Lipids with a single glycerol molecule esterified to a single fatty acid molecule are referred to as monoglycerides/monoacylglycerides. Similarly, lipids which contain a single glycerol molecule esterified to two fatty acids are referred to as diglycerides/diacylglycerides (Koolman and Roehm, 2013). Fatty acids are essential constituents of numerous lipids. The chain length and level of saturation (number of double bonds) of the fatty acids within lipid molecules contribute to the physical and chemical characteristics of the individual lipids (Meeker, 2009).

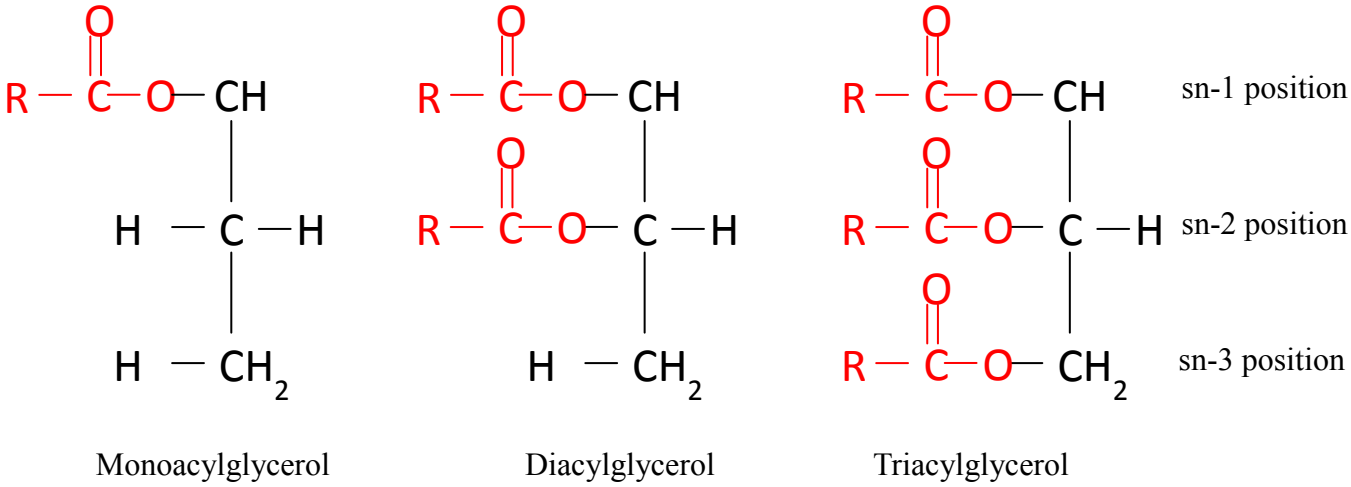



**Figure 1.1** Diagram illustrating the general structure of monoacylglycerols, diacylglycerols and triacylglycerols.

Fatty acid chain length is dependent on the number of carbon atoms within the unbranched hydrocarbon chain attached to the carboxylic acid, which together constitute a single fatty acid (Voet and Voet, 1995; Koolman and Roehm, 2013). Fatty acids can have hydrocarbon chains of between 3 – 24 carbon atoms (Voet and Voet, 1995; Koolman and Roehm, 2013). Short-chain fatty acids have between three and six carbon atoms. Medium-chain fatty acids have between eight and ten carbon atoms. Long-chain fatty acids have between twelve and eighteen carbon atoms and very long-chain fatty acids have greater than eighteen carbon atoms within their hydrocarbon chain (Koolman and Roehm, 2013).

In addition to differences in the number of carbon atoms within their hydrocarbon chain, fatty acids may also differ from one another based on the presence or absence of one or more double bonds within their hydrocarbon chain. If the fatty acid does not contain any double bonds, it is termed a saturated fatty acid (Baião and Lara, 2005; Meeker, 2009). The majority of the fatty acids which make up the lipids in both plant and animal species contain one or more double bonds and are termed unsaturated fatty acids (Voet and Voet, 1995) (Figure 1.2). Oleic acid and linoleic acid are examples of common unsaturated fatty acids (Koolman and Roehm, 2013). If the fatty acid only contains one double bond it is classified as a monounsaturated fatty acid, whereas if more than one

double bond is present it is classified as a polyunsaturated fatty acid (Baião and Lara, 2005; Meeker, 2009).

A specialised shorthand notation which makes use of numbers is commonly used to characterise the various fatty acids according to their structure. The number of carbons in the hydrocarbon chain of the fatty acid is indicated by the first number, which is then followed by a colon and a second number (Koolman and Roehm, 2013). The second number refers to the number of double bonds present within the hydrocarbon chain (Koolman and Roehm, 2013). The positions of the double bonds within the hydrocarbon chain are then indicated following a semicolon (Koolman and Roehm, 2013) (See Figure 1.2).

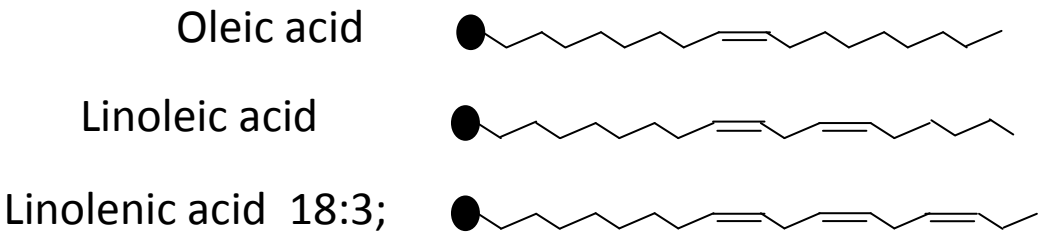



**Figure 1.2** Diagram illustrating the nomenclature used to characterise various fatty acids according to the number of carbon atoms, the number of double bonds and the position of any double bonds within the hydrocarbon chain. Adapted from "Colour atlas of biochemistry", third edition. Koolman and Roehm, 2013.

The carbon atoms are usually numbered from the carbon with the highest oxidation state, thus the carbon within the carboxyl group is referred to as C-1 (Koolman and Roehm, 2013). Greek letters are sometimes also used, such that the carbon furthest away from the carboxyl group is referred to as omega (ω) and the position of the double bonds, counting from the omega or methyl end of the hydrocarbon chain, are then indicated with numbers (Koolman and Roehm, 2013). It is from this nomenclature system that the terms omega-3 and omega-6 fatty acids originate from. The omega-3 and omega-6 polyunsaturated fatty acids are known as essential fatty acids as they cannot be

synthesized *de novo* in both humans and other monogastric animals and are thus required in the diet (Webb and O'Neill, 2008). The type of lipid added to poultry diets is of importance as previous studies have shown that the fatty acid profile and thus the degree of saturation of the dietary lipid influences the amount of overall body fat deposition, as well as the fatty acid profile of the resulting edible bird tissues (Gonzalez-Ortiz et al., 2013).

Thus, in order to optimise poultry meat quality for human consumption, the type of lipid used for the formulation of poultry diets need to be carefully considered. Fats or oils from both animal and plant sources are commonly used in the formulation of poultry feeds (Monfaredi et al., 2011). Pork lard, soyabean oil, sunflower oil, palm oil and coconut oil, amongst others, are some of the animal fats and vegetable oils which are commonly used in the formulation of poultry feeds.

#### 1.2.3.2 The use of pork lard in the formulation of high-fat poultry diets

Pork lard refers to the fat obtained from pigs, of which approximately 8 million tons per year are produced worldwide (Cheong and Xu, 2011). Despite the unique flavours associated with lard, its use within the food industry for culinary purposes such as baking and frying has significantly waned over time (Cheong and Xu, 2011). The lessened usage of lard within the food industry is largely due to its high calorie and saturated fatty acid content and thus low digestibility (Cheong et al., 2009). Previous studies which have made use of lard in the formulation of HFDs have shown that lard is composed of almost equal amounts of saturated and monounsaturated fatty acids, each ranging between 40-45% of total fatty acids (Narciso-Gaytán et al., 2010; Trushenski et al., 2011). Polyunsaturated fatty acids comprise approximately between 12-16 % of the total fatty acids present in lard (Narciso-Gaytán et al., 2010; Trushenski et al., 2011).

Despite its relatively high saturated fatty acid content, lard continues to play a major role within the animal meat production industry due to its positive influence on the texture and flavour of meat products (Cheong et al., 2009). In addition to lard, various vegetable fat sources including soyabean oil, sunflower oil, palm oil and coconut oil are also commonly used in the formulation of poultry diets.

#### 1.2.3.3 The use of soyabean oil in the formulation of high-fat poultry diets

Soyabeans and soyabean products are also commonly used as sources of both energy and protein in the formulation of feeds for production animals such as poultry and swine (Dourado et al., 2011). Soyabeans, *Glycine max*, are legumes of the *Fabaceae* or *Leguminosae* family which are primarily used for the production of vegetable oil (soyabean oil) and oil-seed meal (Dei, 2011; Dourado et al., 2011). Soyabean crops are successfully grown worldwide, with the United States, China, India, Brazil and Argentina being the top producers (Dei, 2011; Dourado et al., 2011). Soyabean oil is a popular component of high-fat poultry diets due to its high digestibility and metabolisable energy content in comparison to other vegetable oils (Dei, 2011). The increased digestibility and metabolisable energy content of soyabean oil can be attributed to its very high content of unsaturated fatty acids, which constitute approximately 80 % of the total fatty acids present (USDA, 2015). Thus, the fatty acids are easily digested, absorbed and utilised as an energy source by the birds (Huyghebaert et al., 1988). In addition, the popularity of soyabean oil as an ingredient in animal feeds can also be attributed to its ready availability all year-round, as well as the minimal variation in nutrient content (Dei, 2011). In addition to soyabean oil, sunflower oil is also commonly used as an added source of energy in poultry feed formulation. Sunflower oil is also comprised of approximately 80 % unsaturated fatty acids, with a very similar fatty acid profile to that of soyabean oil (USDA, 2015).

#### 1.2.3.4 The use of sunflower oil in the formulation of high-fat poultry diets

Sunflower oil is acquired from the seeds of the sunflower plant, *Helianthus annus* (O'Brien, 2009), the oil yield of which is relatively high at approximately 50 % (San Juan and Villamide, 2001). Unextracted, whole sunflower oilseeds have been used as feed ingredients in poultry diets (Rodriguez et al., 2005). However, its utilization in poultry feeding is somewhat restricted compared to other oilseeds, particularly soyabean, due to the relatively high fibre and low lysine content (San Juan and Villamide, 2000). Sunflower oil however, is a valuable fat source for poultry, specifically for laying hens, as a result of the high linoleic (18:2) fatty acid content (San Juan and Villamide, 2000; San Juan and Villamide, 2001). The degree of unsaturation of sunflower oil can differ significantly depending on the growing climate and temperature. The higher the temperature at which the sunflowers are grown, the lower the linoleic (18:2; 9, 12) content and the higher the oleic acid (18:1; 9) content and visa-versa (Robertson, 1972; Campbell, 1983). This large variation

in degree of unsaturation of the fatty acids in sunflower oil may pose a problem in the supplementation of sunflower oil to poultry feeds, with regards to standardising the fatty acid profile of the sunflower oil and thus the fatty acid profiles of the diets fed to poultry. Another vegetable oil which is popular for its use in the formulation of poultry diets is palm oil.

#### 1.2.3.5 The use of palm oil in the formulation of high-fat poultry diets

Palm oil is obtained from the fruit of the tropical oil palm tree, *Elaeis guineensis* (Hayes and Khosla, 2007; Oluba et al., 2011). Palm oil is high in saturated fatty acids (approximately 50 % of total fatty acids are saturated) (Wing-Keong, 2002; Das et al., 2014a), which makes it less susceptible to oxidative damage compared to other vegetable oils (Wing-Keong, 2002). Additionally as a result of its high saturated fatty acid content, palm oil has also been associated with a positive influence on the firmness of poultry meat (Renner and Hill, 1961; Das et al., 2014a). There is a dearth of information concerning the use of palm oil in the formulation of poultry feeds compared to that available for other animal fats and vegetable oils (Das et al., 2014a). Palm oil has overtaken soyabean oil as the most widely produced vegetable oil in the world. It is also cheaper than soyabean oil and thus would ultimately lower the expenses associated with poultry production, with respect to feed costs (Das et al., 2014a). Therefore, as a result of the affordability of palm oil and its reduced susceptibility to oxidation which in turn decreases the occurrence of rancidity of the poultry meat, the possible use of palm oil in the formulation of poultry feed is attractive and needs to be further investigated. Another vegetable oil which is commonly used in the formulation of poultry feed and is very high in saturated fatty acids, even more so than palm oil, is coconut oil.

#### 1.2.3.6 The use of coconut oil in the formulation of high-fat poultry diets

Coconut oil comes from the coconut palm tree, *Cocos nucifera*, which is grown in many parts of the world (Aderolu and Akinremi, 2009; Batool, 2012). Over 90 % of the total fatty acids present in coconut oil are saturated fatty acids (Guarte et al., 1996), with lauric acid (12:0) representing between 40-50 % of total fatty acids (Luo et al., 2014). The high saturated fatty acid content and thus low unsaturated fatty acid content of coconut oil makes it the most stable oil with respect to heat (coconut oil has a high melting point) and resistance to lipid peroxidation/oxidative rancidity, thus ensuring a long shelf-life (Guarte et al., 1996; Aderolu and Akinremi, 2009; Luo et al., 2014).

Thus the use of coconut oil in the formulation of poultry feeds would be advantageous in that the feed would not easily spoil, reducing feed loss.

Approximately 60 % of the total fatty acids present in coconut oil are of the medium chain triglyceride variety and are thus easily absorbed directly into the portal circulation (Bhatnagar et al., 2009; Wang et al., 2015). As a result of their rapid metabolism and reduced storage in adipose tissue, ingestion of diets rich in medium chain triglycerides have been shown to reduce lipid deposition and have favourable effects with regards to the serum lipid profile of broilers (Wang et al., 2015). Despite the positive effects associated with coconut oil supplementation to broiler diets, the use of coconut oil on a large scale in the formulation of poultry feed might not be worthwhile as a result of the high cost of coconut oil. Since coconut oil is relatively expensive compared to other vegetable oils, blending coconut oil with other lower cost vegetable oils may be beneficial, not only in terms of reducing production costs but also in terms of increasing the stability of other polyunsaturated vegetable oils which are susceptible to oxidation (Bhatnagar et al., 2009).

Inclusion of the above-mentioned animal and vegetable fats to standard poultry diets does not necessarily mean that the added dietary fat will be absorbed by the birds. In order to understand how fats are handled and absorbed in avian species, we first have to understand the anatomy, as well as the physiological functioning of the avian gastrointestinal tract.

## 1.3 The avian digestive system and the digestion, absorption and deposition of fat

### 1.3.1 Anatomical organisation of the avian digestive system

The avian gastrointestinal tract is different compared to that of mammals in that it has a larger number of organs with substantial inter-organ cooperation. The tract consists of the mouth, oesophagus, crop, proventriculus (glandular stomach), ventriculus/gizzard (muscular stomach), intestine, caeca, rectum and cloaca (Klasing, 1999).

The beak, mouth and tongue form the upper part of the digestive tract which functions in the grasping or acquisition of food, as well as in the commencement of the digestive process through the mechanical processing and lubrication of ingested food (Klasing, 1999; Slomka-McFarland, 2011). The beak of birds replaces the lips and teeth found in mammals and is composed of a keratin sheath which extends from the upper and lower jaw bones (Duke, 1997; Klasing, 1999). Salivary glands which are stimulated upon feeding are present within the oral cavity of most avian species; however the glands tend to be better developed in birds which consume predominantly dry food (Duke, 1997). Like the beak, the avian tongue also functions in the mechanical manipulation of ingested food, breaking up large food particles and pushing food backwards, towards the oesophagus. Depending on the species of bird and the food which they eat, the size, length and muscularity of the tongue differs (Klasing, 1999).

Upon leaving the mouth, ingested food enters into the oesophagus, a muscular tube connecting the oral cavity to the stomach (Slomka-McFarland, 2011). Since food passes through the oral cavity relatively unchewed, due to the absence of teeth in birds, the oesophagus is somewhat distensible and is able to accommodate the swallowing of large food particles. The oesophagus is typically composed of three parts, including the cervical oesophagus, the crop (a dilation of the oesophagus, forming a pouch-like structure) and the thoracic oesophagus (Hena et al., 2012). The crop functions in the storage and lubrication of ingested food and also plays a role in the regulation of the passage of food further down the digestive tract (Slomka-McFarland, 2011). From the crop, food particles pass through the thoracic oesophagus and into the stomach.

The stomach of birds is divided into two main sections, namely the proventriculus or glandular stomach and the ventriculus/gizzard or muscular stomach (Orosz, 2011). Chemical digestion of ingested food particles is initiated in the proventriculus, through the secretion of hydrochloric acid and pepsin by the gastric glands (chief cells) and mucous by the tubular glands (Klasing, 1999; Duke, 1989). The food, together with the secretions of the proventriculus, passes quickly into the muscular ventriculus. It is here where the relatively large food particles are broken up mechanically by contractions of distinct striated muscular bands which make up the muscular wall of the gizzard (Slomka-McFarland, 2011). The mechanical grinding of the food into smaller particles increases the

available surface area upon which the proteolytic enzymes secreted from the proventriculus carry out their function, digesting the food (Orosz, 2011; Klasing, 1999). The gizzard usually contains small pieces of gravel or stones which become trapped and assist in the grinding of food particles (Flammer, 2007; Klasing, 1999). The ventriculus/gizzard opens up into the small intestine, which, in most birds, is relatively short (Flammer, 2007).

The small intestine serves as the primary site for the chemical digestion (involving both intestinal and pancreatic digestive enzymes) and absorption of ingested nutrients (Duke, 1989). The avian small intestine can be histologically divided into two main sections, the duodenal loop and the ileum, as there in no clear distinction between the jejenum and ileum in birds (Duke, 1997; Klasing, 1999; Hena et al., 2012). The pancreas lies within the loop formed by the duodenum and both the pancreatic ducts and the bile ducts open into the duodenum (Halnan, 1949). The duodenum leads into the ileum, which then opens up into the large intestine or colon. At the junction of the ileum and the colon, about half of all avian species have single or paired finger-like lateral projections from the small intestine, forming pouch-like structures which differ in size depending on the species of bird, known as the caecum or caeca (Hena et al., 2012; Duke, 1989). The avian caecum performs a number of different functions, the most important of which includes the regulation of water balance, the fermentation of fibre by the resident caecal microflora and the recycling of nitrogenous wastes for the synthesis of amino acids (Duke, 1997; Hena et al., 2012; Svihus et al., 2013).

The rectum or colon extends from the ileocaecal junction to the terminal chamber of the digestive tract, known as the cloaca (Klasing, 1999). The colon in birds, unlike those of mammals, is relatively small in diameter and not very long. The colon functions primarily in the absorption of water from colon contents (Duke, 1997; Klasing, 1999). The cloaca is divided into three different sections, namely the coprodeum, the urodeum and the proctodeum, by transverse folds of tissue (Halnan, 1949). The rectum opens into the coprodeum, the ureters and reproductive tracts (oviduct in females and ductus deferens in males) open into the urodeum and the proctodeum opens up to the exterior through a transverse slit known as the vent (Klasing, 1999; Duke, 1989).

Now that we have examined the anatomical organisation of the avian digestive system, as well as the overall functioning of each compartment, we can now examine the fate of ingested lipids more closely, with respect to the various processes involved in the digestion, absorption and handling of fat throughout the avian gastrointestinal tract.

### 1.3.2 Fat digestion in avian species

The digestion of dietary lipids, in the form of triglycerides, essentially begins in the avian intestine. Upon arrival into the duodenum, after leaving the gizzard/muscular stomach, the presence of these lipid particles within the intestinal tract stimulates the release of cholecystokinin from the intestinal epithelium into the intestinal lumen (Baião and Lara, 2005). Cholecystokinin induces the contraction of the gallbladder which results in the release of bile into the duodenum. The bile salts present in the bile secretion emulsify the large lipid particles into smaller lipid droplets, thus increasing the available surface area for hydrolysis by the digestive enzymes to occur (Freeman, 1984). Emulsification of the large dietary fat particles upon entry of the chyme into the duodenum also enhances the mixture of the dietary fat together with the secretions from the intestine and pancreas (Krogdahl, 1985). In addition to emulsification, the bile salts also enable the adsorption of colipase, a polypeptide cofactor, at the lipid-water interface, which is required for the attachment of pancreatic lipase, a digestive enzyme produced and secreted by the exocrine pancreas (Freeman, 1984; Doreau and Chilliard, 1997).

The production and secretion of digestive enzymes, as well as bicarbonate-rich pancreatic juice from the exocrine pancreas into the intestinal lumen occurs in response to a number of stimuli including secretin, vaso-active intestinal peptide, hydrochloric acid, vagal stimulation, duodenal distention and cholecystokinin (Baião and Lara, 2005; Gelis, 2015). The digestive enzymes released from the avian exocrine pancreas are similar to those present in mammals and include amylase, trypsin, chymotrypsin, carboxypeptidases and various lipases, one of which is pancreatic lipase (Gelis, 2015). Pancreatic lipase, together with other pancreatic enzymes including cholesterol esterase and phospholipase, hydrolyse/digest the ingested lipids yielding free fatty acids and 2-monoglycerides, as well as cholesterol, 1,2-diglycerides, glycerol and lysophospholipids (Freeman, 1984; Sklan et al., 1984). In monogastric/non-ruminant animals such as birds, the digestibility of

dietary fat is often more than 80 % (Doreau and Chilliard, 1997). However, factors such as age, fatty acid chain length, the position of fatty acids on the glycerol backbone and the degree of unsaturation of the fatty acids , can affect fat digestibility.

Fat digestibility has been shown to increase with increasing age in poultry due to the insufficient production of digestive enzymes and bile salts in young chicks (Wiseman, 1984). Digestibility of saturated fatty acids increases with decreasing chain length, thus saturated fats rich in short chain fatty acids are more digestible than those rich in medium chain fatty acids, which, in turn, are more digestible than those rich in long chain fatty acids (Smink et al., 2008). Fat digestibility is also dependent on the position of the fatty acid on the glycerol backbone. In the majority of animal and vegetable fats, saturated fatty acids, if present, occupy the external sn-1 and sn-3 (according to the stereospecificity numbering system) positions on the glycerol backbone; whereas the middle sn-2 position is typically occupied by an unsaturated fatty acid (Bracco, 1994). Pancreatic lipase and the polypeptide cofactor, colipase, predominantly hydrolyse the ester bonds in the sn-1 and sn-3 positions of dietary triglycerides, as the ester bond in the sn-2 position is somewhat resistant to hydrolysis (Bracco, 1994). Thus, following hydrolysis by pancreatic lipase the fatty acids that were esterified in the sn-1 and sn-3 positions are liberated into the small intestinal lumen, together with the remaining 2-monoglyceride (Bracco, 1994). As a result of the hydrophilic nature of the 2-monoglyceride in comparison to fatty acids hydrolysed from the sn-1 and sn-3 positions on the glycerol backbone, the fatty acids esterified in the sn-1 and sn-3 positions are less efficiently absorbed from the intestinal lumen and are thus said to have a lower digestibility compared to the fatty acid esterified in the sn-2 position Doreau and Chilliard, 1997; Smink et al., 2008). Saturated fatty acids are also known to have a lower digestibility compared to unsaturated fatty acids (Lessire and Leclercq, 1982) as saturated fatty acids are less easily incorporated into micelles compared to unsaturated fatty acids as a result of their non-polarity (Zhang et al., 2011a). The non-polar saturated fatty acids are thus less soluble within the micelle and are believed to be incorporated into the centre of the micelle (Garrett and Young, 1975), which in turn can affect their subsequent absorption from the gastrointestinal tract. Thus, it is clear that the quality of ingested dietary fat plays a role in avian fat digestion and absorption.

### 1.3.3 Fat absorption across the avian gastrointestinal tract epithelium

Following digestion, the products of fat hydrolysis need to be absorbed from the intestinal lumen, across the gastrointestinal tract epithelium. The fat digestion products, including the free fatty acids and 2-monoglycerides, together with bile salts form mixed micelles which in turn facilitate the absorption of the fat digestion products by providing the intestinal mucosal cells with large concentrations of lipids within the unstirred water layer next to the mucosa (Krogdahl, 1985). Upon translocation of the micelles to the intestinal brush border, the fat hydrolysis products are passively absorbed into the intestinal mucosal cell (Freeman, 1984; Doreau and Chilliard, 1997).

The passive absorption of the lipid products across the intestinal brush border membrane is dependent on the chain length and unsaturation level of the lipids (Krogdahl, 1985). In mammals, fatty acid absorption takes place primarily in the jejunum, whereas in birds fatty acid absorption has been observed in both the duodenum and ileum (Drackley, 2000). Once within the mucosal epithelial cell, the fatty acids and monoglycerides reassemble to form triglycerides and together with both free and esterified cholesterol, as well as phospholipids and lipoproteins, they form large lipoprotein particles known as portomicrons (Baião and Lara, 2005). These portomicrons in birds are equivalent to the mammalian chylomicrons and are similar in both size and composition (Hermier, 1997). However, due to the poorly developed avian lymphatic system, absorbed lipids are transported mainly as triglycerides of the very low density lipoprotein (VLDL) fraction, which are secreted directly into the portal system and are thus termed portomicrons (Freeman, 1984; Hermier, 1997; Baião and Lara, 2005). These portomicrons first pass through the liver before reaching the rest of the circulation (Hermier, 1997).

### 1.3.4 Fat metabolism in the avian liver

The liver plays a very important role in lipid metabolism. Lipid metabolism involves a number of different pathways which are for the most part interdependent and function together in regulating hepatic lipid content (Nguyen et al., 2008). Hepatic lipid content is the net result of various processes including *de novo* fatty acid synthesis, the esterification of free fatty acids to form triglycerides, the export of triglycerides from the liver, hepatic fatty acid uptake, as well as hepatic fatty acid oxidation (Nguyen et al., 2008). Alterations in any of the above mentioned processes

could result in the accumulation of triglycerides within the liver, ultimately affecting overall liver function. Each of the processes/pathways mentioned above will now be discussed in more detail.

#### 1.3.4.1 *De novo* synthesis of free fatty acids in the avian liver

The synthesis of fatty acids *de novo* is a fundamental metabolic pathway in the maintenance of energy homeostasis (Nguyen et al., 2008). When total energy intake is higher than total energy expenditure, fatty acids as well as triglycerides are synthesized (Muniz, 2003). It is important to note that *de novo* fatty acid synthesis or lipogenesis refers strictly to the synthesis of fatty acids and is not the same process by which fatty acids are esterified to glycerol to form triglycerides, which can be referred to as esterification or glycerolipid synthesis (Drackley, 2000) and will be discussed in more detail following this section.

*De novo* fatty acid synthesis is the process by which fatty acids are synthesized through the successive condensation of two-carbon units, which are initially derived from acetyl-CoA (Muniz, 2003). There are two major tissues within the body which are capable of synthesizing fatty acids *de novo*, namely the liver and the adipose tissue. Fatty acids which are synthesized in the liver are exported via the assembly of lipoproteins, which then function as both structural components for the construction of membranes and as an energy source (Nguyen et al., 2008). The fatty acids synthesized within the adipose tissue directly contribute to the deposition of fat within the adipose tissue itself and thus play a role in long term energy storage (Nguyen et al., 2008). In birds, as in humans, the primary site of *de novo* fatty acid synthesis is the liver, whereas in pigs, ruminants and rodents, the adipose tissue is the main lipogenic tissue (Drackley, 2000; Bergen and Mersmann, 2005; Nafikov and Beitz, 2007). Despite some differences in the primary lipogenic tissues between certain mammalian species compared to avian species, the pathways involved in the process of *de novo* fatty acid synthesis are similar for both mammals and birds (Newman, 2000).

Palmitic acid (16:0) is the most abundant fatty acid and the first fatty acid to be produced during *de novo* fatty acid synthesis. Palmitic acid acts as a precursor for the synthesis of longer-chain fatty acids through special elongation reactions and shorter chain fatty acids can be produced if the fatty acid chain is released before it reaches sixteen carbon atoms in length (Muniz, 2003). Palmitic acid

is synthesized from acetyl-CoA through the activity of acetyl-CoA carboxylase and fatty acid synthase, in the cytoplasm of the cell (Muniz, 2003). Acetyl-CoA carboxylase is the enzyme which catalyzes the conversion of acetyl-CoA to malonyl-CoA, which in turn acts as the 'donor' of acetyl units during the fatty acid chain elongation process (Drackley, 2000). In addition to acetyl-CoA carboxylase, fatty acid synthase also plays an important role in *de novo* fatty acid synthesis.

Fatty acid synthase is a multifunctional, multi-enzyme complex which contains the seven individual enzyme activities required to catalyse the synthesis of palmitic acid from malonyl-CoA (Newman, 2000; Muniz, 2003; Nguyen et al., 2008). In animal cells, the individual enzyme components which make up the fatty acid synthase complex are all covalently linked on a single polypeptide chain in the following order, from the N to the C-terminal of fatty acid synthase: β-ketoacyl synthetase, acetyl-CoA transacylase, malonyl-CoA transacylase, dehydratase, enoyl reductase, β-ketoacyl reductase, acyl carrier protein and thioesterase (Muniz, 2003). The first six reaction steps involve a successive increase in fatty acid chain length through the addition of a two-carbon unit per reaction and the last step involves the release of the free fatty acid (Stevens, 1996). Regulation of the amount of *de novo* fatty acid synthesis which occurs within the liver is dependent on both nutritional and hormonal factors.

The acetyl-CoA which is used as a substrate for *de novo* fatty acid synthesis is derived from dietary carbohydrate and the process is thus highly dependent on the nutritional status of the animal, with *de novo* fatty acid synthesis being enhanced in fed animals and in animals consuming a diet rich in carbohydrates (Hermier, 1997; Muniz, 2003). In contrast, starvation or the consumption of a diet rich in lipids with a very low carbohydrate concentration reduces the functioning of the *de novo* fatty acid synthesis pathway (Muniz, 2003). In addition to the nutritional aspect of control of *de novo* fatty acid synthesis, various hormones such as insulin, glucagon, triiodothrionine ($T_3$), the catecholamines and the glucocorticoids influence hepatic *de novo* fatty acid synthesis.

Insulin and triiodothrionine have been shown to promote the synthesis of fatty acids *de novo*, by increasing the activity and/or abundance of both acetyl-CoA carboxylase, the enzyme which

catalyzes the rate-limiting step of the *de novo* fatty acid synthesis pathway, as well as that of the fatty acid synthase enzyme complex (Drackley, 2000; Newman, 2000). Glucagon, the catecholamines and the glucocorticoids however, have antilipogenic effects and result in a decrease in acetyl-CoA carboxylase and fatty acid synthase activity and/or abundance (Drackley, 2000; Newman, 2000). The hormone-induced changes in enzyme activity are a form of short-term regulation, whereas the changes in abundance of enzyme protein are a form of long-term regulation (Drackley, 2000). The regulation of the expression of the lipogenic genes is primarily facilitated by transcription factors such as sterol-responsive element binding protein-1c (SREBP-1c) and nuclear factors such as the liver X receptors (Nguyen et al., 2008). The increased expression of SREBP-1c results in a significant increase in the expression of lipogenic genes, such as fatty acid synthase. Overexpression of SREBP-1c is induced by insulin, whereas glucagon and cAMP cause a decrease in the expression of SREBP-1c (Nguyen et al., 2008).

With various hormonal and nutritional controls in place, *de novo* fatty acid synthesis is inhibited during times of low energy status, where the mobilisation of stored fats as an energy source is required. Fats are stored as triglycerides, which are formed through the process of esterification of free fatty acids.

#### 1.3.4.2 Esterification of free fatty acids to form triglycerides

Fatty acids are seldom present as non-esterified, free fatty acids. Most fatty acids are esterified to glycerol to form what is known as a glycerolipid. Triglycerides and phospholipids are examples of glycerolipids. In the liver, the majority of free fatty acids are preferentially esterified to form phospholipids which can then be incorporated into plasma and intracellular membranes (Drackley, 2000; Nguyen et al., 2008). Triglycerides are actively synthesized by the liver in the presence of very high concentrations of free fatty acids or when the transfer of phospholipids to the membranes is overloaded (Drackley, 2000; Nguyen et al., 2008).

Triglyceride synthesis or esterification is initiated by a two-step reaction in which acyl chains from acyl-CoA are successively transferred to glycerol-3-phosphate, firstly to form lysophosphatidic acid

and then to form phosphatidic acid (Coleman et al., 2000). The hydrolysis of phosphatidic acid then results in the formation of a diglyceride, which then undergoes a final acylation step to form a triglyceride (Coleman et al., 2000). The glycerol-3-phosphate used in the triglyceride synthesis reaction is produced either via glycolysis or from the glycerol that originates from adipose tissue lipolysis, which is then phosphorylated (Drackley, 2000). The enzymes involved in the synthesis of triglycerides, which include glycerol-3-phosphate acyltransferase, lysophosphatidate acyltransferase, phosphotidate phosphohydrolase and diacylglycerol acyltransferase, may serve as important regulatory points for triglyceride synthesis, as well as for the accumulation of triglycerides in the liver (Nguyen et al., 2008). These enzymes however, have not been well characterized in farm animals, including poultry and cattle (Drackley, 2000). Figure 1.3 indicates the steps and enzymes involved in triglyceride synthesis.

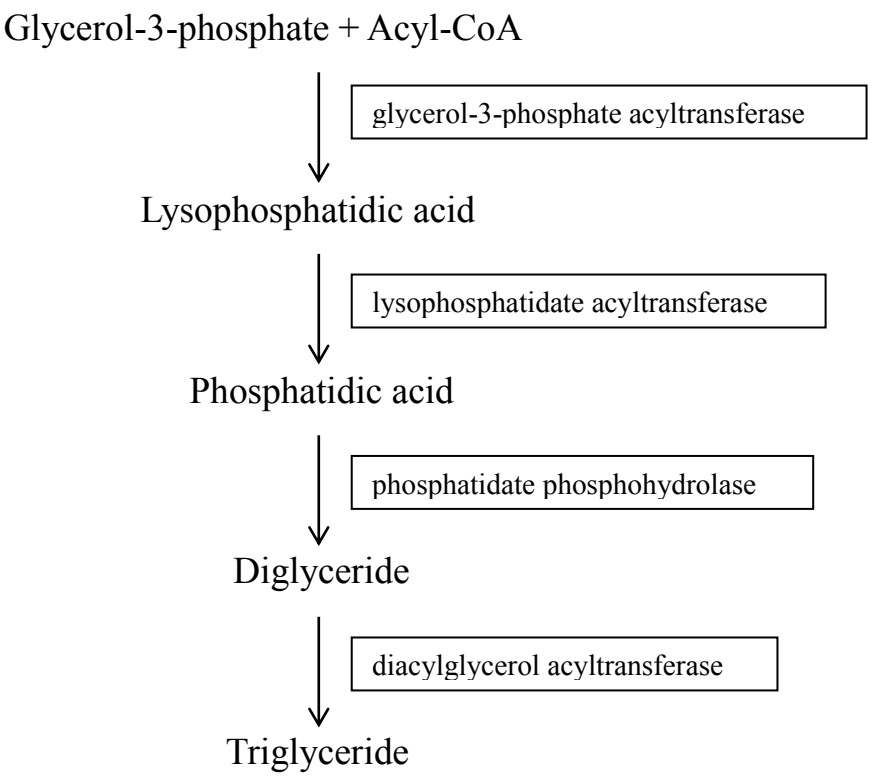



**Figure 1.3** Diagram illustrating the steps and enzymes involved in the synthesis of triglycerides. Adapted from "Physiological and nutritional regulation of enzymes of triacylglycerol synthesis" by Coleman et al., 2000.

In mammals, triglyceride synthesis is regulated by various transcription factors and hormones, as well as by the state of cellular energy supply (Coleman et al., 2000; Nguyen et al., 2008). SREBP-1c is a transcription factor which upregulates the synthesis of mRNAs for important enzymes involved in lipid biosynthesis pathways (Brown and Goldstein, 1997; Coleman et al., 2000). Increased expression of SREBP-1c has been shown to result in the increased expression of genes which code for enzymes such as fatty acid synthase and glycerol-3-phosphate acyltransferase (Pegorier et al., 2004; Nguyen et al., 2008). The expression of SREBP-1c is regulated by insulin as well as the nutritional status of the animal (Kim et al., 1998). When overall nutritional status is poor and energy levels are low, SREBP-1c mRNA levels are reduced (Kim et al., 1998). This in turn would result in lower levels of fatty acid synthase and glycerol-3-phosphate acyltransferase and thus reduce lipid biosynthesis. Peroxisome proliferator-activated receptor alpha (PPARα) is another transcription factor involved in the regulation of triglyceride synthesis. PPARα is a nuclear transcription factor that has been shown to reduce the expression of lipogenic genes by decreasing the SREBP-1c mediated activation of lipogenic genes, through a direct decrease in the overall quantity of SREBP-1c present in the hepatocyte nuclei (Worgall et al., 1998; Coleman et al., 2000). Thus, increased levels of PPARα would result in reduced triglyceride synthesis.

The regulation of triglyceride synthesis is also affected by the balance of the counter-regulatory hormones, insulin and glucagon (Coleman et al., 2000). Increases in circulating insulin levels have been shown to directly increase the activity of lipogenic enzymes, specifically that of glycerol-3-phosphate acyltransferase (Sul and Wang, 1998). Thus, insulin promotes the synthesis of triglycerides. In addition to insulin and glucagon, thyroid hormone has also been shown to have some sort of regulatory role with respect to triglyceride synthesis, however, contrasting evidence has been observed with regards to its' overall effect. Hypothyroidism has been shown to result in increased activity of microsomal glycerol-3-phosphate acyltransferase and diacylglycerol acyltransferase; whereas reduced mitochondrial activity of glycerol-3-phosphate acyltransferase and phosphotidate phosphohydrolase have been observed during hypothyroidism (Coleman et al., 2000). In addition to the long-term regulation of triglyceride synthesis at the gene transcription level by various hormones and transcription factors, various acute mechanisms play a role in the regulation of triglyceride synthesis primarily in response to cellular energy supply and demand.

Adenosine monophosphate-activated protein kinase (AMPK) is a sensor of cellular energy supply (Nguyen et al., 2008). AMPK is activated when energy supply to the cell is relatively low, thus ATP levels are low and AMP levels begin to rise (Nguyen et al., 2008). Activation of AMPK by the increasing levels of AMP results in the channelling of acyl-CoA molecules away from triglyceride synthesis and towards β-oxidation, in order to increase cellular energy supply. This is achieved through inhibition of glycerol-3-phosphate acyltransferase and thus the triglyceride synthesis pathway is blocked (Nguyen et al., 2008). In addition to glycerol-3-phosphate acyltransferase, phosphatidate phosphohydrolase is also acutely regulated in response to cellular energy supply. Increased levels of glucagon and AMP have been shown to result in the translocation of phosphatidate phosphohydrolase from the microsomal membrane of hepatocytes into the cytosol, thus rendering the enzyme inactive (Brindley, 1991; Coleman et al., 2000).

Having looked at the various steps involved, as well as the various points of regulation in the synthesis of triglycerides from fatty acyl-CoA molecules, which is enhanced in times of high cellular energy supply, we will now do the same for the process of β-oxidation, which involves the breakdown of free fatty acids, a process which is enhanced in times of low cellular energy supply.

#### 1.3.4.3 The uptake and oxidation of free fatty acids by the avian liver

In times of low cellular energy supply, stored fats/lipids in the adipose tissue are mobilised as an alternative energy source and transported to the liver to be metabolized for energy. The uptake of free fatty acids into the liver from the bloodstream, via transport proteins or diffusion, is directly proportional to the concentration of the free fatty acids in the bloodstream (Drackley, 2000; Nguyen et al., 2008). Once inside the hepatocytes, long-chain fatty acids which contain more than 14 carbon atoms in their hydrocarbon chain are bound to an acyl-CoA molecule through the action of the acyl-CoA synthetase enzymes (Drackley, 2000; Nguyen et al., 2008). These fatty acyl-CoA molecules and the other shorter chain free fatty acids are then transported via an acyl-CoA binding protein or a fatty acid binding protein, respectively, into the intracellular compartments of the hepatocytes to be metabolised (Drackley, 2000; Nguyen et al., 2008).

Once inside the liver, the oxidation of free fatty acyl-CoA molecules can take place either in the mitochondria or in the peroxisomes within liver hepatocytes. Both oxidative processes are similar however, mitochondrial oxidation is primarily responsible for the oxidation of short, medium and long-chain fatty acids, whereas peroxisomal oxidation is responsible for the oxidation of very long-chain fatty acids (Nguyen et al., 2008). The oxidation of fatty acids within the mitochondria occurs via a process known as β-oxidation, which plays a central role in the production of energy (Drackley, 2000; Skiba-Cassy et al., 2007; Nguyen et al., 2008).

Oxidation of fatty acids via the β-oxidation pathway results in the formation of acetyl-CoA. The multi-step pathway involves the transfer of electrons to both flavin-adenine dinucleotide (FAD) and the oxidised form of nicotinamide-adenine dinucleotide ($NAD^+$), which results in the formation of the reduced forms of these coenzymes (Drackley, 2000; Nguyen et al., 2008). These reduced coenzymes can then donate electrons to the electron transport chain which promotes the synthesis of ATP (Drackley, 2000; Nguyen et al., 2008). The acetyl-CoA formed during the oxidation of fatty acids can then enter into the tricarboxylic acid cycle to be completely oxidised to carbon dioxide, or it can be used to form ketone bodies (Drackley, 2000; Nguyen et al., 2008). Ketogenesis is heightened during times of increased lipolysis in adipose tissue and thus, increased uptake of free fatty acids by the liver (Drackley, 2000; Nguyen et al., 2008).

Following the uptake of free fatty acids into the liver, in order to undergo mitochondrial β-oxidation, the fatty acids have to enter into the mitochondria. Entry of the fatty acids into the mitochondria is dependent on the fatty acid chain length. Short and medium-chain fatty acids (12 carbon atoms or less in their hydrocarbon chain) are able to pass through the mitochondrial membrane unassisted, whereas entry of long chain fatty acids into the mitochondria is dependent on the activity of the carnitine palmitoyltransferase-1 enzyme (CPT-1) (Drackley, 2000; Nguyen et al., 2008). CPT-1 is an integral outer mitochondrial membrane protein which catalyzes the transfer of the acyl group from fatty acyl-CoA complexes to carnitine, to form acylcarnitine (Skiba-Cassy et al., 2007). The acylcarnitine molecule is then transported to the inner mitochondrial membrane by a protein known as carnitine:acylcarnitine translocase (McGarry et al., 1989; Skiba-Cassy et al., 2007). The transacylation reaction is then reversed on the inner mitochondrial membrane by the

carnitine palmitoyltransferase-2 enzyme (CPT-2), which results in the regeneration of acyl-CoA (McGarry et al., 1989; Skiba-Cassy et al., 2007). The long-chain fatty acyl-CoA molecules can then enter into the β-oxidation pathway. Figure 1.4 indicates the transport of a fatty acid across the mitochondrial membrane, into the mitochondria.

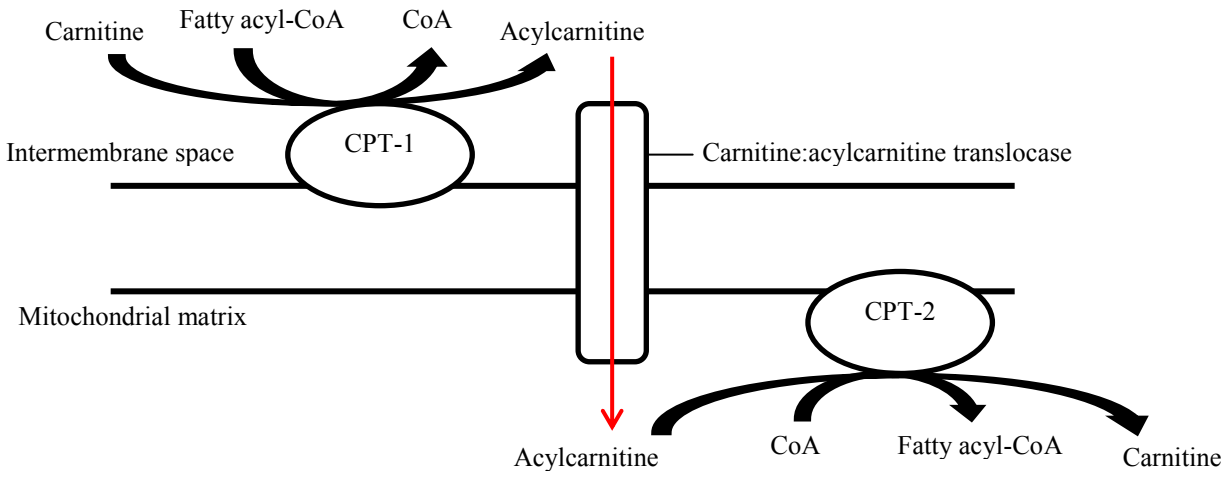



**Figure 1.4** Diagram illustrating the transport of a fatty acid across the outer and inner mitochondrial membranes, into the mitochondria for β-oxidation. Adapted from "Fatty acids: Metabolism" by P Muniz, 2003. Elsevier Science Ltd.

The activity of the CPT-1 enzyme and thus the entry of long-chain fatty acids into the mitochondria for β-oxidation are inhibited by interaction with the product of the first step of *de novo* fatty acid synthesis, malonyl-CoA (Drackley, 2000; Skiba-Cassy et al., 2007; Nguyen et al., 2008). Malonyl-CoA levels are low during times of negative energy balance (Drackley, 2000; Skiba-Cassy et al., 2007; Nguyen et al., 2008), thus β-oxidation, as opposed to lipogenesis, is favoured. The fatty acids that are catabolised are delivered to the extrahepatic tissues as very low-density lipoproteins (VLDLs).

### 1.3.5 Very low density lipoprotein catabolism

Triglycerides within VLDLs which are not used within the liver are transported to extra-hepatic tissues, including the muscles and heart tissue for utilisation, or to the adipose tissue for storage

(Baião and Lara, 2005). Transfer of triglycerides from within the VLDLs/portomicrons to the tissues involves their release from the protein portion of the lipoprotein particles, followed by triglyceride hydrolysis which results in the formation of free fatty acids and glycerol (Butler, 1976; Hermier, 1997). Lipoprotein lipase is the enzyme responsible for the hydrolysis of the triglyceride-rich VLDL/portomicron core (Cooper et al., 1992). The majority of the lipoprotein lipase present within the body is synthesised and contained within the skeletal muscle and adipose tissue. However, only once the enzyme is secreted and attached to the capillary walls, is it active (Hermier, 1997). Following triglyceride hydrolysis, the free fatty acids are able to enter the surrounding tissues and are either oxidised to obtain energy or, in the case of the adipose tissue, re-esterified and stored as triglycerides (Hermier, 1997; Ferrini et al., 2010).

Since the entry of fatty acids into the tissues is directly dependent on the activity of lipoprotein lipase, lipoprotein lipase activity represents a limiting step (Ferrini et al., 2010), the regulation of which is important in the overall nutritional status of the animal. The expression and thus activity of lipoprotein lipase has been shown to fluctuate according to the energy needs of various individual tissues, specifically in mammals (Bensadoun, 1991; Fielding and Frayan, 1998). In the fed state, lipoprotein lipase activity is high in adipose tissue and low in skeletal muscle and heart tissue, thus promoting the storage of dietary fatty acids. In contrast, in the fasted state lipoprotein lipase activity is low in adipose tissue and high in skeletal muscle and heart tissue (depending on the nutritional status of the tissue), thus dietary fatty acids are directed towards the tissues for oxidation to provide energy (Bensadoun, 1991; Fielding and Frayan, 1998). Contrasting results exist concerning the regulation of lipoprotein lipase in avian adipose tissue. Some studies suggest that the regulation of lipoprotein lipase expression and activity in avian adipose tissue is somewhat less sensitive to the nutritional status of the bird (Hermier, 1997). However, other studies have shown that the expression of lipoprotein lipase in the abdominal fat pad of chickens can be modified by changes in the quality of ingested dietary fat (Ferrini et al., 2010). Ferrini et al. (2010) observed decreased lipoprotein lipase expression and thus lower resultant fat deposition within the abdominal fat pad of chickens fed a basal diet compared to those fed diets rich in n-3 polyunsaturated fatty acids (diets supplemented with either tallow or linseed oil, at a 10 % level of inclusion). The reduced lipoprotein lipase expression was attributed to the lower level of apparent metabolisable energy intake in chickens fed the basal diet compared to those receiving the supplemented diets, thus more

energy was required for processes such as protein deposition and regular metabolic maintenance compared to that of fat deposition and ingested fatty acids were probably oxidised for energy and not stored (Ferrini et al., 2010).

### 1.3.6 Fat deposition/triglyceride storage in avian species

Fat deposition/triglyceride storage in avian species is dependent on the available exogenous or endogenous lipid substrates which result from the diet or from *de novo* lipogenesis in the liver, respectively (Hermier, 1997). In contrast to mammals, where the primary site of lipogenesis is the adipose tissue, in birds, the liver is the primary site of *de novo* lipogenesis/fatty acid synthesis, with very limited fatty acid synthesis occurring in the adipose tissue (Griffin et al., 1992; Mossab et al., 2002). Thus, the adipose tissue contains accumulated fat almost exclusively derived from liver lipogenesis or the diet. The degree of hepatic lipogenesis and VLDL secretion therefore plays an important role in the overall amount of fat deposition in avian species (Hermier, 1997; Mossab et al., 2002).

In addition to the hepatic influence on body fat deposition, previous studies have shown that body fat deposition can also be regulated by various nutritional factors, including dietary energy and protein levels as well as the level and quality/source of dietary fat (Fouad and El-Senousey, 2014). Previous studies have shown that a reduction in the dietary energy levels of chickens leads to an overall decrease in total body fat deposition, which was attributed to a decrease in activity levels of various enzymes involved in hepatic lipogenesis, including fatty acid synthase (Tanaka et al., 1983). Fatty acid synthase is one of the key lipogenic enzymes involved in the hepatic synthesis of long-chain fatty acids *de novo* (Back et al., 1986; Fouad and El-Senousey, 2014). Changes in the level of fatty acid synthase, induced by various nutritional influences, are regulated at the level of fatty acid synthase gene transcription. Fatty acid synthase gene transcription is the determining factor for the content of fatty acid synthase mRNA within the liver (Back et al., 1986; Clarke, 1993). Dietary protein levels have also been found to have a direct influence on body fat deposition, with the ingestion of low protein diets by broiler chickens being associated with significantly increased percentage abdominal fat (Collin et al., 2003; Fouad and El-Senousey, 2014). This reinforces the

fact that dietary macronutrient content specifically that of protein, plays an important role in the regulation of protein and fat deposition in chickens.

The amount and source of dietary fat have also been shown to influence body fat deposition. Crespo and Esteve-Garcia (2002a) observed that increased dietary fat inclusion levels resulted in reduced efficiency of fat deposition, due to the higher levels of energy used for heat production. In addition, the type of dietary fatty acids has been shown to affect the overall amount as well as the fatty acid composition of abdominal and intrahepatic fat deposition in broilers. Velasco et al. (2010) observed increased abdominal fat deposition and increased hepatic triglyceride content in female broiler chicks fed diets supplemented with palm oil (rich in saturated fatty acids) compared to those receiving sunflower oil (rich in polyunsaturated fatty acids). The varying lipid deposition was attributed to the ability of individual fatty acids to regulate gene transcription levels of various lipogenic genes, depending on the length of the fatty acid as well as the number and position of any double bonds (Clarke, 1993; Velasco et al., 2010). Saturated fatty acids are known to up-regulate the expression and synthesis of lipogenic genes, whereas unsaturated fatty acids are potent inhibitors of lipogenic genes (Clarke, 1993; Velasco et al., 2010). Thus, in general, fat deposition is considered the net result of three main processes, namely: dietary fat absorption, hepatic *de novo* lipogenesis and β-oxidation of fatty acids (see Figure 1.5). The lower body fat deposition generally observed following ingestion of diets rich in polyunsaturated fatty acids is attributed to reduced hepatic lipogenesis in conjunction with heightened β-oxidation.

After examining the anatomical arrangement and physiological functioning of the avian gastrointestinal tract, specifically with respect to the digestion, absorption and overall handling of ingested dietary fat, we can now investigate the metabolic disturbances which may be induced following the consumption of a HFD in avian species.

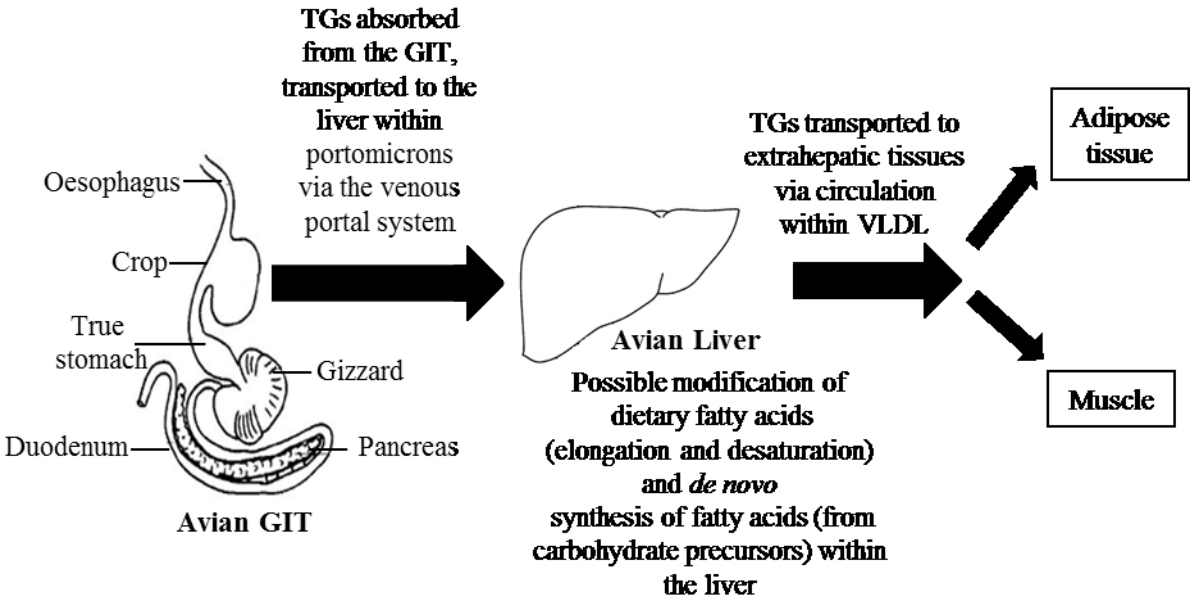



**Figure 1.5** Diagram illustrating the process by which fat deposition occurs in avian species. Adapted from Price, 2010. TGs = triglycerides; VLDL = very low density lipoproteins.

## 1.4 Possible high-fat diet induced metabolic disturbances in avian species

Research into HFD-induced metabolic disturbances in avian species, specifically in alternative poultry species such as the Guinea fowl, Muscovy duck and Japanese quail, is somewhat limited. In humans and other mammals, the consumption of energy-dense, HFDs has been shown to promote the development of a number of metabolic disturbances, which are collectively referred to as the metabolic syndrome (Roche, 2005; Tierney et al., 2011). The metabolic syndrome is characterised by dyslipidaemia (increased plasma triglycerides and reduced plasma high density lipoprotein cholesterol), hypertension, hyperglycaemia, insulin resistance, obesity and an overall pro-inflammatory state (Gillingham et al., 2011; Kassi et al., 2011; Fava et al., 2013; Boers et al., 2014). The diagnosis of the metabolic syndrome is accompanied by an increased risk for developing type II diabetes mellitus, as well as cardiovascular disease (Kassi et al., 2011; Fava et al., 2013). Since the metabolic syndrome is a heterogeneous condition, the treatment of this syndrome necessitates multiple therapies which are targeted towards the various metabolic abnormalities usually observed (Tierney et al., 2011).

Lifestyle modifications, involving exercise interventions and dietary adjustments are the treatment of choice for the metabolic syndrome, however no specific treatment guidelines have been established (Boers et al., 2014; Kaur, 2014). Dietary adjustments are often recommended as the composition of the diet consumed, specifically with respect to the fatty acid profile, has been shown to play an important role in the pathogenesis of the syndrome. Saturated fatty acid intake has been shown to increase the overall risk of developing components of the metabolic syndrome, whilst the intake of monounsaturated and polyunsaturated fatty acids has been shown to be beneficial in reducing the risk of developing the metabolic syndrome (Melanson et al., 2009).

We will now examine the normal status and regulation of blood glucose levels, erythrocyte osmotic fragility, gut microbiota balance, bone health and fat deposition in avian species, as well as the possible HFD-induced alterations in these parameters which could contribute to the development of various metabolic conditions in birds which resemble components of the metabolic syndrome in mammals.

### 1.4.1 Blood glucose regulation in avian species

The main dietary carbohydrate consumed by poultry is in the form of starch, which is digested by the enzyme α-amylase and then ultimately hydrolysed to glucose (Braun and Sweazea, 2008; Weideman et al., 2012). Glucose is utilised by birds mainly for energy production (Braun and Sweazea, 2008) and regardless of the amount of dietary starch/glucose intake, avian blood glucose concentrations are maintained at a relatively constant level (Klasing, 1998). Fasting blood glucose concentrations in birds average approximately 15.6 mM, which is around two-fold higher than the blood glucose concentrations observed in non-diabetic humans and other mammals (Polakof et al., 2011; Sumners et al., 2014). This constantly elevated level of blood glucose within avian species, compared to that of mammals, is indicative of a difference in the regulation of blood glucose homeostasis between the two species, with the pancreatic hormones insulin and glucagon not functioning in the same manner (Polakof et al., 2011).

In birds, normal insulin/glucagon ratios are roughly half those observed in mammals, or lower (Pollock, 2002). Thus, birds are usually in a catabolic state, similar to that observed in diabetic, exercising or starving mammals, which ensures the availability of sufficient fuel in order to maintain their high metabolic rate (Pollock, 2002; Polakof et al., 2011). The low insulin/glucagon ratio contributes to the significantly increased blood glucose concentrations observed in birds, as well as the predominantly 'glucagon-driven' metabolism (Polakof et al., 2011).

Glucagon, which is secreted by the avian pancreatic α-cells, is the dominant pancreatic hormone in the regulation of avian glucose metabolism, as birds are more sensitive to glucagon than they are to insulin (Braun and Sweazea, 2008). Glucagon has similar actions in birds and mammals and has been shown to stimulate lipolysis, hepatic gluconeogenesis and glycogenolysis and inhibit hepatic lipogenesis (Scanes and Braun, 2013). In doing so, glucagon causes a significant increase in circulating blood glucose concentrations (Scanes and Braun, 2013). Various stimuli for glucagon secretion by the avian exocrine pancreas exist, including cholecystokinin, somatostatin, insulin and free fatty acids (Pollock, 2002). Similarly to the situation in mammals, following the ingestion of glucose, the release of glucagon from the avian pancreas is strongly inhibited (Ruffier et al., 1998). In addition to glucagon, insulin also plays an important regulatory role in avian blood glucose homeostasis; however it is not the dominant pancreatic hormone in birds, as is the case in mammals (Hazelwood, 1984).

Insulin is secreted by the β-cells of the avian pancreas. Contrary to insulin's actions in mammals, in birds insulin has no effect on hepatic glycogenolysis or gluconeogenesis, or on the glycolysis which occurs at the level of the liver and skeletal muscles (Scanes and Braun, 2013). Additionally, avian insulin does not suppress lipolysis (Pollock, 2002). Insulin functions in decreasing blood glucose concentrations and has been shown to enhance the uptake of glucose into the skeletal muscles, kidneys and the brain (Scanes and Braun, 2013). Despite the significant increase in insulin secretion observed in birds, following increases in blood glucose concentrations, previous studies have shown that the glucose molecules themselves are not the primary trigger for insulin release (Hazelwood, 1984; Pollock, 2002). In fact, the avian pancreas is relatively insensitive to glucose and the release of insulin by the β-cells is stimulated by amino acids, glucagon and cholecystokinin instead

(Hazelwood, 1984; Pollock, 2002). Insulin-induced blood glucose regulation is largely dependent on insulin sensitivity, as well as the overall amount of insulin released by pancreatic β-cells (Kaiyala et al., 1999).

After having examined the normal hormonal regulation of blood glucose concentrations in avian species, we will now explore the possible changes in blood glucose regulation and glucose tolerance which could be induced by the consumption of a HFD.

#### 1.4.1.1 High-fat diet-induced changes in glucose tolerance

Previous studies in mammals have shown that HFD feeding is associated with changes in the relationship between insulin sensitivity and β-cell function, which in turn results in impaired glucose tolerance and the development of insulin resistance (Kaiyala et al., 1999; Honors et al., 2012). Whether the same phenomenon is observed in birds, following the ingestion of a HFD, is not certain.

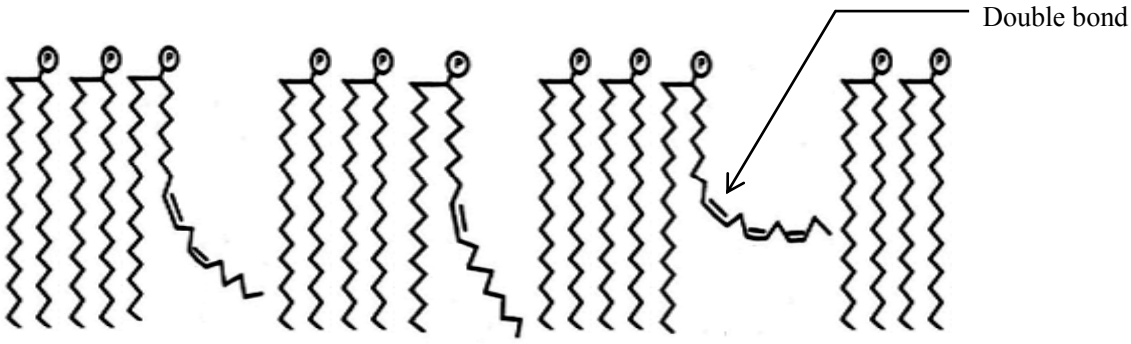
Signs of impaired glucose tolerance in mammals include increased fasting blood glucose and insulin concentrations, as well as an increased insulin area under the curve (AUC); following a glucose tolerance test (Honors et al., 2012). The oral glucose tolerance test (OGTT) is deemed the most suitable analytical technique for the assessment of changes in glucose tolerance (Bergman, 1989). Following the ingestion of a glucose load, the glucose is absorbed from the intestine and into the blood stream. The amount of glucose absorbed from the intestine, as well as the rate of glucose absorption largely affects the changes in blood glucose concentrations following glucose ingestion (Weideman et al., 2012). Once the ingested glucose is absorbed from the intestine into the bloodstream, blood glucose concentrations will increase and then over time will decrease, eventually returning back to basal concentrations, depending on the rate of clearance of the glucose from the bloodstream. Thus, blood glucose concentrations which remain elevated for a longer period of time following glucose ingestion are indicative of a reduced or slower clearance of glucose from the blood stream, which in turn is suggestive of a reduced/poorer glucose tolerance.

Various mechanisms have been shown to play a role in the HFD-induced reduction in insulin sensitivity and glucose tolerance previously observed in mammals. Increased dietary fat intake has been shown to result in increased lipid oxidation within insulin-sensitive tissues, which in turn inhibits insulin-stimulated glucose uptake (Boden, 1997; Kaiyala et al., 1999). In addition, reduced insulin receptor binding and diminished activation of the insulin receptor tyrosine kinase, as well as a reduction in the expression of insulin-dependent glucose transporters, have all been related to the changes in insulin sensitivity observed following HFD feeding (Grundleger and Thenen, 1982; Kaiyala et al., 1999). The fatty acid profile of ingested dietary fat has also been shown to be a powerful modulator of insulin action in humans, other mammals and birds, with both fatty acid chain length and degree of saturation modifying insulin release (Storlien et al., 1997; Newman et al., 2005).

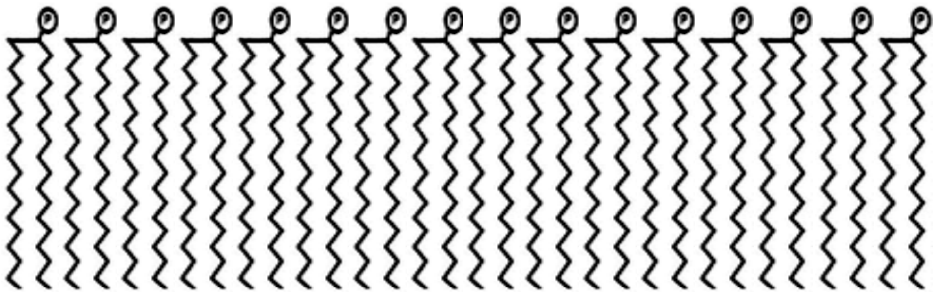
Saturated fatty acids have been shown to impair insulin action, whereas omega-3 polyunsaturated fatty acids have been shown to improve insulin action (Riccardi et al., 2004; Newman et al., 2005). The effects of various dietary fatty acids in the regulation of insulin function in both mammals and avian species is thought to be facilitated via dietary-induced changes in the fatty acid composition of various cell membranes (Riccardi et al., 2004; Newman et al., 2005). Cell membrane lipid profiles are particularly sensitive to dietary changes in omega-3:omega-6 polyunsaturated fatty acid ratio, with omega-3 polyunsaturated fatty acids preferentially incorporated into cell membranes as compared to omega-6 polyunsaturated fatty acids (Haag and Dippenaar, 2005). Changes in membrane fatty acid composition could affect insulin action and glucose tolerance by inducing changes in overall membrane fluidity, which in turn could result in changes in insulin receptor binding and affinity, as well as in the activity of glucose transporters (Riccardi et al., 2004; Newman et al., 2005).

Membrane fluidity refers to the degree of molecular motion and molecular disorder within the membrane lipid bilayer (Los and Murata, 2004). The membrane lipid bilayer is largely composed of glycerophospholipids and cholesterol which together forms a matrix in which various ion channels, receptors and proteins involved in cell signalling are embedded (Fenton et al., 2000). Membrane fluidity is highly dependent on the degree of membrane fatty acid unsaturation, with an increasing

level of unsaturated fatty acids present in the membrane leading to an increase in membrane fluidity (Los and Murata, 2004). The higher the content of unsaturated fatty acids present within the membrane, the more "kinky" and flexible the membrane becomes, leading to increased membrane fluidity and responsiveness. In contrast, the higher the saturated fatty acid content within the membrane, the more rigid and unresponsive the membrane becomes (Haag and Dippenaar, 2005). The effect of the degree of unsaturation on the rigidity of the membrane is illustrated in Figure 1.6. The presence of the double bonds in the unsaturated fatty acids allows for the fatty acids to be packed further apart from one another, as well as introducing some flexibility into the membrane due to their "bendiness" (Haag and Dippenaar, 2005; Hagen et al., 2010). The absence of double bonds within the saturated fatty acids allows for the fatty acids to be packed closely together, making the membrane more rigid, less flexible and less responsive (Haag and Dippenaar, 2005; Hagen et al., 2010).




Membrane containing both unsaturated and saturated fatty acids.



Membrane containing only saturated fatty acids.

**Figure 1.6** Diagram illustrating how the degree of unsaturation of membrane fatty acids determine the fluidity of the membrane. Adapted from "Dietary fats, fatty acids and insulin resistance: short review of a multifaceted connection" by Haag and Dippenaar, 2005.

Changes in membrane fluidity brought about by changes in membrane fatty acid composition have been shown to affect the binding of insulin to receptors on the plasma membrane of adipocytes. Field et al. (1990) observed a significant increase in the polyunsaturated fatty acid content of adipocyte membrane phospholipids in rats fed a diet with a high polyunsaturated to saturated fatty acid ratio. The increased polyunsaturated fatty acid content of the adipocyte membrane phospholipids was associated with increased insulin receptor binding, as well as increased rates of insulin-stimulated glucose transport (Field et al., 1990). The improved insulin binding was attributed to possible conformational changes or actual structural changes within the receptor, as well as altered mobility within the lipid membranes, all induced by the alterations in membrane fatty acid composition (Ginsberg et al., 1981; Gould et al., 1982). The accompanying increase in the amount of insulin-stimulated glucose transport into the cell was attributed to the positive influence of the increased dietary intake of polyunsaturated fatty acids on the coupling between the receptor and transporter (Field et al., 1990). The effects of dietary fatty acid intake on insulin/glucose metabolism are not only confined to mammals, diet-induced effects on insulin/glucose metabolism have also been observed in avian species.

Newman et al. (2005) observed an increase in the maximal insulin release in response to a glucose infusion in broiler chickens fed a diet rich in saturated fatty acids compared to those fed a diet rich in polyunsaturated fatty acids. Thus, a lower concentration of insulin was required to decrease plasma glucose concentrations to basal levels in birds fed the diet high in polyunsaturated fatty acids compared to those fed the diet high in saturated fatty acids (Newman et al., 2005). The improved clearance of glucose from the blood stream was attributed to an increase in insulin receptor affinity or an increase in insulin receptor binding sites, which is brought about due to an increase in the polyunsaturated nature of the membrane environment (Newman et al., 2005).

The HFD-induced changes in membrane fatty acid composition and thus in membrane fluidity are not only reflected in adipocyte and skeletal muscle membranes, a similar situation is also observed in the membranes of erythrocytes following HFD feeding. Changes in erythrocyte membrane fatty acid composition has been shown to result in alterations in erythrocyte osmotic fragility, which

could have consequences in terms of the health of the birds consuming the high-fat poultry diets which are commonly used during production.

### 1.4.2 Erythrocyte osmotic fragility in avian species

Erythrocyte osmotic fragility is a measure of the ability of erythrocytes to resist osmotically-induced lysis, when incubated in solutions of serially diluted concentrations of phosphate-buffered saline (Perk et al., 1964; Oyewale and Durotoye, 1988; Kolanjiappan et al., 2002; Cools et al., 2011; Moyo et al., 2012). Erythrocyte osmotic fragility is influenced by various intrinsic and extrinsic factors, including erythrocyte membrane-related factors, erythrocyte size, volume and age, as well as blood storage conditions and the osmolality, temperature and pH of the test medium used to test osmotic fragility (Oyewale and Durotoye, 1988; Oyewale, 1991; Oyewale, 1992; Oyewale, 1993; Mafuvadze and Erlwanger, 2007; Moyo et al., 2012). The measurement of haematological parameters, such as erythrocyte osmotic fragility, is an important clinical tool for the assessment of the overall health status of the animal or human (Azeez et al., 2011).

Like mammalian erythrocytes, avian erythrocytes are also composed of a phospholipid-bilayer, which is interspersed with various membrane proteins. Structural factors such as the fatty acid composition of membrane phospholipids, membrane cholesterol content, membrane cholesterol/phospholipid ratio, the protein matrix and the degree of unsaturation and length of fatty acyl phospholipid chains, have been shown to affect erythrocyte membrane function (Vajreswari and Narayanareddy, 1992; Kempaiah and Srinivasan, 2006; Sengupta and Ghosh, 2011).

Phospholipids are important structural constituents of both subcellular organelles and cell surface membranes. The phospholipid bilayer of the erythrocyte membrane plays an important role in determining the permeability and fluidity of the membrane (Ferlazzo et al., 2011). Cholesterol, an essential component of all cell membranes, also plays a role in determining membrane fluidity, by regulating the movement of phospholipid fatty acyl chains and thus maintaining the membrane in an ‘intermediate fluid state’ (Owen et al., 1982; Uydu et al., 2012). Increased erythrocyte membrane cholesterol concentrations, relative to membrane phospholipids, have been shown to result in

reduced membrane fluidity and thus reduced erythrocyte deformability (Uydu et al., 2012) due to alterations in erythrocyte surface area and distortions in erythrocyte shape (Kolanjiappan et al., 2002). In addition to alterations in erythrocyte membrane cholesterol and phospholipid concentrations, erythrocyte membrane fluidity/deformability is also influenced by the phospholipid fatty acid composition of the membrane and thus the resulting unsaturation level of the membrane (Nagahuedi et al., 2009; Sengupta and Ghosh, 2011). Increases in the amount of saturated fatty acids within membrane phospholipids are associated with reduced membrane fluidity. As previously mentioned, the membrane becomes stiff as the saturated fatty acids are able to pack closely together, due to the absence of any double bonds (Hagen et al, 2010), thus reducing erythrocyte deformability.

The ability of the erythrocyte to deform is one of the most important determinants of erythrocyte function overall and is essential for the survival of erythrocytes in circulation (Kempaiah and Srinivasan, 2006; Uydu et al., 2012). The osmotic fragility of erythrocytes is an influential factor in determining the erythrocyte deformability (Kempaiah and Srinivasan, 2006). Alterations in the ratio of membrane cholesterol to phospholipids, as well as in the fatty acid composition of the phospholipids within the erythrocyte membrane, which ultimately lead to changes in the deformability/fragility of erythrocytes, can be brought about by dietary modifications.

#### 1.4.2.1 High-fat diet-induced changes in erythrocyte osmotic fragility

Avian erythrocytes, unlike those of mammals, are nucleated, have a relatively short lifespan and are metabolically active (Thrall et al., 2004). Thus, as a result of their high cell membrane turnover and high metabolic capacity, they are likely to be more susceptible to dietary modifications, such as HFDs. Owing to the constant exchange of lipids between the erythrocyte membrane and plasma phospholipids, as well as through *de novo* membrane phospholipid synthesis and phospholipid acyl group turnover, dietary fatty acids can be incorporated into the erythrocyte membrane (Sengupta and Ghosh, 2011; Clandinin et al., 1991). Thus, dietary fatty acids have been shown to affect the fatty acid composition of the erythrocyte membrane which in turn can result in changes in erythrocyte morphology and thus the ability of the erythrocyte to resist haemolysis (KirchgeBner et al., 1994; Cools et al., 2009). The consumption of HFDs containing added dietary fats of differing

saturation levels by poultry birds may induce changes in the cholesterol:phospholipid ratio and fatty acid composition of the erythrocyte membrane. Alterations in erythrocyte membrane cholesterol:phospholipid ratio and fatty acid composition may in turn lead to changes in the ability of the erythrocytes to deform, thus affecting their susceptibility to osmotic lysis, which could ultimately affect the health of production birds.

Another factor which has been shown to affect the health status of animals and humans alike besides the food that they ingest, is that of the gut microbiota. The role of the gut microbiota in avian health and the possible disruption of the gut microbiota profile in response to HFD feeding will now be discussed.

### 1.4.3 The gut microbiota balance in avian species

In both birds and mammals the gut microbiota plays an important role in the regulation of host health and physiology (Vasai et al., 2014) including ensuring the appropriate development of intestinal morphology and digestive function, protection against pathogens and detoxification of certain compounds (Lan et al., 2005; Kohl, 2012; Stanley et al., 2014). The gut microbiota also plays a role in host nutrition and growth performance, as well as in overall immune modulation and the maintenance of intestinal immune homeostasis (Amit-Romach et al., 2004; Lan et al., 2005; Stanley et al., 2014; Waite and Taylor, 2014). In addition to the constructive contributions of the gut microbiota to the welfare of the host, there are also some disadvantages associated with the presence of bacteria within the gastrointestinal tract. The gut microbiota competes with the host for nutrients within the gastrointestinal tract, thus affecting the efficiency of energy utilisation by the host (Lan et al., 2005; Stanley et al., 2014). The gut microbiota can also induce a constant inflammatory response within the gastrointestinal tract, as well as secrete various toxic compounds which adversely affect the health of the host (Stanley et al., 2014). Thus the maintenance of an appropriate balance between the various gut microbiota is imperative.

Similarly to the mammalian gastrointestinal tract, the avian gastrointestinal tract is colonised by a large number of bacterial species (Vasai et al., 2014). The three dominant bacterial phyla present

within the avian gastrointestinal tract include *Bacteroidetes* (gram-negative), *Firmicutes* (gram-positive) and *Proteobacteria* (gram-negative) (Waite and Taylor, 2014). These bacterial phyla are not unique to the avian gastrointestinal tract and are frequently observed in many gut environments. At higher taxonomic levels the microbiota are in fact very similar between mammals and birds, however at lower taxonomic levels, there are some species which occur exclusively in birds (Kohl, 2012).

A functional gastrointestinal tract with an ideal gut microbiota balance is essential to ensure maintenance of bird health and growth performance (Baurhoo et al., 2009; Nkukwana et al., 2015). In the absence of a healthy gut microbiota composition, despite the formulation of quality feed, production birds will not reach optimal performance (Yegani and Korver, 2008; Nkukwana et al., 2015). Gut microbiota composition is influenced by age, environmental factors and diet (Torok et al., 2011). Studies using broiler chickens have shown that establishment of the gut microbiota commences soon after hatching and in the case of the small intestine, the microbial community is established by two weeks post hatch (Amit-Romach et al., 2004; Lan et al., 2005). Establishment of the caecal microbiota however, requires between 6-7 weeks (Lan et al., 2005). Thus, the number and diversity of the microbes which make up the microbiota increases rapidly as the birds age (Chambers and Gong, 2011). The composition of the microbial community established within the gastrointestinal tract of the birds is largely dependent on the type of bacteria/microbes which are first introduced into the gut from the environment and by means of the diet (Apajalahti et al., 2012). Thus, the environment in which the birds are hatched has a significant influence on the gut bacterial profile. On commercial poultry farms, eggs are usually removed from the layers and hatched in a separate area (Stanley et al., 2014). Additionally, various precautions are taken by poultry farmers to avoid the spread of bacterial pathogens. Thus, the newly hatched birds are exposed to a large number of environmental bacteria (from bedding material, human handlers, feed etc.) rather than that from parental sources, resulting in widely varying colonization of the gastrointestinal tract (Stanley et al., 2014). Over and above the various environmental factors, the main contributing factor to gut microbiota composition is diet. The physical structure of feed particles, including particle size and preparation procedure, as well as the levels of dietary carbohydrates, protein and fat within the diet, all influence the microbiota profile within the gastrointestinal tract (Torok et al., 2011).

#### 1.4.3.1 High-fat diet-induced changes in gut microbiota composition

Previous studies in humans as well as in various animal models have shown that increased dietary fat intake in the form of a HFD results in alterations in both the type and number of bacteria present within the gut (Shen et al., 2014). In general, the consumption of a HFD is associated with a reduced *Bacteroidetes:Firmicutes* ratio, resulting from a decrease in *Bacteroidetes* and an increase in *Firmicutes* (Kim et al., 2012b; Moreira et al., 2012; Patterson et al., 2014; Shen et al., 2014), a profile characteristic of that associated with obese individuals or animals (Greiner and Backhed, 2011). The HFD-induced changes in abundance of the two dominant bacterial phyla, *Bacteroidetes* and *Firmicutes* are thought to arise as a result of the overflow of dietary fat to the distal small intestine, following HFD feeding (De Wit et al., 2012). The increased amount of fatty acids in the distal small intestine is thought to evoke an antimicrobial effect on the gut microbiota, resulting in a reduction in the *Bacteroidetes:Firmicutes* ratio, as well as reduced bacterial diversity (De Wit et al., 2012). Saturated fatty acids have been shown to induce a more pronounced overflow of fat to the distal small intestine and thus have a more substantial effect on the composition of the gut microbiota than unsaturated fatty acids (De Wit et al., 2012).

The HFD-induced changes in the *Bacteroidetes:Firmicutes* ratio, have been associated with differences in the ability of the bacteria to harvest energy from the diet (Musso et al., 2010; Torok et al., 2011). This increased ability to harvest energy induced by the changes in quantity and composition of the gut microbiota is a result of the increased expression of various genes associated with dietary energy harvest (Musso et al., 2010; De Wit et al., 2012). The HFD-induced gut microbiome is rich in β-fructosidase, phosphotransferases and glycoside hydrolases, thus increasing the degradation of fructose-containing carbohydrates, the import of simple sugars and the breakdown of otherwise indigestible alimentary polysaccharides (Musso et al., 2010), respectively. All of the above mentioned processes contribute to increased dietary energy harvest.

In addition to the changes in dietary energy harvest, the HFD-induced alteration in gut microbiota balance is also associated with the development of intestinal inflammation, which precedes the weight gain and other metabolic disorders which usually develop upon consumption of a HFD (Shen et al., 2014). The intestinal inflammation observed following HFD feeding is thought to

occur in response to the increased production of pro-inflammatory mediators by immune cells, upon stimulation by lipopolysaccharide (Moreira et al., 2012). Lipopolysaccharide is an important constituent of the outer cell wall of gram-negative bacteria, the levels of which are significantly increased following HFD feeding (Greiner and Backhed, 2011; Moreira et al., 2012; Tehrani et al., 2012). A group of bacteria known as *Bifidobacteria* (gram-positive) which have been shown to reduce intestinal lipopolysaccharide levels are decreased following HFD feeding, thus resulting in increased intestinal levels of lipopolysaccharide (Tehrani et al., 2012). Previous studies have shown that dietary fat, through the activation of mast cells, indirectly increases the intracellular and paracellular intestinal permeability, which together with reduced expression of proteins in tight junctions within the intestinal mucosa following HFD feeding, favours the translocation of lipopolysaccharide from the intestine into the blood stream (Moreira et al., 2012). The presence of increased levels of lipopolysaccharide in the bloodstream is referred to as 'metabolic endotoxemia', which leads to the development of inflammation (De La Serre et al., 2010; Kim et al., 2012b). The binding of lipopolysaccharide to the CD14 toll-like receptor 4 complex initiates downstream inflammatory events, through the activation of various transcription factors including nuclear factor-κβ, which then increases the production of pro-inflammatory cytokines, interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) (De La Serre et al., 2010; Musso et al., 2010; Kim et al., 2012b; Tehrani et al., 2012). Not only does an increase in overall dietary fat content lead to increased intestinal permeability following HFD feeding, but the type of dietary fatty acids consumed have been shown to affect intestinal permeability differently, thus resulting in differential increases in lipopolysaccharide and varying degrees of intestinal inflammation (Moreira et al., 2012). The intake of diets containing fats high in saturated fatty acids generally results in a higher inflammatory response (Moreira et al., 2012).

Thus, the consumption of HFDs by birds during production, through alterations in the various proportions of gut microbiota, may predispose the birds to an obese phenotype affecting not only the health and well-being of the birds but also the subsequent carcass fat deposition, which could influence the quality of meat obtained from the birds. This, in turn, may have implications for poultry production practices/production costs, as well as for the health of humans who consume poultry products. In addition to the HFD-induced alterations in gut microbiota profile, HFD feeding has also been associated with changes in bone tissue.

### 1.4.4 Bone health in avian species

The bone health of poultry production birds is an important factor which affects the overall economic profitability of the poultry production industry. The development of avian osteoporosis in production birds resulting in increased incidence of bone fractures, fragments of bone within the meat products, as well as increased mortality of the birds during production, ultimately increases economic losses (Rath et al., 2000; Whitehead and Fleming, 2000). Bone is a type of connective tissue which is composed of a mineralised organic structural framework, which consists of collagen, non-collagenous proteins, cartilage, osteocytes, osteoblasts and osteoclasts, as well as various inorganic minerals including calcium and phosphorus (Al-Nouri and Al-Khalifa, 2011; Rezq et al., 2010). Bone tissue is continuously remodelled, involving processes of bone formation and bone resorption (Rezq et al., 2010). Bone formation is carried out by the mono-nucleated osteoblasts which produce collagen and non-collagenous proteins that form part of the organic matrix (Al-Nouri and Al-Khalifa, 2011; Crockett et al., 2011). Bone resorption is carried out by the multi-nucleated osteoclasts, which secrete several factors that degrade the organic matrix resulting in the release of minerals from the bone tissue (Al-Nouri and Al-Khalifa, 2011; Crockett et al., 2011).

The regulation of bone formation and bone resorption is carried out by a number of locally produced factors which include amongst others: prostaglandins, cytokines, leukotrienes, thromboxanes, lipoxins and resolvins, all of which are formed from polyunsaturated fatty acid precursors (Liu, 2000; Virtanen et al., 2012; Tarlton et al., 2013). The synthesis of these regulatory factors and thus the overall regulation of bone formation and bone resorption is highly dependent on the type of polyunsaturated fatty acid precursors (omega-6 or omega-3), as well as the fatty acid chain length (Tarlton et al., 2013). The regulatory factors produced from omega-6 polyunsaturated fatty acids are generally pro-inflammatory mediators, whereas those produced from omega-3 polyunsaturated fatty acids function in resolving inflammation and thus they have been thought to have opposing effects on bone metabolism (Virtanen et al., 2012; Tarlton et al., 2013).

Prostaglandin $E_2$ ($PGE_2$) is the primary regulator of bone tissue function and metabolism (Watkins et al., 2001; Virtanen et al., 2012). At high concentrations, $PGE_2$ has been shown to inhibit bone formation, whereas at low concentrations $PGE_2$ stimulates bone formation (Watkins et al., 2001;

Albertazzi and Coupland, 2002). $PGE_2$ is formed from the 20-carbon omega-6 polyunsaturated fatty acid, arachidonic acid (James et al., 2000; Watkins et al., 2001). Arachidonic acid and the 20-carbon omega-3 polyunsaturated fatty acid, eicosapentanoic acid, are formed through the desaturation and elongation of the 18-carbon, primary dietary omega-6 and omega-3 polyunsaturated fatty acids, linoleic acid and α-linolenic acid, respectively (James et al., 2000). Following the production of arachidonic acid and eicosapentanoic acid, these 20-carbon polyunsaturated fatty acids are then oxidised by either cyclooxygenase or lipoxygenase to give rise to series-2 (eg. $PGE_2$) or series-3 prostaglandins (eg. $PGE_3$), respectively (Tarlton et al., 2013). Since eicosanoids produced from omega-6 polyunsaturated fatty acids have more of an agonistic effect on eicosanoid receptors compared to those formed from omega-3 polyunsaturated fatty acids and arachidonic acid is the preferred substrate for cyclooxygenase (compared to eicosapentanoic acid) (Lands, 1992; Watkins et al., 2001), one could hypothesize that changes in dietary ratios of omega-6: omega-3 polyunsaturated fatty acids could ultimately affect prostaglandin biosynthesis and thus bone metabolism.

#### 1.4.4.1 High-fat diet-induced changes in bone health

Previous studies in humans (Paunescu et al., 2013), rats (Li et al., 2010; Lukas et al., 2011), rabbits (Al-nouri et al., 2014) and birds (Liu et al., 2003; Tarlton et al., 2013) have shown in general, that diets rich in omega-3 polyunsaturated fatty acids or a reduction in the dietary omega-6: omega-3 polyunsaturated fatty acid ratio is associated with positive effects on bone quality and strength, with increases in bone mineral content and density, which are attributed, for the most part, to the omega-3 polyunsaturated fatty acid-induced reduction in $PGE_2$ synthesis and thus an overall decrease in bone resorption processes (Liu et al., 2003; Li et al., 2010; Lukas et al., 2011; Paunescu et al., 2013; Tarlton et al., 2013; Al-nouri et al., 2014). Thus, the type of dietary fat (specifically omega-6 polyunsaturated fatty acids) used in the formulation of poultry diets should be carefully considered as the use of fats rich in omega-6 polyunsaturated fatty acids could negatively affect the skeletal health of production birds, which as previously mentioned could result in adverse effects on overall bird health and on the economic profitability of the poultry production industry. Additionally, the inclusion of high levels of fat in poultry diets has been shown to result in the formation of insoluble soaps between the fatty acids and minerals such as calcium, rendering it unavailable to the birds which may have consequences in terms of bone mineral retention (Atteh and Leeson, 1983; Abdulla

et al., 2016). Thus, when high levels of fat are used to formulate poultry diets, calcium should also be supplemented (Abdulla et al., 2016). The ratio of calcium to phosphorus in poultry diets is also of importance. Whilst decreasing dietary calcium has been shown to improve phosphate utilisation, an increase in dietary calcium may aggravate a phosphate definciency since the calcium may reduce the amount of phosphate available for absorption through formation of $Ca_2PO_4$ precipitates in the gastrointestinal tract (Letourneau-Montminy et al., 2008; Hamdi et al., 2015).

Another factor which should be kept in mind when formulating high-fat poultry diets is that of the resulting fatty acid profile of the edible bird tissues, following consumption of these diets.

### 1.4.5 Fat deposition in avian species

Fat deposition is the net outcome of the absorption of dietary fat, the *de novo* synthesis of endogenous fatty acids and the break down or oxidation of fatty acids (Smink et al., 2010). Excessive fat deposition in broiler chickens and other 'table birds' is a major problem for the poultry production industry as besides being an unfavourable trait for consumers, it also results in decreased feed efficiency and decreased carcass yield (Tůmová and Teimouri, 2010; Fouad and El-Senousey, 2014). Previous studies have shown that the overall amount of adiposity can be influenced by nutritional manipulations such as HFDs and that the composition of the resulting body fat is dependent on the fatty acid profile of the dietary fat consumed (Dvorin et al., 1998).

#### 1.4.5.1 High-fat diet-induced changes in fat deposition

The influence of dietary fat sources on the fatty acid composition of bird tissues is thought to be associated with differences in the digestibility and metabolisable energy of the various fats, thus affecting the overall energy available to the birds (Nitsan et al., 1997). Fat digestibility is dependent on a variety of factors including the length of the hydrocarbon chain, the level of saturation of the fatty acids and the position of the fatty acids on the glycerol molecule (Renner and Hill, 1961; Dvorin et al., 1998). Long-chain saturated fatty acids are less digestible than medium-chain saturated or unsaturated fatty acids (Smink et al., 2008). Saturated fatty acids are less easily incorporated into micelles compared to unsaturated fatty acids as a result of their non-polarity

(Zhang et al., 2011a). The non-polar saturated fatty acids are thus less soluble within the micelle and are believed to be incorporated into the centre of the micelle (Garrett and Young, 1975), which in turn can affect their subsequent absorption from the gastrointestinal tract. Animal fats are generally rich in saturated fatty acids and are thus less easily digested and have a lower metabolisable energy content compared to vegetable oils, which are rich in unsaturated fatty acids (Monfaredi et al., 2011; Tancharoenrat et al., 2014).

Previous studies involving dietary supplementation with various animal or vegetable fats/oils, have shown that in general, supplementation with fats/oils rich in unsaturated fatty acids result in reduced skeletal muscle and abdominal fat deposition compared to those rich in saturated fatty acids (Sanz et al., 2000a; Ferrini et al., 2008; Gonzalez-Ortiz et al., 2013). The proposed mechanism for the above-mentioned observation involves the preferential oxidation of polyunsaturated fatty acids compared to saturated fatty acids, which in turn may lead to increased synthesis of endogenous fatty acids from carbohydrates, which is less efficient metabolically and has a higher energy cost, than the synthesis of triglycerides, for storage, from dietary fatty acids (Crespo and Esteve-Garcia, 2001; Wongsuthavas et al., 2008). Thus, feeding of diets high in polyunsaturated fatty acids compared to saturated fatty acids results in less body fat deposition in poultry (Crespo and Esteve-Garcia, 2001; Wongsuthavas et al., 2008).

Additionally, the rates of lipid synthesis (*de novo* fatty acid synthesis) and lipid oxidation (β-oxidation) have been shown to differ, with differing dietary fatty acid profiles (Ferrini et al., 2008; Smink et al., 2010; Poorghasemi et al., 2013) which also subsequently affects fat deposition. At high levels of fat intake, particularly of polyunsaturated fatty acids, the expression and activities of various enzymes involved in β-oxidation, such as acyl-CoA oxidase and carnitine palmitoyl transferase 1, are increased, thus enhancing the β-oxidative pathway overall (Rise and Galli, 1999; Crespo and Esteve-Garcia, 2001). Acyl-CoA oxidase is the enzyme which catalyses the first step of the peroxisomal β-oxidative pathway. Initiation of mitochondrial β-oxidation involves the uptake of long-chain fatty acids into the mitochondria, which is facilitated by carnitine palmitoyl transferase 1 (Haug et al., 2014). Despite suggestions that increased intake of polyunsaturated fatty acids results in increased expression and activity of various enzymes involved in β-oxidation, contradictory

results have also been observed. Haug et al. (2014) found no significant differences in gene expression of both acyl-CoA oxidase and carnitine palmitoyl transferase 1 in chickens fed diets supplemented with linseed oil (rich in polyunsaturated fats) at 24g/kg feed for 4 weeks, compared to those receiving only the standard diet (Haug et al., 2014). Thus, the results from studies investigating the effects of dietary supplementation with various different fats/oils on lipid metabolism and the resulting lipid deposition are inconsistent.

The ease with which the fatty acid composition of both storage tissues and skeletal muscle are modified, as a result of modifications in dietary fat intake, differ and this difference has been attributed to the different functions of the tissues (Bou et al., 2009). Fat storage tissues include the abdominal fat, which includes all the fat surrounding the proventriculus, gizzard, cloaca and adjacent muscles, as well as the subcuticular fat in the cranial thigh region and in the ventro-caudal neck region, along with the mesenteric fat (Tůmová and Teimouri, 2010). These storage tissues act as a fat reservoir, the fatty acid composition of which is a relatively accurate reflection of the dietary fatty acid profile and is somewhat easier to modify through diet than the fat within skeletal muscle (Bou et al., 2009). Modification of the fatty acids within skeletal muscle by diet, are somewhat limited. Majority of the fatty acids in skeletal muscle are usually found as part of the phospholipids of cell membranes, thus a relatively stable fatty acid composition is maintained in order to conserve both the physical properties and biological activities of the membranes (Bou et al., 2009). In addition to the phospholipids, skeletal muscle lipids also include the triglycerides within adipocytes situated in the interfascicular region along the muscle fibres (intermuscular fat), as well as the triglycerides present as cytosolic droplets in the muscle fibres (intramuscular fat) (De Smet et al., 2004). The polyunsaturated fatty acid content of the above mentioned triglycerides, albeit relatively low, can be influenced by dietary modifications, particularly in monogastric animals such as poultry birds (De Smet et al., 2004; Duraisamy et al., 2013).

Now that we understand the processes by which lipids are metabolised in birds, and the adverse metabolic disturbances which could result following the consumption of a HFD in birds, we will now look at the organoleptic consequences resulting from altered lipid deposition in the meat of poultry birds.

## 1.5 Fat and poultry meat quality

Commonly studied characteristics of poultry meat quality include flavour, juiciness, texture and colour or appearance of the meat (Petracci and Baéza, 2011). Most meat quality traits are in some way influenced by the overall fat content or fatty acid composition of the meat. Meat fat content includes subcutaneous fat, intermuscular fat and intramuscular fat (Culioli et al., 2003). The amount of intramuscular fat has been shown to be a major determinant of some of the sensory qualities of meat, including flavour, juiciness and texture or tenderness (Hocquette, 2010).

As previously mentioned, intramuscular fat is predominantly comprised of triglycerides (De Smet et al., 2004). Meats with reduced levels of intramuscular fat are rich in polyunsaturated fatty acids, due to the increased proportion of membrane phospholipids, which contain a large amount of polyunsaturated fatty acids, compared to triglycerides (De Smet, 2012). Poultry meat is well known for its relatively low fat content and high unsaturated fatty acid content compared to other meats (Barroeta, 2007). Increased intramuscular fat deposition is correlated with enhanced meat quality, typically resulting in improved texture, flavour and juiciness (Li et al., 2013). In contrast, lean meats with low intramuscular fat content are generally somewhat dry and less tasty (Hocquette, 2010; De Smet, 2012). Intramuscular fat deposition within avian and mammalian muscles typically occurs intracellularly, as droplets within the cytoplasm of myofibres, and between muscle fibre fasciculi in intramuscular adipocytes (Gondret et al., 1998; Hocquette, 2010). Expansion of the adipocytes found between the muscle fibres has been shown to increase meat tenderness, by disrupting the structure of the endomysium and forcing the muscle fibres apart (Wood et al., 2003; Hocquette, 2010). The various distinguishing flavours of different meat species also stem from the differences in lipid proportions within the meat (Mottram, 1998). Poultry meat flavour encompasses both the odour and taste of the meat (Jayasena et al., 2013).

Meat flavour is to a large extent affected by the fatty acid composition of the meat as a result of the production of several odorous, volatile compounds, through a series of complex reactions between the fatty and lean tissue components of the meat, induced by cooking (Jayasena et al., 2013). The main reactions induced through cooking include the thermal degradation or auto-oxidation of fatty acids and the reaction between amino acids and reducing sugars commonly referred to as the

Maillard reaction (Mottram, 1998). Products of both reactions and the resulting compounds formed by the interaction between these products contribute to overall meat flavour (Mottram, 1998; Wood et al., 2003; Jayasena et al., 2013). Unsaturated fatty acids are particularly important in the development of flavour during the cooking process, as a result of their susceptibility to oxidation, compared to that of saturated fatty acids (Wood et al., 2003).

Lipid peroxidation is the process by which lipids containing carbon-carbon double bonds, particularly those with numerous double bonds (polyunsaturated fatty acids), are attacked by oxidants (Ayala et al., 2014). The reaction involves the removal of the hydrogen atom attached to the carbon atom adjacent to a double bond, and the replacement of that hydrogen atom with an oxygen atom (Ayala et al., 2014). The double bond adjacent to the methylene group makes the carbon-hydrogen bond weaker and thus the hydrogen atom is easily removed (Repetto et al., 2012). The primary products of the lipid peroxidation process are known as lipid hydroperoxides. Numerous secondary products are also formed during the lipid peroxidation process including malondialdehyde, hexanal, propanal and 4-hydroxynonenal (Ayala et al., 2014). Both the primary and secondary products of lipid peroxidation are cytotoxic and induce oxidative stress and oxidative damage and ultimately cell death (Repetto et al., 2012). The oxidative breakdown of the unsaturated fatty acids within poultry meat products results in the formation of off-flavours and off-odours, as well as a loss in the nutritional value of the meat products (Bou et al., 2009).

In addition to influencing overall meat flavour development during cooking, the susceptibility of unsaturated fatty acids to oxidation also plays an important role in deterioration of meat quality and thus the shelf life of poultry meat products. Reactive oxygen species and free radicals produced during the lipid peroxidation process, promotes the oxidation of red oxymyoglobin to brown metmyoglobin, resulting in surface discolouration of the meat (Wood et al., 2003; Filgueras et al., 2010). Myoglobin is the haeme protein present within muscle tissue, which gives meat its colour (Faustman et al., 2010). Different cuts of meat discolour at different rates, depending on the relative proportions of red versus white muscle fibres. Oxidative (red) muscle fibres display greater oxygen consumption rates and higher lipid content than white muscle fibres and thus discolour at a faster

rate (Faustman, 2010; Hocquette, 2010). Myoglobin oxidation also supports the formation of off flavours and rancid odours (Filgueras et al., 2010).

Thus, not only does the fat content of meat play an important role in the development of flavour, but the presence of specific fatty acids (i.e. polyunsaturated fatty acids) can significantly affect deterioration rates of meat products, in turn affecting consumer buying activities. Therefore it is important to monitor the addition of various fats/oils (of differing fatty acid compositions) to the diet of meat-producing animals, such as poultry, as the edible tissues are often reflective of the dietary fatty acid profile, which can have consequences in terms of human health, following consumption of the meat products.

### 1.6 Implications for humans following the consumption of poultry meat products from poultry birds fed high-fat diets

Meat and meat products serve as an important supply of foodstuffs of high nutritional value in most developing and developed countries alike (Lofgren, 2013). Animal meat serves as an important source of nutrients for human diets including protein of high biological value, zinc, iron, selenium and B-vitamins, amongst others (Decker and Park, 2010; Pereira and Vicente, 2013; Chulayo and Muchenje, 2015). Despite the favourable nutritional composition of meat, with respect to protein, vitamins and minerals, meat also represents a major source of fat within the human diet, specifically of saturated and trans fatty acids (Wood et al., 2003; Salter, 2013; Milićević et al., 2014).

The consumption of saturated and trans fatty acids by humans has been associated with the development of a number of chronic diseases, including cardiovascular disease, as well as various cancers and metabolic diseases (Wood et al., 2003; Pereira and Vicente, 2013; Milićević et al., 2014). The intake of saturated and trans fatty acids has been shown to increase the ratio of low-density lipoprotein cholesterol to high-density lipoprotein cholesterol, which in turn increases the risk of development of cardiovascular disease (Livingston et al., 2012; Salter, 2013; Mapiye et al., 2015). According to the World Health Organisation, by the year 2020, approximately 75 % of all deaths worldwide, will be attributable to chronic diseases (WHO/FAO, 2003; Givens et al., 2006;

Givens, 2009). Thus, a number of nutritional recommendations have been made in order to try and reduce human saturated fatty acid intake. One such recommendation states that total fat intake should not comprise more than between 15 and 30 % of overall dietary calories, with saturated fatty acids only comprising 10 % of these calories (Jime´nez-Colmenero et al., 2001). The intake of lean protein sources such as fish and poultry, as alternatives to red meat, is also highly recommended (Daniel et al., 2011). Like red meat, poultry meat also contains significant amounts of good quality protein, vitamins, iron and zinc. However, poultry meat in general has a lower overall fat content and contains significantly higher amounts of beneficial polyunsaturated fatty acids compared to beef, pork, lamb and veal (Barroeta, 2007; Lofgren, 2013). In contrast to the effects of saturated fatty acid intake on the development of various chronic diseases, the consumption of increased amounts of polyunsaturated fatty acids, specifically of the omega-3 variety, have been shown to reduce the risk of several chronic diseases (Pisulewski, 2005; Milićević et al., 2014).

Despite the significantly increased overall amounts of polyunsaturated fatty acids in poultry meat compared to meat from mammals such as beef, pork, lamb and veal, the ratio of omega-6: omega-3 polyunsaturated fatty acids in the skeletal muscle of birds and mammals, differs. Birds have a much lower content of omega-3 polyunsaturated fatty acids and a much higher content of omega-6 polyunsaturated fatty acids in skeletal muscle, compared to mammals (Hulbert et al., 2003; Rymer and Givens, 2005). The recommended ratio of omega-6: omega-3 polyunsaturated fatty acids for optimal human health and nutrition, is below 5. However, the ratios of omega-6: omega-3 polyunsaturated fatty acids in most animal products are between 10 and 15 (Kouba and Mourot, 2011). Thus, the enrichment of edible animal tissues, specifically that of poultry, with beneficial omega-3 polyunsaturated fatty acids and the development of ‘functional foods’ has become an area of interest for researchers. Various strategies have been employed in an attempt to successfully modify the fatty acid composition of animal meat and improve the ‘functional’, health-related properties of the meat, such that they are more in-line with dietary guidelines for humans (Pisulewski, 2005).

One such strategy involves changing the fatty acid composition of animal meat products through enrichment of the diet fed to production animals, with omega-3 polyunsaturated fatty acids. The

fatty acid composition of poultry meat, together with that from other monogastrics is relatively easily modified by dietary means, compared to that from ruminants (Rymer and Givens, 2005; Woods and Fearon, 2009). In monogastric animals, the fatty acids are absorbed unaltered from the small intestine and then transported to various tissues to be utilised or to the adipose tissue for storage (Baião and Lara, 2005; Woods and Fearon, 2009). In ruminants however, the fatty acids ingested via the diet are hydrogenated in the rumen (Wood et al., 2003; Kouba and Mourot, 2011), thus making it more difficult to ensure the direct transfer of dietary fatty acids, in their original state, to tissue lipids. Previous studies have demonstrated the successful transfer of omega-3 polyunsaturated fatty acids into the tissues of poultry. However, the majority of studies have focussed on the chicken and not many studies have looked at the transfer of beneficial omega-3 polyunsaturated fatty acids from the diet into the tissues of birds, other than chickens, which are becoming popular as table birds for human consumption.

One of the main disadvantages associated with the enrichment of animal meat products, including poultry meat, with polyunsaturated fatty acids (omega-3 and omega-6 polyunsaturated fatty acids) is the resulting decrease in oxidative lipid stability of the meat (Decker and Park, 2010; Kouba and Mourot, 2011; Ribeiro et al., 2013; Puvača et al., 2014). Unsaturated fatty acids are highly susceptible to oxidation, due to the presence of double bonds (Kouba and Mourot, 2011). Thus, the greater the content of unsaturated fatty acids within the animal meat product, the more susceptible it is to oxidation (Decker and Park, 2010; Kouba and Mourot, 2011). This in turn results in deleterious changes with respect to the organoleptic qualities of the meat, such as meat flavour and colour, as well as in the shelf life of meat products (Bhalerao et al., 2014). In order to counteract the oxidative changes which usually result, subsequent to increasing the content of unsaturated fatty acids within animal meat products, a number of substances, including vitamin E, vitamin C, carotenoids and selenium, have been used as dietary supplements for production animals which consume diets rich in unsaturated fatty acids, in order to increase the resulting antioxidant activity within animal meat (Jime´nez-Colmenero et al., 2001; Barroeta, 2007; Schiavone et al., 2010; Delles et al., 2014). These antioxidants exert their effects by scavenging lipid peroxides and free radicals, as well as delaying the autoxidation process and decreasing the rate at which oxidation occurs and in doing so they ultimately inhibit the deterioration of meat products by oxidation (Choe and Min, 2007; Ganesan et al., 2014). Thus, when formulating high-fat poultry diets, the type of fat included in the

diet, as well the inclusion of an appropriate antioxidant needs to be carefully considered, since the dietary fatty acids fed to poultry can be successfully transferred into their edible tissues, which in turn can affect the health of human consumers as well as the oxidative stability of the meat products.

In spite of the association of HFD consumption, specifically those rich in saturated and trans fatty acids, with the development of a number of metabolic diseases (Wood et al., 2003; Pereira and Vicente, 2013; Salter, 2013; Milićević et al., 2014), certain dietary trends are promoting an increased fat intake. Low-carbohydrate, high-fat diets such as the Paleo and Banting diets, are popular for their weight-loss results in certain populations, with numerous randomised control trials observing significantly greater weight-loss achieved following the low-carbohydrate, high-fat diets than following a diet high in carbohydrates (Brehm et al., 2003; Foster et al., 2003; Samaha et al., 2003; Yancy et al., 2004; Noakes et al., 2006). With the growing preference or 'want' for foods high in lipids, the modification of overall lipid content of meat obtained from production animals, such that it meets the increased dietary lipid requirements of certain people, is a possibility. Various strategies exist in terms of modifying animal carcass composition, including, as previously mentioned, the management or modification of the diet fed to production animals (Jime´nez-Colmenero et al., 2001). Thus, by increasing the amount of lipids fed to poultry birds, it might be possible to increase the overall lipid content of the edible bird tissues.

### 1.7 Rationale for study/ problem statement

Poultry feeds are often formulated with added dietary lipids, as a source of concentrated energy. The consumption of a high-fat, energy-dense diet in humans, other mammals and some birds has been shown to result in a number of metabolic disorders. The key metabolic disturbances include glucose intolerance and obesity, with further disturbances often observed in erythrocyte osmotic fragility, bone health and gut microbiota balance. The effects of these HFDs on the health status of birds during production is rarely investigated and most research involving current poultry production practices is performed in the domestic chicken and extrapolated to alternative poultry species. Thus, there is a dearth of information concerning the metabolic effects and physiological responses to diets used in the production of alternative poultry species, which could have a negative

impact on the health of the birds during production. Broiler chicken meat currently serves as the primary source of poultry meat and eggs consumed by humans. In order to broaden the scope of poultry meat and egg supply, alternative poultry species such as the Guinea fowl (*Numida meleagris*), Muscovy duck (*Cairina moschata*) and Japanese quail (*Coturnix coturnix japonica*) are being investigated. The present research aimed to investigate the physiological responses of Guinea fowl, Muscovy ducks and Japanese quail to the consumption of various HFDs, of varying fatty acid profiles, as a means of assessing the diet-induced consequences with respect to the overall health status of the birds. Additionally, the present research aimed to assess the transfer of the dietary fatty acids provided to the birds into the edible bird tissues, as well as the overall tissue lipid content following the significantly increased dietary lipid supplementation (Japanese quail). Previous studies have shown that not only is the total amount of fat added to poultry diets important in terms of the resulting meat fatty acid profile, but also the type of fats consumed by the birds during production. The fatty acid profile of the edible bird tissues has been shown to reflect that of the diet which the birds consume during production. Thus, the type of fats, as well as the overall quantity, used to formulate poultry diets are of importance with respect to the effect of the diets on the fatty acid profile of the edible bird tissues, which in turn can affect the health of the humans which consume the poultry meat products

To our knowledge, no studies have examined the effects of different HFDs, with varying fatty acid profiles, on the overall health status of the birds (metabolic effects of the consumption of the different HFDs and the resulting physiological responses), whilst also assessing the transfer of the dietary fatty acids consumed by the birds into the edible bird tissues by examining the resulting fatty acid profiles and total lipid content of the liver and breast and thigh muscles of the birds. Previous studies involving HFD feeding in birds have observed HFD-induced metabolic disturbances following between 4-6 weeks of HFD feeding (Crespo and Esteve-Garcia, 2001; Ferrini et al., 2008; Mohammadi et al., 2011; Poorghasemi et al., 2013). Study 1 and study 2 aimed to investigate the progressive (study 1) and long-term (study 2) metabolic effects of HFD feeding in alternative poultry species and thus a feeding period of 12 weeks was used.

### 1.8 Objectives, aims and hypotheses

The overall objectives and the specific aims assessed in order to achieve the objectives, as well as the hypotheses, for both Study 1 and Study 2 are listed below:

**Study 1 objective**: To investigate the metabolic effects of a HFD, based on palm oil and lard (high in saturated fats) on the overall health status of Guinea fowl (*Numida meleagris*) and Muscovy ducks (*Cairina moschata*).

Specific aims: The current study aimed to investigate the effects of a HFD rich in saturated fatty acids on the:

- growth performance of the birds, by assessing the HFD-induced changes in body mass during the feeding period.
- glucose tolerance of the birds, by assessing the HFD-induced changes in various oral glucose tolerance test (OGTT) parameters.
- osmotic fragility of the avian erythrocytes as an indirect means of assessing the HFD-induced changes in erythrocyte membrane fatty acid composition.
- general health profile of the birds by assessing the HFD-induced changes in various serum metabolic markers of health.
- absolute and relative organ masses and intestine lengths of the birds following the HFD feeding period.
- liver of the birds by assessing the HFD-induced changes in liver mass, liver lipid content and liver histology.
- caecal microbiota profile by assessing the HFD-induced effects on the variability and number of a few select bacterial species in the caecal content of the birds.

Hypotheses:

$H_0$: The HFD, rich in saturated fatty acids, fed to Guinea fowl and Muscovy ducks for either four, eight or twelve weeks, has no effect on the growth performance, glucose tolerance, erythrocyte osmotic fragility, liver mass, liver lipid content, caecal microbiota profile, serum biochemistry/general health profile and the absolute and relative organ masses and intestine lengths of the birds.

**Study 2 objective**: To investigate the metabolic effects of different HFDs, based on coconut oil, lard, palm oil, soyabean oil or sunflower oil, on the overall health status as well as the bone health and overall lipid content and fatty acid profiles of the edible tissues (liver, breast and thigh muscle) of male, Japanese quail (*Coturnix coturnix japonica*).

Specific aims: The current study aimed to investigate the effects of different HFDs, of varying fatty acid profiles, on the:

- growth performance of the birds, by assessing the HFD-induced changes in body mass during the feeding period.
- glucose tolerance of the birds, by assessing the HFD-induced changes in various oral glucose tolerance test (OGTT) parameters.
- osmotic fragility of the avian erythrocytes as an indirect means of assessing the HFD-induced changes in erythrocyte membrane fatty acid composition.
- general health profile of the birds by assessing the HFD-induced changes in various serum metabolic markers of health.
- absolute and relative organ masses and intestine lengths of the birds following the HFD feeding period.
- liver of the birds by assessing the HFD-induced changes in liver mass, liver lipid content and liver histology.
- fatty acid profiles of the liver, breast muscle and thigh muscle of the Japanese quail.
- masses, lengths and densities of the femora of the Japanese quail.

Hypotheses:

$H_0$: The different HFDs, of varying fatty acid profiles, fed to male Japanese quail for a period of twelve weeks, have no effect on the growth performance, glucose tolerance, erythrocyte osmotic fragility, serum biochemistry/general health profile, absolute and relative organ masses and intestine lengths, liver mass, liver lipid content, bone health parameters and the fatty acid profiles of the liver, breast and thigh muscles of the birds.

# CHAPTER TWO: THE EFFECTS OF A HIGH-FAT DIET BASED ON SATURATED FATS, ON THE GROWTH PERFORMANCE, GLUCOSE TOLERANCE, ERYTHROCYTE OSMOTIC FRAGILITY, SERUM BIOCHEMISTRY, LIVER AND GUT MICROBIOTA PROFILE OF GUINEA FOWL AND MUSCOVY DUCKS

## 2.1 Introduction

As outlined in section 1.8 of this thesis, the main objective of Study 1 was to investigate the metabolic effects of a HFD, based on palm oil and lard (high in saturated fats) on the overall health status of Guinea fowl (*Numida meleagris*) and Muscovy ducks (*Cairina moschata*), which are becoming popular as alternative sources of protein for human consumption (Adzitey, 2012; Moreki and Seabo, 2012).

Poultry meat is a favourite animal protein source amongst consumers, as a result of its affordable price compared to red meat (Al-Nasser, 2006; Petracci et al., 2013) and its perceived health benefits which include a high protein content, low fat content and low cholesterol content (Vukasovic, 2010; Daniel et al., 2011; Petracci et al., 2013). The use of Guinea fowl and Muscovy ducks as alternatives to the domestic chicken as sources of protein for human consumption, offer a number of advantages in terms of poultry production. Both bird species are relatively hardy and are resistant to a number of common poultry diseases (Mwale et al., 2008; Madzimure et al., 2011; Yakubu, 2013). During production, poultry birds are generally fed HFDs, formulated using a variety of either animal or vegetable fats, which have been shown to result in improved feed conversion and increased growth rates (Kratzer et al., 1994; Nobakht, 2012; Sahito et al., 2012). The effect of HFD-feeding of this nature on the overall health status of poultry birds is rarely investigated.

The consumption of HFDs, specifically those rich in saturated fatty acids, in humans, other mammals and some birds, has been shown to result in the development of a number of metabolic disturbances collectively referred to as the metabolic syndrome (Roche, 2005; Melanson et al., 2009; Tierney et al., 2011). Since the majority of studies involving poultry production practices and poultry nutrition are performed using the domestic chicken, there is a need for studies which examine the efficacy and safety of these HFDs, as well as the resulting metabolic effects on the overall health status of alternative poultry species such as the Guinea fowl and Muscovy duck. Recently, there has been an increased consumer demand for welfare-friendly animal products and consumers are interested in the methods used to raise production animals (Chulayo and Muchenje, 2015). In order to address public concerns about the health and well-being of animals during production, which in turn could affect consumer attitudes towards purchasing animal meat products (Troy and Kerry, 2010), the resulting metabolic health effects of the HFDs commonly used in poultry production need to be further investigated.

The use of palm oil and lard in the formulation of diets fed to meat-type animals, including poultry, is advantageous. Since palm oil is less susceptible to oxidative damage compared to other vegetable oils, the addition of palm oil to poultry feed is a popular practice as the high saturated fatty acid content of palm oil reduces the occurrence of rancidity of the poultry feed (Wing-Keong, 2002). The consumption of lard by animals bred for their meat has been shown to have a positive influence on the texture and flavour of the meat products (Cheong et al., 2009). The consumption of saturated fats has been associated with impaired or altered insulin action (Newman et al., 2005), increased erythrocyte osmotic fragility (Hagen et al, 2010), increased skeletal muscle and abdominal fat deposition (Sanz et al., 2000a; Ferrini et al., 2008; Gonzalez-Ortiz et al., 2013), alterations in gut microbiota composition (De Wit et al., 2012) and alterations in serum lipid profiles (Viveros et al., 2009; Velasco et al., 2010) in various animal species. Thus, the current study undertook to investigate the possible HFD-induced effects on the overall health status of the Guinea fowl and Muscovy ducks through the assessment of serum biochemistry, glucose tolerance, erythrocyte osmotic fragility, liver lipid deposition and the gut microbiota profile.

## 2.2 Mateials and Methods

### 2.2.1 Ethical approval

Ethical approval for the study was granted by the University of the Witwatersrand Animal Ethics Screening Committee (Ethics clearance number: 2011/08/2A).

### 2.2.2 Animals, housing and diets

Thirty six, four week old, Guinea fowl (*Numida meleagris*) (452.4 g ± 91.2) and 36, four week old, Muscovy ducks (*Cairina moschata*) (671.4 g ± 194.0) were used in the study. The birds were randomly divided into two groups (per species of bird) of 18 birds each and allocated to one of two dietary groups. The standard diet (STD) group which received standard, commercial poultry feed (Epol®, Centurion, Pretoria, South Africa) or the high-fat diet (HFD) group which received the commercial poultry feed enriched with additional fat in the form of palm oil (SupaCrisp, Super Olein Palm Oil, Felda Bridge Africa (PTY) Ltd, Johannesburg, South Africa) at 20 % of the mass of the feed and lard (Norbert's German butchery, Wilropark, Roodepoort, South Africa) at 2 % of the mass of the feed (in total 22 % added dietary fat, on a *weight/weight* basis). Both palm oil and pork

lard are rich in saturated fatty acids (Wing-Keong, 2002; Cheong et al., 2009; Cheong and Xu, 2011; Das et al., 2014a). Table 2.1 shows the composition of the standard, commercial poultry feed used in the study. The birds received their respective diets *ad libitum* for the duration of the experimental period, which consisted of twelve weeks. The birds also had free access to water at all times. For a few minutes each day, the birds were handled, in order to accustom them to being handled, such that any stress-induced hyperglycaemia during the interventions was prevented (Simon and Rosselin, 1979). The birds were housed together with their respective groups in the Central Animal Service's animal unit, at the Faculty of Health Sciences, University of the Witwatersrand. Lighting was restricted to 12 hours in each 24 hour period, lights on from 06:00. Environmental enrichment of the bird enclosures, in the form of perching logs for the Guinea fowl and paddle pools filled with water for the Muscovy ducks, was provided.

**Table 2. 1** Composition of standard, commercial poultry feed fed to Guinea fowl and Muscovy ducks for between four to twelve weeks.

| Ingredient | Quantity ($g.kg^{-1}$) |
|---|---|
| Protein | 160 |
| Moisture | 120 |
| Fat | 25 |
| Fibre | 70 |
| Calcium | 35 |
| Phosphorus | 5.5 |
| Total Lysine | 6.6 |

2.2.3 General experimental procedures

Upon receiving the birds from the supplier (Platinum Pets, Johannesburg, South Africa), the birds were allowed a two week adaptation period, to enable them to become acclimated with the housing, handling and feeding conditions. Following the adaptation period, the twelve week experimental period commenced, during which the birds received their respective standard or HFDs. The birds were weighed twice weekly. Every four weeks, six birds from each group (STD and HFD Guinea fowl and STD and HFD Muscovy ducks) were fasted for approximately 15 hours overnight and then subjected to an oral glucose tolerance test (OGTT). Details of the OGTT are presented below. Following the OGTT, the birds were re-fed and allowed a 72 hr recovery period, after which they were euthanized using an anaesthetic overdose of Sodium Pentobarbital (Eutha-naze, Centaur Labs, South Africa) (200 $mg.kg^{-1}$), administered via the wing vein. Blood samples were collected by cardiac puncture (using 20 G hypodermic needles) into vacutainer tubes (Vacuette, Greiner Bio-One, Kremsmunster, Austria) containing either lithium heparin (for osmotic fragility determinations) or into serum separator, clot activator vacutainer tubes (Vacuette) (for serum determinations), and mixed gently. The bird's livers were excised and excess connective was

removed. The livers were then rinsed in ice-cold saline and blotted dry and weighed (liver mass represented as a percentage of body mass). Samples of liver tissue were then collected and processed for histology and liver lipid content. The pancreas, proventriculus, ventriculus, small intestine, large intestine and caecum were also excised, rinsed in ice-cold saline, blotted dry and weighed (all organ masses are represented as both absolute organ mass and relative organ mass (as a percentage of body mass)). The lengths of the small and large intestine were also measured (all small and large intestine lengths are represented as both absolute length and relative length (as unit length per unit body mass)).

### 2.2.4 Specific experimental procedures

#### i) Body mass measurements

The birds were weighed twice weekly by placing them in pre-weighed cages on a scale (Presica 310 M, Laser, Johannesburg, South Africa). Weight gain was then calculated using the formula below:

$$\frac{\text{(Final body mass – Initial body mass)}}{\text{Initial body mass}} \times 100$$

Feed intake and feed conversion ratio/feed efficiency form part of the indices which are usually assessed as part of growth performance, however these are not taken into consideration in this study.

#### ii) Oral glucose tolerances tests (OGTTs)

Following an overnight fast, fasting blood glucose concentrations of blood samples collected via venipuncture to the wing vein, after four, eight and twelve weeks of STD or HFD feeding, were measured using a glucometer (Ascensia Elite ™ Blood glucose meter, Bayer Corporation, Mishawaka, USA). Prior to the venipuncture, the area surrounding the wing vein was clipped of its feathers and sterilized with alcohol-soaked gauze swabs. Following the fasting blood glucose concentration measurements, birds were then administered a single dose of glucose solution ($5g.kg^{-1}$, 50 % w/v glucose solution; Sigma-Aldrich ®, Seelze, Germany) orally, via orogastric intubation into the crop. Blood glucose concentrations ($mmol.l^{-1}$) were then determined using a calibrated glucometer (Ascensia Elite ™ Blood glucose meter, Bayer

Corporation, Mishawaka, USA) at fixed time intervals (15, 30, 60, 90 and 120 minutes) following glucose administration (Loxham et al., 2007). Glucose tolerance curves, for both species of birds in the STD and HFD groups were then constructed. Baseline glucose, peak glucose and glucose concentrations two hours following administration of the glucose load were then determined. Area under the glucose curve (AUC) was also determined.

iii) Blood parameters

*a) Erythrocyte osmotic fragility*

Erythrocyte osmotic fragility was determined using methods modified from those described by Suess et al. (1948), Oyewale and Durotoye (1988), Adenkola et al. (2010) and Moyo et al. (2012). In brief: 50 µl of whole blood was added to tubes, each containing 5 ml of serially diluted concentrations (between 0 % and 0.85 % saline) of phosphate-buffered saline (PBS) (pH 7.4).The tubes were gently inverted a few times, in order to allow mixing of the blood and saline solutions. After standing at room temperature (24 °C) for 30 min, the mixtures were then centrifuged (Sorvall RT 6000 B, Du Pont, Hertfordshire, UK) at 370 *g* and 22 °C for 5 min. The supernatant was transferred into plastic cuvettes (Cuvette Macro PS, Lasec), and the absorbance of the haemoglobin released into solution upon haemolysis of the erythrocytes was determined indirectly, using a spectrophotometer (Ultrospec II, LKB Biochrom, Cambridge, England), at 540 nm. Distilled water was used as a blank. Assuming maximal or complete haemolysis (maximum haemoglobin released) in the PBS solution with the highest absorbance, the percentage haemoglobin released in each PBS solution, for each bird, was calculated. The point at which erythrocytes in solution started haemolysing (initial haemolysis), at which half the erythrocytes in solution were haemolysed (mean corpuscular fragility (MCF) / 50 % haemolysis) and at which all erythrocytes in solution were haemolysed (maximal haemolysis) was assessed (Suess et al., 1948).

*b) General health/clinical biochemistry profile*

Blood samples collected via cardiac puncture, into serum separator, clot activator vacutainer tubes, were centrifuged (Sorvall RT 6000 B, Du Pont) at 370 *g* and 22 °C for 15 min. The serum was collected and serum uric acid, total protein, albumin, aspartate aminotransferase (AST), total bilirubin, calcium, cholesterol and triglyceride concentrations were determined

using an IDEXX Vetlab Analysis Machine (IDEXX Laboratories, Westbrook, ME, USA), according to manufacturer's instructions.

iv) Liver parameters

*a) Liver lipid content*

Liver lipid content analysis was only performed using liver samples, from half of the birds after four and eight weeks of either STD or HFD feeding. Lipid extraction was performed using procedures described by Bligh and Dyer (1959). Briefly, liver samples of approximately 5 g were placed into a chloroform: methanol (2:1) solution, overnight at 4°C. The samples were then filtered through filter paper (Albert®, Pore 7-11, Size 185 mm) and 30 ml 0.9 % saline was added. The samples were then gently mixed by inverting and allowed to stand overnight at 4 °C, to allow separation into two phases. The bottom (chloroform) phase was then collected and reduced to dryness under vacuum, at 37 °C, using a water bath (Labex®, Krugersdorp, South Africa) and then made up to 20 ml with chloroform. An aliquot of 2 ml of the extracts was then placed into dried, pre-weighed vials, and re-dried at 50 °C for 30 minutes, cooled and then reweighed to determine the lipid content.

*b) Histology*

Immediately after euthanasia, the liver was excised, rinsed in ice-cold saline (0.9 % NaCl) solution and weighed. It was trimmed of all connective tissue and a section of tissue was fixed in 10% formaldehyde (in PBS) (0.1 M phosphate-buffered saline, pH 7.4) for at least10 hr and embedded in paraffin. 5 μm-thick paraffin sections were cut and stained with haematoxylin and eosin for histological analysis. All sections were examined for steatosis (the accumulation of fat droplets within the liver tissue) and assigned a score, relative to the level of lipid deposits in the sample. The score was assigned according to the histological classification by Brunt et al. (1999), where a score of 1 indicated mild steatosis, a score of 2 indicated moderate steatosis and a score of 3 indicated severe steatosis.

v) Microflora analysis of the caecal content

Following euthanasia of the birds the caecum was carefully removed from the carcass and the contents were opened into a petri dish. The caecal content was then analysed for the presence

of various gram-positive and gram-negative bacteria, including: *Lactobacillus*, *Escherichia coli*, *Clostridium, Campylobacter*, *Salmonella and Bifidobacterium*. Bacterial DNA was extracted from the caecum content samples using a QIAamp® DNA stool minikit (Qiagen®), according to manufacturer's instructions. The concentration and purity of the DNA was established spectrophotometrically. The primer sets (Inqaba Biotechnical Industries, Hatfield, South Africa) for *Lactobacillus*, *Escherichia coli*, *Clostridium, Campylobacter*, *Salmonella and Bifidobacterium* as published by Amit-Romach et al. (2004) were used in the present study and are shown in Table 2.2.

Bacterial targets within the caecal content were amplified using standard PCR methods. Briefly, 10 µl of DNA extract was added to a PCR mixture to reach a total reaction volume of 25 µl. The PCR mixture contained 12.5 µl of EconoTaq (Lucigen® Corporation, Middleton, USA) master mix, 2 µl of nuclease-free water and 0.25 µl of each primer (i.e. 0.25 µl of forward primer and 0.25 µl of reverse primer). A T100™ Thermal Cycler (Biorad) was used to perform the PCR for each bacterial primer set, using the following amplification conditions: 1 cycle of 94ºC for 3 min, 35 cycles of 94ºC for 30 s, 60ºC for 1 min and 68ºC for 2 min and then 1 final cycle of 68ºC for 7 min (Amit-Romach et al., 2004). Agarose gel (2%) electrophoresis, containing ethidium bromide, was then used to visualise the PCR products. A FastRuler™ low range, DNA ladder (Fermentas) was used for scale.

**Table 2. 2** PCR primer sets used in the study.

| Bacterial group | Primers Sequence (5'- 3') | Length (bp) |
|---|---|---|
| **Lactobacillus** | **LAA-f** CATCCAGTGCAAACCTAAGAG<br>**LAA-r** GATCCGCTTGCCTTCGCA | 286 |
| **Escherichia coli** | **ECO-f** GACCTCGGTTTAGTTCACAGA<br>**ECO-r** CACACGCTGACGCTGACCA | 585 |
| **Clostridium** | **Clos58-f** AAAGGAAGATTAATACCGCATAA<br>**Clos780-r** ATCTTGCGACCGTACTCCCC | 722 |
| **Campylobacter** | **Camp-f** ATCTAATGGCTTAACCATTAAAC<br>**Camp-r** GGACGGTAACTAGTTTAGTATT | 857 |
| **Salmonella** | **Sal201-f** CGGGCCTCTTGCCATCAGGTG<br>**Sal597-r** CACATCCGACTTGACAGACCG | 396 |
| **Bifidobacterium** | **Bif164-f** GGGTGGTAATGCCGGATG<br>**Bif662-r** CCACCGTTACACCGGGAA | 510 |

PCR primer sequences used to manufacture the primer sets were obtained from a previous publication by Amit-Romach et al. (2004), entitled: "Microflora ecology of the chicken intestine using 16S ribosomal DNA primers" Poultry Science 83: 1093-1098.

### 2.2.5 Statistical analysis

All data are expressed as the mean ± SD, unless otherwise stated. The data were analysed and plotted using Graphpad 7 Prism software (Graph-pad Software Inc, San Diego, USA). $p < 0.05$ was considered significant.

*Body mass*: A Student's t-test was used to compare initial body mass (at time point: 0 weeks) between the two dietary groups (STD and HFD) as a whole, before allocation to the various time point groups. A Student's t-test was also used to compare initial body mass to final body mass (after either four, eight or twelve weeks of feeding) within each dietary group, for both Guinea fowl and Muscovy ducks. A two-way ANOVA was used to compare the final body mass and percentage body mass gain of the birds after four, eight and twelve weeks of feeding, between the STD and HFD groups, per species of bird. Differences between the STD and HFD groups (diet effect) at each particular time point were identified using a Sidak's multiple comparisons post-hoc test (following the two-way ANOVA). Differences between the various time points within a dietary group (time effect) were identified using a Tukey's multiple comparisons post-hoc test (following the two-way ANOVA).

*Oral glucose tolerance tests (OGTT)*: Differences in blood glucose values measured at fixed time intervals following administration of the glucose load, within a single dietary group, after either four, eight or twelve weeks of STD or HFD feeding, were analysed using a repeated measures ANOVA with a Bonferroni post-hoc test, for each bird species. Differences in baseline blood glucose concentrations, peak blood glucose concentrations, blood glucose concentrations two hours following administration of the glucose load and area under the glucose curve, between birds in the STD and HFD groups at each time point (after four, eight or twelve weeks of feeding) were analysed using a two-way ANOVA. Differences between the STD and HFD groups (diet effect) at each particular time point were identified using a Sidak's multiple comparisons post-hoc test (following the two-way ANOVA). Differences between the various time points within a dietary group (time effect) were identified using a Tukey's multiple comparisons post-hoc test (following the two-way ANOVA).

*Osmotic fragility*: The mean ± SD for the data concerning the percentage haemoglobin released from erythrocytes upon haemolysis, for each PBS solution, was calculated and used to construct the fragiligrams for each time point (after four, eight and twelve weeks of STD or HFD feeding). This process was repeated for both dietary group (STD and HFD) and for both bird species. The range of PBS solutions at which initial haemolysis (IH), 50% haemolysis (MCF) and maximal haemolysis (MH) of the erythrocytes occurred was then read off the graphs.

*General health/clinical biochemistry profile, organ masses (intestine lengths) and liver lipid content*: Differences in serum parameters, organ masses *(*absolute and relative), intestine lengths and liver lipid content, between birds in the STD and HFD groups at each time point (after four, eight or twelve weeks of feeding) were analysed using a two-way ANOVA. Differences between the STD and HFD groups (diet effect) at each particular time point were identified using a Sidak's multiple comparisons post-hoc test (following the two-way ANOVA). Differences between the various time points within a dietary group (time effect) were identified using a Tukey's multiple comparisons post-hoc test (following the two-way ANOVA).

One Guinea fowl and one Muscovy duck fell ill during the experimental period and were excluded from the study. Thus, for most of the statistical analyses (unless otherwise stated), n = 6 for both species of bird, at all time points, except for the Guinea fowl on the HFD at eight weeks and the Muscovy ducks on the STD at eight weeks, where n = 5. For the analysis of the liver lipid content, n = 3 for both species of bird, at all time points, except for the Muscovy ducks on the STD at eight weeks, where n = 2.

The statistical models used for the two-way ANOVA was as follows:

**Two-way ANOVA:**

$y_{ijk} = \mu + \alpha_i + b_j + (\alpha b)_{ij} + e_{ijk}$

$y_{ijk}$ = dependent variable of interest [body mass, glucose tolerance test parameters, serum parameters, organ masses (absolute and relative), intestine lengths]

$\mu$ = overall mean effect

$\alpha_i$ = the $i^{th}$ fixed effect of the dietary treatments (2 dietary treatments)

$b_j$ = the $j^{th}$ fixed effect of time

$(\alpha b)_{ij}$ = the fixed interaction effect between diet and time

$e_{ijk}$ = the random residual error

## 2.3 Results

### 2.3.1 Guinea fowl

#### i) Body mass

Figure 2.1 shows the initial (time: 0 weeks) and final (after either four, eight or twelve weeks) body mass (g) for each dietary group (A), as well as the average body mass gain (%) per dietary group (B) of Guinea fowl following either four, eight or twelve weeks of STD or HFD feeding. No significant differences ($p > 0.05$, Student's t-test) in initial body mass (following the two week adaptation period) were observed between birds allocated to the STD and HFD groups. Final body mass of birds in both the STD and HFD groups, at all time points examined (after four, eight or twelve weeks), was significantly higher than initial body mass ($p < 0.001$, Student's t-test). A significant diet ($p = 0.02$, $F_{(1, 29)} = 6.57$, Two-way ANOVA) and time ($p < 0.0001$, $F_{(2, 29)} = 13.6$, Two-way ANOVA) effect was observed with regards to the final body mass of the birds. Following eight weeks of feeding, the birds on the STD had a significantly higher ($p = 0.03$, Two-way ANOVA) final body mass than those on the HFD. No significant differences in final body mass were observed ($p > 0.05$, Two-way ANOVA) between STD and HFD groups following four and twelve weeks of either STD or HFD feeding. Within the HFD group, final body mass was significantly greater ($p = 0.004$, Two-way ANOVA) following twelve weeks of feeding versus that observed following four weeks of feeding. In the STD group, final body mass was significantly greater following both eight ($p = 0.005$, Two-way ANOVA) and twelve weeks ($p = 0.003$, Two-way ANOVA) of feeding compared to that observed following four weeks of feeding.

With respect to percentage body mass gain, a significant diet ($p = 0.02$, $F_{(1, 29)} = 6.497$, Two-way ANOVA) and time ($p < 0.0001$, $F_{(2, 29)} = 65.24$) effect were observed. Upon post-hoc analysis no significant differences were observed between birds in the STD and HFD groups following four, eight and twelve weeks of feeding. When comparing the percentage body mass gain across the three different time points within the HFD group, percentage body mass gain was significantly lower after eight ($p = 0.0001$, Two-way ANOVA) and twelve ($p < 0.0001$, Two-way ANOVA) weeks of feeding compared to that after four weeks of feeding. Percentage body mass gain was also significantly lower following twelve weeks of HFD feeding ($p = 0.02$, Two-way ANOVA) compared to that observed after eight weeks of HFD feeding. Similarly, in the STD group, percentage body mass gain was also significantly lower following eight ($p < 0.0001$, Two-way ANOVA) and twelve ($p < 0.0001$, Two-way ANOVA) weeks of feeding versus that observed following four weeks of feeding. No significant differences ($p > 0.05$, Two-way ANOVA) were observed between the percentage body mass gain observed following eight and twelve weeks of STD feeding.

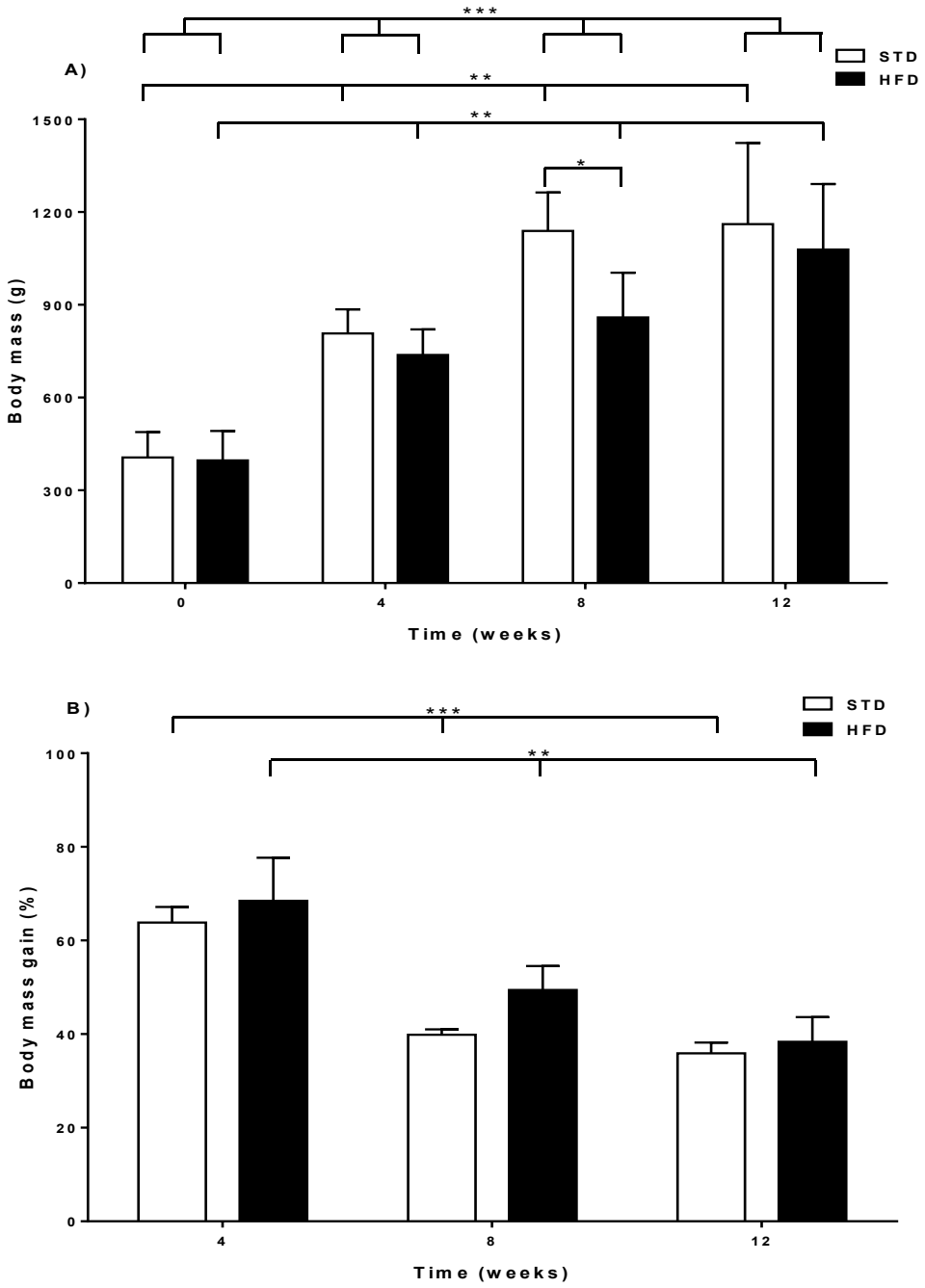



**Figure 2.1** Initial (time: 0 weeks) and final (after either 4, 8 or 12 weeks) body mass (g) per dietary group (A) and average body mass gain (%) per dietary group (B) of Guinea fowl following either 4, 8 or 12 weeks of STD or HFD feeding. STD = standard diet; HFD = high-fat diet. Data represented as means ± SD. n = 6 at all time points studied, for Guinea fowl in both the STD and HFD groups (except n = 5 for Guinea fowl on the HFD at 8 weeks). Figure 2.1 (A): *** $p < 0.001$ when comparing final body mass (at either 4, 8 or 12 weeks) to initial body mass (at 0 weeks) for both dietary groups; ** $p < 0.01$ when comparing final body mass following 4, 8 and 12 weeks of feeding, within a single dietary group; * $p < 0.05$ when comparing final body mass between the STD and HFD groups following 8 weeks of feeding. Figure 2.1 (B): *** $p < 0.001$ and ** $p < 0.01$ when comparing body mass gain in the STD and HFD groups, respectively, following 8 and 12 weeks of feeding to body mass gain following 4 weeks feeding.

ii) Oral glucose tolerance

Figures 2.2 and 2.3 show the glucose tolerance curves and area under the glucose curve for Guinea fowl after four (A), eight (B) and twelve (C) weeks of STD or HFD feeding. No significant ($p > 0.05$, Two-way ANOVA) diet or time effects were observed with regards to fasting blood glucose concentrations, peak blood glucose concentrations, blood glucose concentrations 120 min following the glucose load and glucose AUC between STD and HFD groups, after either four, eight or twelve weeks of feeding. Blood glucose concentrations peaked at 30 min following gavage in Guinea fowl on the HFD versus 15 min for those on the STD following four weeks of feeding and at 30 min following gavage for Guinea fowl in the HFD and STD groups after eight weeks of feeding. Following twelve weeks of feeding, blood glucose concentrations peaked at 30 min following gavage in Guinea fowl on the HFD versus 15 min for those on the STD diet. Peak blood glucose concentrations were significantly higher than fasting blood glucose concentrations ($p < 0.01$, repeated measures ANOVA) for both STD and HFD groups after four weeks of feeding and for the STD group after eight weeks of feeding. Following eight weeks of HFD feeding and twelve weeks of both STD and HFD feeding, peak blood glucose concentrations were not significantly different from fasting blood glucose concentrations ($p > 0.05$, repeated measures ANOVA). Blood glucose concentrations returned to normal 60 min after gavage in Guinea fowl in both the STD and HFD groups at all time points assessed.

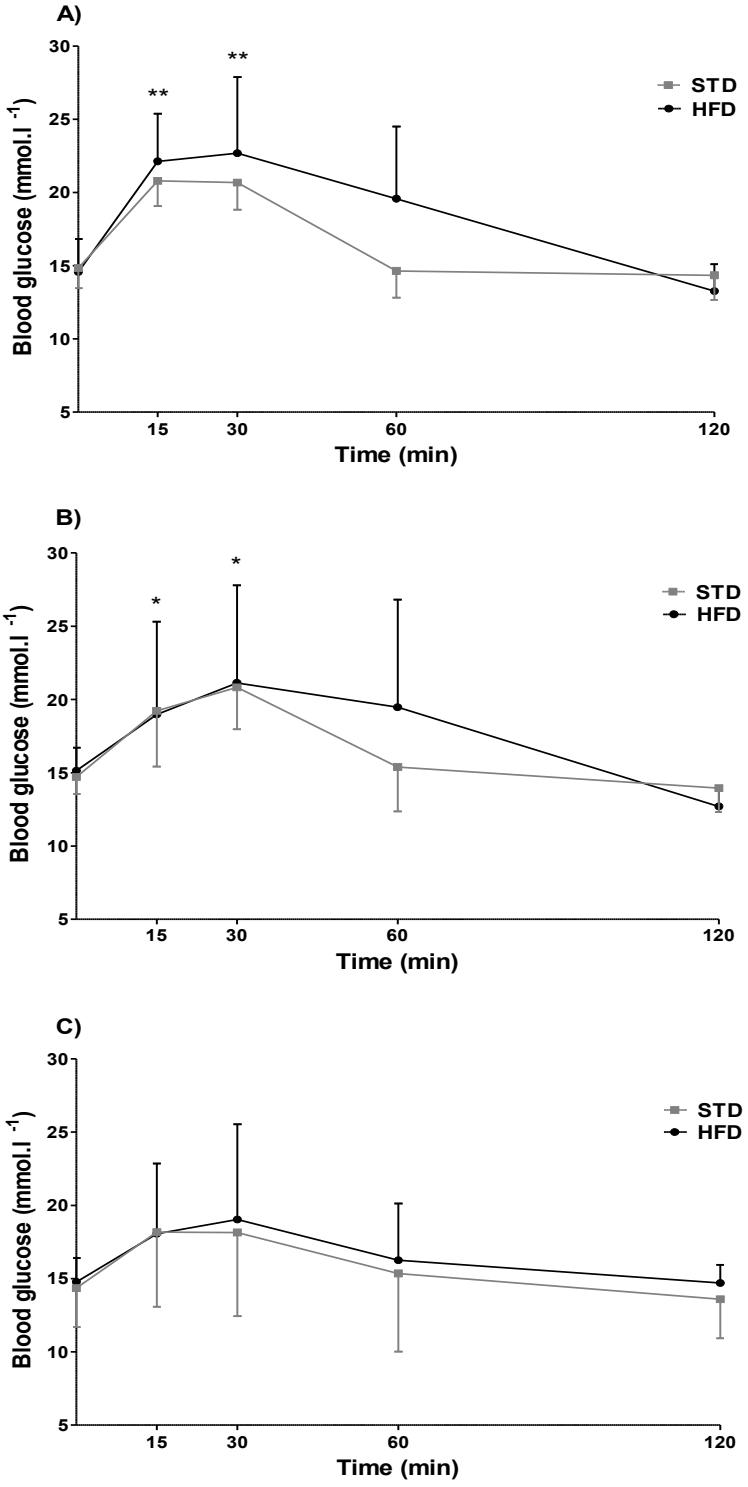



**Figure 2.2** Glucose tolerance curves for Guinea fowl after 4 (A), 8 (B) and 12 (C) weeks of STD or HFD feeding. STD = standard diet; HFD = high-fat diet. Data represented as means ± SD. n = 6 at all time points studied, for Guinea fowl in both the STD and HFD groups (except n = 5 for Guinea fowl on the HFD at 8 weeks). ** $p < 0.01$ and * $p < 0.05$ significance when comparing peak blood glucose concentrations to fasting blood glucose concentrations, within individual dietary groups.

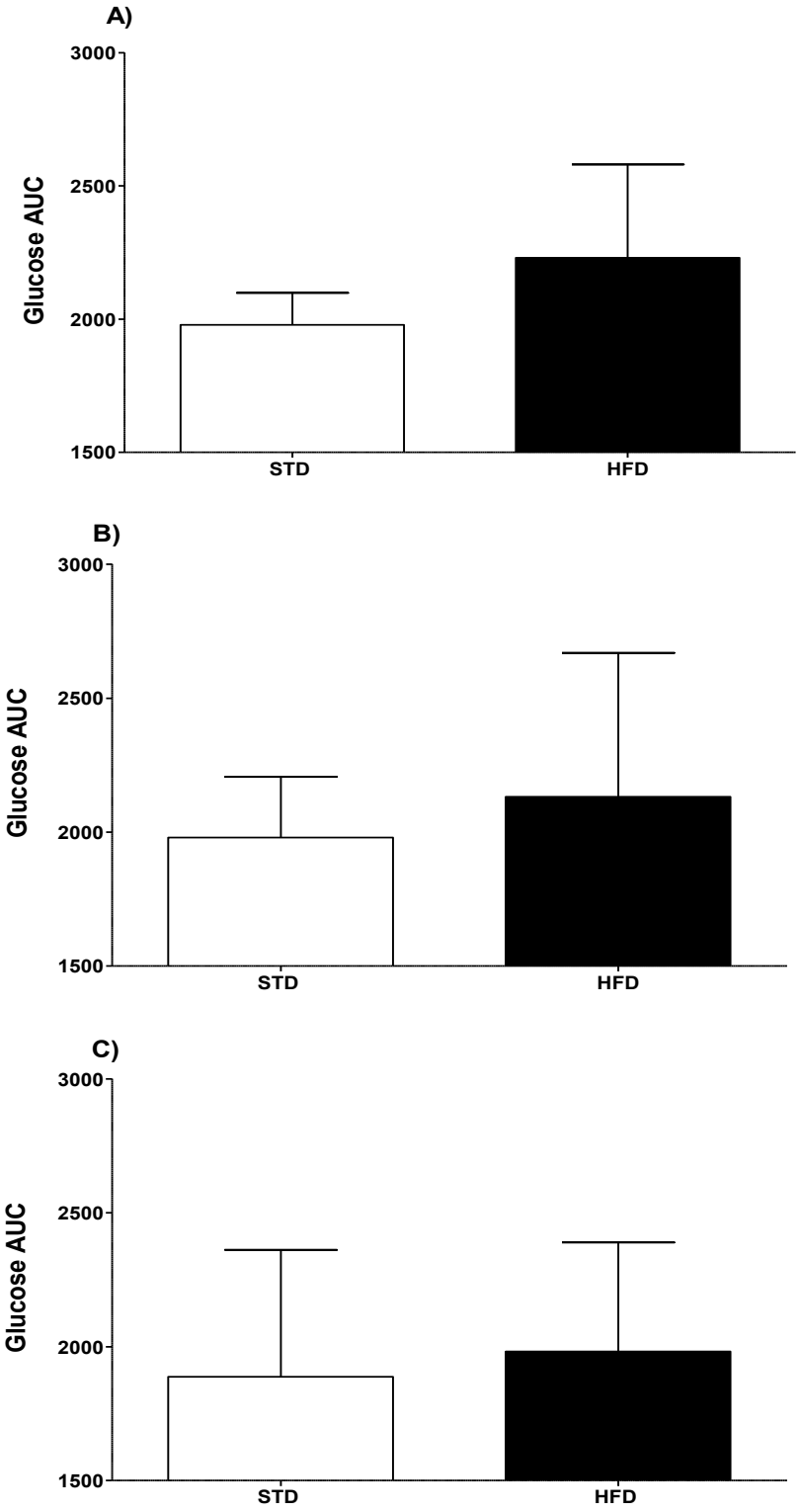



**Figure 2.3** Area under the glucose curves for Guinea fowl after 4 (A), 8 (B) and 12 (C) weeks of STD or HFD feeding. STD = standard diet; HFD = high-fat diet. Data represented as means ± SD. n = 6 at all time points studied, for Guinea fowl in both the STD and HFD groups (except n = 5 for Guinea fowl on the HFD at 8 weeks).

iii) Erythrocyte osmotic fragility

Figure 2.4 shows the fragiligrams of the erythrocytes from Guinea fowl following four (A), eight (B) or twelve (C) weeks of STD or HFD feeding. The fragiligrams of the erythrocytes from the Guinea fowl in the STD group were not different from those of the erythrocytes from the Guinea fowl in the HFD group after four (A), eight (B) and twelve weeks (C) of STD or HFD feeding. Table 2.3 indicates the range of % PBS solutions at which initial haemolysis (IH), 50% haemolysis (MCF) and maximal haemolysis (MH) occurred for the erythrocytes from the Guinea fowl in the STD and HFD groups, after four, eight and twelve weeks of STD or HFD feeding. There were no differences in any of the osmotic fragility indices examined for the erythrocytes from the birds in the STD group compared with those for the erythrocytes from the birds in the HFD group, at each time point.

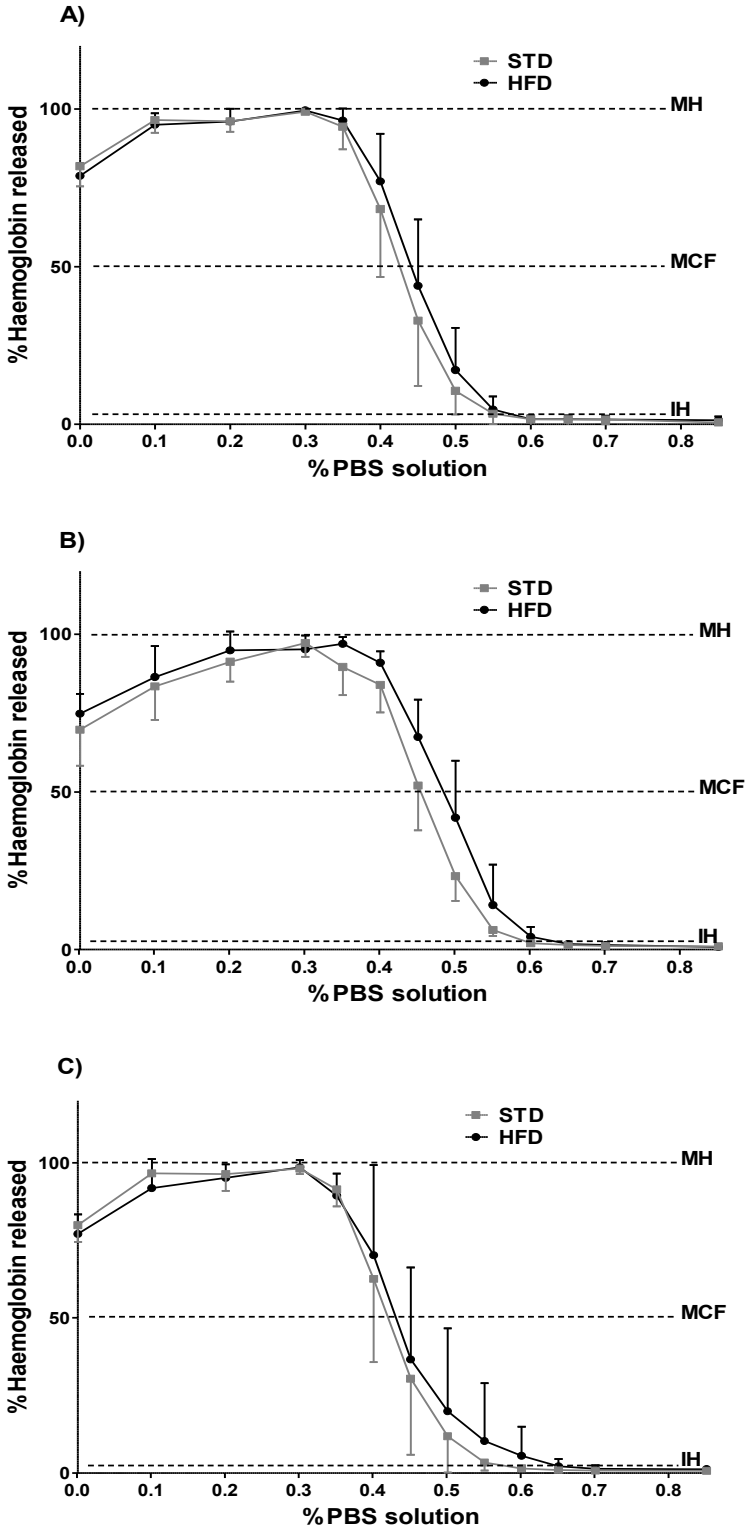



**Figure 2.4** Fragiligrams obtained from the erythrocytes of Guinea fowl after 4 (A), 8 (B) and 12 (C) weeks of STD or HFD feeding. STD = standard diet; HFD = high-fat diet. Data represented as means ± SD. n = 6 at all time points studied, for Guinea fowl in both the STD and HFD groups (except n = 5 for Guinea fowl on the HFD at 8 weeks).

**Table 2. 3** The range of phosphate-buffered saline solutions (%) at which initial haemolysis, 50 % haemolysis and maximal haemolysis occurred for the erythrocytes from Guinea fowl after 4, 8 and 12 weeks of standard or high-fat diet feeding.

| | % PBS solution | |
|---|---|---|
| | **STD** | **HFD** |
| **Initial haemolysis** | | |
| 4 weeks | 0.5-0.55 | 0.5-0.55 |
| 8 weeks | 0.55-0.6 | 0.55-0.6 |
| 12 weeks | 0.5-0.6 | 0.5-0.6 |
| **MCF** | | |
| 4 weeks | 0.4-0.45 | 0.4-0.45 |
| 8 weeks | 0.45-0.5 | 0.45-0.5 |
| 12 weeks | 0.4-0.45 | 0.4-0.45 |
| **Maximal haemolysis** | | |
| 4 weeks | 0.3-0.35 | 0.3-0.35 |
| 8 weeks | 0.3-0.4 | 0.3-0.4 |
| 12 weeks | 0.3-0.35 | 0.3-0.35 |

PBS = phosphate-buffered saline solution; STD = standard diet; HFD = high-fat diet; MCF = mean corpuscular fragility / 50 % haemolysis.

iv) General health/clinical biochemistry profile

Serum parameters for Guinea fowl in both the STD and HFD groups are presented in Table 2.4. No significant effects of diet or time ($p > 0.05$, Two-way ANOVA) were observed in serum uric acid, total protein, albumin, AST, calcium, total bilirubin and triglyceride concentrations between birds on the STD and HFD, after either four, eight or twelve weeks of feeding. A significant main effect of diet ($p < 0.0001$, $F_{(1, 29)} = 27.33$, Two-way ANOVA) was observed with regards to the serum cholesterol concentrations (Figure 2.5). Serum cholesterol concentrations were significantly higher in the HFD group following four ($p = 0.02$, Two-way ANOVA), eight ($p = 0.004$, Two-way ANOVA) and twelve ($p = 0.05$, Two-way ANOVA) weeks of feeding compared to that observed in the STD group.

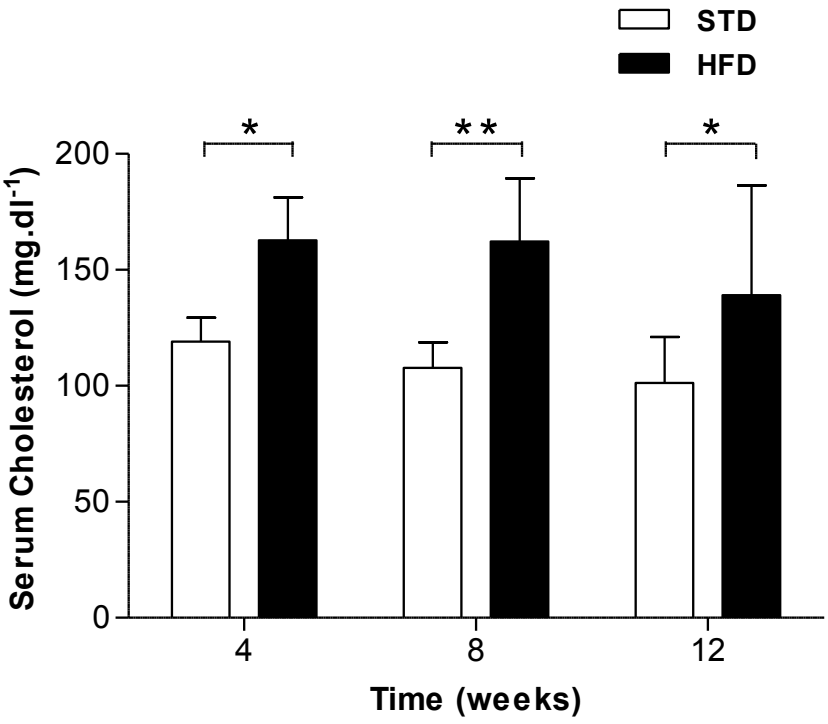



**Figure 2.5** Serum cholesterol concentrations ($mg.dl^{-1}$) of Guinea fowl after 4, 8 and 12 weeks of STD or HFD feeding. STD = standard diet; HFD = high-fat diet. Data represented as means ± SD. * $p < 0.05$ and ** $p < 0.01$ when comparing HFD group to STD group at specific time point.

**Table 2. 4** Serum parameters from Guinea fowl after 4, 8 and 12 weeks of standard or high-fat diet feeding.

| Serum parameters: | 4 weeks | | 8 weeks | | 12 weeks | |
|---|---|---|---|---|---|---|
| | **STD** | **HFD** | **STD** | **HFD** | **STD** | **HFD** |
| Uric ($mg.dl^{-1}$) | 4.1 ± 1.0 | 2.6 ± 1.4 | 3.8 ± 1.2 | 4.0 ± 1.3 | 5.1 ± 2.0 | 3.4 ± 0.4 |
| TPro ($g.dl^{-1}$) | 2.3 ± 0.3 | 2.4 ± 0.4 | 2.4 ± 0.1 | 2.4 ± 0.6 | 3.4 ± 2.3 | 2.6 ± 0.7 |
| Alb ($g.dl^{-1}$) | 0.3 ± 0.2 | 0.4 ± 0.4 | 0.3 ± 0.1 | 0.3 ± 0.2 | 0.3 ± 0.2 | 0.4 ± 0.4 |
| AST ($U.l^{-1}$) | 344.3 ± 105.2 | 407.3 ± 199.8 | 225.8 ± 16.9 | 260.6 ± 35.3 | 397.5 ± 232.9 | 301.0 ± 143.1 |
| TBil ($mg.dl^{-1}$) | 0.4 ± 0.4 | 0.4 ± 0.4 | 0.3 ± 0.2 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.2 |
| Calc ($mg.dl^{-1}$) | 10.7 ± 1.8 | 10.1 ± 1.3 | 11.1 ± 1.1 | 10.8 ± 1.3 | 9.8 ± 3.3 | 11.3 ± 2.4 |
| Trig ($mg.dl^{-1}$) | 68.7 ± 40.2 | 65.2 ± 23.2 | 90.3 ± 40.1 | 66.2 ± 49.9 | 38.8 ± 14.65 | 39.3 ± 19.2 |

Data represented as mean ± SD. Uric = uric acid, TPro = total protein, Alb = Albumin, AST = aspartate aminotransferase, Tbil = total bilirubin, Calc = Calcium, Trig = triglycerides. n = 6 for both HFD and STD groups at 4 weeks, n = 5 for HFD group at 8 weeks and n = 6 for STD group at 8 weeks, n = 6 for both HFD and STD groups at 12 weeks. STD = standard diet, HFD = high-fat diet.

v) Organ masses and small and large intestine lengths

The absolute and relative masses (mass represented as a percentage of body mass) of the liver, pancreas, proventriculus, ventriculus, small intestine, large intestine and caecum, along with the absolute and relative lengths of the small and large intestines (as unit length per unit body mass) of the Guinea fowl are represented in Tables 2.5 (absolute) and 2.6 (relative), respectively. No significant differences ($p > 0.05$, Two-way ANOVA) in the absolute proventriculus, ventriculus and small intestine masses, as well as in the absolute small intestine length were observed between Guinea fowl in the STD and HFD groups, following either four, eight or twelve weeks of feeding. A significant main effect of time was observed in the absolute liver ($p = 0.0421$, $F_{(2, 29)} = 3.542$, Two-way ANOVA), pancreas ($p = 0.0069$, $F_{(2, 29)} = 5.936$, Two-way ANOVA), large intestine ($p = 0.0041$, $F_{(2, 29)} = 6.677$, Two-way ANOVA) and caecum mass ($p = 0.0004$, $F_{(2, 29)} = 10.52$, Two-way ANOVA) of the Guinea fowl. A significantly lower ($p = 0.0096$, Two-way ANOVA) absolute liver mass was observed in the STD group following four weeks of feeding than that observed following eight weeks of feeding. A significantly lower ($p = 0.0269$, Two-way ANOVA) absolute pancreas mass was observed in the HFD group following four weeks of feeding than that observed following twelve weeks of feeding. A significantly lower ($p = 0.0012$, Two-way ANOVA) absolute large intestine mass was observed in the STD group following four weeks of feeding than that observed following twelve weeks of feeding. The absolute caecum mass was significantly lower in the STD group ($p = 0.0025$, Two-way ANOVA) following four weeks of feeding compared to that observed following eight weeks of feeding and in the HFD following four weeks of feeding compared to that observed following eight ($p = 0.0431$, Two-way ANOVA) and twelve weeks ($p = 0.0131$, Two-way ANOVA) of feeding. A significant main effect of time was also observed in the absolute length of the large intestine ($p = 0.0017$, $F_{(2, 29)} = 7.971$, Two-way ANOVA), with a significantly lower absolute large intestine length observed in the STD group following four ($p = 0.0032$, Two-way ANOVA) and eight weeks ($p = 0.0088$, Two-way ANOVA) of feeding compared to that observed following twelve weeks of STD feeding.

No significant differences ($p > 0.05$, Two-way ANOVA) in the relative pancreas, small intestine, large intestine and caecum mass were observed between Guinea fowl in the STD and HFD groups, following either four, eight or twelve weeks of feeding. A significant time effect ($p = 0.0375$, $F_{(2, 29)} = 3.685$, Two-way ANOVA) was observed in the relative liver

mass of the Guinea fowl; however no significance was observed following post-hoc analysis. Significant diet ($p = 0.0108$, $F_{(1, 29)} = 7.413$, Two-way ANOVA) and interaction effects ($p = 0.0407$, $F_{(2, 29)} = 3.584$, Two-way ANOVA) were observed in the relative proventriculus mass of the Guinea fowl. Guinea fowl in the HFD group had a significantly larger ($p = 0.0096$) relative proventriculus mass following eight weeks of feeding compared to that observed in the STD group. A significant diet effect ($p = 0.0002$, $F_{(1, 29)} = 18$, Two-way ANOVA) was also observed in the relative ventriculus of the Guinea fowl, with birds in the HFD group displaying a significantly larger ($p = 0.0389$, Two-way ANOVA) relative ventriculus mass following eight weeks of feeding compared to those in the STD group. Significant time and diet effects were observed in the relative lengths of the small (time: $p = 0.0032$, $F_{(2, 29)} = 7.051$, Two-way ANOVA; diet: $p = 0.0076$, $F_{(1, 29)} = 8.23$, Two-way ANOVA) and large intestines (time: $p = 0.0084$, $F_{(2, 29)} = 5.663$, Two-way ANOVA; diet: $p = 0.0373$, $F_{(1, 29)} = 4.762$, Two-way ANOVA) of the Guinea fowl. Guinea fowl in the STD group had significantly longer relative small intestines lengths following four weeks of feeding compared to that observed following eight ($p = 0.0160$, Two-way ANOVA) and twelve weeks ($p = 0.0101$, Two-way ANOVA) of STD feeding. Relative small intestine length was significantly higher ($p = 0.0054$, Two-way ANOVA) in birds in the HFD group compared to those in the STD group following eight weeks of feeding. Despite the significant main effect of diet on the relative large intestine length, post-hoc analysis showed no significant differences ($p > 0.05$, Two-way ANOVA). Guinea fowl in the STD group had significantly longer relative large intestine lengths ($p = 0.0456$, Two-way ANOVA) following four weeks of feeding compared to that observed following eight weeks of feeding. In the HFD group, significantly longer relative large intestine lengths ($p = 0.0164$, Two-way ANOVA) were observed following four weeks of feeding compared to that observed following twelve weeks of feeding.

**Table 2. 5** The absolute organ masses and lengths of the small and large intestines of Guinea fowl after 4, 8 and 12 weeks of standard or high-fat diet feeding.

| Organ mass (g): | 4 weeks | | 8 weeks | | 12 weeks | |
|---|---|---|---|---|---|---|
| | STD | HFD | STD | HFD | STD | HFD |
| Liver | 11.68 ± 1.52[a] | 12.16 ± 2.78 | 17.12 ± 3.43[b] | 13.17 ± 4.64 | 13.58 ± 2.87[a,b] | 14.56 ± 1.98 |
| Pancreas | 1.52 ± 0.19 | 1.42 ± 0.39[a] | 1.92 ± 0.15 | 1.67 ± 0.35[a,b] | 1.93 ± 0.43 | 1.97 ± 0.45[b] |
| Proventriculus | 2.65 ± 0.41 | 2.32 ± 0.58 | 2.76 ± 0.69 | 3.15 ± 0.96 | 2.82 ± 0.82 | 3.36 ± 0.71 |
| Ventriculus | 22.68 ± 3.01 | 27.88 ± 4.84 | 29.20 ± 11.42 | 31.19 ± 8.00 | 29.72 ± 10.49 | 39.23 ± 10.89 |
| Small intestine | 14.23 ± 1.83 | 15.51 ± 4.75 | 20.99 ± 3.74 | 24.51 ± 6.82 | 50.08 ± 81.91 | 22.81 ± 5.84 |
| Large intestine | 1.36 ± 0.22[a] | 1.54 ± 0.51 | 2.15 ± 0.62[a,b] | 1.63 ± 0.36 | 2.78 ± 1.16[b] | 1.96 ± 0.25 |
| Caecum | 2.45 ± 0.45[a] | 2.10 ± 0.40[a] | 3.81 ± 0.58[b] | 3.07 ± 0.41[b] | 3.04 ± 0.93[a,b] | 3.21 ± 0.80[b] |
| **Intestine lengths (m):** | | | | | | |
| Small intestine | 0.86 ± 0.12 | 0.80 ± 0.10 | 0.88 ± 0.07 | 0.94 ± 0.08 | 0.85 ± 0.09 | 0.92 ± 0.07 |
| Large intestine | 0.09 ± 0.01[1] | 0.10 ± 0.004 | 0.09 ± 0.01[1] | 0.09 ± 0.01 | 0.11 ± 0.01 | 0.10 ± 0.01[2] |

Data represented as mean ± SD. Different letters indicate significant differences between the various time points within a single dietary group. STD = standard diet; HFD = high-fat diet.

**Table 2. 6** The relative organ masses and lengths of the small and large intestines of Guinea fowl after 4, 8 and 12 weeks of standard or high-fat diet feeding.

| Organ mass (% of body mass): | 4 weeks | | 8 weeks | | 12 weeks | |
|---|---|---|---|---|---|---|
| | STD | HFD | STD | HFD | STD | HFD |
| Liver | 1.45 ± 0.13 | 1.64 ± 0.24 | 1.49 ± 0.16 | 1.52 ± 0.35 | 1.20 ± 0.24 | 1.39 ± 0.29 |
| Pancreas | 0.19 ± 0.03 | 0.19 ± 0.04 | 0.17 ± 0.02 | 0.20 ± 0.05 | 0.17 ± 0.03 | 0.19 ± 0.06 |
| Proventriculus | 0.33 ± 0.05 | 0.31 ± 0.05 | 0.24 ± 0.04[1] | 0.38 ± 0.14[2] | 0.24 ± 0.04 | 0.32 ± 0.07 |
| Ventriculus | 2.85 ± 0.57 | 3.80 ± 0.71 | 2.52 ± 0.76[1] | 3.72 ± 1.19[2] | 2.55 ± 0.54 | 3.64 ± 0.68 |
| Small intestine | 1.77 ± 0.21 | 2.08 ± 0.53 | 1.84 ± 0.19 | 3.00 ± 1.38 | 3.87 ± 5.75 | 2.19 ± 0.74 |
| Large intestine | 0.17 ± 0.03 | 0.21 ± 0.06 | 0.19 ± 0.05 | 0.20 ± 0.07 | 0.24 ± 0.08 | 0.19 ± 0.02 |
| Caecum | 0.30 ± 0.03 | 0.28 ± 0.04 | 0.34  0.08 | 0.37 ± 0.09 | 0.27 ± 0.06 | 0.31 ± 0.10 |
| **Intestine lengths ($mm.g^{-1}$):** | | | | | | |
| Small intestine | 1.10 ± 0.13[a] | 1.10 ± 0.13 | 0.78 ± 0.10[b, 1] | 1.13 ± 0.26[2] | 0.76 ± 0.20[b] | 0.88 ± 0.17 |
| Large intestine | 0.11 ± 0.02[a] | 0.13 ± 0.01[a] | 0.08 ± 0.01[b] | 0.11 ± 0.02[a,b] | 0.10 ± 0.03[a,b] | 0.10 ± 0.01[b] |

Data represented as mean ± SD. Different letters indicate significant differences between the various time points within a single dietary group. Different numbers indicate significant differences between the STD and HFD groups at specific time point. STD = standard diet; HFD = high-fat diet.

vi) Liver lipid content and liver histology

Figure 2.6 shows the lipid yield (as a % of liver tissue sample mass) of liver tissue from Guinea fowl following four or eight weeks of either STD or HFD feeding. A significant main effect of time ($p = 0.0047$, $F_{(1, 8)} = 14.99$, Two-way ANOVA was observed in the liver lipid yield of Guinea fowl, however upon post-hoc analysis no significance was identified.

No evidence of steatosis was observed in any of the liver histology sections examined from birds in both dietary groups, at all time points. Thus, a score of 0 was allocated to all Guinea fowl liver histology sections. Images of the histological sections obtained from the liver of a representative bird from each dietary group, at each time point are shown in Figure 2.7. The images were obtained using a computer-based image acquisition and analysis system at 200 times magnification (Axiovision 3, Carl Zeiss, Gottingen, Germany).

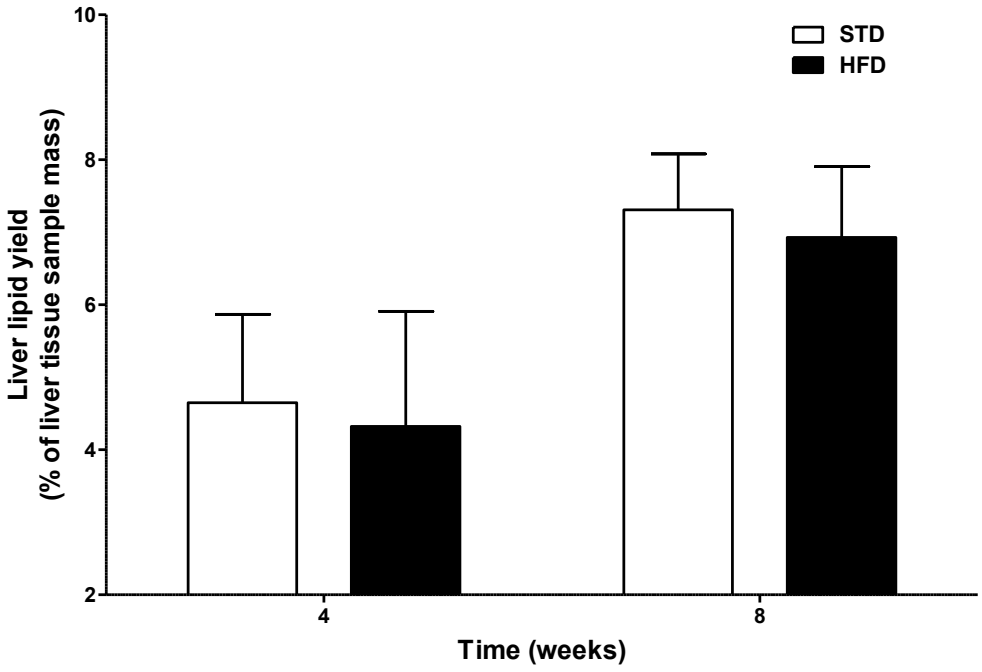



**Figure 2.6** Liver lipid yield (as a % of liver tissue sample mass) of Guinea fowl after 4 and 8 weeks of STD or HFD feeding. STD = standard diet; HFD = high-fat diet. Data represented as means ± SD.

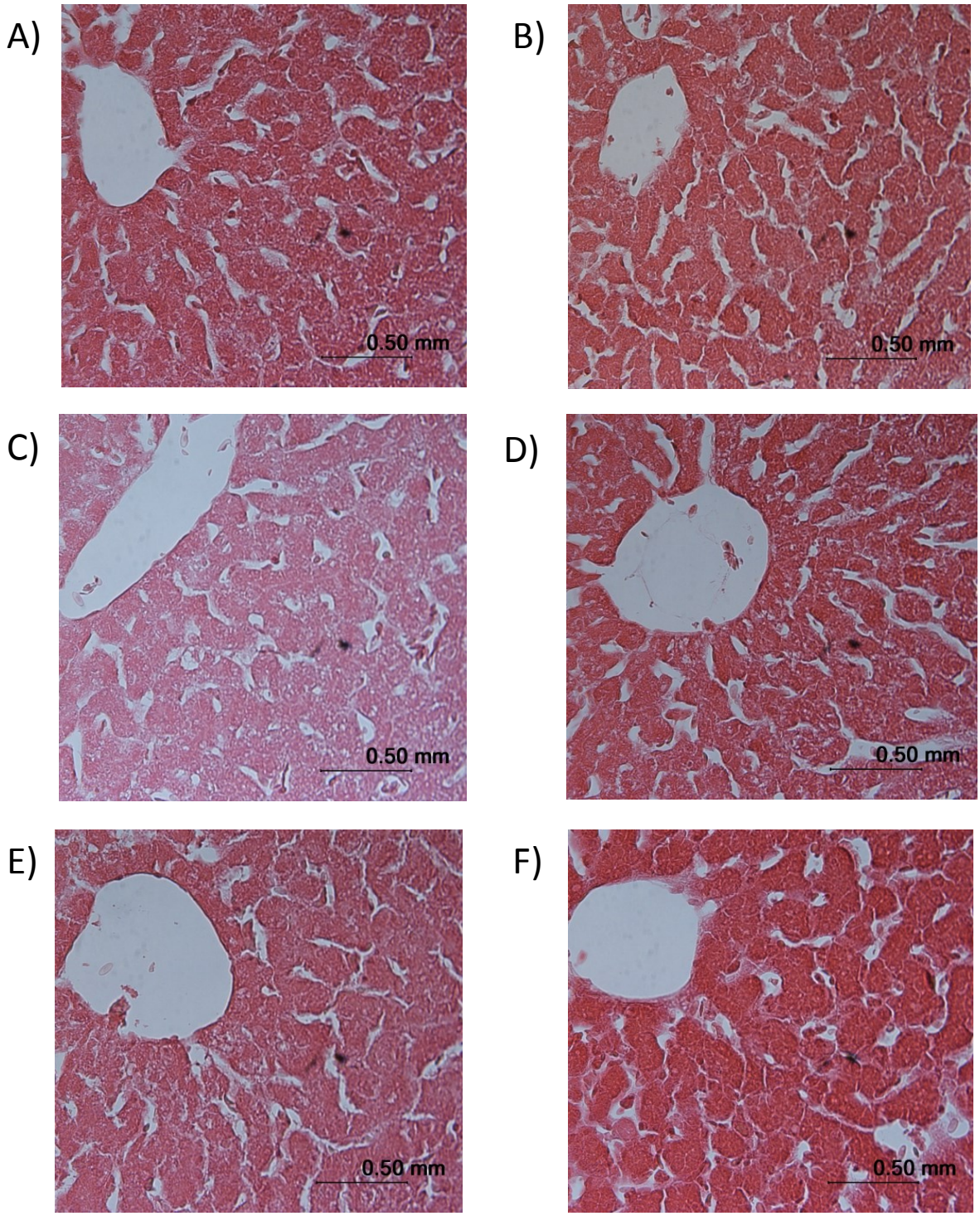



**Figure 2.7** Images of the histological sections, stained with haematoxylin and eosin, obtained from the liver of a representative Guinea fowl from each dietary group, at each time point. Images were obtained using a computer-based image acquisition and analysis system at 200 times magnification (Axiovision 3, Carl Zeiss, Gottingen, Germany). A) STD group after 4 weeks of feeding; B) HFD group after 4 weeks of feeding; C) STD group after 8 weeks of feeding; D) HFD group after 8 weeks of feeding; E) STD group after 12 weeks of feeding; F) HFD group after 12 weeks of feeding. STD = standard diet; HFD = high-fat diet.

vii) Microflora analysis of caecal content

The PCR products for *Lactobacillus* (A), *Escherichia coli* (B), *Clostridium* (C), *Campylobacter* (D), *Salmonella* (E) *and Bifidobacterium* (F) , in a 2 % agarose gel, from the caecal content of representative Guinea fowl from both the STD and HFD groups, after four, eight or twelve weeks of feeding are represented in Figure 2.7. The multiple bands observed in the gels containing the PCR products for *Lactobacillu*s (A), *Escherichia coli* (B), *Clostridium* (C) and *Salmonella* (E) are indicative of poor primer specificity, resulting in non-specific DNA binding and the display of various 'off-target' primer effects. With respect to the *Campylobacte*r (D) PCR products, the expected DNA product of 857 bp was observed in most of the birds in both dietary groups, at all-time points studied. There was a tendency for the bands to become more prominent with increasing length of feeding period, specifically in the HFD group. The expected DNA product of 510 bp for *Bifidobacterium* (F) was also observed in most of the birds from both the STD and HFD groups, at all time points studied. Like with the *Campylobacter*, the presence of the *Bifidobacterium* PCR products increased with increasing length of feeding period, with bands observed in all birds from both the STD and HFD groups, following twelve weeks of feeding.

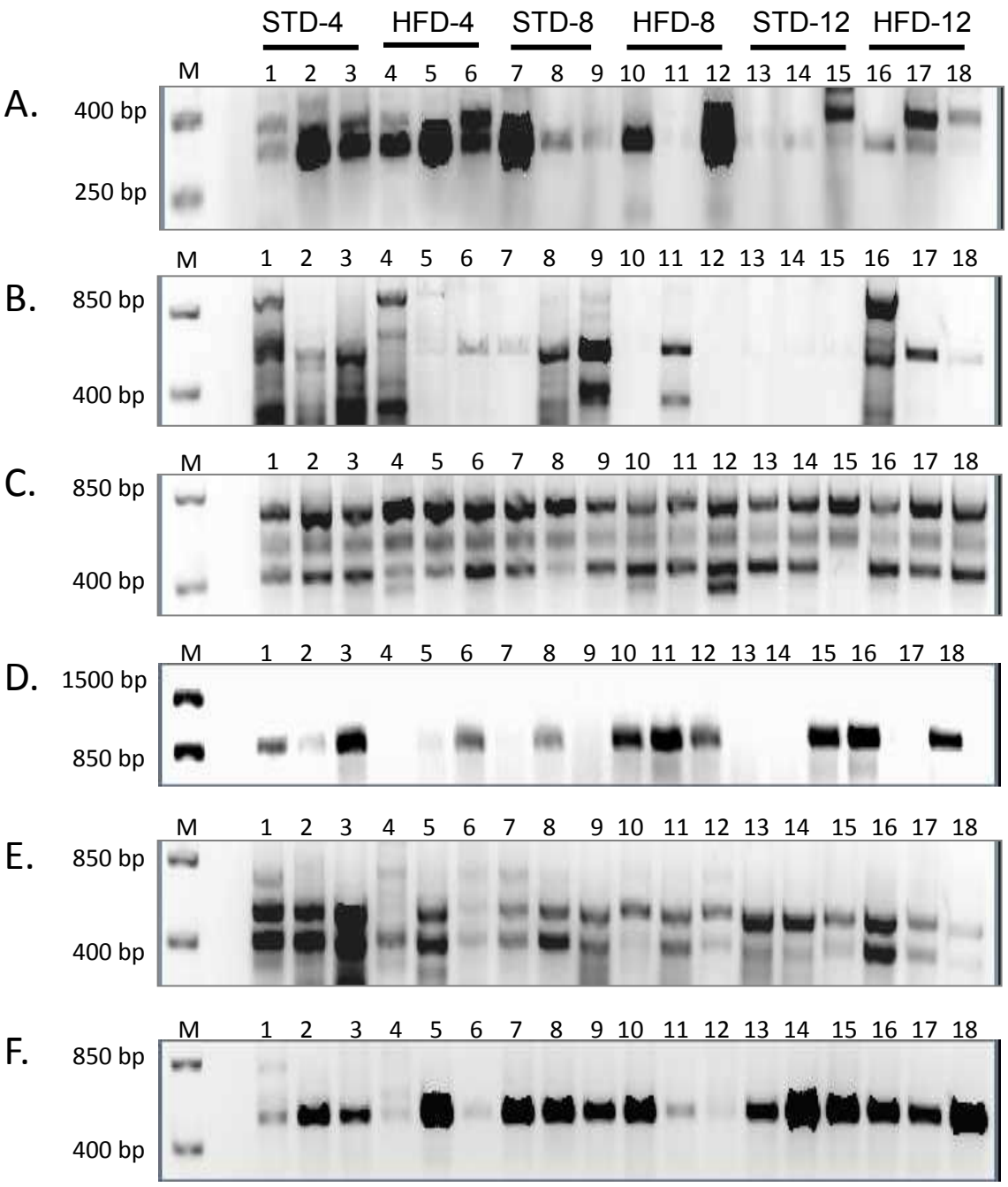



**Figure 2.8** PCR products in a 2% agarose gel from the caecum of representative Guinea fowl from the standard (STD) and high-fat (HFD) diet groups, following 4, 8 and 12 weeks of feeding. A = *Lactobacillus* (286 bp); B = *Escherichia coli* (585 bp); C = *Clostridium* (722 bp); D = *Campylobacter* (857 bp); E = *Salmonella* (396 bp); F = *Bifidobacterium* (510 bp); M = DNA size marker; bp = base pairs.

### 2.3.2 Muscovy ducks

#### i) Body mass

Figure 2.9 shows the initial (time: 0 weeks) and final (after either four, eight or twelve weeks) body mass (g) for each dietary group (A), as well as the average body mass gain (%) per dietary group (B) of Muscovy ducks following either four, eight or twelve weeks of STD or HFD feeding. Initial body mass (following the two week adaptation period) of the birds allocated to the STD and HFD groups as a whole, prior to their allocation to the time point groups, was not significantly different from one another ($p > 0.05$, Student's t-test). Final body mass of birds in both the STD and HFD groups, at all time points examined (after four, eight or twelve weeks), was significantly higher than initial body mass ($p < 0.05$, Student's t-test). No significant differences ($p > 0.05$, Two-way ANOVA) in final body mass of the birds in the STD and HFD groups were observed following four, eight or twelve weeks of feeding. Percentage body mass gain was not significantly different ($p > 0.05$, Two-way ANOVA) between birds in the STD and HFD groups following four, eight and twelve weeks of feeding. Similarly, no significant differences ($p > 0.05$, Two-way ANOVA) were observed when comparing percentage body mass gain across the three different time points, within a single dietary group.

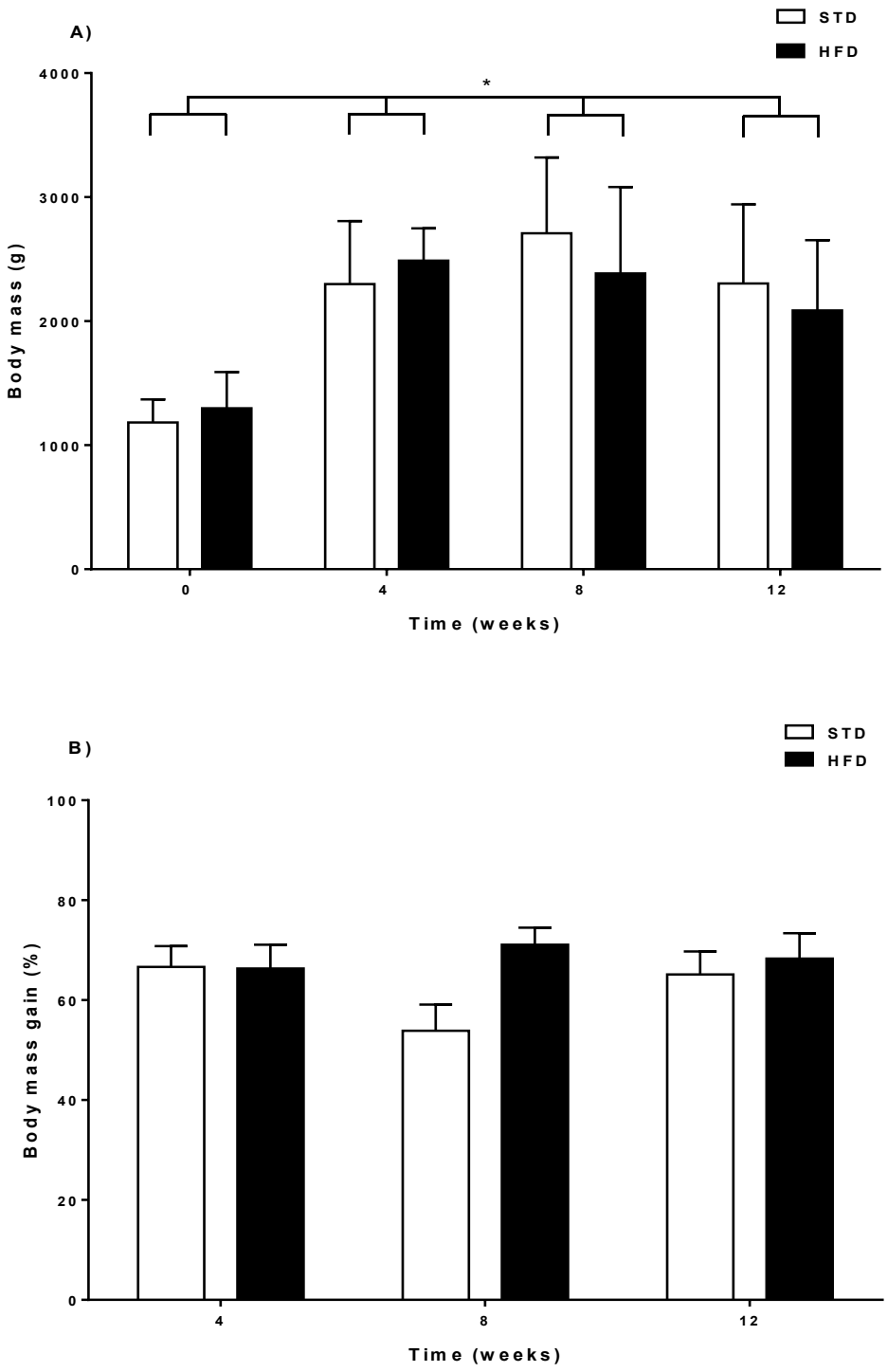



**Figure 2. 9** Initial (time: 0 weeks) and final (after either 4, 8 or 12 weeks) body mass (g) per dietary group (A) and average body mass gain (%) per dietary group (B) of Muscovy ducks following either 4, 8 or 12 weeks of STD or HFD feeding. STD = standard diet; HFD = high-fat diet. Data represented as means ± SD. n = 6 at all time points studied, for Muscovy ducks in both the STD and HFD groups (except n = 5 for Muscovy ducks on the STD at 8 weeks). Figure 2.8 (A): * $p < 0.05$ when comparing final body mass (at either 4, 8 or 12 weeks) to initial body mass (at 0 weeks) for both dietary groups.

ii) Oral glucose tolerance

Figures 2.10 and 2.11 show the glucose tolerance curves and area under the glucose curves for Muscovy ducks after four (A), eight (B) and twelve (C) weeks of STD or HFD feeding. No significant ($p > 0.05$, Two-way ANOVA) effects of diet or time were observed regarding fasting blood glucose concentrations, peak blood glucose concentrations and the area under the glucose curve. A significant main effect of time ($p = 0.04$, $F_{(2, 29)} = 3.562$) was observed in the blood glucose concentration 120 min following the glucose load. However following post-hoc analysis, no significant difference ($p > 0.05$, Two-way ANOVA) in blood glucose concentrations 120 min following the glucose load were observed following four, eight or twelve weeks of feeding, within a single dietary group. Peak blood glucose concentrations were reached 30 min after administration of the glucose load, following four, eight and twelve weeks of either STD or HFD feeding. All peaks were significantly higher ($p < 0.01$, repeated measures ANOVA) than fasting blood glucose concentrations in both dietary groups, after four, eight and twelve weeks of feeding. Blood glucose concentrations only returned to normal 120 min after gavage, at all time points assessed, for birds in both the STD and HFD groups.

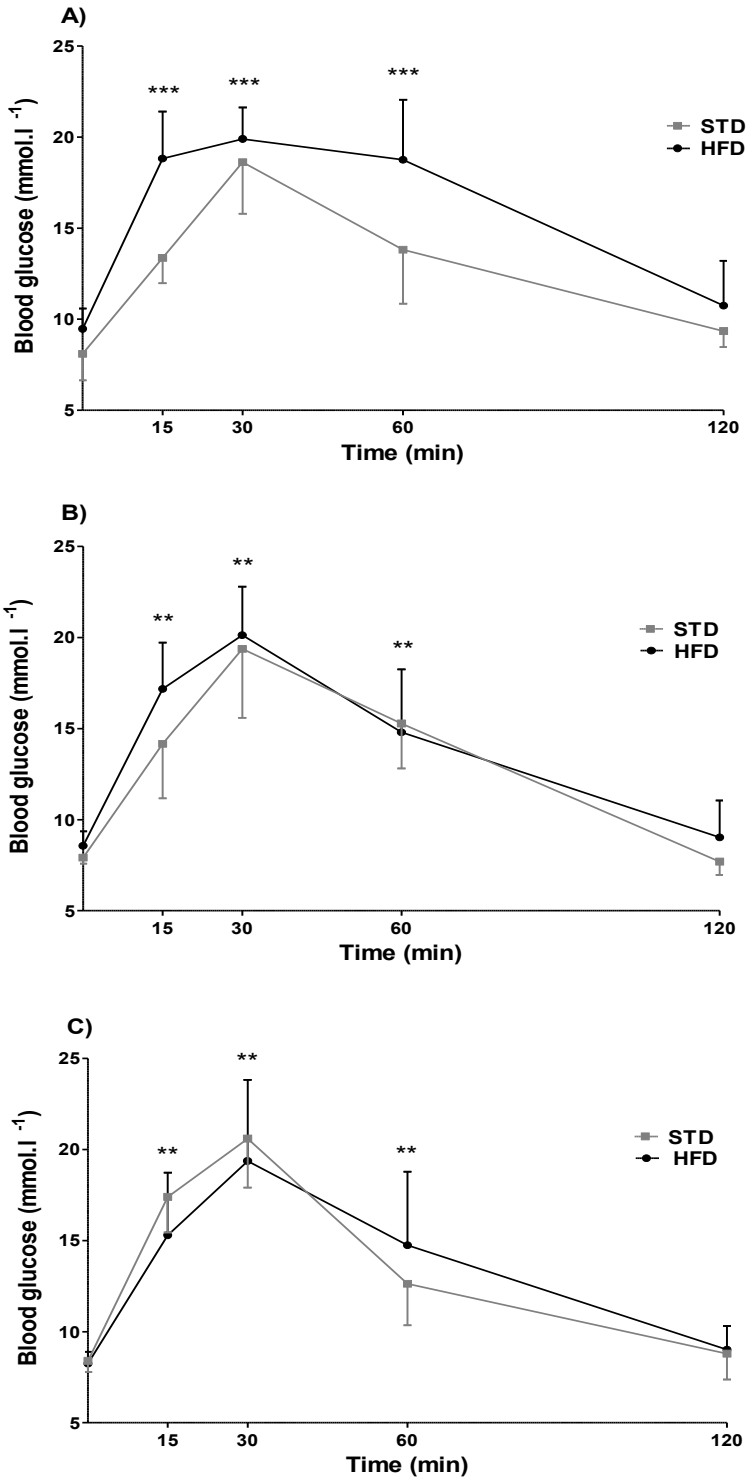



**Figure 2.10** Glucose tolerance curves for Muscovy ducks after 4 (A), 8 (B) and 12 (C) weeks of STD or HFD feeding. STD = standard diet; HFD = high-fat diet. Data represented as means ± SD. n = 6 at all time points studied, for Muscovy ducks in both the STD and HFD groups (except n = 5 for Muscovy ducks on the STD at 8 weeks). *** $p < 0.001$ and ** $p < 0.01$ significance when comparing peak blood glucose concentrations to fasting blood glucose concentrations, within individual dietary groups.

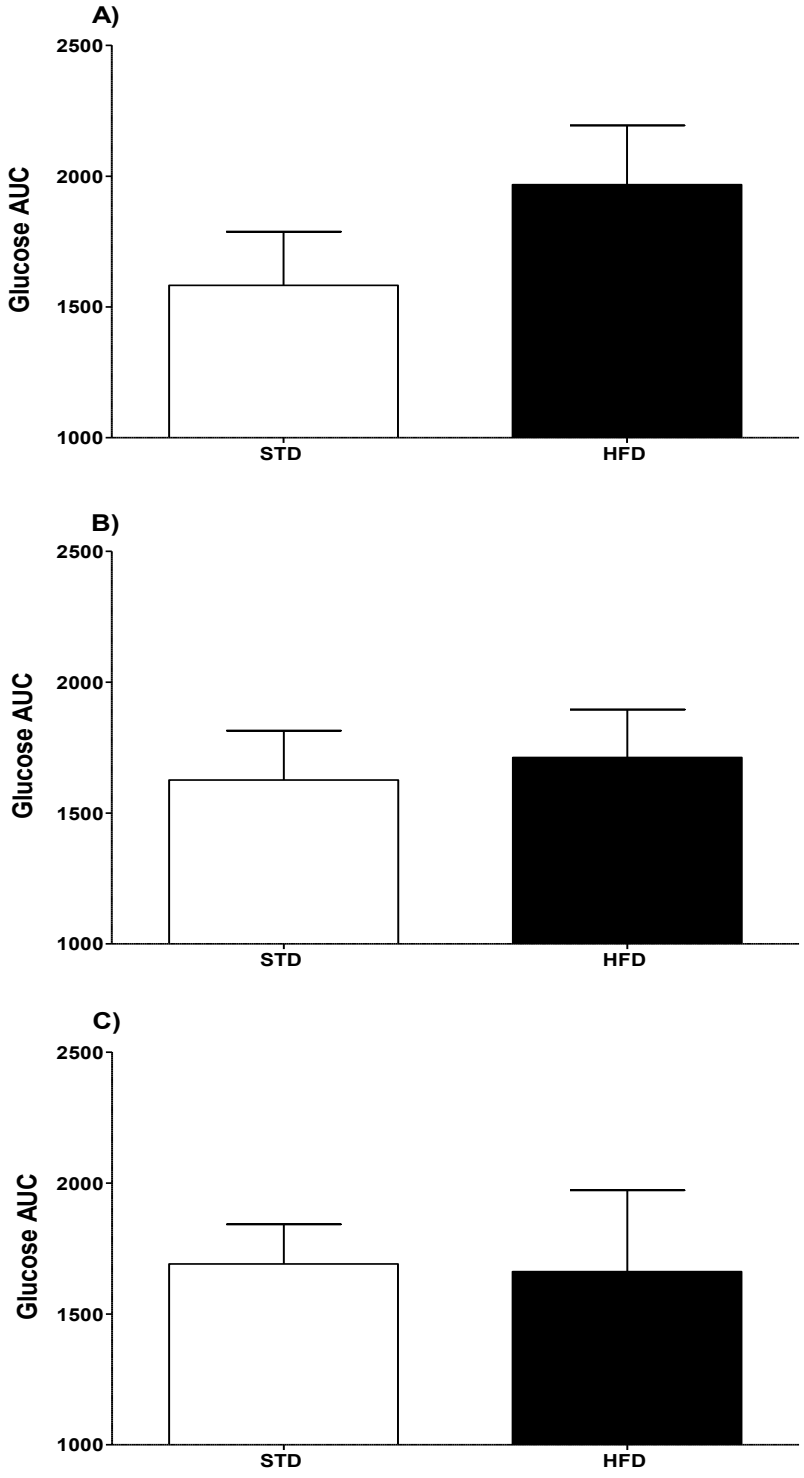



**Figure 2.11** Area under the glucose curves for Muscovy ducks after 4 (A), 8 (B) and 12 (C) weeks of STD or HFD feeding. STD = standard diet; HFD = high-fat diet. Data represented as means ± SD. n = 6 at all time points studied, for Muscovy ducks in both the STD and HFD groups (except n = 5 for Muscovy ducks on the STD at 8 weeks).

iii) Erythrocyte osmotic fragility

Figure 2.12 shows the fragiligrams of the erythrocytes from the Muscovy ducks following four (A), eight (B) and twelve (C) weeks of STD or HFD feeding. The fragiligrams of the erythrocytes from the Muscovy ducks in the STD group were not different from those of the erythrocytes from the Muscovy ducks in the HFD group after four (A), eight (B) and twelve weeks (C) of STD or HFD feeding. Table 2.7 indicates the range of % PBS solutions at which IH, 50% haemolysis/MCF and MH occurred for the erythrocytes from the Muscovy ducks in the STD and HFD groups, after four, eight and twelve weeks. There were no differences in any of the osmotic fragility indices examined for the erythrocytes from the birds in the STD group compared with those for the erythrocytes from the birds in the HFD group, at each time point.

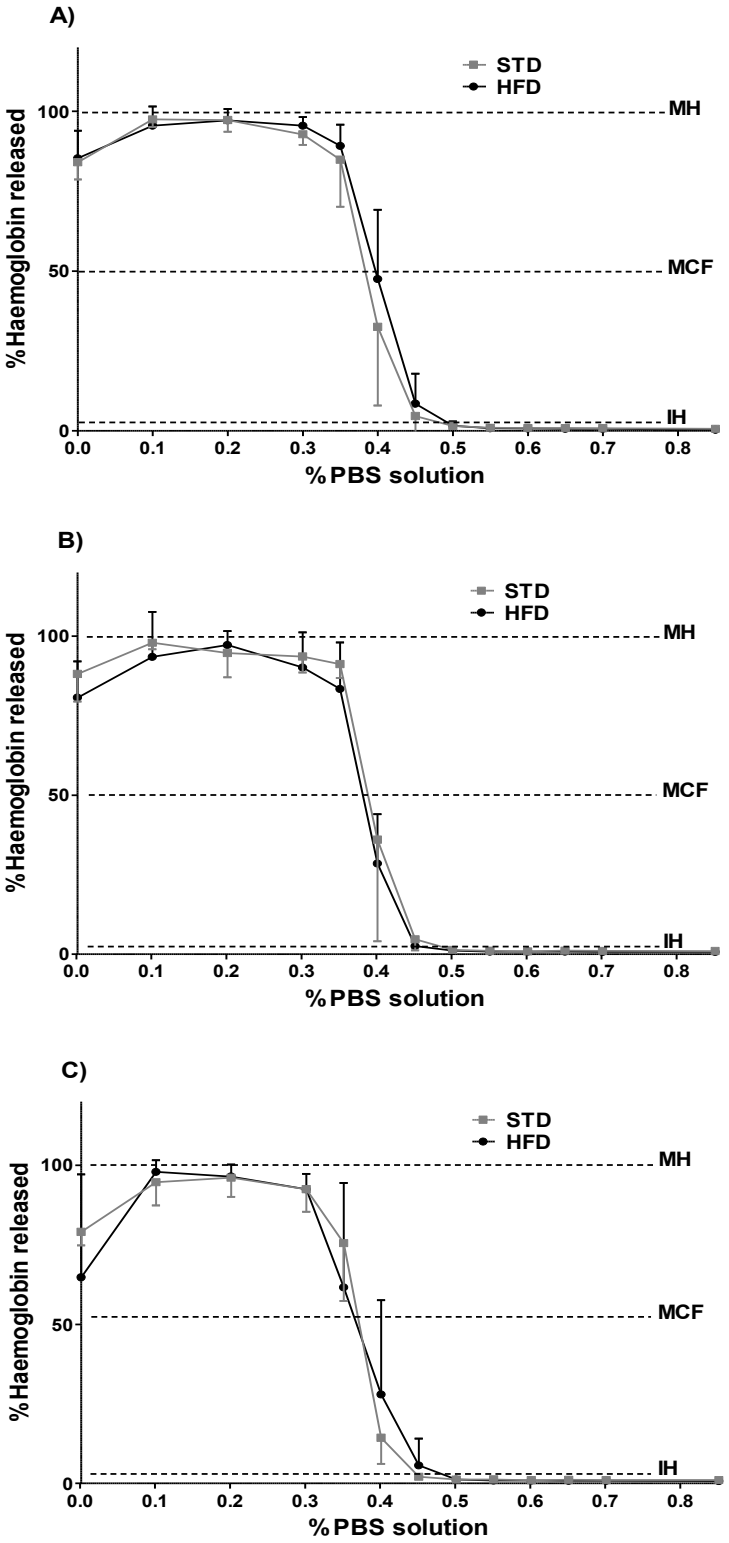



**Figure 2.12** Fragiligrams obtained from the erythrocytes of Muscovy ducks after 4 (A), 8 (B) and 12 (C) weeks of STD or HFD feeding. STD = standard diet; HFD = high-fat diet. Data represented as means ± SD. n = 6 at all time points studied, for Muscovy ducks in both the STD and HFD groups (except n = 5 for Muscovy ducks on the STD at 8 weeks).

**Table 2. 7** The range of phosphate-buffered saline solutions (%) at which initial haemolysis, 50 % haemolysis and maximal haemolysis occurred for the erythrocytes from Muscovy ducks after 4, 8 and 12 weeks of standard or high-fat diet feeding.

| | % PBS solution | |
|---|---|---|
| | **STD** | **HFD** |
| **Initial haemolysis** | | |
| 4 weeks | 0.45-0.5 | 0.45-0.5 |
| 8 weeks | 0.45-0.5 | 0.45-0.5 |
| 12 weeks | 0.4-0.5 | 0.4-0.5 |
| **MCF** | | |
| 4 weeks | 0.35-0.4 | 0.35-0.4 |
| 8 weeks | 0.35-0.4 | 0.35-0.4 |
| 12 weeks | 0.35-0.4 | 0.35-0.4 |
| **Maximal haemolysis** | | |
| 4 weeks | 0.1-0.2 | 0.1-0.2 |
| 8 weeks | 0.1-0.2 | 0.1-0.2 |
| 12 weeks | 0.1-0.2 | 0.1-0.2 |

PBS = phosphate-buffered saline solution; STD = standard diet; HFD = high-fat diet; MCF = mean corpuscular fragility / 50 % haemolysis.

iv) General health/clinical biochemistry profile

Serum parameters for Muscovy ducks in both the STD and HFD groups are presented in Table 2.8. A significant main effect of diet ($p = 0.003$, $F_{(1, 29)} = 10.42$, Two-way ANOVA) was observed in serum uric acid concentrations, however following post-hoc analysis no significant differences in serum uric acid concentrations were observed between the STD and HFD groups, following either four, eight or twelve weeks of feeding. No significant effects of diet or time ($p > 0.05$, Two-way ANOVA) were observed with regards to serum total protein, albumin, AST and total bilirubin concentrations in Muscovy ducks in both the STD and HFD groups, following four, eight or twelve weeks of feeding. A significant time ($p = 0.002$, $F_{(2, 29)} = 7.462$, Two-way ANOVA) and interaction ($p = 0.03$, $F_{(2, 29)} = 3.994$, Two-way ANOVA) effect was observed with regards to serum calcium concentrations. Significantly lower serum calcium concentrations were observed following eight ($p = 0.002$, Two-way ANOVA) and twelve ($p = 0.0004$, Two-way ANOVA) weeks of HFD feeding compared to that observed following four weeks of HFD feeding. No significant difference in serum calcium concentrations between the various time points were observed in the STD group. A significant diet ($p < 0.0001$, $F_{(1, 29)} = 29.26$, Two-way ANOVA) and interaction ($p = 0.02$, $F_{(2, 29)} = 4.317$, Two-way ANOVA) effect were observed with regards to serum cholesterol concentrations (Figure 2.13). Serum cholesterol concentrations were significantly higher in birds in the HFD group compared to those in the STD group after four ($p < 0.0001$. Two-way ANOVA) and twelve ($p = 0.01$, Two-way ANOVA) weeks of feeding, however no significant differences ($p > 0.05$, Two-way ANOVA) in serum cholesterol concentrations were observed between the two dietary groups after eight weeks of feeding. A significant main effect of time ($p = 0.04$, $F_{(2, 28)} = 3.758$, Two-way ANOVA) was observed with regards to serum triglyceride concentrations. Upon post-hoc analysis, no significant differences ($p > 0.05$, Two-way ANOVA) in serum triglyceride concentrations were observed following four, eight or twelve weeks of feeding, within a single dietary group.

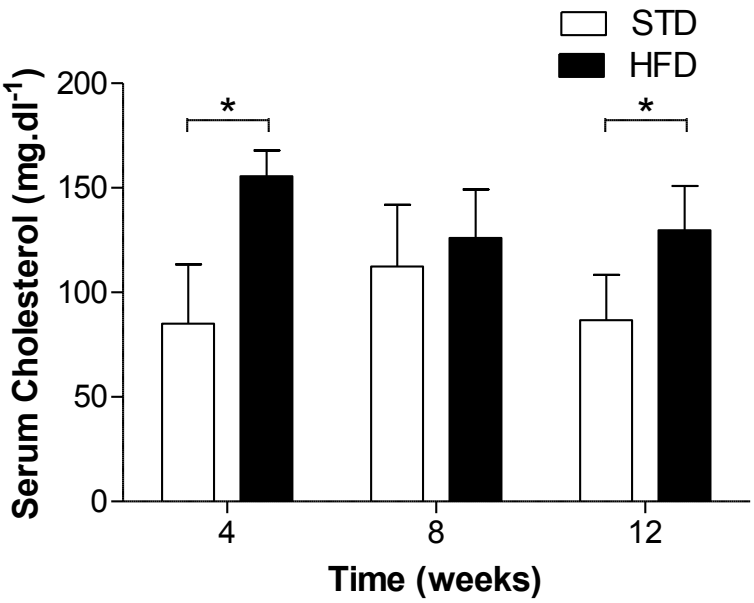



**Figure 2.13** Serum cholesterol concentrations ($mg.dl^{-1}$) of Muscovy Ducks after 4, 8 and 12 weeks of STD or HFD feeding. STD = standard diet; HFD = high-fat diet. Data represented as means ± SD. * $p < 0.05$ when comparing HFD group to STD group at specific time point.

**Table 2. 8** Serum parameters from Muscovy ducks after 4, 8 and 12 weeks of standard or high-fat diet feeding.

| Serum Parameters: | 4 weeks | | 8 weeks | | 12 weeks | |
|---|---|---|---|---|---|---|
| | **STD** | **HFD** | **STD** | **HFD** | **STD** | **HFD** |
| Uric ($mg.dl^{-1}$) | 2.7 ± 1.0 | 2.3 ± 0.9 | 3.1 ± 0.7 | 2.0 ± 1.0 | 2.9 ± 0.8 | 1.7 ± 0.2 |
| TPro ($g.dl^{-1}$) | 3.1 ± 0.7 | 3.4 ± 0.5 | 3.4 ± 0.2 | 3.3 ± 0.3 | 3.4 ± 0.5 | 3.1 ± 0.2 |
| Alb ($g.dl^{-1}$) | 0.6 ± 0.4 | 0.7 ± 0.2 | 0.8 ± 0.3 | 0.8 ± 0.3 | 0.7 ± 0.1 | 0.7 ± 0.3 |
| AST ($U.l^{-1}$) | 21.2 ± 12.4 | 29.0 ± 17.0 | 18.8 ± 2.5 | 16.8 ± 15.42 | 19.2 ± 11.1 | 18.2 ± 9.2 |
| TBil ($mg.dl^{-1}$) | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.4 ± 0.3 | 0.2 ± 0.2 | 0.2 ± 0.1 | 0.2 ± 0.1 |
| Calc ($mg.dl^{-1}$) | 9.4 ± 1.3 | $10.7 \pm 0.8^{1}$ | 9.1 ± 0.2 | $9.0 \pm 0.5^{2}$ | 9.0 ± 0.7 | $8.7 \pm 0.5^{2}$ |
| Trig ($mg.dl^{-1}$) | 38.2 ± 12.8 | 46.8 ± 7.5 | 49.0 ± 13.3 | 55.0 ± 3.9 | 44.2 ± 4.0 | 44.0 ± 7.8 |

Data represented as mean ± SD. Different numbers indicate significant differences between the various time points within a single dietary group. HFD = high-fat diet, STD = standard diet, Uric = uric acid, Tpro = total protein, Alb = albumin, AST = aspartate aminotransferase, Tbil = total bilirubin, Calc = Calcium, Trig = triglycerides. n = 6 for both HFD and STD groups at 4 weeks (except for Trig, where n = 5 in STD group), n = 6 for HFD group at 8 weeks (except for AST, where n = 4 in HFD group) and n = 5 for STD group at 8 weeks (except for AST, where n = 4 in STD group), n = 6 for both HFD and STD groups at 12 weeks.

v) Organ masses and small and large intestine lengths

The absolute and relative masses (represented as a percentage of body mass) of the liver, pancreas, proventriculus, ventriculus, large intestine and caecum, along with the absolute and relative lengths of the small and large intestines (as unit length per unit body mass) of the Muscovy ducks are represented in Tables 2.9 (absolute) and 2.10 (relative), respectively. No significant differences ($p > 0.05$, Two-way ANOVA) in the absolute masses of the pancreas and proventriculus as well as in the absolute lengths of the small and large intestines were observed between Muscovy ducks in the STD and HFD groups, following four, eight and twelve weeks of feeding. A significant main effect of time was observed in the absolute liver ($p = 0.0191$, $F_{(2, 29)} = 4.548$, Two-way ANOVA), ventriculus ($p = 0.0176$, $F_{(2, 29)} = 4.658$, Two-way ANOVA), small intestine ($p = 0.0022$, $F_{(2, 29)} = 7.583$, Two-way ANOVA) and large intestine ($p = 0.0143$, $F_{(2, 29)} = 4.936$, Two-way ANOVA) mass of the Muscovy ducks. A significantly larger ($p = 0.0099$, Two-way ANOVA) absolute liver mass was observed in the HFD group following four weeks of feeding compared to that observed following twelve weeks of feeding. The absolute small intestine mass was also significantly larger in the HFD group following four weeks of feeding compared to that observed following eight ($p = 0.0439$, Two-way ANOVA) and twelve ($p = 0.0025$, Two-way ANOVA) weeks of feeding. Significantly larger absolute large intestine ($p = 0.0456$, Two-way ANOVA) and ventriculus ($p = 0.0191$, Two-way ANOVA) masses were observed in the HFD group following four weeks of feeding compared to that observed following twelve weeks of feeding. The absolute large intestine and ventriculus masses observed in the HFD group following eight weeks of feeding were not significantly different from those observed following either four or twelve weels of HFD feeding. A significant time ($p = 0.0031$, $F_{(2, 29)} = 7.076$, Two-way ANOVA) and interaction effect ($p = 0.0351$, $F_{(2, 29)} = 3.767$, Two-way ANOVA) were observed in the absolute caecum mass of the Muscovy ducks. The absolute caecum mass of the birds in the HFD group was significantly larger following four weeks of feeding compared to that observed following eight ($p = 0.0130$, Two-way ANOVA) and twelve ($p = 0.0003$, Two-way ANOVA) weeks of feeding.

No significant differences ($p > 0.05$, Two-way ANOVA) in relative proventriculus mass and the relative lengths of the small and large intestines were observed between Muscovy ducks in the STD and HFD groups, following either four, eight or twelve weeks of feeding. A significant main effect of time was observed in the relative liver ($p = 0.0001$, $F_{(2, 29)} = 12.63$,

Two-way ANOVA), pancreas ($p = 0.0215$, $F_{(2, 29)} = 4.398$, Two-way ANOVA), ventriculus ($p = 0.0021$, $F_{(2, 29)} = 7.712$, Two-way ANOVA), large intestine ($p = 0.0059$, $F_{(2, 29)} = 6.167$, Two-way ANOVA) and caecum masses ($p = 0.0007$, $F_{(2, 29)} = 9.542$, Two-way ANOVA) of the Muscovy ducks. Significantly increased relative liver masses were observed in the STD group following four weeks of feeding compared to that following twelve weeks ($p = 0.0463$, Two-way ANOVA) of feeding and in the HFD group following four weeks of feeding compared to that following eight ($p = 0.0073$, Two-way ANOVA) and twelve weeks ($p = 0.0007$, Two-way ANOVA) of feeding. The relative pancreas ($p = 0.0338$, Two-way ANOVA), ventriculus ($p = 0.0121$, Two-way ANOVA) and large intestine ($p = 0.0382$, Two-way ANOVA) masses were significantly larger in the STD group following four weeks of feeding compared to that observed following eight weeks of feeding. A significantly larger relative caecum mass was observed in the HFD group following four weeks of feeding compared to that observed following eight ($p = 0.0041$, Two-way ANOVA) and twelve weeks ($p = 0.0004$, Two-way ANOVA) of feeding. A significant time ($p = 0.0023$, $F_{(2, 29)} = 7.56$, Two-way ANOVA) and diet effect ($p = 0.0498$, $F_{(1, 29)} = 9.542$, Two-way ANOVA) were observed in the relative small intestine mass of the Muscovy ducks, however upon post-hoc analysis no significance was identified.

**Table 2. 9** The absolute organ masses and lengths of the small and large intestines of Muscovy ducks after 4, 8 and 12 weeks of standard or high-fat diet feeding.

| Organ mass (g): | 4 weeks | | 8 weeks | | 12 weeks | |
|---|---|---|---|---|---|---|
| | STD | HFD | STD | HFD | STD | HFD |
| Liver | 33.35 ± 7.20 | 39.50 ± 7.61[a] | 33.64 ± 9.55 | 30.38 ± 9.90[a,b] | 28.16 ± 9.12 | 24.41 ± 5.64[b] |
| Pancreas | 4.34 ± 0.50 | 4.52 ± 1.08 | 3.89 ± 0.44 | 3.70 ± 1.15 | 3.74 ± 1.02 | 3.48 ± 0.71 |
| Proventriculus | 7.63 ± 2.27 | 8.04 ± 1.65 | 7.60 ± 2.11 | 7.26 ± 2.71 | 6.91 ± 1.94 | 5.63 ± 0.56 |
| Ventriculus | 64.46 ± 12.05 | 66.19 ± 13.26[a] | 57.63 ± 15.23 | 53.00 ± 13.58[a,b] | 54.22 ± 13.79 | 44.72 ± 8.82[b] |
| Small intestine | 41.47 ± 6.32 | 51.95 ± 7.88 | 37.90 ± 11.54 | 39.33 ± 11.82 | 32.60 ± 6.41 | 33.49 ± 6.60 |
| Large intestine | 5.01 ± 1.42 | 5.12 ± 1.30[a] | 4.37 ± 1.03 | 3.92 ± 1.08[a,b] | 3.79 ± 0.80 | 3.49 ± 1.01[b] |
| Caecum | 3.55 ± 0.63 | 5.10 ± 0.82[a] | 3.40 ± 0.97 | 3.44 ± 1.31[b] | 3.13 ± 1.12 | 2.65 ± 0.61[b] |
| **Intestine lengths (m):** | | | | | | |
| Small intestine | 1.41 ± 0.13 | 1.62 ± 0.14 | 1.47 ± 0.24 | 1.50 ± 0.23 | 1.40 ± 0.17 | 1.32 ± .013 |
| Large intestine | 0.11 ± 0.02 | 0.13 ± 0.01 | 0.12 ± 0.02 | 0.11 ± 0.01 | 0.11 ± 0.01 | 0.12 ± 0.03 |

Data represented as mean ± SD. Different letters indicate significant differences between the various time points within a single dietary group. STD = standard diet; HFD = high-fat diet.

**Table 2. 10** The relative organ masses and lengths of the small and large intestines of Muscovy ducks after 4, 8 and 12 weeks of standard or high-fat diet feeding.

| Organ mass (% of body mass): | 4 weeks | | 8 weeks | | 12 weeks | |
|---|---|---|---|---|---|---|
| | **STD** | **HFD** | **STD** | **HFD** | **STD** | **HFD** |
| Liver | 1.46 ± 0.23[a] | 1.58 ± 0.18[a] | 1.25 ± 0.22[a,b] | 1.26 ± 0.08[b] | 1.22 ± 0.16[b] | 1.18 ± 0.08[b] |
| Pancreas | 0.22 ± 0.05[a] | 0.20 ± 0.03 | 0.16 ± 0.02[b] | 0.17 ± 0.02 | 0.17 ± 0.04[a,b] | 0.17 ± 0.02 |
| Proventriculus | 0.33 ± 0.03 | 0.32 ± 0.06 | 0.29 ± 0.06 | 0.30 ± 0.05 | 0.30 ± 0.04 | 0.28 ± 0.04 |
| Ventriculus | 2.86 ± 0.50[a] | 2.65 ± 0.42 | 2.18 ± 0.51[b] | 2.26 ± 0.27 | 2.37 ± 0.16[a,b] | 2.17 ± 0.22 |
| Small intestine | 1.87 ± 0.50 | 2.09 ± 0.25 | 1.41 ± 0.31 | 1.66 ± 0.17 | 1.46 ± 0.23 | 1.65 ± 0.34 |
| Large intestine | 0.22 ± 0.05[a] | 0.20 ± 0.03 | 0.16 ± 0.02[b] | 0.17 ± 0.02 | 0.17 ± 0.04[a,b] | 0.17 ± 0.02 |
| Caecum | 0.16 ± 0.02 | 0.21 ± 0.04[a] | 0.12 ± 0.02 | 0.15 ± 0.03[b] | 0.14 ± 0.03 | 0.13 ± 0.03[b] |
| **Intestine lengths (mm/g):** | | | | | | |
| Small intestine | 0.63 ± 0.10 | 0.66 ± 0.10 | 0.56 ± 0.12 | 0.66 ± 0.10 | 0.63 ± 0.10 | 0.66 ± 0.12 |
| Large intestine | 0.05 ± 0.01 | 0.05 ± 0.01 | 0.04 ± 0.01 | 0.05 ± 0.01 | 0.05 ± 0.01 | 0.06 ± 0.01 |

Data represented as mean ± SD. Different letters indicate significant differences between the various time points within a single dietary group. STD = standard diet; HFD = high-fat diet.

vi) Liver lipid content and histology

Figure 2.14 show the lipid yield (as a % of liver tissue sample mass) of liver tissue from Muscovy ducks following four or eight weeks of either STD or HFD feeding. A significant main effect of time ($p = 0.0362$, $F_{(1, 7)} = 6.683$, Two-way ANOVA) was observed in the liver lipid yield of Muscovy ducks, however upon post-hoc analysis no significance was identified.

Photographs of the histological sections obtained from the liver of a representative bird from each dietary group, at each time point are shown in Figure 2.15. No evidence of steatosis was observed in any of the liver histology sections examined for birds from both dietary groups, at all time points. Thus, a score of 0 was allocated to all Muscovy duck liver histology sections.

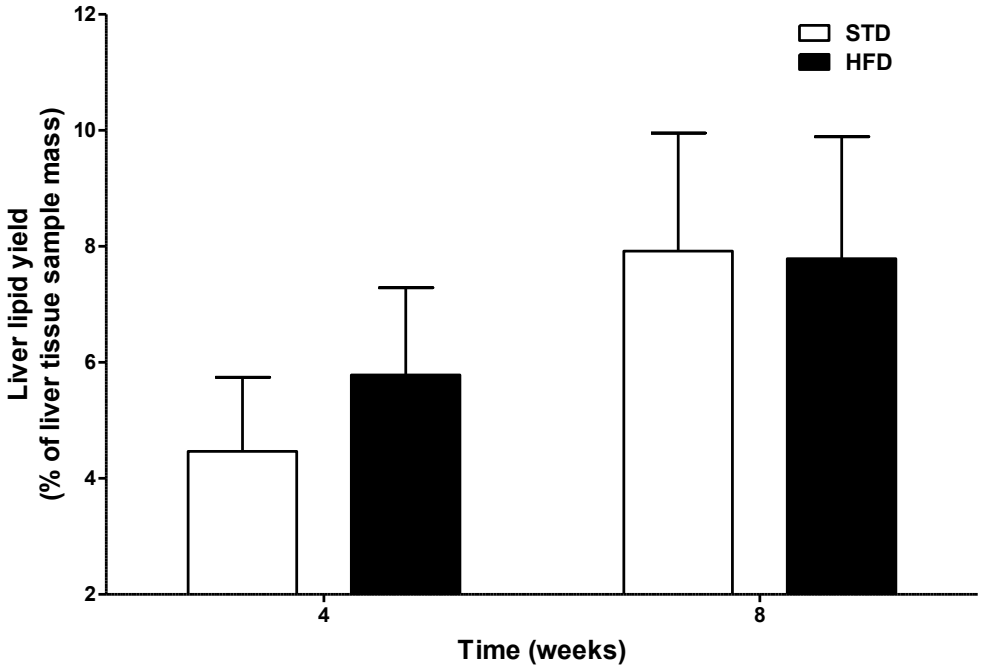



**Figure 2. 14** Liver lipid yield (as a % of liver tissue sample mass) of Muscovy ducks after 4 and 8 weeks of standard or high-fat diet feeding. STD = standard diet; HFD = high-fat diet. Data represented as means ± SD.

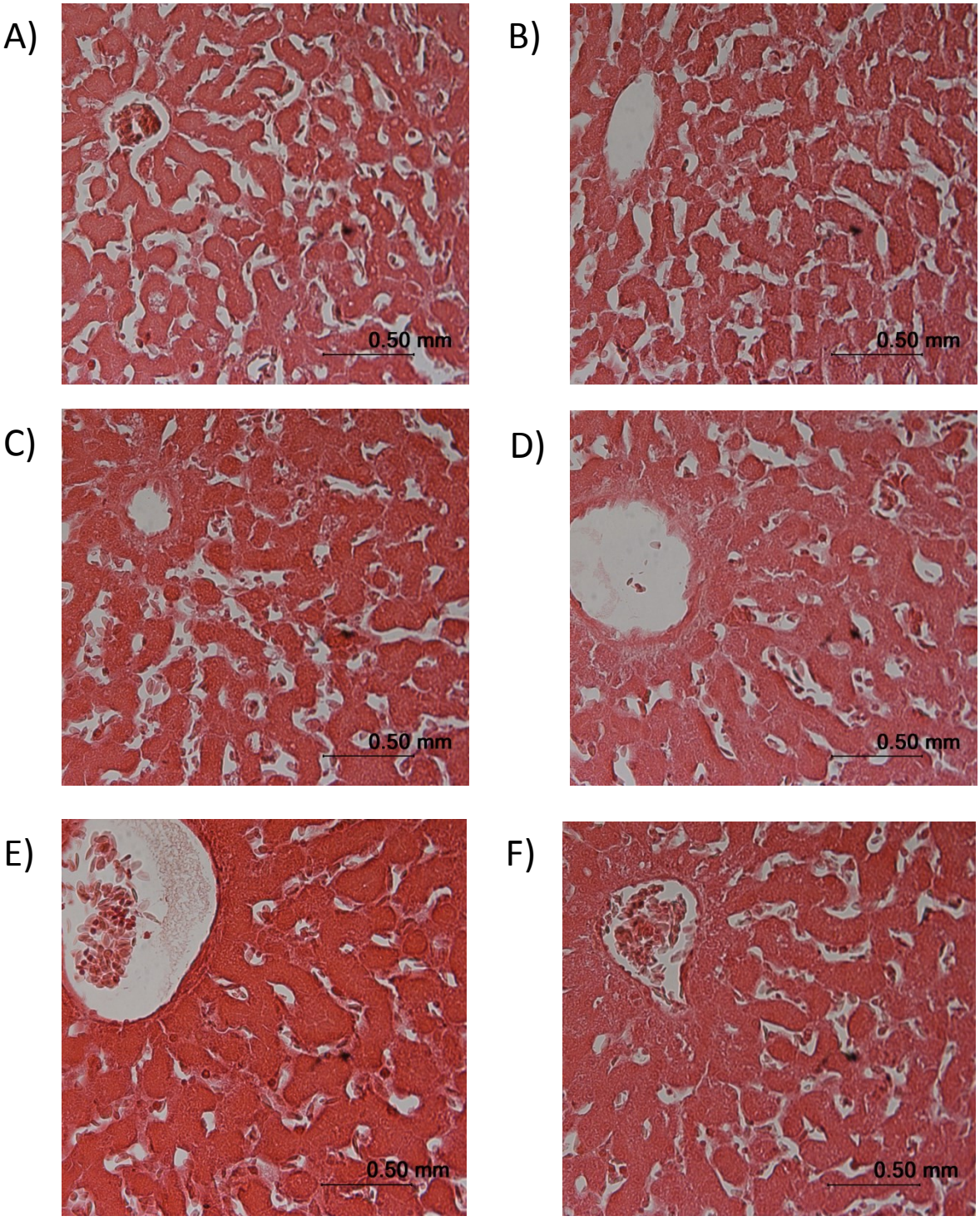



**Figure 2.15** Images of the histological sections, stained with haematoxylin and eosin, obtained from the liver of a representative Muscovy duck from each dietary group, at each time point. Images were obtained using a computer-based image acquisition and analysis system at 200 times magnification (Axiovision 3, Carl Zeiss, Gottingen, Germany). A) STD group after 4 weeks of feeding; B) HFD group after 4 weeks of feeding; C) STD group after 8 weeks of feeding; D) HFD group after 8 weeks of feeding; E) STD group after 12 weeks of feeding; F) HFD group after 12 weeks of feeding. STD = standard diet; HFD = high-fat diet.

vii) Microflora analysis of caecal content

The PCR products for *Lactobacillus* (A), *Escherichia coli* (B), *Clostridium* (C), *Campylobacter* (D), *Salmonella* (E) *and Bifidobacterium* (F) , in a 2 % agarose gel, from the caecal content of representative Muscovy ducks from both the STD and HFD groups, after four, eight or twelve weeks of feeding are represented in Figure 2.16. The primer specificity for *Lactobacillu*s (A), *Escherichia coli* (B), *Clostridium* (C) and *Salmonella* (E) was poor, resulting in non-specific DNA binding and multiple bands being observed in the respective gels. With respect to the *Campylobacte*r (D) PCR products, the expected DNA product of 857 bp was observed in most of the birds in the standard diet group, after four weeks of feeding (lanes 1-3). Very light bands were observed in one of the birds from the HFD group after four weeks of feeding (lane 6) and in one of the birds from the STD group, after eight weeks of feeding (lane 7). No PCR product was observed in any of the other remaining lanes (lanes 8-18), in fact some DNA smearing seems to have taken place which might have affected the results. The expected DNA product of 510 bp for *Bifidobacterium* (F) was observed in some of the birds from both the STD and HFD groups, at various time points, however no clear pattern of presence of the PCR product was observed.

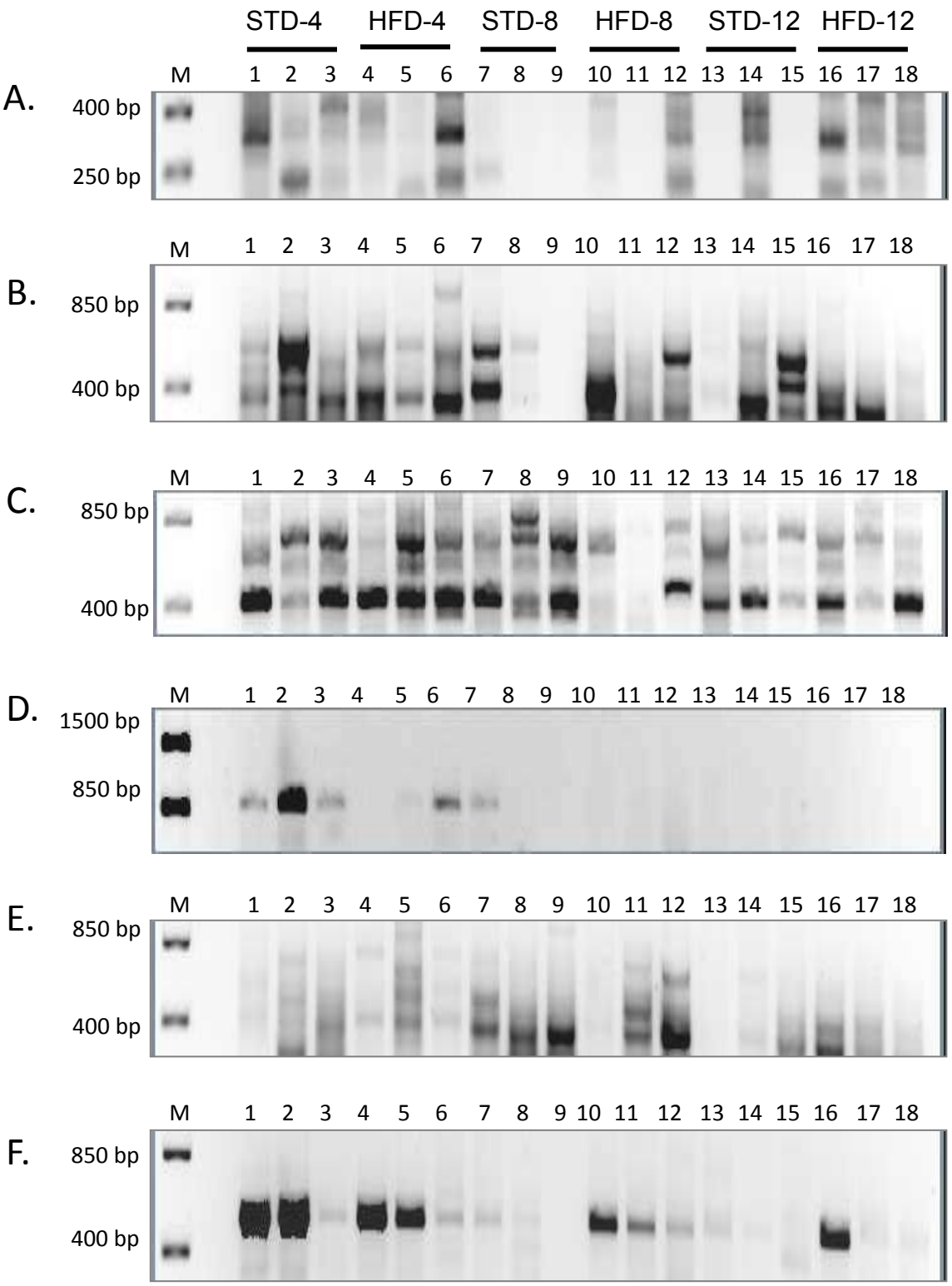



**Figure 2.16** PCR products in a 2% agarose gel from the caecum of representative Muscovy ducks from the standard (STD) and high-fat (HFD) diet groups, following 4, 8 and 12 weeks of feeding. A = *Lactobacillus* (286 bp); B = *Escherichia coli* (585 bp); C = *Clostridium* (722 bp); D = *Campylobacter* (857 bp); E = *Salmonella* (396 bp); F = *Bifidobacterium* (510 bp); M = DNA size marker; bp = base pairs.

## 2.4 Discussion

The main objective of the current study was to investigate the metabolic effects of a HFD rich in saturated fatty acids on the overall health status of Guinea fowl and Muscovy ducks, following either four, eight or twelve weeks of HFD feeding. In general, the HFD was well-tolerated by both bird species with no adverse effects observed with respect to the growth performance, glucose tolerance, erythrocyte osmotic fragility, liver lipid deposition and gut microbiota profile of the birds. The general health profile/clinical biochemistry of the birds was also relatively unaffected by the HFD, except for the significantly increased serum cholesterol levels observed in birds receiving the HFD. Despite the raised cholesterol levels in circulation, the birds seemed to remain healthy throughout the feeding period. My null hypothesis ($H_0$) stated that the HFD, rich in saturated fatty acids, would have no influence on the growth performance, glucose tolerance, erythrocyte osmotic fragility, liver mass, liver lipid content, caecal microbiota profile, serum biochemistry/general health profile and the absolute and relative organ masses and intestine lengths of the Guinea fowl and Muscovy ducks. This was the case with regards to the growth performance, glucose tolerance, erythrocyte osmotic fragility and liver lipid content, thus leading me to accept my null hypothesis in these instances. I was unable to accept or reject my null hypothesis with regards to the diet-induced changes observed in the caecal microbiota profile and the absolute and relative organ masses and intestine lengths, as there were no clear diet-induced trends observed. I accepted my null hypothesis with regards to the serum biochemistry/general health profile of the birds, except for in the case of the serum cholesterol measurements which were affected by the HFD, thus leading me to reject the null hypothesis in this instance.

Poultry diets are often formulated with additional lipid sources in the form of either animal fat or vegetable oils. These lipids are added to poultry diets with the purpose of increasing the energy density of the diets thus resulting in reduced feed intake, as well as improving overall feed conversion efficiency and growth performance of the birds (Mahesar et al., 2011; Nobakht and Mehmannavaz, 2012). Feed intake is an important determinant of the levels of nutrients consumed by production birds and has a significant impact on the productivity of poultry farms (Mbajiorgu et al., 2011). One of the limitations of the current study was the lack of accurate measurement of the feed intake of the birds, due to them being housed as groups. In addition to the group housing, the mixture of the some of the feed with the water in the water baths provided to the ducks, also proved to be problematic. Nevertheless, the birds were

observed to be eating the majority of the standard and HFDs provided to them. Since I did not measure feed intake, I am unable to comment on the effects of the HFD used in the current study on the feed intake and feed conversion efficiency of the birds, however the growth performance of the birds was assessed.

*Growth performance*

The HFD used in the current study had no adverse effects on the growth performance of both bird species, however no significant improvement in growth performance following HFD feeding was observed. Most previous studies involving the effects of HFD feeding on body mass gain in poultry are focussed on the differences in body mass gain achieved by birds receiving different types of dietary fat and often do not have a control dietary group, receiving no additional dietary fat. The lack of control group makes it difficult to assess their interpretation of improved body mass gain following HFD feeding, for comparison to my study. Das et al. (2014a) observed a significant improvement in live body mass gain of broiler chickens supplemented with 2.5% of palm oil, soyabean oil or fish oil, between the 3$^{rd}$ and 5$^{th}$ weeks of age. The increase in live body mass gain was accompanied by a decrease in feed consumption of the oil-supplemented groups compared to that of the control group (Das et al., 2014a). Our results are in contrast with this study, as even at a much higher level of lipid supplementation we did not observe any significant differences between the percentage body mass gain between birds on the STD and those on the HFD. Thus, it might not be necessary to supplement diets intended for Guinea fowl and Muscovy ducks during production with additional lipid sources and thus production would be cheaper in terms of feed formulation costs. To confirm the possible reduction in feed formulation costs, a detailed cost-benefit analysis would have to be performed, taking into account the feed intake of the birds, which was not measured in the current study, as well as the feed conversion efficiency and cost of the feed.

*Glucose tolerance*

Studies involving the addition of various lipid sources to the diets of poultry birds are conventionally focussed on the effects of the diets on the growth performance, feed intake, feed conversion efficiency and resulting tissue fatty acid profile of the birds. Very few studies have investigated the metabolic effects of these HFDs on parameters related to the health

status of the birds during production. The current study is unique in that aspect. HFD feeding in mammals and some birds has been associated with alterations in glucose tolerance. In the current study, no significant differences in any of the oral glucose tolerance test parameters examined were observed between birds on the STD and HFDs. Similarly, in a study by Lembede et al. (2014), no significant differences in glucose tolerance were observed between Japanese quail receiving a HFD composed of 10 % canola oil or a STD for a period of 6 weeks. The Japanese quail were subjected to both an oral and intravenous glucose tolerance test. These findings are in disagreement with previous studies which have shown that the consumption of dietary lipids rich in saturated and monounsaturated fatty acids, such as palm oil, lard and canola oil, causes an imbalance in the plasma concentration of plasma glucose and insulin in broiler chickens (Crespo and Esteve-Garcia, 2003; Newman et al., 2005).

Crespo and Esteve-Garcia (2003) observed significantly raised plasma insulin concentrations that were not accompanied by a depletion of plasma glucose in broilers fed diets supplemented with 10% tallow or olive oil compared to those fed diets supplemented with 10% sunflower or linseed oil. These results suggest the development of some degree of insulin resistance in the birds fed diets rich in saturated and monounsaturated fatty acids compared to those fed diets rich in polyunsaturated fatty acids (Crespo and Esteve-Garcia, 2003). The birds in this study were not subjected to any form of glucose tolerance test, thus the authors did not comment on their ability to handle an exogenous glucose load. However, given the role of avian insulin in promoting the uptake of glucose from the bloodstream (Scanes and Braun, 2013), one would expect the HFD-induced changes in the insulin/glucose balance and the development of insulin resistance in the birds fed the diets rich in saturated and monounsaturated fatty acids to have an effect on glucose tolerance.

Newman et al. (2005) subjected male broiler chickens to an intravenous glucose tolerance test, during which plasma glucose and insulin concentrations were measured, following 6 weeks of HFD feeding. The HFDs were composed of 80 g/kg of tallow (rich in saturated and monounsaturated fatty acids), fish oil (rich in polyunsaturated fatty acids) or sunflower oil (rich in polyunsaturated fatty acids). No significant differences in plasma glucose response to the glucose tolerance test were observed between the three different HFD groups; however the birds receiving the tallow had a markedly increased plasma insulin response. Thus, the birds

receiving the fish oil and sunflower oil required a much lower level of insulin to achieve the same response. The improved clearance of glucose from the blood stream in the birds receiving the diets rich in polyunsaturated fatty acids was attributed to a likely increase in the amount of polyunsaturated fatty acids within the tissue membranes of these birds (Newman et al., 2005). This in return results in an increase in the number of insulin receptor binding sites and an increase in the affinity of the insulin receptor for binding (Newman et al., 2005). These findings corroborate those of Crespo and Esteve-Garcia (2003), confirming the development of a degree of insulin resistance induced by the ingestion of saturated and monounsaturated fatty acids. Since the present study did not measure the insulin response of the birds following the oral glucose load, it is possible that we too may have observed alterations in the insulin response in the birds receiving the HFD. Neither of the above mentioned studies included a control group (receiving no additional lipids), so the authors did not comment on the differences observed in context of normal glucose regulation in broiler chickens. Given the HFD-induced changes in the insulin function in chickens observed in previous studies, the lack of any observable changes in glucose tolerance in the present study may suggest some inter-species differences in the regulation of lipid metabolism amongst avian species.

*Erythrocyte osmotic fragility*

The lack of HFD-induced changes in glucose tolerance is mirrored in the absence of any HFD-induced changes in the osmotic fragility of the avian erythrocytes assessed in the current study. In addition to altering the fatty acid composition of various tissue membranes and thus modifying insulin function, the fatty acid profile of the diet has also been shown to influence the composition of the erythrocyte membrane which in turn affects the fluidity of the membrane and its ability to resist osmotic lysis (Clandinin et al., 1991; Kempaiah and Srinivasan, 2006). In general, an increase in the polyunsaturated fatty acid content of the erythrocyte membrane reduces osmotic fragility, whereas an increase in the saturated fatty acid content of the membrane makes the membrane more rigid and thus fragile and susceptible to lysis (Raz and Livne, 1973; Wing-Keong et al., 2001; Sengupta and Gosch, 2011; Bazzano et al., 2015). The lack of any significant differences in erythrocyte osmotic fragility between birds on the HFD and STD in the current study suggests that consumption of the HFD (rich in saturated fatty acids) did not result in any significant modifications with respect to erythrocyte membrane fatty acid composition, which was indirectly assessed using erythrocyte osmotic fragility.

The influence of factors including age (Azeez et al., 2009; Azeez et al., 2011), sex (Azeez et al., 2011), water deprivation (Baloyi et al., 2006; Mafuvadze et al., 2008); transportation stress (Minka and Ayo, 2013), temperature (Sinkalu et al., 2015), blood storage conditions (Mafuvadze and Erlwanger, 2007) and dietary protein levels (Tuleun et al., 2013) on erythrocyte osmotic fragility have previously been investigated in a variety of avian species. There is a dearth of literature regarding the effects of the consumption of a HFD on avian erythrocyte osmotic fragility. Abou-Ashour and Edwards (1972) observed a slight increase in the fragility of erythrocytes from White Leghorn laying hens, which received a basal diet supplemented with graded concentrations of *Sterculia foetida* oil at 0.1%, 0.3% and 1% for a period of approximately 10 weeks, compared to those consuming only the basal diet. The increased fragility was attributed to changes in the physico-chemical composition of the erythrocyte membrane (Abou-Ashour and Edwards, 1972). *Sterculia foetida* oil contains cyclopropene fatty acids, the ingestion of which has been shown to result in disruption of normal fatty acid metabolism and the deposition of increasing amounts of saturated fatty acids within animal tissues (Evans et al., 1962; Abou-Ashour and Edwards, 1972). The increased fragility of the erythrocytes observed by Abou-Ashour and Edwards (1972) in the hens consuming *Sterculia foetida* oil is consistent with the presence of an increased amount of saturated fatty acids within the erythrocyte membrane, which would in turn make it rigid, less deformable and thus more susceptible to haemolysis. The results of the present study are not in agreement with these results. Even at a much higher level of dietary inclusion of lipids rich in saturated fatty acids, significant diet-induced changes in the fragility of the Guinea fowl and Muscovy ducks erythrocytes were not observed. It should be noted however, that the hens used by Abou-Ashour and Edwards (1972) were much older (28 weeks old) when commencing the study, compared to the birds in the current study (4 weeks old). Since age has been shown to influence the osmotic fragility of avian erythrocytes (Azeez et al., 2009; Azeez et al., 2011), it is possible that this may have played a role in the different results obtained.

Erythrocyte osmotic fragility in response to HFD feeding has been investigated in other species including fish, pigs and rats, with varying results. Results similar to that of the current study were obtained from a study by Wing-Keong et al. (2001), in which no significant

differences in osmotic fragility were observed in Tilapia fish (*Tilapia oreochromis*) erythrocytes (nucleated erythrocytes, like in birds) following a feeding trial with various dietary lipids, including palm oil, for a period of 8 weeks. Cools et al. (2011) also observed no significant alterations in erythrocyte osmotic fragility in pigs fed diets containing varying ratios of pork lard to fish oil, for a period of 9 days, despite changes in the erythrocyte membrane fatty acid profile. In contrast, Kempaiah and Srinivasan (2006), observed reduced erythrocyte osmotic fragility in rats (non-nucleated erythrocytes) fed a HFD composed of 30 % hydrogenated vegetable fat for a period of 8 weeks.

The lack of HFD-induced effects on erythrocyte osmotic fragility in the current study, as well as that observed in previous studies, may be due to some sort of adaptation to the HFD which occurs at the level of the small intestines. HFD feeding has been shown to result in various modifications with regards to the transport processes and metabolic activities of the enterocytes of the small intestine (Clandinin et al., 1991). Thus, the composition of the products of lipid digestion, to be absorbed into the blood stream and then delivered to various tissues, may be modified (Clandinin et al., 1991). So any changes in erythrocyte membrane fatty acid composition which may have been induced by alterations in dietary fatty acid profile, thus affecting fragility, may have been prevented. Additionally, previous studies have shown that the degree to which the fatty acid composition of various membranes throughout the body is influenced by the diet may be limited. Membranes, to some extent, are able to regulate the composition of fatty acids within the membrane phospholipids in order to circumvent any large changes in the properties of the membrane, including that of membrane fluidity (Kummerow, 1983). This may be the case in the present study. Taken together with the lack of HFD-induced changes in the body mass gain and glucose tolerance of the Guinea fowl and Muscovy ducks, the absence of any changes in erythrocyte osmotic fragility of the birds also supports the notion that there might be some inter-species differences with respect to the metabolism and subsequent membrane/tissue deposition of ingested dietary lipids.

*General health/clinical biochemistry profile*

The current study assessed the overall health status of the birds following consumption of the HFD, through the assessment of various serum metabolic health markers including serum lipid concentrations. No studies to our knowledge have examined serum metabolic health

markers and the serum lipid profile of alternative poultry species such as Guinea fowl and Muscovy ducks in response to HFD feeding of this nature. Dietary fat consumption can result in changes in the overall serum lipid profile (Monfaredi et al., 2011), which in turn is reflective of various steady-state metabolic regulatory processes (Mossab et al., 2002; Zhang et al., 2011a). In general, the consumption of saturated fatty acids results in increased concentrations of serum triglyceride and cholesterol compared to that of polyunsaturated fatty acids (Viveros et al., 2009; Velasco et al., 2010).

In the current study, the consumption of the HFD, rich in saturated fatty acids, had no significant effect on serum triglyceride concentrations in both the Guinea fowl and Muscovy ducks. However, serum cholesterol concentrations were significantly increased following HFD feeding in both the Guinea fowl and Muscovy ducks at most time points examined. The HFD-induced increase in serum cholesterol concentrations seemed to be well-tolerated by the birds as they remained healthy throughout the feeding period. Our results are in agreement with a study by Monfaredi et al. (2011), who also observed no significant differences in serum triglyceride concentrations between broiler chickens receiving beef tallow (rich in saturated and monounsaturated fatty acids) at either 20g/kg or 40/kg, compared to those on a control diet. The serum cholesterol concentrations of the broilers receiving the added dietary lipids were also significantly increased compared to the broilers on the control diet (Monfaredi et al., 2011). Celebi and Utlu (2006) however, observed significant increases in both serum cholesterol and triglyceride concentrations in laying hens supplemented with 4 % beef tallow compared to those not receiving additional lipids. Similarly, Ayala et al. (2009) also observed significant increases in both serum triglyceride and cholesterol concentrations in chickens, following the consumption of a diet enriched with 20 % cholesterol and 2 % palm oil (rich in saturated fatty acids). There seems to be some controversy with respect to the HFD-induced effects on serum triglyceride concentrations, which is probably due to possible differences in energy status of the birds (i.e. fed or fasted) as well as differences in the utilization of triglycerides as an energy source. Overall, the consumption of a diet rich in saturated fatty acids tends to result in increased serum cholesterol concentrations. The hypercholesterolemic effects of saturated fatty acids have been attributed to decreases in hepatic low-density lipoprotein receptor expression and activity, as well as increases in the production of low-density lipoproteins (Kuo et al., 1990; Tripodi et al., 1991; Mustad et al., 1996; Fenandez and West, 2005). Thus an overall increase in blood concentrations of low-

density lipoprotein cholesterol and reduced low-density lipoprotein cholesterol turnover is observed following consumption of lipids high in saturated fatty acids (Fenandez and West, 2005). Certain saturated fatty acids such as palmitic acid, myristic acid and lauric acid are more hypercholesterolemic than others (Fenandez and West, 2005), thus the fatty acid composition of the animal fats and vegetable oils added to poultry diets needs to be carefully considered, with regards to maintaining the overall health status of the birds during production.

In addition to evaluating the lipidaemic status of the Guinea fowl and Muscovy ducks in the present study, various other serum metabolic markers of health were also assessed. A significant decrease in serum calcium concentrations in Muscovy ducks following eight and twelve weeks of HFD feeding compared to that observed following four weeks of feeding was observed. Thus, HFD feeding for longer than four weeks resulted in significantly reduced serum calcium concentrations in the Muscovy ducks. This may be attributed to the formation of insoluble soaps within the gut lumen. Free fatty acids formed during the lipid digestion process are able to bind with dietary calcium to form insoluble soap complexes, which in turn make the absorption of the fatty acids and calcium ions impossible (Atteh and Leeson, 1983). Previous studies have indicated that the formation of insoluble soaps is more likely in the case of diets rich in saturated fatty acids, such as the HFD used in the present study, versus those rich in unsaturated fatty acids (Atteh and Leeson, 1983; Atteh and Leeson, 1984; Lin and Chiang, 2010). Since no significant HFD-induced changes were observed in the majority of the serum parameters measured in both the Guinea fowl and the Muscovy, it seems the birds remained healthy throughout the duration of the HFD feeding trial, with no obvious disturbances in serum health markers.

Similar results have been obtained from previous studies involving the effects of supplementation of oils/fats, such as those used in the present study, on various serum health markers in birds. Burlikowska et al. (2010) observed no significant differences in serum total protein, albumin and uric acid concentrations in male broiler chickens receiving either soyabean oil, a vegetable lipid concentrate or lard. No control group (not receiving any additional lipids) was included in the above mentioned study, but the authors did state that the serum parameters assessed fell within the range of reference values for chickens (Burlikowska

et al., 2010). Similarly, Febel et al. (2008) observed no significant differences in serum concentrations of uric acid and AST in male broiler chickens supplemented with 6 % lard compared to those receiving 6 % sunflower oil or soyabean oil, for a period of 5 weeks. Ayala et al. (2009) also observed no significant differences in plasma AST concentrations in male, White Leghorn chickens supplemented with 20 % palm oil and 2 % lard compared to those receiving the STD.

The birds assessed in the present study, as well as those investigated in previous studies, seemed to handle the respective HFDs relatively well with no obvious adverse health disturbances observed.

*Organ masses and lengths*

With regards to the diet-induced effects on the absolute and relative organ masses and lengths of the small and large intestines assessed in the birds, no clear trends were observed in both the Guinea fowl and Muscovy ducks. Previous studies involving dietary lipid supplementation and relative organ masses and lengths have yielded conflicting results. Peebles et al. (1997) observed no diet-induced effects on the relative liver, pancreas and gallbladder mass of broiler chickens fed diets supplemented with either 3 % or 7 % lard (high in saturated fatty acids). Poorghasemi et al. (2013) observed a significantly increased relative pancreas mass in broiler chickens fed 4 % tallow (high in saturated fatty acids) and a significantly reduced relative proventriculus mass in broiler chickens fed 2 % tallow + 2 % sunflower oil (high in polyunsaturated fatty acids) compared to those receiving either 4 % canola oil (high in monounsaturated fatty acids), 4 % sunflower oil or 2 % tallow + 2 % canola oil. Mohammed and Horniakova, (2012) observed no significant differences in relative liver and heart mass in male chickens receiving varying levels and combinations of animal packed fat (high in saturated fatty acids), rapeseed oil or sunflower oil. Relative bowel (combined small and large intestines) mass was significantly increased in the group receiving 2.5 % animal packed fat with 2.5 % sunflower oil and significantly decreased in the group receiving 2.5 % animal packed fat with 2.5 % rapeseed oil, compared to those receiving either only 5 % animal packed fat or 2.5 % animal packed fat with 1.25 % rapeseed oil and 1.25 % sunflower oil (Mohammed and Horniakova, 2012). No clear trends have been observed in the available

literature or in the current study, with regards to the effects of HFD feeding on relative organ mass. Thus, further investigation into the resulting effects is required.

*Liver lipid deposition*

The deposition of lipids within animal body tissues is the net result of the absorption of dietary fatty acids, the oxidation of fatty acids for energy and *de novo* fatty acid synthesis (Smink et al., 2010). The dietary fatty acid profile has been shown to affect the resulting lipid deposition within the various body tissues. Diets high in saturated fatty acids generally result in increased lipid accumulation, whereas the consumption of polyunsaturated fatty acids tends to reduce lipid deposition (Crespo and Esteve-Garcia, 2002a; Smink et al., 2010; Velasco et al., 2010). Liver lipid content was only analysed using liver samples, from half of the Guinea fowl and half of the Muscovy ducks, after four and eight weeks of either STD or HFD feeding, as a result of the loss of some of the liver samples due to a faulty refrigerator. I was unable to repeat the study with another batch of birds due to problems with the supplier, in that he was unable to supply me with more birds, specifically Muscovy ducks, from the same source. Since the environment from which the birds were sourced can have an influence on various parameters, including the gut microbiota profile and thus overall metabolic function (Torok et al., 2011; Apajalahti et al., 2012), I decided against repeating the liver lipid measurements with a new batch of birds from a different source. This was one of the limitations of the current study. No significant differences in liver lipid content were observed in the current study between birds on the HFD and those on the STD. Our results are in agreement with those of Magubane et al. (2013) who also observed no HFD-induced effects on liver lipid content in Japanese quail supplemented with 10 % canola oil (rich in monounsaturated fatty acids) for a period of 7 weeks. Majority of the previous studies involving dietary lipid supplementation and the resulting liver lipid deposition in birds have been reported in chickens. Additionally, very often these studies do not have a control group (not receiving any additional fat) which makes comparing their results to the results of the current study somewhat difficult. Nevertheless, the consumption of the various HFDs have been shown to have an effect on the total amount of lipid deposited within the liver tissue, which is in contrast to the results of the current study.

An et al. (1997) observed significantly reduced liver lipid content in growing chicks fed diets supplemented with oils rich in n-3 polyunsaturated fatty acids compared to those receiving n-6 polyunsaturated and saturated fatty acids. Velasco et al. (2010) observed significantly increased hepatic triacylglycerol content in female broiler chicks receiving a diet supplemented with 90g/kg of palm oil (rich in saturated fatty acids), compared to those receiving 90g/kg of sunflower oil (rich in polyunsaturated fatty acids). Smink et al. (2010) observed a trend for reduced liver lipid content in female broilers receiving diets containing either 4 % or 8 % sunflower oil compared to those receiving palm oil, although the results were not statistically significant. Pinchasov and Nir (1992) observed reduced liver lipid content in male broiler chickens with increasing levels of inclusion of polyunsaturated fatty acids. The effects of supplementation with polyunsaturated fatty acids on lowering liver lipid deposition have been attributed either to an increase in the oxidation of fatty acids or a reduction in hepatic lipogenesis, or a combination of both (Pinchasov and Nir, 1992; Smink et al., 2010; Velasco et al., 2010).

The HFD used in the current study was composed of lipids rich in saturated fatty acids. Thus, based on the results of previous studies involving dietary supplementation with lipids of varying fatty acid profiles, one would expect to observe an increase in total liver lipid deposition in the birds consuming the HFD compared to those receiving the STD, which was not the case. The lack of HFD -induced differences in liver lipid deposition, in both the Guinea fowl and Muscovy ducks in the present study, are in contrast to that observed in previous studies, performed using predominantly chickens. This may suggest some inter-avian species differences with respect to lipid metabolism. Genetic differences in lipid metabolism between different avian species and even between different breeds of the same avian species have been observed in studies involving the induction of liver lipid accumulation by overfeeding. Hermier et al. (1991) observed differences in the susceptibility of two different breeds of geese, the Landes breed and the Rhine breed, to the development of hepatic steatosis (accumulation of lipids within the liver). The increased susceptibility of the Landes goose to the development of hepatic steatosis when overfed was ascribed to the reduced capacity of this breed for the export of VLDLs from the liver, thus hepatic lipid accumulation was favoured (Hermier et al., 1991). Fournier at al., (1997) observed a hyporesponsiveness to the induction of hepatic steatosis following overfeeding in the Poland goose compared to the Landes goose. The Poland goose was said to respond to the

overfeeding with greater exportation of all liver lipids, thus resulting in increased extrahepatic fattening as opposed to hepatic steatosis (Fournier at al., 1997). Hermier et al. (2003) also observed inter-species variation in the susceptibility of two different breeds of ducks to liver lipid accumulation. The Muscovy ducks displayed a higher degree of hepatic steatosis following overfeeding compared to the common ducks, which was attributed to less efficient channelling of liver lipids towards secretion via VLDLs and thus less storage of lipids in extrahepatic tissues (Hermier et al., 2003). Since differences in hepatic lipid metabolism following overfeeding have previously been observed between different avian species/breeds, it is possible that core differences exist in the processing of dietary lipids and their subsequent fate following HFD feeding as well. This could explain the inconsistencies observed between the liver lipid deposition in the birds used in the present study and those reported on in previous studies.

*Caeca microbiota composition*

In addition to investigating the effects of a diet rich in saturated fatty acids on the liver lipid deposition of the Guinea fowl and Muscovy ducks, the HFD-induced effects on the composition of the caeca microbiota of the birds was also investigated. The microbiota within the caeca of poultry is the most extensively investigated microbiome within the poultry gastrointestinal tract (Pan and Yu, 2014). The blind-ended pouches which constitute the caeca provide the ideal environment for bacterial growth and thus contain a very diverse microbiome (Pan and Yu, 2014). Diet-induced alterations in the composition of gut microbiota within production animals, including poultry, have a profound influence on animal nutrition and overall health status (O'Hara and Shanahan, 2006).

Alterations in diet composition have been shown to influence gut microbiota balance. In the present study, due to various technical difficulties with regards to primer specificity, we were unable to obtain any conclusive results with respect to the effect of the HFD on the caeca gut microbiota composition of the birds. Of the bacterial species investigated, only the primers for *Campylobacter* (gram-negative bacteria) and *Bifidobacterium* (gram-positive bacteria) showed specific binding, resulting in the presence of the expected PCR product within the agarose gels. With regards to the Guinea fowl caecal content there seemed to be a trend for an increase in the amount of *Campylobacter* and *Bifidobacterium* product observed, with

increasing length of feeding period, in both the STD and HFD groups. The increased presence of PCR product with time in the case of *Campylobacter* seemed to be more prominent in the HFD groups. Analysis of the presence or absence of *Campylobacter* and *Bifidobacterium* PCR products in the Muscovy ducks caecal content yielded inconclusive results, with no clear pattern of presence observed in either the STD or HFD groups.

Most previous studies involving modulation of the gut microbiota in poultry have been performed in chickens and have focussed on the effects of various non-starch polysaccharides, present in the cereals used to formulate poultry diets (Yegani and Korver, 2008), on the composition of the gut microbiota. Very few studies have investigated the modulation of the poultry gut microbiota following ingestion of various different lipid sources which are commonly added to poultry diets as a concentrated energy source. Geier et al. (2009) observed no significant changes in overall microbial communities present in the ileum and cecum of broiler chickens, following dietary supplementation with fish oil, together with starch. Zhang et al. (2011b) also observed no significant differences in the populations of various bacteria in the caecal content of broiler chickens fed either soyabean oil or fish oil, both rich in polyunsaturated fatty acids. The lack of diet-induced changes in the caecal bacterial populations were attributed to the possible masking of the lipid source effects by the high lipid content in the maize, which was used as the cereal base of the diets ingested by the birds (Zhang et al., 2011b). The consumption of diets rich in saturated fatty acids, by mammals; results in an overflow of dietary fat to distal parts of the gastrointestinal tract, which in turn elicits changes in the composition of the gut microbiota and alterations in overall lipid metabolism (De wit et al., 2012). The effects of saturated fatty acids on the gut microbiota of birds have not been well studied and their ability to modify gut microbiota balance is inconclusive.

Van der Hoeven-Hangoor et al. (2013) found that a diet rich in medium-chain saturated fatty acids inhibited the presence of various gram-positive bacteria, whilst promoting the growth of various gram-negative bacteria in the ileum of broiler chickens. The resulting change in microbiota balance was associated with improved feed utilization efficiency within the birds (Van der Hoeven-Hangoor et al., 2013). Contradictory to these results, Zeitz et al. (2015) observed no significant change in the DNA copy numbers of various gram-positive and gram-

negative bacteria in broilers supplemented with medium-chain saturated fatty acids. The authors hypothesized that the medium-chain fatty acid-induced effect may have been present shortly after the birds began the lipid supplementation but it was short-lived and thus not observed at the end of the feeding period (Zeitz et al., 2015).

Besides diet alterations which are the primary cause for the modulation of poultry gut microbiota composition, substantial changes in the composition of the gut microbiome and thus its function take place as the birds age (Oakley et al., 2014). The trend for an increase in the amount of *Campylobacter* and *Bifidobacterium* PCR products with time observed in the Guinea fowl of the current study, could suggest a developmental age effect on overall microbiota composition. The importance of the exploration of the progressive changes (Oakley et al., 2014), as well as the diet-induced changes (Geier et al., 2009; Van der Hoeven-Hangoor et al., 2013) in the gut microbiota composition of poultry have been highlighted previously. Since lipids are important energy sources commonly used in the formulation of poultry diets (Zhang et al., 2011a) it is imperative that more in-depth investigations are carried out into the resultant changes in poultry gut microbiota composition and how these changes affect poultry performance in terms of production. These studies need to be extended to include alternative poultry species as well, so that appropriate, species-specific feeding regimes can be developed which promote the development of a beneficial balance of gut microbiota, which in turn will contribute to the maintenance of a good health status in the birds during production.

## 2.5 Conclusion

In conclusion, the results of this study show that the HFD, rich in saturated fatty acids, was well-tolerated by both the Guinea fowl and Muscovy ducks, with no obvious adverse health effects observed. Thus, the use of such diets in a poultry production setting would in fact be safe in terms of maintaining the overall health status of these alternative poultry species during production. However, the lack of any positive HFD-induced effects on growth performance of the birds highlights the fact that it might not be necessary to supplement the diets of Guinea fowl and Muscovy ducks with added dietary fat during production. Nevertheless, since feed intake and feed conversion efficiency was not assessed in the current study, I cannot conclude whether the supplementation of additional dietary fat would reduce

feed intake and thus improve feed conversion efficiency, which would have an implication on overall poultry production costs. Further studies into the use of HFDs in alternative poultry species are required in order to establish the physiological mechanisms behind the resistance of these alterative poultry species to the metabolic effects of HFD.

Following on from the first study, a study to investigate the parameters that were assessed in the Guinea fowl and Muscovy ducks, in another alternative poultry species which is also becoming popular as a table bird, the Japanese quail was carried out. Both Guinea fowl and Muscovy ducks are seasonally reproducing birds, whereas the Japanese quail breeds all year around and thus the quail were also a lot easier to acquire. For the second study I decided to feed the quail a broader variety of HFDs, with varying saturated and unsaturated fatty acid content, for a period of 12 weeks. Since the Guinea fowl and Muscovy ducks proved to be resistant to the adverse effects of the saturated fatty acids contained within the HFD, I thought it would be interesting to investigate whether this resilience is conserved within the Japanese quail. In addition, I also wanted to investigate whether the beneficial effects of polyunsaturated fatty acids with respect to the lipidaemic status and tissue lipid deposition which have been observed in previous studies (mainly in domestic chickens), would be evident in this alternative poultry species. The resulting fatty acid profiles of the liver, breast and thigh muscle tissue of the Japanese quail were assessed to confirm the successful transfer of the dietary fatty acids into the edible bird tissues. Despite my unsuccessful attempt at assessing the gut microbiota profile of the Guinea fowl and Muscovy ducks, I had planned to assess the diet-induced alterations in the quail gut microbiota profile, however I wasn't able to sample a sufficient amount of caecal content in the quails.

# CHAPTER THREE: THE EFFECTS OF DIFFERENT HIGH-FAT DIETS ON GROWTH PERFORMANCE, GLUCOSE TOLERANCE, ERYTHROCYTE OSMOTIC FRAGILITY, SERUM BIOCHEMISTRY AND THE LIPID CONTENT AND FATTY ACID PROFILE OF THE LIVER, BREAST AND THIGH MUSCLES OF MALE, JAPANESE QUAIL.

### 3.1 Introduction

As outlined in section 1.8, the main objective of Study 2 was to investigate the metabolic effects of different HFDs, based on coconut oil, lard, palm oil, soyabean oil or sunflower oil, on the overall health status as well as the bone health and overall lipid content and fatty acid profiles of the edible tissues (liver, breast and thigh muscles) of male, Japanese quail (*Coturnix coturnix japonica*).

The Japanese quail is another alternative poultry species which is becoming popular as a table bird is the Japanese quail. The Japanese quail also offers a number of advantages in terms of poultry production compared to the domestic chicken. The Japanese quail is a fairly small bird and requires minimal housing space (Haruna et al., 1997) and has lower feed requirements compared to that of chickens, thus reducing overall production costs (Owen and Dike, 2013). They also reach sexual maturity at a relatively early age and thus have short generation intervals, allowing for the production of many generations of quail throughout the year (Afrose et al., 2011; Owen and Dike, 2013). Quail hens can lay approximately 340-350 eggs in a period of between 1-2 years (Afrose et al., 2011). As previously mentioned in section 2.1 of this thesis, it is common practice for animal and vegetable fats to be added to diets formulated for poultry as a means to improve their growth performance and feed efficiency (Kratzer et al., 1994; Nobakht, 2012; Sahito et al., 2012). Additional lipids are also commonly added to poultry diets in order to modify the fatty acid profile of the edible bird tissues, making them more acceptable to health conscious consumers (Pisulewski, 2005; Woods and Fearon, 2009; Uchewa, 2013).

The consumption of increased quantities of polyunsaturated fatty acids by humans, specifically omega-3 polyunsaturated fatty acids, has been associated with reduced risk of development of a number of chronic diseases including cardiovascular disease (Pisulewski, 2005; Milićević et al., 2014). There has been increased consumer awareness regarding the health risks associated with the consumption of various foods (Fresco, 2009), thus the development of "functional foods" for the benefit of health conscious consumers has become quite popular (Nantapo et al., 2015). Poultry meat enriched with beneficial polyunsaturated fatty acids is one such food (Woods and Fearon, 2009). Vegetable fats rich in polyunsaturated fatty acids are also commonly used as lipid sources to be added to poultry diets.

In the current study five HFDs, composed of different lipid sources at 22 % of the mass of the feed, that is: palm oil, lard, coconut oil, soyabean oil and sunflower oil. So in addition to the palm oil and lard which were used to formulate the HFD used in study 1, coconut oil which is another oil rich in saturated fatty acids was used. Enrichment of meat products with coconut oil ensures a relatively long product shelf-life, due to the resistance of coconut oil to lipid peroxidation/oxidative rancidity (Guarte et al., 1996; Aderolu and Akinremi, 2009; Luo et al., 2014). Also included were two vegetable oil sources namely, soyabean oil and sunflower oil, which despite their unfavourable omega-3: omega-6 polyunsaturated fatty acid ratio, are still commonly used in the preparation of HFDs for poultry. The use of soyabean and sunflower oil is still popular in the formulation of HFDs for poultry as a result of their high digestibility and metabolisable energy content, which can be attributed to the very high content of unsaturated fatty acids present in the oils (Huyghebaert et al., 1988; Dei, 2011; USDA, 2015).

As previously mentioned, the consumption of HFDs has been shown to result in the development of various metabolic disorders in humans and other mammals (Roche, 2005; Melanson et al., 2009; Tierney et al., 2011). Very few studies concerning HFD-feeding have been performed using birds and those which have been performed typically involve the domestic chicken and are focussed on improving meat quality by modification of the fatty acid profile of the edible bird tissues (Crespo and Esteve-Garcia 2001; Velasco et al. 2010). There is a dearth of information concerning the physiological responses and metabolic effects of HFDs, commonly formulated for poultry, on the overall health status of alternative poultry species such as the Japanese quail. Thus, the current study was designed to investigate the possible HFD-induced effects on the overall health status of the Japanese quail through the assessment of serum biochemistry, glucose tolerance, erythrocyte osmotic fragility and bone health of the birds, whilst at the same time assessing the overall lipid content and fatty acid profiles of the edible bird tissues (liver, breast and thigh muscles).

## 3.2 Materials and Methods

### 3.2.1 Ethical approval

Ethical approval for the study was granted by the University of the Witwatersrand Animal Ethics Screening Committee (Ethics clearance number: 2013/02/05).

### 3.2.2 Animals, housing and diets

Fifty-seven, four-week old, male Japanese, jumbo quail (*Coturnix coturnix japonica*) (150.25 g ± 17.73), obtained from a commercial supplier (SA Quail breeders, Rockliff Farm, East London, South Africa) were used in the study. The birds were randomly divided into six groups (with a minimum of n = 8 birds in each group) and allocated to either a STD group which received standard, commercial poultry feed (Epol®, Centurion, South Africa) or to one of five HFD groups which received the commercial poultry feed enriched with either coconut oil (Organic Cold-pressed Virgin coconut oil, Dis-Chem Pharmacies, Glen Austin, South Africa), lard (Norbert's German butchery, Wilropark, Roodepoort, South Africa), palm oil (SupaCrisp, Super Olein Palm Oil, Felda Bridge Africa (PTY) Ltd, Johannesburg, South Africa), soyabean oil (d'lite, Willowton Group, Pietermaritzburg, South Africa) or sunflower oil (Sunfoil, Willowton Group, Pietermaritzburg, South Africa) at 22 % of the mass of the feed (22 % of the total diet was composed of fat, on a weight/weight basis). The birds were fed their respective diets *ad libitum*, with free access to water for the duration of the experimental period. All birds were housed individually in cages, with straw for bedding, in the animal unit of the Central Animal Service, Faculty of Health Sciences, University of the Witwatersrand. Lighting was restricted to 12 hours in each 24 hour period, lights on from 06:00. To provide environmental enrichment of the bird cages, the birds were provided with perching logs.

### 3.2.3 General experimental procedures

Following a two-week adaptation period, to familiarise the birds with the housing, handling and feeding conditions, the experimental period commenced and continued for twelve weeks, during which the birds were fed their respective diets. Body mass was measured twice weekly. Following the twelve week feeding period, the birds were fasted for approximately 15 hours overnight and then subjected to an oral glucose tolerance test (OGTT). Details of the

OGTT are presented below. Following the OGTT, the birds were re-fed and allowed a 72 hr recovery period, after which they were euthanized using an anaesthetic overdose of Sodium Pentobarbital (Eutha-naze, Centaur Labs, South Africa) (200 $mg.kg^{-1}$), administered via the wing vein. Blood samples were collected by cardiac puncture (using 20 G hypodermic needles) into vacutainer tubes (Vacuette, Greiner Bio-One, Thailand) containing either lithium heparin (for osmotic fragility determinations) or into serum separator, clot activator vacutainer tubes (Vacuette, Greiner Bio-One, Thailand) (for serum determinations), and mixed gently. The livers were excised and excess connective tissue was removed. The livers were rinsed in ice-cold saline and blotted dry, before they were weighed. Samples of the liver tissue were then stored (-70°C) until further analysis (total lipid content, fatty acid profile and histology). Samples of breast and thigh muscle tissue were cut from each whole muscle and stored (-70°C) until further analysis (total lipid content and fatty acid profile). The pancreas, proventriculus, ventriculus, small intestine, large intestine, caecum, abdominal fat pad and testes were also excised, rinsed in ice-cold saline, blotted dry and weighed (all organ masses are represented as both absolute organ mass and relative organ mass (as a percentage of body mass)). The lengths of the small and large intestine were also measured (all small and large intestine lengths are represented as both absolute length and relative length (as unit length per unit body mass)). The left leg from each bird was excised and the femur was isolated, trimmed of surrounding soft tissue and stored until further analysis.

### 3.2.4 Specific experimental procedures

#### i) Diet proximate analysis and fatty acid profile of added fat

Samples of each of the six diets were collected at the time of each feed preparation and then pooled (per diet) once the feeding trial was completed. Samples were then mixed/milled and sent to the Agricultural Research Council's Irene Analytical Services Laboratories, South Africa (ARC laboratory) for analysis. Proximate components, that is, crude protein and fat content were determined as outlined by the Official Methods of Analysis of Analytical Chemists (AOAC) (2005: method numbers 954.01 and 920.39, respectively). Gross energy content (MJ/kg) of each diet was determined using an MC-1000 Modular Calorimeter (Energy Instrumentation, 135 Knoppieslaagte, Centurion, South Africa), according to manufacturer's instructions. Samples of each of the five added dietary fat sources (coconut

oil, palm oil, lard, soyabean oil and sunflower oil) were collected and sent to the ARC laboratory for total fat and fatty acid profile analyses.

ii) Body mass measurements

The birds were weighed twice weekly by placing them in pre-weighed cages on a scale (Presica 310 M, Laser, Johannesburg, South Africa) and weight gain was calculated using the formula below:

$$\frac{\text{(Final body mass – Initial body mass)}}{\text{Initial body mass}} \times 100$$

Feed intake and feed conversion ratio/feed efficiency form part of the indices which are usually assessed as part of growth performance, however these are not taken into consideration in this study.

iii) Oral glucose tolerance tests (OGTTs)

After the twelve week feeding period, following an overnight fast, the birds were subjected to an OGTT, as described previously in section 2.2.4 ii) of this thesis. Glucose tolerance curves for birds in each dietary group were then constructed. Baseline glucose, peak glucose and glucose concentrations three hours following administration of the glucose load, were then determined. Area under the glucose curve (AUC) was also determined.

iv) Blood parameters

*a) Erythrocyte osmotic fragility*

The osmotic fragility determinations were carried out using methods modified from those described by Suess et al. (1948), Oyewale and Durotoye (1988), Adenkola et al. (2010) and Moyo et al. (2012). The methodology is described briefly in section 2.4.2 iii) of this thesis.

*b) General health/clinical biochemistry profile*

The blood samples collected into serum separator, clot activator vacutainer tubes were centrifuged (Sorvall RT 6000 B, Du Pont, United kingdom) at 370 g and 22˚ C for 15 min. The serum was then collected and serum uric acid, total protein, albumin, AST, total bilirubin, calcium, cholesterol and triglyceride concentrations were determined using an IDEXX Vetlab Analysis Machine (IDEXX Laboratories, Westbrook, Maine), according to manufacturer's instructions.

v) Liver parameters

*a) Liver lipid content and fatty acid profile*

Samples of liver tissue were collected from each bird, in each specific dietary group. The samples from all birds in a specific dietary group were pooled and sent as a composite sample (representative sample from each group) to the ARC laboratory for total fat and fatty acid profile analyses. Prior to the total fat content and fatty acid profile analyses the liver samples from each of the dietary groups were freeze dried and then milled together, such that the analyses were carried out on only one (composite) sample per dietary group.

Total fat content was extracted from both the added dietary fat samples and the tissue samples by the Soxhlet method as described by AOAC (2005; method number 920.39). For the fatty acid profile analyses, methyl esters for capillary gas chromatography were prepared according to methods described by Christopherson & Glass (1969). Briefly, the fat extracts were transmethylated with 2 M methanol–sodium hydroxide. The resulting fatty acid methyl esters were extracted in heptane, filtered and dried under nitrogen. The fatty acids were separated by a temperature gradient over 45 min on a gas chromatograph, with nitrogen as a carrier gas on a DB-23 capillary column (90 cm x 250 μm x 0.25 μm) (Supelco; Sigma-Aldrich). The gas chromatograph consisted of a HP6890 GC (Hewlett Packard, Bristol, UK) with flame ionisation detector (FID). Both detector and injector temperatures were set at 300°C. A computer equipped with Chemistation software was used for quantification of the fatty acids (Chemistations Deutschland GmbH, Augustastrasse, Wesel, Germany) and nonadecanoic acid (C19:0) was used as the internal standard.

*b) Liver histology*

Liver samples for histology were prepared according to the methodology described previously in section 2.4.2 iv) b) of this thesis.

vi) Breast and thigh muscle parameters

*a) Muscle lipid content and fatty acid profile*

Sections of breast muscle tissue and sections of thigh muscle tissue were collected from each bird, in each specific dietary group. The breast muscle samples from all birds in a specific dietary group were pooled and sent as a composite sample (representative sample from each group) to the ARC laboratory for total fat and fatty acid profile analyses. The procedure was repeated for the thigh muscle samples. Prior to the total fat content and fatty acid profile analyses the breast and thigh muscle samples from each of the dietary groups were freeze dried and then milled together, such that the analyses were carried out on only one (composite) sample per dietary group.

vii) Femur mass, length and relative density (modified Seedor index)

Following removal of the left femora from each bird carcass the bones were defleshed and then dried in an oven (Salvis ®, Salvis Lab, Switzerland) at 50°C, for approximately 3-4 days. The bones were then weighed and the length of the femur was measured from the medial condyle to the greater trochanter, using a pair of vernier calipers (Hi-impact, Dejuca, South Africa). A modification of the 'Seedor index' (Seedor et al., 1991, Almeida et al., 2008) was used as an estimation of bone density, calculated using the following formula:

Seedor index = mass of bone (mg)/length of bone (mm)

Relative bone density was then further subjectively assessed by performing radiographs of the bones, using a Fuji film X-ray machine (Industrial X-ray film FR, Fuji Photo Film Co, Ltd, Tokyo, Japan). The bones were placed on the photographic plate at a distance of 1 metre away from the X-ray light source with settings of 4.8 kVp, 0.71 mA per plate was used.

### 3.2.5 Statistical analysis

All data are expressed as the mean ± SD, unless otherwise stated. The data were analysed and plotted using Graphpad 5 Prism software (Graph-pad Software Inc, San Diego, USA). $p < 0.05$ was considered significant. For all analyses on body mass, percentage body mass gain, oral glucose tolerance test, liver mass and osmotic fragility, quail numbers per group are as follows: n = 10 (soyabean oil and palm oil groups); n = 9 (sunflower oil and STD groups); n = 11 (coconut oil group); n = 8 (lard group). For all analyses on serum biochemistry parameters: n = 10 (soybean oil, palm oil and coconut oil groups); n = 9 (sunflower oil and STD groups); n = 7 (lard group).

*Diet proximate and mineral composition*: A one-way ANOVA with a Bonferroni post-hoc test was used to compare the proximate and mineral components of each diet, between the six diets used in the present study. n = 2 for each proximate/mineral component within each diet, as the assays were performed in duplicate.

*Body mass*: A one-way ANOVA with a Bonferroni post-hoc test was used to compare the body mass of the quail between the different dietary groups at the start of the experimental procedure (initial body mass) and after the twelve-week feeding period (final body mass), as well as the differences in percentage body mass gain. Differences between initial and final body mass of the birds within each specific dietary group was assessed using a paired Student's t-test.

*Oral glucose tolerance tests (OGTT)*: Differences in blood glucose values measured at fixed time intervals following administration of the glucose load, within a single dietary group, following the twelve-week feeding period, were analysed using a repeated measures ANOVA with a Bonferroni post-hoc test. Differences in baseline blood glucose concentrations, peak blood glucose concentrations, blood glucose concentrations two hours following administration of the glucose load and area under the glucose curve, between birds in the different dietary groups were analysed using a one-way ANOVA with a Bonferroni post-hoc test.

*Osmotic fragility*: The mean ± SD for the data concerning the percentage haemoglobin released from erythrocytes upon haemolysis, for each PBS solution, for each dietary group (STD group and the various HFD groups) was calculated and used to construct the fragiligrams. The range of PBS solutions at which initial haemolysis (IH), 50% haemolysis (MCF) and maximal haemolysis (MH) of the erythrocytes occurred was then read off the graphs.

*General health/clinical biochemistry profile and organ masses (intestine lengths):* Differences in serum parameters, organ masses (absolute and relative) and intestine lengths between birds in the various dietary groups were analysed using a one-way ANOVA with a Bonferroni post-hoc test.

*Liver and muscle (breast and thigh) lipid content and fatty acid profile:* The data concerning the fatty acid profile and total lipid content of the tissue samples (liver and breast and thigh muscles) are presented as percentage of total fatty acids and percentage of tissue sample, respectively. Each assay was performed only once on the composite sample from each of the dietary groups.

*Femur mass, length and relative density:* Differences in the mass, length and relative bone density of the femora between birds in various dietary groups were analysed using a one-way ANOVA with a Bonferroni post-hoc test.

The statistical model used for the one-way ANOVA was as follows:

**One-way ANOVA:**

$$y_{ij} = \mu + \alpha_i + e_{ij}$$

$y_{ij}$ = dependent variable of interest [diet proximate components, body mass, glucose tolerance test parameters, serum parameters, organ masses (absolute and relative), intestine lengths and femur mass, length and relative bone density]

$\mu$ = overall mean effect

$\alpha_i$ = the $i^{th}$ fixed effect of the dietary treatments (6 dietary treatments)

$e_{ij}$ = random residual error

## 3.3 Results

### 3.3.1 Diet proximate analysis and fatty acid profile of added fat sources

Results from the proximate content and energy analyses of both the STD and the five HFDs are shown in Table 3.1. No significant differences ($p > 0.05$, ANOVA) in crude protein, fibre and phosphorus content were observed between the diets. Crude protein content ranged between 11.66 ± 0.34 % and 16.03 ± 0.51 % of total diet composition. All HFDs had a significantly higher ($p < 0.0001$, ANOVA) fat content (ranging between 22.28 ± 0.70 % and 26.13 ± 0.39 % of total diet composition) compared to the STD (3.66 ± 0.08 % of total diet composition). Dry matter content of the various diets differed by < 1%. The SuO diet had a significantly higher ($p < 0.05$, ANOVA) ash content compared to that of the STD. All HFDs had a significantly higher ($p < 0.001$, ANOVA) calcium content compared to that observed in the STD. The HFDs composed of coconut oil (CO), palm oil (PO), soyabean oil (SO) and sunflower oil (SuO) had a significantly higher ($p < 0.05$, ANOVA) energy content (ranging between 19.51 ± 1.03 $MJ.kg^{-1}$ and 20.87 ± 1.42 $MJ.kg^{-1}$) compared to that of the STD (15.85 ± 0.03 $MJ.kg^{-1}$). The energy content of the HFD composed of lard (19.51 ± 1.03 $MJ.kg^{-1}$) was not significantly different ($p > 0.05$, ANOVA) from that of the STD.

**Table 3. 1** Added dietary fat inclusion and proximate content of the standard and high-fat diets fed to male, Japanese quail for 12 weeks.

| Proximate component (%) | STD | CO-diet | L-diet | PO-diet | SO-diet | SuO-diet |
|---|---|---|---|---|---|---|
| Dry matter | 90.21 ± 0.02[a] | 91.54 ± 0.13[a,b] | 91.26 ± 0.75[a,b] | 91.12 ± 0.08[a,b] | 91.85 ± 0.07[b] | 91.74 ± 0.20[b] |
| Moisture | 9.80 ± 0.02[a] | 8.47 ± 0.13[a,b] | 8.74 ± 0.75[a,b] | 8.88 ± 0.08[a,b] | 8.15 ± 0.07[b] | 8.26 ± 0.20[b] |
| Ash | 5.06 ± 0.13[a] | 7.59 ± 0.18[a,b] | 6.44 ± 0.49[a,b] | 6.52 ± 0.17[a,b] | 6.77 ± 1.15[a,b] | 8.49 ± 0.69[b] |
| Protein | 16.03 ± 0.51 | 11.66 ± 0.34 | 14.91 ± 2.64 | 14.61 ± 0.47 | 11.71 ± 0.15 | 11.90 ± 0.13 |
| Fat ^ | 3.66 ± 0.08[a] | 23.28 ± 0.79[b] | 22.44 ± 1.07[b] | 24.39 ± 0.49[b] | 26.13 ± 0.39[b] | 22.28 ± 0.70[b] |
| Fibre | 4.49 ± 0.24 | 3.91 ± 0.24 | 4.31 ± 0.08 | 5.36 ± 1.17 | 4.77 ± 0.58 | 3.66 ± 0.17 |
| Calcium | 0.52 ± 0.01[a] | 1.51 ± 0.01[b] | 1.21 ± 0.01[b] | 1.14 ± 0.01[b] | 0.96 ± 0.04[b] | 1.69 ± 0.03[b] |
| Phosphorus | 0.55 ± 0.00 | 0.39 ± 0.03 | 0.40 ± 0.01 | 0.46 ± 0.10 | 0.45 ± 0.04 | 0.44 ± 0.01 |
| **Energy (MJ.kg$^{-1}$)** | 15.85 ± 0.03[a] | 19.68 ± 0.28[b] | 19.51 ± 1.03[a,b] | 20.87 ± 1.42[b] | 20.02 ± 0.76[b] | 20.45 ± 0.20[b] |

Proximate component data presented as mean ± SD, n = 2 composite samples of each diet. Different letters indicate significant differences when compared to STD group. STD = standard diet; CO = coconut oil; L = lard; PO = palm oil; SO = soybean oil; SuO = sunflower oil. $ $p < 0.0001$ when various dietary groups compared to STD (one-way ANOVA); * $p < 0.05$ when various dietary groups compared to STD (one-way ANOVA). ^ Fat was added at 22 % on a *weight/weight* basis.

Table 3.2 shows the results of the fatty acid profile analysis (fatty acids represented as a percentage of total fatty acids) for each of the added dietary fats. The CO contained the highest percentage of saturated fatty acids, with tridecyclic acid being the dominant saturated fatty acid, followed by myristic acid. Similar amounts of saturated fatty acids were observed in the L and PO, with palmitic acid being the dominant saturated fatty acid in both fats, followed by stearic acid. L and PO also had similar amounts of monounsaturated and polyunsaturated fatty acids. Oleic acid was the dominant monounsaturated fatty acid observed in both L and PO. Linoleic acid was the dominant polyunsaturated fatty acid observed in L and PO. The highest percentage of polyunsaturated fatty acids was observed in the SO and SuO, with linoleic acid being the dominant polyunsaturated fatty acid in both fats. SO had the lowest ratio of omega-6: omega-3 fatty acids and the highest ratio of omega-6: omega-3 fatty was observed in the SuO. The lowest saturated to unsaturated fatty acid ratio was observed in the SuO. The highest saturated to unsaturated fatty acid ratio was observed in the CO.

**Table 3. 2** Fatty acid profiles of the added dietary fats used to formulate the high-fat diets fed to male, Japanese quail for 12 weeks.

| | Added dietary fats: | | | | |
|---|---|---|---|---|---|
| | **CO** | **L** | **PO** | **SO** | **SuO** |
| **Fatty acid:** | | | | | |
| C8:0 (Caprylic acid) | 5.55 | nd | nd | nd | nd |
| C10:0 (Capric acid) | 6.21 | 0.11 | 0.02 | nd | nd |
| C14:0 (Myristic acid) | 19.47 | 1.52 | 1.07 | 0.08 | 0.06 |
| C16:0 (Palmitic acid) | 8.54 | 27.46 | 37.65 | 10.00 | 5.72 |
| C16:1 (Palmitoleic acid) | 0.01 | 1.77 | 0.20 | 0.08 | 0.06 |
| C18:0 (Stearic acid) | 3.14 | 17.54 | 3.85 | 5.04 | 5.44 |
| C18:1 ω-9 (Oleic acid) | 4.91 | 36.40 | 44.41 | 25.25 | 30.54 |
| C18:2 ω-6 (Linoleic acid) | 0.82 | 11.90 | 11.35 | 52.87 | 55.96 |
| C18:3 ω-3 (Alpha-linolenic acid) | nd | 0.60 | 0.23 | 4.88 | 0.09 |
| C22:0 (Behenic acid) | 0.03 | 0.01 | 0.06 | 0.59 | 1.08 |
| C13:0 (Tridecyclic acid) | 51.07 | nd | nd | nd | nd |
| Saturated FA | 94.16 | 47.69 | 43.56 | 16.58 | 13.12 |
| Monounsaturated FA | 4.97 | 38.90 | 44.76 | 25.57 | 30.79 |
| Polyunsaturated FA | 0.87 | 13.03 | 11.61 | 57.82 | 56.08 |
| Cis FA | 5.73 | 47.91 | 55.69 | 78.08 | 86.48 |
| ω-3 FA | 0.01 | 0.65 | 0.23 | 4.88 | 0.09 |
| ω-6 FA | 0.84 | 12.10 | 11.93 | 52.89 | 55.96 |
| ω-9 FA | 4.92 | 36.41 | 44.41 | 25.25 | 30.55 |
| ω6:ω3 | 59.93:1 | 18.67:1 | 52.56:1 | 10.85:1 | 643.26:1 |
| SFA:UFA | 16.12:1 | 0.92:1 | 0.77:1 | 0.20:1 | 0.15:1 |

Fatty acid data presented as a percentage of total fatty acids within the added dietary fats. CO = coconut oil; L = lard; PO = palm oil; SO = soybean oil; SuO = sunflower oil; FA = fatty acids; SFA = saturated fatty acids; UFA = unsaturated fatty acids; nd = not detected (below detectable threshold); ω = omega.

### 3.3.2 Body mass

Figure 3.1 shows the initial (time: 0 weeks) and final (after twelve weeks) body mass (g) (A), as well as the percentage body mass gain (B) per dietary group of Japanese quail fed either a STD or one of five different HFDs for a period of twelve weeks. No significant differences in initial body mass ($p > 0.05$, ANOVA) were observed between birds in the different dietary groups. Following the twelve-week feeding period, the final body masses and percentage body mass gain of birds in the SO and SuO groups were significantly greater ($p < 0.05$, ANOVA) than that of birds in the STD group. The final body mass (following the twelve-week feeding period) of the birds in all five HFD groups was significantly greater ($p < 0.01$, Student's t-test) than their initial body mass (before starting the feeding period). The final body mass of birds in the STD group however, was not significantly different ($p > 0.05$, Student's t-test) from their initial body mass.

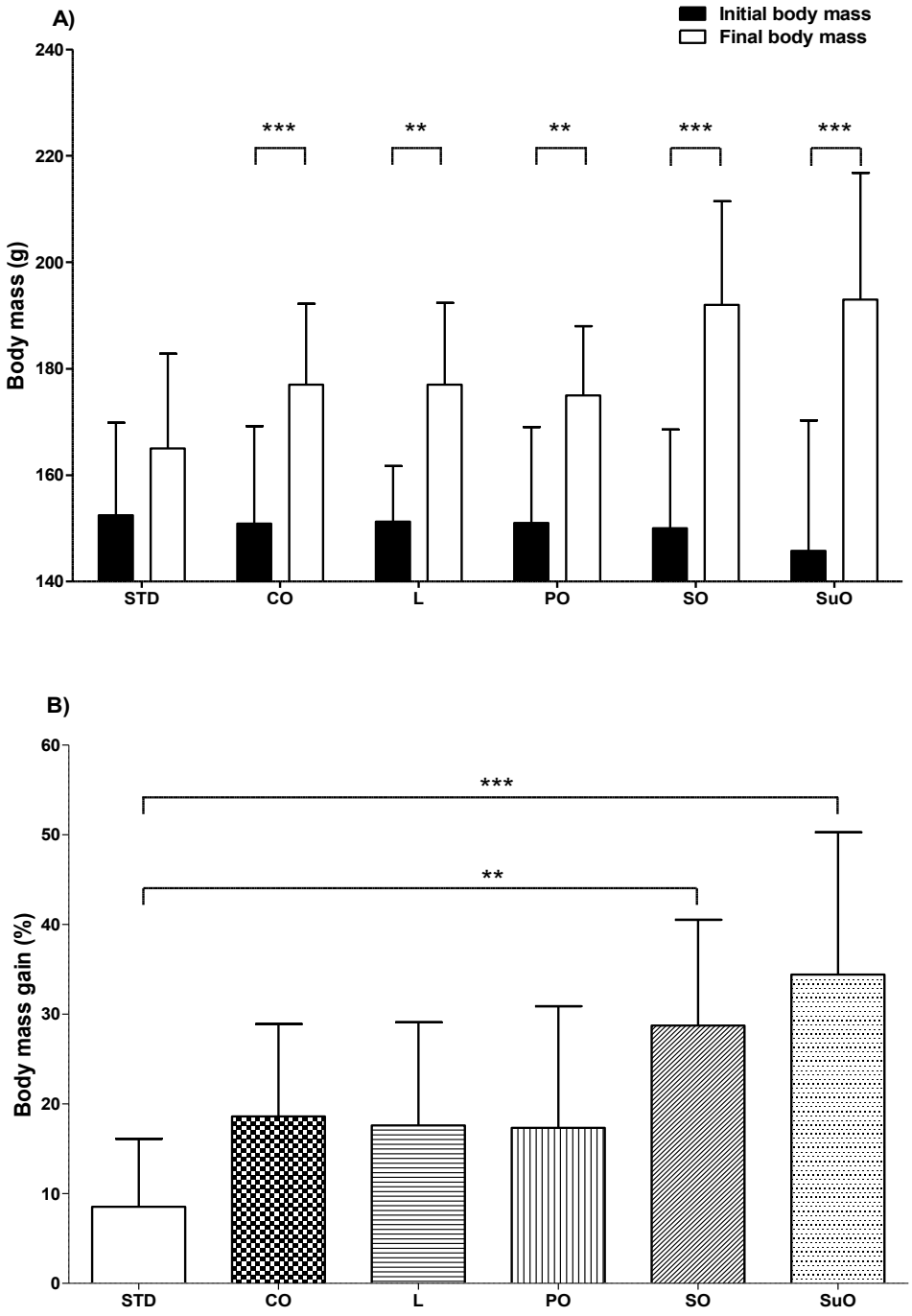



**Figure 3.1** The initial (time: 0 weeks) and final (after 12 weeks of feeding) body masses (A) and percentage body mass gain (B) of male, Japanese quail (*Coturnix coturnix japonica*) fed either a STD or one of five different HFDs, for a period of 12 weeks. Data represented as means ± SD. STD = standard diet (n=9); CO = Coconut oil (n=11); L = Lard (n=8); PO = Palm oil (n=10); SO = soyabean oil (n=10); SuO = Sunflower oil (n= 9). Figure 1A: ** $p < 0.01$ when comparing initial versus final body mass within each individual dietary group. Figure 1B: ** $p < 0.01$ and *** $p < 0.001$ when comparing birds in the SO and SuO groups, respectively, to the STD group.

### 3.3.3 Oral glucose tolerance tests

Figure 3.2 shows the glucose tolerance curves (A) and area under the glucose curves (AUC) (B) obtained for the birds in each of the six different dietary groups, following twelve weeks of STD or HFD feeding. There were no significant differences ($p > 0.05$, ANOVA) observed in baseline blood glucose concentrations, peak blood glucose concentrations, blood glucose concentrations 3 hrs following the glucose load or area under the glucose curve (AUC), between the different dietary groups. Peak blood glucose concentrations were reached 15 min after administration of the glucose load and were significantly higher ($p < 0.05$, repeated measures ANOVA) than baseline blood glucose concentrations in all dietary groups. Blood glucose concentrations returned to normal 60 min following administration of the glucose load in all HFD groups versus at 120 min following administration of the glucose load in the STD diet group.

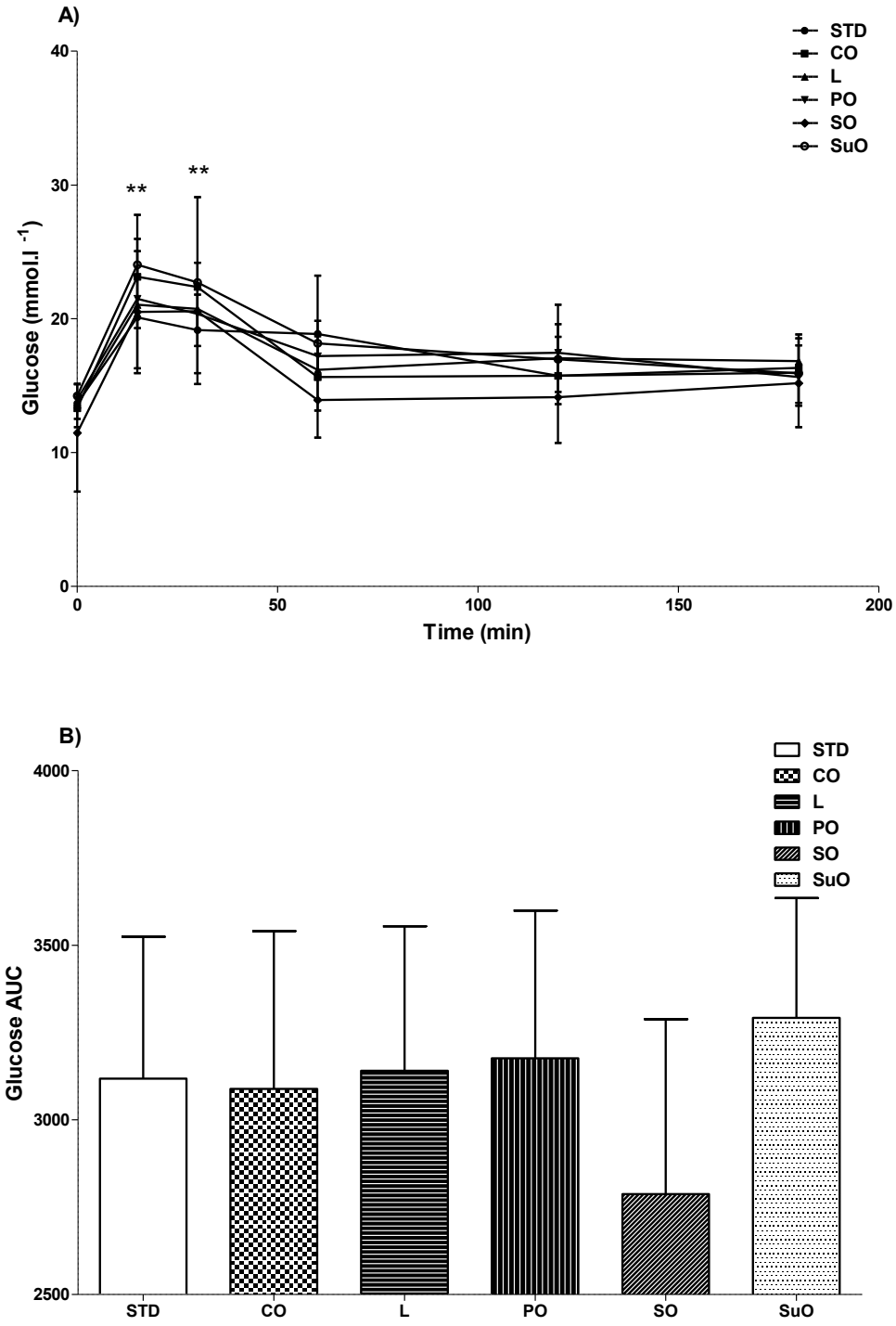



**Figure 3.2** The glucose tolerance test curves (A) and area under the glucose curve (B) of male, Japanese quail (*Coturnix coturnix japonica*) fed either a STD or one of five different HFDs, for 12 weeks. Data represented as means ± SD. STD = standard diet (n=9); CO = Coconut oil (n=11); L = Lard (n=8); PO = Palm oil (n=10); SO = soyabean oil (n=10); SuO = Sunflower oil (n= 9). Figure 1A: ** $p < 0.01$ when comparing peak blood glucose concentrations, following an oral glucose load, to baseline blood glucose concentrations within each individual dietary group.

3.3.4 Erythrocyte osmotic fragility

Figure 3.3 shows the fragiligrams of the erythrocytes from birds in all dietary groups, following twelve weeks of feeding. The fragiligrams obtained were similar for the erythrocytes from birds in all dietary groups, after the twelve-week feeding period. The range of percentage PBS solutions at which initial haemolysis (IH), mean corpuscular fragility (MCF) and maximal haemolysis (MH) occurred for erythrocytes from birds in the various dietary groups, after twelve weeks of feeding, are shown in Table 3.3. There were no differences in the range of PBS solutions at which initial haemolysis (0.5 - 0.55 % PBS) occurred in all groups of birds. Mean corpuscular fragility (MCF) and maximal haemolysis (MH), for erythrocytes from the birds in the various dietary groups also occurred within the same range of PBS solutions (0.4 - 0.45 % PBS and 0.2 - 0.4 % PBS, respectively).

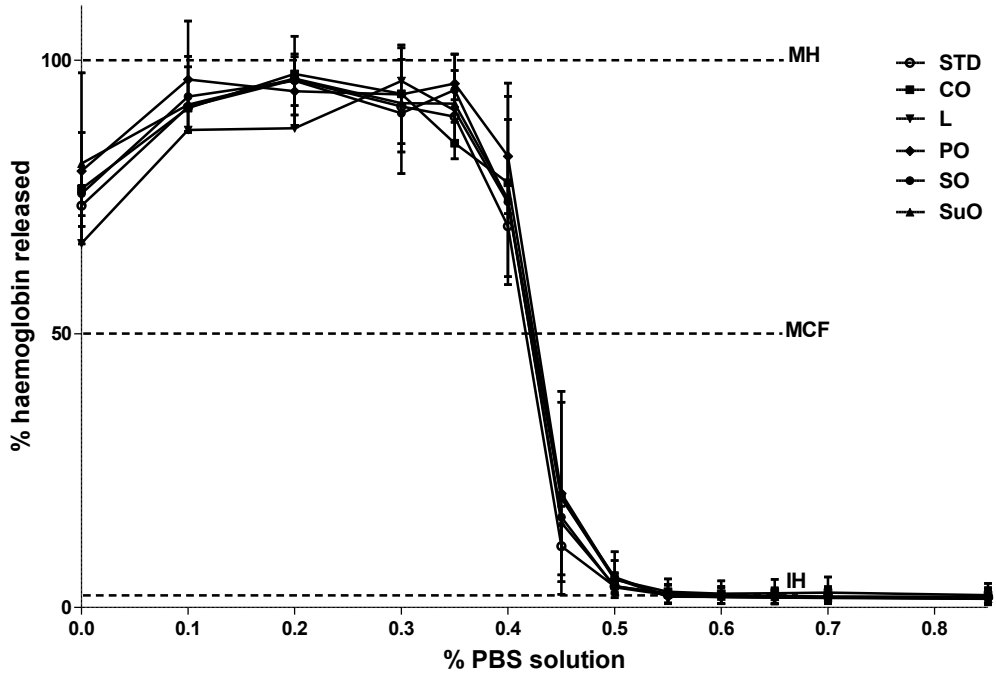



**Figure 3.3** Fragiligrams obtained from erythrocytes of male, Japanese quail (*Coturnix coturnix japonica*) after 12 weeks of either STD or HFD feeding. Data represents the means ± SD. STD = standard diet (n=9); CO = Coconut oil (n=11); L = Lard (n=8); PO = Palm oil (n=10); SO = soyabean oil (n=10); SuO = Sunflower oil (n= 9); IH = initial haemolysis; MCF = mean corpuscular fragility; MH = maximal haemolysis.

**Table 3. 3** Range of concentrations of phosphate-buffered saline solutions (%) at which initial haemolysis, mean corpuscular fragility and maximal haemolysis of erythrocytes from Japanese quail occurred, after 12 weeks of feeding either a standard diet or one of five different high-fat diets.

| | % PBS solution | | | | | |
|---|---|---|---|---|---|---|
| | **STD-group** | **CO-group** | **L-group** | **PO-group** | **SO-group** | **SuO-group** |
| **Initial haemolysis** | 0.50 - 0.55 | 0.50 - 0.55 | 0.50 - 0.55 | 0.50 - 0.55 | 0.50 - 0.55 | 0.50 - 0.55 |
| **MCF** | 0.40 – 0.45 | 0.40 – 0.45 | 0.40 – 0.45 | 0.40 – 0.45 | 0.40– 0.45 | 0.40 – 0.45 |
| **Maximal haemolysis** | 0.20 – 0.30 | 0.20 – 0.30 | 0.30 – 0.35 | .35 – 0.40 | 0.20 – 0.30 | 0.20 – 0.30 |

STD = standard diet; CO = coconut oil; L = lard; PO = palm oil; SO = soyabean oil; SuO = sunflower oil; MCF = Mean corpuscular fragility.

3.3.5 General health/clinical biochemistry profile

Table 3.4 shows the serum parameters for Japanese quail in both the STD and five different HFD groups. No significant differences ($p > 0.05$, ANOVA) in serum uric acid, total protein, albumin, AST, total bilirubin and calcium were observed between the different dietary groups. Serum cholesterol concentrations were significantly lower ($p < 0.05$, ANOVA) in birds from the PO group compared to those in the STD group. Serum triglyceride concentrations were significantly lower ($p < 0.05$, ANOVA) in all the HFD groups compared to that observed in the STD group (Figure 3.4).

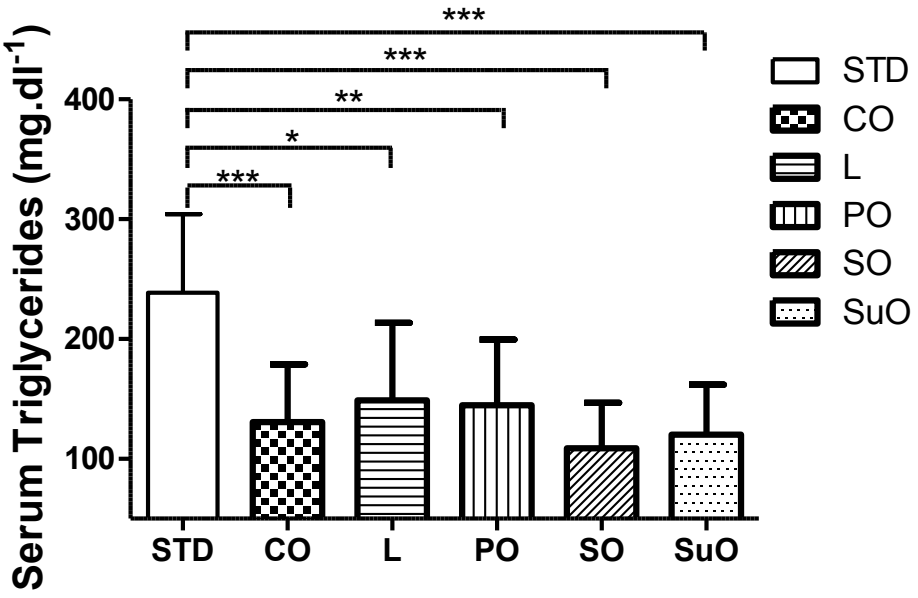



**Figure 3.4** Serum triglyceride concentrations ($mg.dl^{-1}$) of Japanese quail fed either a STD or one of five different HFDs, for 12 weeks. STD = standard diet; HFD = high-fat diet; CO = coconut oil; L = Lard; PO = Palm oil; SO = Soyabean oil; SuO = Sunflower oil. Data represented as means ± SD. * $p < 0.05$ and ** $p < 0.01$ and *** $p < 0.001$ when comparing HFD groups to STD group.

**Table 3. 4** Japanese quail (*Coturnix coturnix japonica*) serum parameters after 12 weeks of either standard or high-fat diet feeding.

| Serum parameters: | Dietary groups: | | | | | |
|---|---|---|---|---|---|---|
| | **STD-group** | **CO-group** | **L-group** | **PO-group** | **SO-group** | **SuO-group** |
| Uric ($mg.dl^{-1}$) | 7.3 ± 1.9 | 7.3 ± 3.1 | 6.2 ± 3.4 | 5.4 ± 3.1 | 7.5 ± 5.1 | 7.4 ± 2.9 |
| TPro ($g.dl^{-1}$) | 3.1 ± 0.3 | 3.6 ± 0.8 | 3.4 ± 0.9 | 3.5 ± 1.3 | 4.1 ± 1.7 | 3.2 ± 0.7 |
| Alb ($g.dl^{-1}$) | 0.6 ± 0.2 | 0.8 ± 0.4 | 0.7 ± 0.4 | 0.8 ± 0.5 | 1.3 ± 1.0 | 0.7 ± 0.4 |
| AST ($U.l^{-1}$) | 608.5 ± 123.1 | 457.4 ± 160.8 | 494.0 ± 196.6 | 400.2 ± 101.3 | 521.0 ± 207.1 | 408.5 ± 138.2 |
| TBil ($mg.dl^{-1}$) | 0.3 ± 0.3 | 1.8 ± 3.8 | 0.5 ± 1.0 | 0.9 ± 1.6 | 1.8 ± 3.8 | 0.4 ± 0.8 |
| Calc ($mg.dl^{-1}$) | 9.9 ± 1.1 | 10.1 ± 1.4 | 10.5 ± 1.3 | 9.4 ± 1.1 | 9.9 ± 2.3 | 9.2 ± 1.3 |
| Chol ($mg.dl^{-1}$) | $263.8 \pm 117.4^{a}$ | $230.1 \pm 42.8^{a,b}$ | $209.7 \pm 44.9^{a,b}$ | $177.4 \pm 30.6^{b}$ | $199.9 \pm 19.2^{a,b}$ | $204.0 \pm 19.7^{a,b}$ |
| Trig ($mg.dl^{-1}$) | 238.4 ± 65.8 | $130.7 \pm 48.0^{\#}$ | 148.9 ± 64.7* | $144.7 \pm 54.7^{\$}$ | $108.7 \pm 38.0^{\#}$ | $120.1 \pm 41.8^{\#}$ |

Data represented as mean ± SD. Different letters indicate significant differences when compared to STD group. STD = standard diet (n=9); CO = Coconut oil (n=11); L = Lard (n=8); PO = Palm oil (n=10); SO = soyabean oil (n=10); SuO = Sunflower oil (n= 9). Uric = uric acid, TPro = total protein, Alb = Albumin, AST = aspartate aminotransferase, Tbil = total bilirubin, Calc = Calcium, Chol = cholesterol, Trig = triglycerides.

### 3.3.6 Organ masses and small and large intestine lengths

The absolute and relative masses (represented as a percentage of body mass) of the liver, pancreas, proventriculus, ventriculus, small intestine, large intestine, caecum, abdominal fat pad and testes, along with the absolute and relative (as unit length per unit body mass) lengths of the small and large intestines are represented in Table 3.5 (absolute) and Table 3.6 (relative), respectively. No significant differences ($p > 0.05$, ANOVA) in the absolute masses of the liver, pancreas, proventriculus, ventriculus, small intestine, large intestine, caecum, abdominal fat pad and testes were observed between birds in the various dietary groups, following twelve weeks of feeding.

The relative liver mass of birds in the SuO group was significantly lower ($p < 0.05$, ANOVA) than that observed in the STD group, following twelve weeks of feeding. No significant differences ($p > 0.05$, ANOVA) in relative liver mass were observed between the remaining dietary groups following twelve weeks of feeding. No significant differences ($p > 0.05$, ANOVA) in the relative mass of the pancreas, proventriculus, ventriculus, small intestine, large intestine, caecum, abdominal fat pad and testes were observed between birds in the various dietary groups, following twelve weeks of feeding. There were also no significant differences ($p > 0.05$, ANOVA) observed in the absolute and relative lengths of the large intestines between various dietary groups, however birds in the PO group had significantly greater ($p < 0.01$, ANOVA) absolute small intestine lengths than those in the STD group. Relative small intestine lengths were not significantly different ($p > 0.05$, ANOVA) between the various dietary groups.

**Table 3. 5** The absolute organ masses and lengths of the small and large intestines of Japanese quail after 12 weeks of either standard or high-fat diet feeding.

| | Dietary groups: | | | | | |
|---|---|---|---|---|---|---|
| **Organ mass (g):** | **STD-group** | **CO-group** | **L-group** | **PO-group** | **SO-group** | **SuO-group** |
| Liver | 3.1 ± 0.67 | 2.93 ± 0.59 | 2.94 ± 0.40 | 3.02 ± 0.61 | 3.16 ± 0.88 | 2.51 ± 0.44 |
| Pancreas | 0.33 ± 0.06 | 0.30 ± 0.07 | 0.35 ± 0.09 | 0.32 ± 0.07 | 0.33 ± 0.06 | 0.32 ± 0.06 |
| Proventriculus | 0.50 ± 0.08 | 0.57 ± 0.13 | 0.58 ± 0.12 | 0.56 ± 0.08 | 0.57 ± 0.09 | 0.61 ± 0.09 |
| Ventriculus | 3.1 ± 0.40 | 2.98 ± 1.03 | 3.12 ± 0.25 | 3.14 ± 0.56 | 3.52 ± 0.42 | 3.30 ± 0.36 |
| Small intestine | 2.78 ± 0.38 | 3.23 ± 0.52 | 3.10 ± 0.54 | 3.04 ± 0.49 | 2.89 ± 1.10 | 2.83 ± 0.33 |
| Large intestine | 0.37 ± 0.14 | 0.49 ± 0.09 | 0.52 ± 0.09 | 0.48 ± 0.09 | 0.51 ± 0.10 | 0.48 ± 0.07 |
| Caecum | 0.44 ± 0.14 | 0.51 ± 0.12 | 0.39 ± 0.11 | 0.44 ± 0.16 | 0.56 ± 0.26 | 0.42 ± 0.10 |
| Abdominal fat pad | 2.0 ± 1.56 | 2.68 ± 1.53 | 2.49 ± 2.89 | 2.28 ± 2.31 | 1.97 ± 1.37 | 1.33 ± 0.83 |
| Testes | 4.89 ± 1.80 | 5.51 ± 0.90 | 6.47 ± 1.34 | 5.18 ± 0.85 | 5.62 ± 0.82 | 5.37 ± 1.87 |
| **Intestine lengths (m):** | | | | | | |
| Small intestine | 0.48 ± 0.05[a] | 0.54 ± 0.03[a,b] | 0.54 ± 0.06[a,b] | 0.57 ± 0.03[b] | 0.54 ± 0.08[a,b] | 0.52 ± 0.04[a,b] |
| Large intestine | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.06 ± 0.03 | 0.06 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.01 |

Data represented as mean ± SD. Different letters indicate significant differences when compared to STD group. STD = standard diet; CO = Coconut oil; L = Lard; PO = Palm oil; SO = Soyabean oil; SuO = Sunflower oil.

**Table 3. 6** The relative organ masses and lengths of the small and large intestines of Japanese quail after 12 weeks of either standard or high-fat diet feeding.

| Organ mass (% of body mass): | Dietary groups: | | | | | |
|---|---|---|---|---|---|---|
| | **STD-group** | **CO-group** | **L-group** | **PO-group** | **SO-group** | **SuO-group** |
| Liver | 1.87 ± 0.33[a] | 1.65 ± 0.28[a,b] | 1.68 ± 0.33[a,b] | 1.72 ± 0.31[a,b] | 1.68 ± 0.59[a,b] | 1.30 ± 0.24[b] |
| Pancreas | 0.40 ± 0.59 | 0.17 ± 0.04 | 0.20 ± 0.06 | 0.18 ± 0.04 | 0.17 ± 0.03 | 0.16 ± 0.02 |
| Proventriculus | 0.31 ± 0.06 | 0.32 ± 0.08 | 0.33 ± 0.07 | 0.32 ± 0.04 | 0.30 ± 0.03 | 0.31 ± 0.03 |
| Ventriculus | 1.87 ± 0.18 | 1.65 ± 0.55 | 1.76 ± 0.09 | 1.80 ± 0.34 | 1.85 ± 0.28 | 1.71 ± 0.17 |
| Small intestine | 1.69 ± 0.21 | 1.81 ± 0.22 | 1.75 ± 0.39 | 1.74 ± 0.28 | 1.52 ± 0.61 | 1.47 ± 0.39 |
| Large intestine | 0.23 ± 0.09 | 0.28 ± 0.06 | 0.25 ± 0.11 | 0.27 ± 0.05 | 0.27 ± 0.07 | 0.25 ± 0.05 |
| Caecum | 0.26 ± 0.09 | 0.29 ± 0.07 | 0.22 ± 0.06 | 0.25 ± 0.09 | 0.30 ± 0.17 | 0.22 ± 0.05 |
| Abdominal fat pad | 1.16 ± 0.80 | 1.49 ± 0.76 | 1.34 ± 1.47 | 1.25 ± 1.22 | 0.91 ± 0.72 | 0.68 ± 0.44 |
| Testes | 2.96 ± 1.09 | 3.10 ± 0.43 | 3.67 ± 0.83 | 2.96 ± 0.49 | 2.60 ± 0.99 | 2.67 ± 0.68 |
| **Intestine lengths (mm/g):** | | | | | | |
| Small intestine | 2.94 ± 0.45 | 3.03 ± 0.24 | 3.05 ± 0.41 | 3.25 ± 0.33 | 2.82 ± 0.55 | 2.70 ± 0.34 |
| Large intestine | 0.40 ± 0.04 | 0.36 ± 0.05 | 0.34 ± 0.14 | 0.35 ± 0.05 | 0.34 ± 0.05 | 0.34 ± 0.06 |

Data represented as mean ± SD. Different letters indicate significant differences when compared to STD group. STD = standard diet; CO = Coconut oil; L = Lard; PO = Palm oil; SO = Soyabean oil; SuO = Sunflower oil.

### 3.3.7 Liver lipid content and fatty acid profile

Figure 3.5 shows the lipid yield (as a % of liver tissue sample mass) of liver tissue samples from male, Japanese quail, following twelve weeks of either STD or HFD feeding. A lower percentage lipid yield was observed in the livers of all birds from the HFD groups compared to that of the STD group. Of the HFD groups, birds from the CO group had the highest percentage liver lipid yield, whilst birds from the SuO group had the lowest.

The results of the liver fatty acid profile analyses are presented in Table 3.7 (fatty acids represented as a percentage of total fatty acids). The livers of the birds receiving CO had the highest percentage of saturated fatty acids, with palmitic acid being the dominant saturated fatty acid, followed by stearic acid. The livers of the birds receiving the STD had the highest percentage of monounsaturated fatty acids, whereas all groups receiving the HFDs had a lower percentage of monounsaturated fatty acids. Oleic acid was the dominant monounsaturated fatty acid in all dietary groups. The livers of the birds receiving SO had the highest percentage of polyunsaturated fatty acids, followed by those receiving SuO, whilst the remaining dietary groups had a lower percentage of polyunsaturated fatty acids. Linoleic acid was the dominant polyunsaturated fatty acid in all dietary groups. The lowest ratio of omega-6: omega-3 fatty acids was observed in the livers of the birds receiving SO, while the highest ratio of omega-6: omega-3 fatty was observed in the livers of the birds receiving SuO. The livers of the birds receiving SO had the lowest saturated to unsaturated fatty acid ratio, while the highest saturated to unsaturated fatty acid ratio was observed in the livers of the birds receiving CO.

### 3.3.8 Liver histology

No evidence of steatosis was observed in any of the liver histology sections examined for birds from the various dietary groups. Thus, a score of 0 was allocated to all Japanese quail liver histology sections. Photographs of the histological sections obtained from the liver of a representative bird from each dietary group, at each time point are shown in Figure 3.6.

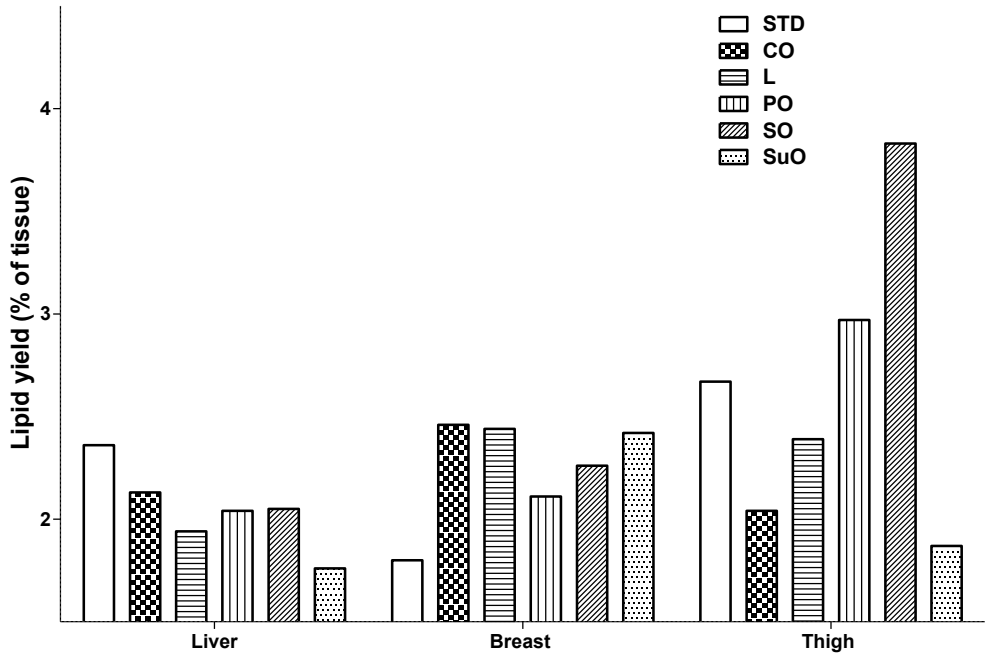



**Figure 3. 5** Total lipid yield (as a percentage of tissue sample) of the liver, breast muscle and thigh muscle of Japanese quail fed either a STD or one of five different HFDs, for 12 weeks. STD = standard diet; CO = coconut oil; L = Lard; PO = Palm oil; SO = Soyabean oil; SuO = Sunflower oil.

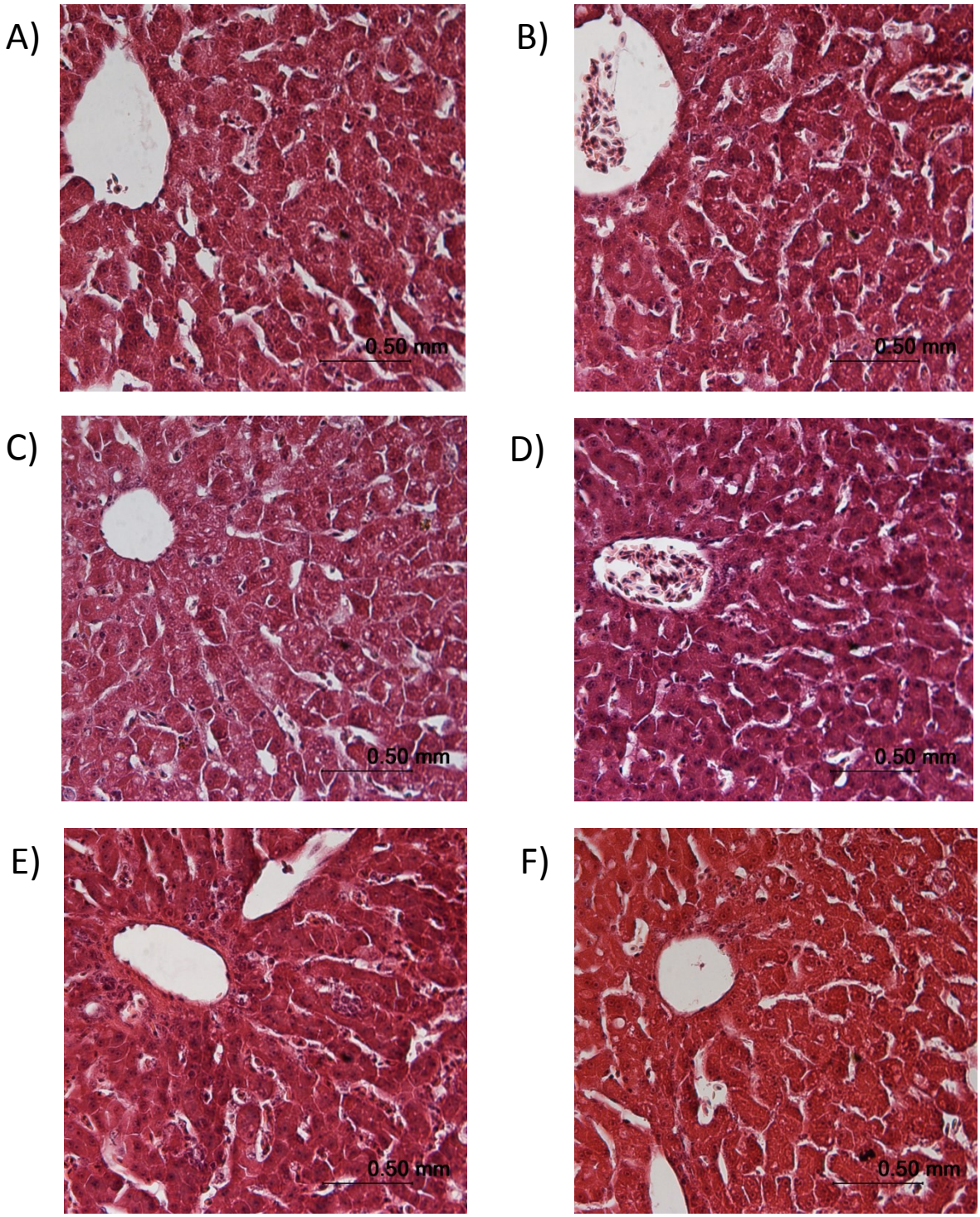



**Figure 3.6** Images of the histological sections, stained with haematoxylin and eosin, obtained from the liver of a representative Japanese quail from each dietary group. Images were obtained using a computer-based image acquisition and analysis system at 200 times magnification (Axiovision 3, Carl Zeiss, Gottingen, Germany). A) Standard diet (STD) group; B) Coconut oil (CO) group; C) Lard (L) group; D) Palm oil (PO) group; E) Soyabean oil (SO) group; F) Sunflower oil (SuO) group.

**Table 3. 7** Fatty acid profiles of the liver samples from male, Japanese quail fed either a standard diet or one of five different high-fat diets, for 12 weeks.

| | Dietary groups: | | | | | |
|---|---|---|---|---|---|---|
| **Fatty acid:** | **STD-group** | **CO-group** | **L-group** | **PO-group** | **SO-group** | **SuO-group** |
| C12:0 (Lauric acid) | 0.10 | 6.33 | 0.14 | 0.11 | 0.08 | 0.09 |
| C14:0 (Myristic acid) | 0.58 | 5.61 | 0.72 | 0.38 | 0.25 | 0.25 |
| C16:0 (Palmitic acid) | 24.90 | 19.57 | 24.06 | 20.35 | 14.41 | 14.13 |
| C16:1 (Palmitoleic acid) | 4.04 | 1.63 | 1.19 | 0.68 | 0.40 | 0.41 |
| C18:0 (Stearic acid) | 13.97 | 17.66 | 16.64 | 16.60 | 18.60 | 20.05 |
| C18:1 ω-9 (Oleic acid) | 31.05 | 18.35 | 26.17 | 29.55 | 14.34 | 15.16 |
| C18:2 ω-6 (Linoleic acid) | 13.51 | 15.45 | 18.64 | 18.71 | 35.56 | 35.71 |
| C18:3 ω-3 (Alpha-linolenic acid) | 0.23 | 0.18 | 0.32 | 0.16 | 1.04 | 0.11 |
| C20:4 ω-6 (Arachidonic acid) | 6.86 | 7.83 | 6.65 | 9.42 | 8.33 | 10.48 |
| C23:0 (Tricosanoic acid) | 0.59 | 2.12 | 0.54 | 0.45 | 0.42 | 0.43 |
| C22:6 ω-3 (Docosahexaenoic acid) | 0.11 | 0.16 | 2.51 | 1.61 | 4.34 | 0.86 |
| Saturated FA | 41.13 | 52.57 | 42.80 | 38.36 | 34.29 | 35.65 |
| Monounsaturated FA | 35.78 | 21.04 | 27.88 | 30.56 | 15.22 | 15.74 |
| Polyunsaturated FA | 22.88 | 26.25 | 29.17 | 31.01 | 50.44 | 48.53 |
| Cis FA | 44.34 | 33.66 | 44.65 | 48.20 | 49.86 | 50.80 |
| ω-3 FA | 1.00 | 1.09 | 3.01 | 1.93 | 5.54 | 1.06 |
| ω-6 FA | 21.27 | 24.41 | 25.86 | 28.97 | 44.52 | 46.94 |
| DHA | 0.11 | 0.16 | 2.51 | 1.61 | 4.34 | 0.86 |
| ω-9 FA | 31.06 | 18.57 | 26.17 | 29.55 | 14.34 | 15.16 |
| ω6: ω3 | 21.27:1 | 22.39:1 | 8.59:1 | 15.01:1 | 8.04:1 | 44.28:1 |
| **SFA:UFA** | 0.70:1 | 1.11:1 | 0.75:1 | 0.62:1 | 0.52:1 | 0.55:1 |

Fatty acid data presented as a percentage of total fatty acids within composite tissue sample. STD = standard diet; CO = coconut oil; L = lard; PO = palm oil; SO = soybean oil; SuO = sunflower oil; FA = fatty acids; SFA = saturated fatty acids; UFA = unsaturated fatty acids; ω = omega.

### 3.3.9 Muscle lipid content and fatty acid profile

Figure 3.5 shows the results of the total lipid yield (as a percentage of total tissue sample) for the breast and thigh muscle samples from each dietary group. A higher percentage lipid yield was observed in the breast muscle of all birds from the HFD groups compared to that of the STD group. The breast muscle from birds in the CO group had the highest percentage lipid yield, whilst that from birds in the PO group had the lowest. The thigh muscles from birds in the PO and SO groups had a higher percentage lipid yield compared to those from birds in the STD. The thigh muscles from the remaining HFD groups all had a lower percentage lipid yield compared to the STD. Birds in the STD, PO and SO dietary groups displayed a lower percentage lipid yield in their breast muscles compared to that in their thigh muscles. The birds in the CO, L and SuO dietary groups displayed a higher percentage lipid yield in their breast muscles versus their thigh muscles.

The results of the fatty acid profile analyses for the breast and thigh muscles of birds from each dietary group are shown in Tables 3.8 and 3.9 (fatty acids represented as a percentage of total fatty acids). The breast and thigh muscles from the birds in the CO group had the highest percentage of saturated fatty acids, with palmitic acid being the dominant saturated fatty acid in the breast muscle, followed by lauric acid. Lauric acid was the dominant saturated fatty acid in the thigh muscle (37.06 %), followed by myristic acid. The highest percentage of monounsaturated fatty acids were observed in the breast and thigh muscles of the birds receiving PO, with oleic acid being the dominant monounsaturated fatty acid in both the breast and thigh muscles. The highest percentage polyunsaturated fatty acids was observed in birds receiving SuO, in both their breast and thigh muscles, followed by those receiving SO. Linoleic acid was the dominant polyunsaturated fatty acid observed in both the breast and thigh muscles of birds receiving SuO. Linoleic acid was also the dominant polyunsaturated fatty acid observed in both the breast and thigh muscles of birds receiving SO. The lowest ratio of omega-6: omega-3 fatty was observed in the breast and thigh muscles of birds receiving L and the highest ratio was observed in birds receiving SuO. The breast and thigh muscles of the bids receiving SuO had the lowest saturated to unsaturated fatty acid ratio, whilst the breast and thigh muscles of the birds receiving CO displayed the highest saturated to unsaturated fatty acid ratio.

**Table 3. 8** The fatty acid profiles of breast muscle samples from male, Japanese quail fed either a standard diet or one of five different high-fat diets, for 12 weeks.

| Fatty acid: | Dietary groups: | | | | | |
|---|---|---|---|---|---|---|
| | **STD-group** | **CO-group** | **L-group** | **PO-group** | **SO-group** | **SuO-group** |
| C12:0 (Lauric acid) | 0.06 | 14.84 | 0.29 | 0.10 | 0.13 | 0.12 |
| C14:0 (Myristic acid) | 0.67 | 10.69 | 0.94 | 0.57 | 0.49 | 0.46 |
| C14:1 (Myristoleic acid) | 0.11 | 1.02 | 0.04 | 0.05 | 0.04 | nd |
| C16:0 (Palmitic acid) | 20.61 | 16.46 | 22.54 | 18.25 | 17.92 | 14.79 |
| C16:1 (Palmitoleic acid) | 7.94 | 4.68 | 2.91 | 2.04 | 1.64 | 1.04 |
| C18:0 (Stearic acid) | 6.72 | 7.44 | 9.55 | 6.73 | 7.92 | 8.06 |
| C18:1 ω-9 (Oleic acid) | 41.89 | 25.24 | 39.39 | 49.34 | 31.46 | 30.58 |
| C18:2 ω-6 (Linoleic acid) | 17.61 | 13.82 | 17.99 | 17.54 | 35.04 | 41.90 |
| C18:3 ω-3 (Alpha-linolenic acid) | 0.44 | 0.33 | 0.45 | 0.24 | 1.37 | 0.25 |
| C20:4 ω-6 (Arachidonic acid) | 2.78 | 3.54 | 2.66 | 3.70 | 2.39 | 1.57 |
| Saturated FA | 28.39 | 50.29 | 34.26 | 25.97 | 27.04 | 23.93 |
| Monounsaturated FA | 50.28 | 31.26 | 43.16 | 51.80 | 31.73 | 30.54 |
| Polyunsaturated FA | 21.17 | 18.33 | 22.46 | 22.23 | 39.51 | 44.09 |
| Trans FA | 0.17 | 0.08 | 0.12 | 0.05 | 1.73 | 1.41 |
| Cis FA | 59.28 | 38.98 | 57.30 | 66.83 | 64.78 | 71.07 |
| ω-3 FA | 0.56 | 0.61 | 1.23 | 0.62 | 1.81 | 0.50 |
| ω-6 FA | 20.56 | 17.60 | 20.86 | 21.42 | 37.57 | 43.60 |
| ω-9 FA | 41.94 | 25.29 | 39.55 | 49.34 | 31.42 | 30.58 |
| ω6:ω3 | 37.00:1 | 28.87:1 | 16.97:1 | 34.77:1 | 20.71:1 | 87.92:1 |
| SFA:UFA | 0.40:1 | 1.01:1 | 0.52:1 | 0.35:1 | 0.38:1 | 0.32:1 |

Fatty acid data presented as a percentage of total fatty acids within composite tissue sample. STD = standard diet; CO = coconut oil; L = lard; PO = palm oil; SO = soybean oil; SuO = sunflower oil; FA = fatty acids; SFA = saturated fatty acids; UFA = unsaturated fatty acids; nd = not detected (below detectable threshold); ω = omega.

**Table 3. 9** The fatty acid profiles of thigh muscle samples from male, Japanese quail fed either a standard diet or one of five different high-fat diets, for 12 weeks.

| Fatty acid: | Dietary groups: | | | | | |
|---|---|---|---|---|---|---|
| | **STD-group** | **CO-group** | **L-group** | **PO-group** | **SO-group** | **SuO-group** |
| C12:0 (Lauric acid) | 0.26 | 37.06 | 0.25 | 2.36 | 0.08 | 0.11 |
| C14:0 (Myristic acid) | 0.75 | 14.90 | 1.21 | 1.48 | 0.29 | 0.27 |
| C16:0 (Palmitic acid) | 19.51 | 11.13 | 26.61 | 18.08 | 10.97 | 9.20 |
| C16:1 (Palmitoleic acid) | 6.29 | 2.65 | 2.05 | 1.28 | 0.89 | 0.54 |
| C18:0 (Stearic acid) | 4.83 | 3.63 | 7.95 | 3.60 | 4.49 | 5.99 |
| C18:1 ω-9 (Oleic acid) | 40.79 | 16.57 | 39.62 | 52.22 | 29.24 | 29.89 |
| C18:2 ω-6 (Linoleic acid) | 25.02 | 10.64 | 19.00 | 19.19 | 50.21 | 51.93 |
| C18:3 ω-3 (Alpha-linolenic acid) | 0.64 | 0.25 | 0.50 | 0.24 | 2.06 | 0.21 |
| Saturated FA | 25.69 | 67.75 | 36.70 | 25.86 | 16.32 | 16.10 |
| Monounsaturated FA | 47.49 | 20.29 | 40.42 | 53.84 | 30.47 | 30.70 |
| Polyunsaturated FA | 26.67 | 11.86 | 21.00 | 20.27 | 53.16 | 53.21 |
| Trans FA | 0.15 | 0.05 | 1.88 | 0.03 | 0.08 | 0.05 |
| Cis FA | 65.66 | 27.16 | 56.70 | 71.38 | 79.43 | 81.77 |
| ω-3 FA | 0.75 | 0.34 | 0.88 | 0.34 | 2.22 | 0.27 |
| ω-6 FA | 25.88 | 15.88 | 20.00 | 19.90 | 50.84 | 52.83 |
| ω-9 FA | 40.79 | 16.57 | 39.62 | 52.22 | 29.27 | 29.89 |
| ω6:ω3 | 34.55:1 | 46.29:1 | 22.76:1 | 59.10:1 | 2.91:1 | 197.60:1 |
| SFA:UFA | 0.35:1 | 2.11:1 | 0.60:1 | 0.35:1 | 0.20:1 | 0.19:1 |

Fatty acid data presented as a percentage of total fatty acids within composite tissue sample. STD = standard diet; CO = coconut oil; L = lard; PO = palm oil; SO = soybean oil; SuO = sunflower oil; FA = fatty acids; SFA = saturated fatty acids; UFA = unsaturated fatty acids; nd = not detected (below detectable threshold), ω = omega.

### 3.3.10 Femur mass, length and relative density

Table 3.10 shows the masses, lengths and relative densities (Seedor index) of the femora from the birds in the various dietary groups, following twelve weeks of feeding. No significant differences ($p > 0.05$, ANOVA) in femur mass, length or density were observed between birds in the various dietary groups. Figure 3.7 shows the radiographs of the femora obtained from the birds in the various dietary groups after twelve weeks of feeding.

**Table 3. 10** Masses, lengths and relative densities of femora of Japanese quail following 12 weeks of standard or high-fat diet feeding.

| | **Dietary groups:** | | | | | |
|---|---|---|---|---|---|---|
| **Bone parameters:** | **STD-group** | **CO-group** | **L-group** | **PO-group** | **SO-group** | **SuO-group** |
| Mass (mg) | 400.9 ± 39.73 | 419.6 ± 39.23 | 420.6 ± 34.12 | 436.1 ± 24.00 | 406.2 ± 60.51 | 416.2 ± 46.46 |
| Length (mm) | 39.81 ± 1.41 | 40.00 ± 1.14 | 40.69 ± 1.38 | 41.08 ± 0.94 | 39.79 ± 1.48 | 40.58 ± 1.65 |
| Relative Density ($mg.mm^{-1}$) | 10.06 ± 0.79 | 10.49 ± 0.87 | 10.34 ± 0.71 | 10.62 ± 0.52 | 10.01 ± 1.37 | 10.24 ± 0.81 |

Data represented as mean ± SD. STD = standard diet, CO = coconut oil, L = lard, PO = palm oil, SO = soyabean oil, SuO = sunflower oil.

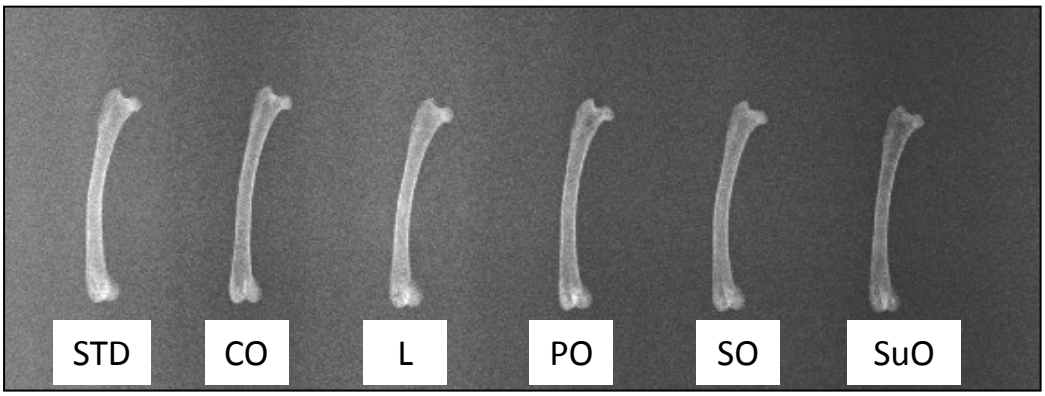



**Figure 3.7** Radiographs of the femora obtained from Japanese quail following 12 weeks of STD or HFD feeding. STD = standard diet group, CO = Coconut oil group, L = Lard group, PO = Palm oil group, SO = Soyabean oil group, SuO = Sunflower oil group, HFD = high-fat diet.

### 3.4 Discussion

The overall objective of the current study was to investigate the metabolic effects of different HFDs, based on coconut oil, lard, palm oil, soyabean oil or sunflower oil, on the overall health status, bone health, overall lipid content and fatty acid profiles of the edible tissues (liver, breast and thigh muscle) of male, Japanese quail (*Coturnix coturnix japonica*). The various HFDs were well-tolerated by the quail with no deleterious effects observed with respect to the growth performance, glucose tolerance, erythrocyte osmotic fragility, liver lipid deposition and bone health of the birds. The serum metabolic health markers were also unaffected by the various HFDs and the birds remained healthy throughout the feeding period. The tissue lipid profiles were reflective of the added dietary lipid sources, confirming the successful transfer of the dietary fatty acids into the edible bird tissues. My null hypothesis stated that the different HFDs, of varying fatty acid profiles would have no effect on the growth performance, glucose tolerance, erythrocyte osmotic fragility, serum biochemistry/general health profile, absolute and relative organ masses and intestine lengths, liver mass, liver lipid content, bone health parameters and the fatty acid profiles of the liver, breast and thigh muscles of the birds. The various HFDs had no significant influence on the growth performance, glucose tolerance, erythrocyte osmotic fragility, serum biochemistry/general health profile, absolute and relative organ masses and intestine lengths, liver mass, liver lipid content and bone health of the birds, thus accepting the null hypothesis in these instances. The liver and muscle lipid profiles were reflective of the dietary lipids ingested and thus were affected by the various HFDs and the null hypothesis was rejected in this instance.

As with the previous study in the Guinea fowl and Muscovy ducks, we were also unable to accurately measure the feed intake of the Japanese quail. Even though the quail were housed individually, they were mixing their feed together with their straw bedding and sometimes out of the cages onto the floor. It was difficult to try and keep track of exactly how much feed the birds were consuming, but the majority of the feed provided to them was consumed.

*Diet proximate analysis*

All the HFDs used in the present study were isonitrogenous to the STD. The fat content of the HFDs was significantly increased compared to that of the STD, as expected. The energy

content of all the HFDs, except for that of the HFD composed of lard, was significantly increased compared to that of the STD, as a result of the added dietary fat. The results of the fatty acid profile analyses of the various lipid sources are for the most part in agreement with previous studies that have made use of some of these animal fats and vegetable oils.

With regards to the coconut oil used in the present study, 94.16 % of the total fatty acids were saturated fatty acids. The saturated fatty acid content of the coconut oil used in a study by Wang et al. (2015) was only 72.52 % of total fatty acids, which is less than that used in the present study. The dominant saturated fatty acid also differed, with lauric acid being dominant, instead of tridecyclic acid, as was the case with the coconut oil used in the present study. The lard used in this study had a higher content of saturated and polyunsaturated fatty acids and a lower content of monounsaturated fatty acids than the lard used by Burlikowska et al. (2010). Palmitic acid was the dominant saturated fatty acid in both cases. The palm oil used in this study had similar amounts of saturated, polyunsaturated and monounsaturated fatty acids to the lard used. The palm oil used in studies by Viveros et al. (2009) and Velasco et al. (2010) had a higher saturated fatty acid content and a lower monounsaturated fatty acid content than that which was used in the present study. The polyunsaturated fatty acid content was similar and the dominant saturated fatty acid was palmitic acid in all cases. Of the total fatty acids within the soyabean oil used in the present study, 57.82 % were polyunsaturated fatty acids. The soyabean oil used in previous studies by Burlikowska et al. (2010), Jankowski et al. (2012) and Wang et al. (2015) also yielded similar polyunsaturated fatty acid proportions, ranging from between 51.60 % to 56.8 % of total fatty acids. Linoleic acid was observed to be the dominant polyunsaturated fatty acid in the present study and in the previous studies mentioned above. The proportion of polyunsaturated fatty acids in the sunflower oil used in the present study was comparable to that of the soyabean oil, with 56.08 % of the total fatty acids in the sunflower oil being polyunsaturated fatty acids. Linoleic acid was also observed to be the dominant polyunsaturated fatty acid. Studies by Celebi and Utlu (2006) and Velasco et al. (2010), yielded similar results, with the sunflower oil they made use of composed of 58.99 % and 58.90 % polyunsaturated fatty acids, respectively. Linoleic acid was also the dominant polyunsaturated fatty acid in both cases. The slight differences observed in the various fatty acid proportions recorded for the different lipid sources between the current study and previous studies is probably as a result of different brands of oils being used.

*Growth performance*

The different HFDs that were used in the current study produced significantly increased final body masses in all the birds receiving the HFDs compared to their initial body masses, following the twelve-week feeding period. Whereas the final body mass of the birds receiving the STD was not significantly different from their initial body mass. However, when comparing percentage body mass gain between the dietary groups, only the birds receiving soyabean oil and those receiving sunflower oil had a significantly increased body mass gain compared to the STD group. It was noted however that the birds receiving the soyabean and sunflower oils had large amounts of feed /oil stuck to their feathers upon termination which could have accounted for the differences in percentage body mass gain observed. The dressed mass/carcass mass of the birds upon termination should be used in future studies, in order to allow for accurate comparison.

Previous studies involving HFD feeding with lipid sources of varying saturation levels have produced contradictory results. Mohammadi et al. (2011) observed significantly increased body mass gain in male broiler chickens receiving either 3 % tallow, 3 % soyabean oil, 1.5 % tallow + 1.5 % soyabean oil, 6 % tallow, 6 % soyabean oil or 3 % tallow + 3 % soyabean oil, compared to the control group. The broiler chickens receiving 6 % tallow (rich in saturated fatty acids) and those receiving 3 % tallow + 3 % soyabean oil (rich in polyunsaturated fatty acids) displayed the greatest body mass gain over the 6-week feeding period (Mohammadi et al., 2011). Poorghasemi et al. (2013) observed significantly increased body mass gain in broiler chickens receiving 2 % tallow + 2 % canola oil compared to those receiving either 4 % tallow; 4 % canola oil; 4 % sunflower oil or 2 % tallow + 2 % sunflower oil. The positive effects of the combination of tallow and canola oil on body mass gain was attributed to the influence of these lipid sources on reducing the rate of passage of the feed through the gastrointestinal tract of the birds, thus improving nutrient absorption and utilization (Latshaw, 2008; Poorghasemi et al., 2013). Crespo and Esteve-Garcia (2001) observed no significant differences in body mass gain of broilers fed diets containing tallow, linseed oil, olive oil or sunflower oil at two different levels of inclusion (6 % or 10 %). Ferrini et al. (2008) observed significantly increased body mass gain in female broiler chickens fed diets containing either

10 % tallow, 10 % sunflower oil rich in oleic acid, 10 % sunflower oil rich in linoleic acid, 10 % linseed oil rich or 10 % mix of fats, compared to the control group.

The contradictory results obtained by previous studies, as well as the lack of significant differences in percentage body mass gain between the various dietary groups in the current study, could be attributed to the age and growth phase of the birds during the feeding periods. The studies by Mohammadi et al. (2011), Poorghasemi et al. (2013) and Ferrinin et al. (2008) discussed above, all of which observed a significant increase in body mass gain in the HFD groups compared to that of the control group or significant differences in body mass gain between different HFD groups; all made use of broiler chickens which were fed various HFDs from the day the chickens were born, for approximately 5-6 weeks following birth. Thus, the birds would have been in a critical linear growth phase. In the current study, I only received the quail from the supplier at four weeks of age. Following a two-week adaptation period, the twelve-week feeding period commenced and thus the quail were six weeks old when beginning the feeding period and 18 weeks old upon being killed. If I had received the birds at an earlier age and begun the feeding period earlier, the results obtained with regards to the influence of both the STD and HFDs on the growth performance of the quail, may have been different.

Male Japanese quail have been shown to reach approximately 98 % of their mature body mass at twelve weeks of age (Raji et al., 2014). The quail in the present study were twelve weeks old following the first six weeks of STD and HFD feeding. Thus, the slow, steady growth of the quail in the last six weeks of feeding most likely led to the overall lack of significant changes in percentage body mass gain between the various dietary groups. Since the supplier was only able to determine the sex of the birds at four weeks of age, I was unable to start the experiment at a younger age, which was a limitation to my study. However, the rationale for starting the study with the quail at six weeks of age was to ensure that the total calories ingested, including the 22 % of added dietary lipids, could be utilised for metabolic activities other than tissue growth. Further studies on the effects of HFD feeding on the growth performance of poultry birds, including alternative poultry species, are required and should take into account the effects of age and growth phase of the birds used in the studies.

*Glucose tolerance*

As previously mentioned in section 2.4 of this thesis, the majority of studies involving dietary lipid supplementation in poultry birds are usually focussed on the effects of the various HFDs on the performance parameters of the birds including the growth performance, feed intake, feed conversion efficiency and resulting tissue fatty acid profile. The two main studies discussed in this thesis are unique in that the effects of the various HFDs on the overall health status of the birds were investigated. The various HFDs fed to the Japanese quail in the current study produced no adverse effects with respect to glucose tolerance, as no significant differences were observed in any of the oral glucose tolerance test parameters examined between birds in the different dietary groups.

The effects of HFDs on glucose tolerance have been investigated in various mammals and some birds. There is a dearth of information concerning the effects of different HFDs, with varying fatty acid profiles, on glucose handling in the Japanese quail. Given that HFDs rich in saturated and monounsaturated fatty acids have been shown to cause an imbalance in plasma glucose and insulin concentrations, whilst diets rich in polyunsaturated fatty acids are associated with normal glucose homeostasis in broiler chickens (Crespo and Esteve-Garcia, 2003; Newman et al., 2005), one would expect to observe varying responses to a glucose load in the quail fed the different HFDs. This was not the case. The previous study discussed in Chapter 2 of this thesis also yielded similar results, with no significant differences observed in any of the glucose tolerance parameters examined in the Guinea fowl and Muscovy ducks on the STD and HFD (rich in saturated and monounsaturated fatty acids).

As discussed in section 2.4 of this thesis, previous studies involving the effects of HFD feeding on glucose tolerance in different avian species have produced conflicting results. In agreement with the results of the current study, Lembede et al. (2014) also observed no significant differences in the glucose tolerance parameters examined in Japanese quail fed a STD or a HFD composed of canola oil (rich in polyunsaturated and monounsaturated fatty acids). The observed similarities in glucose handling between the STD and HFD groups was attributed to a possible synergistic effect of both the polyunsaturated and monounsaturated

fatty acids present in canola oil which may have resulted in increased in vivo β-oxidation, which in turn translated into a state of glucose homeostasis (Lembede et al., 2014). Pal et al. (2002), observed an altered glucagon and insulin sensitivity to plasma glucose in laying hens fed a diet supplemented with 4 % pumpkin seed oil (rich in omega-6 polyunsaturated fatty acids), whereas dietary supplementation with 4 % cod liver oil (rich in omega-3 polyunsaturated fatty acids) had no effect on plasma glucose response to glucagon and insulin. Even though the magnitude of the glucagon-induced rise in plasma glucose concentrations and the insulin-induced fall in plasma glucose concentrations, was not significantly different between the dietary groups, the administration of glucagon and insulin influenced the time course of plasma glucose alterations in the various dietary groups (Pal et al., 2002). The hens receiving the pumpkin oil displayed an extended glucose response to glucagon administration and an altered glucose response to insulin administration, compared to that of the control group and the group receiving cod liver oil (Pal et al., 2002). Even though the hens were not subjected to a glucose tolerance test, one would expect that the diet-induced alterations in plasma glucose response to glucagon and insulin would be mirrored in their response to an exogenous glucose load.

Crespo and Esteve-Garcia (2003) and Newman et al. (2005) observed the development of a degree of insulin resistance in broilers fed diets high in saturated and monounsaturated fatty acids compared to those fed polyunsaturated fatty acids. The broilers in the study by Crespo and Esteve-Garcia (2003) were not subjected to a glucose tolerance test. Those in the study by Newman et al. (2005) were subjected to an intravenous glucose tolerance test, however no significant differences in the glucose tolerance test parameters were observed, despite the significantly increased insulin release in response to the glucose load, observed in the birds receiving the diet high in saturated and monounsaturated fatty acids. Since the present study did not measure the insulin response to the oral glucose load administered to the quail, we are unable to comment on the influence of the HFDs on insulin sensitivity or the possible development of insulin resistance. Given the results from previous studies which indicate that diets rich in saturated and monounsaturated fatty acids induce alterations in insulin resistance, we would expect to see similar changes in the quail consuming the HFDs based on coconut oil, lard and palm oil. The quail consuming the diets based on soyabean oil and sunflower oil should display normal glucose homeostasis.

The results of previous studies investigating the effects of different dietary fats on glucose metabolism and glucose balance are somewhat unclear. This is probably due to differences in the avian species, dietary fats and time course of feeding that the various studies made use of. In order to clarify the regulatory role of various dietary fatty acids on glucose metabolism and the actions of glucagon and insulin in avian species, further investigations need to be carried out.

*Erythrocyte osmotic fragility*

In addition to the various HFDs not inducing any alterations in glucose tolerance in the Japanese quail, no adverse effects with regards to erythrocyte osmotic fragility were observed in any of the quail consuming the HFDs in the present study. Erythrocyte osmotic fragility was used as a measure of the overall health status (Azeez et al., 2011) of the birds during the HFD feeding period and as an indirect measure of the resulting fatty acid composition of the erythrocyte membrane, following the HFD feeding.

The fatty acid profile of the diet ingested by animals and humans can induce changes in the fatty acid composition of the erythrocyte membrane, which in turn affects its' ability to resist haemolysis (Benga et al., 1984; Clandinin et al., 1991; Kempaiah and Srinivasan, 2006). Previous studies in mammals have shown that diets rich in polyunsaturated fatty acids result in reduced erythrocyte osmotic fragility (Wing-Keong et al., 2001; Sengupta and Gosh, 2011; Bazzano et al., 2015), which is brought about by an increase in the polyunsaturated fatty acid content of the membrane, which in turn makes the membrane more flexible. Saturated fatty acid ingestion on the other hand, leads to a stiff, rigid membrane which results in an increase in erythrocyte osmotic fragility (Wing-Keong et al., 2001; Sengupta and Gosch, 2011; Bazzano et al., 2015). Very few studies have investigated the effects of HFD feeding on the osmotic fragility of avian erythrocytes and those that have tend to make use of only one type of HFD. No studies to our knowledge have investigated the effect of different HFDs with varying fatty acid profiles on Japanese quail erythrocyte osmotic fragility.

In a previous study by Abou-Ashour and Edwards (1972), White Leghorn laying hens fed either 0.1 %, 0.3 % or 1 % *Sterculia foetida* oil for 10 weeks, displayed a slight increase in erythrocyte osmotic fragility compared to the hens receiving the basal diet. Ingestion of the cycloprene fatty acids contained in the *Sterculia foetida* oil results in the disruption of normal fatty acid metabolism and the deposition of increasing amounts of saturated fatty acids within animal tissues (Evans et al., 1962; Abou-Ashour and Edwards, 1972). In the current study, one would have expected to observe increased erythrocyte osmotic fragility in the birds consuming the HFDs composed of coconut oil, lard and palm oil (rich in saturated and monounsaturated fatty acids). In the birds consuming the HFDs based on soyabean and sunflower oil (rich in polyunsaturated fatty acids) we expected to observe a reduced erythrocyte osmotic fragility. This was not the case. Thus suggesting that the HFDs used in the present study failed to induce any significant alterations in the fatty acid composition of the erythrocyte membrane and consequently no changes in erythrocyte osmotic fragility were observed.

As was the case with the Guinea fowl and Muscovy ducks (discussed in section 2.4 of this thesis), the lack of HFD-induced changes in erythrocyte osmotic fragility in the Japanese quail may also be as a result of some sort of adaptation to the HFDs by the gastrointestinal tract of the birds. The modification of various transport processes and metabolic activities of the enterocytes of the small intestine, induced by the HFD feeding, may alter the composition of fatty acids absorbed into the bloodstream and passed on to the tissues (Clandinin et al., 1991). As a result, any HFD-induced alterations in erythrocyte membrane fatty acid composition and thus in erythrocyte osmotic fragility, may have been prevented. Previous studies have also shown that membranes are able to maintain an ideal fatty acid composition through a variety of adaptive mechanisms referred to as 'homeoviscous adaptation', which serve to maintain the fluidity of the membrane to ensure optimal membrane functioning (Clamp et al., 1997; Abbott et al., 2012). These adaptive mechanisms serve to resist any drastic changes in membrane fluidity, through their influence on the membrane fatty acid composition in response to any diet-related changes (Clamp et al., 1997). Thus changes in membrane composition can occur as a result of both diet-induced effects, together with 'homeoviscous adaptation' influences (Clamp et al., 1997). Even if we had measured the fatty acid composition of the erythrocyte membranes in the current study, it would be difficult to determine to what extent the changes in membrane fatty acid composition, if any, were due to

ingestion of the HFDs alone or a combination of both the dietary influences and the 'homeoviscous adaptation'(Clamp et al., 1997). Since the twelve-week feeding period in the current study constitutes a relatively long/chronic time period, the lack of changes in erythrocyte osmotic fragility could be due to the mechanisms of 'homeoviscous adaptation' having already counteracted any HFD-induced changes in erythrocyte membrane fatty acid composition. Furthermore, membrane compositional changes following HFD feeding may not always be reflective of the dietary lipid profile (Vajreswari and Narayanareddy, 1992) and may sometimes only occur within certain 'lipid domains' of the membrane, with no overall change in membrane fluidity and osmotic fragility (Clamp et al., 1997). This could be the case in the present study. Further studies are required to investigate the resulting erythrocyte membrane fatty acid composition and the regulation thereof, following HFD feeding.

The lack of any alterations in glucose tolerance and erythrocyte osmotic fragility following consumption of the HFDs by the quail may also suggest some inter-avian species differences with regards to the metabolism of dietary lipids and their subsequent fate. These inter-species differences need to be further investigated.

*General health/clinical biochemistry profile*

Following consumption of the various HFDs, the overall health status of the birds was assessed through the measurement of several serum metabolic health markers, such as the serum lipid profile and markers of both liver and kidney health. As previously mentioned in section 2.4 of this thesis, no studies to our knowledge have investigated the overall health status of alternative poultry species such as the Guinea fowl, Muscovy duck and Japanese quail following HFD diet feeding of this nature. Previous studies performed mostly in chickens, have shown that the consumption of dietary fat brings about changes in the serum lipid profile (Monfaredi et al., 2011), which are dependent on the saturation level of the dietary fatty acids. Saturated fatty acid consumption tends to increase serum triglyceride and cholesterol concentrations, whereas the consumption of polyunsaturated fatty acids is usually favourable with regards to the serum lipid profile (Viveros et al., 2009; Velasco et al., 2010). Contrary to that observed in the study discussed in Chapter 2 of this thesis, in the current study, consumption of the various HFDs resulted in significantly reduced serum triglyceride concentrations in all HFD groups compared to that of the STD group. Serum cholesterol

concentrations were significantly decreased in the birds consuming the HFD composed of palm oil compared to that of the STD group. There were no significant differences in serum triglyceride or serum cholesterol concentrations between the various HFD groups. All the HFDs were well-tolerated by the birds and like the Guinea fowl and Muscovy ducks discussed in Chapter 2, the Japanese quail also remained healthy throughout the feeding period.

In contrast to the results of the present study, Velasco et al. (2010) observed significantly reduced serum concentrations of triglycerides and total cholesterol in broilers fed diets containing 90 g/kg of sunflower oil (rich in polyunsaturated fatty acids) compared to those receiving 90 g/kg of palm oil (rich in saturated and monounsaturated fatty acids). The polyunsaturated fatty acid-induced reduction in serum lipids was ascribed to a possible decrease in hepatic fatty acid and triglyceride synthesis, combined with reduced secretion of very low-density lipoprotein cholesterol and thus triglycerides, from the liver into the blood stream (Velasco et al., 2010). Increased rates of β-oxidation of unsaturated fatty acids have also been suggested, which in turn results in the increased uptake of triglycerides from the blood stream into the tissues (Sanz et al., 2000b; Velasco et al., 2010). Results from a study by Royan et al. (2013) are similar to the results obtained in the current study in that they also observed no significant differences in serum triglyceride concentrations between male, broiler chickens fed 7 % soyabean oil (rich in polyunsaturated fatty acids) compared to those fed 7 % palm oil (rich in saturated and monounsaturated fatty acids). However, the broilers receiving the soyabean oil displayed significantly reduced serum cholesterol concentrations compared to those receiving palm oil (Royan et al., 2013), which was not the case in the present study.

Das et al. (2014b) fed broiler chickens a STD (with no additional lipids) or HFDs composed 2.5 % soyabean oil or palm oil and monitored the resulting serum lipid profile after 3, 4, 5 and 6 weeks of feeding. At most of the time points examined, no significant differences in serum cholesterol concentrations were observed between the various dietary groups (Das et al., 2014b). Serum triglyceride concentrations were not significantly different between the various dietary groups after 3 and 4 weeks of feeding (Das et al., 2014b). After 5 and 6 weeks of feeding all chickens receiving the HFDs based on soyabean oil and palm oil, displayed significantly reduced serum triglyceride concentrations compared to those receiving the STD

(Das et al., 2014b), which was similar to what was observed in the current study. Peebles et al. (1997) also observed significantly reduced serum triglyceride concentrations in broiler chickens supplemented with 7 % added dietary lard compared to those receiving the STD, without additional lipids. The authors attributed the decrease in serum triglyceride concentrations to a metabolic overcompensation by the birds in response to the HFD (Peebles et al., 1997).

Studies performed on isolated hepatocytes have indicated that the concentration of serum triglycerides serves as a measure of *de novo* fatty acid synthesis (Beynen et al., 1983). This would suggest that the reduced serum triglyceride concentrations observed in the present study, following HFD feeding, may indicate a reduced level of *de novo* fatty acid synthesis. Since numerous fatty acids were being provided by the diet of the birds consuming the HFDs, it would make sense that hepatic *de novo* fatty acid synthesis and thus serum triglyceride concentrations would be reduced, compared to that observed in the birds consuming the STD. The ingestion of saturated fatty acids has been shown to upregulate the expression and synthesis of key genes involved in lipogenesis, whereas polyunsaturated fatty acid ingestion has been shown to inhibit lipogenesis (Clarke, 1993; Velasco et al., 2010). The variation in terms of the effects of the different types of fatty acids (saturated versus unsaturated) making up the HFDs on lipogenesis/fatty acid synthesis was not observed in the present study.

In contrast to the HFD-induced effects on serum triglyceride concentrations, no significant alterations in serum cholesterol concentrations were observed following consumption of most of the HFDs. These results are not in agreement with results from our previous study involving the Guinea fowl and Muscovy ducks, in which significantly increased serum cholesterol concentrations were observed in the birds receiving the HFD composed of palm oil and lard, compared to those receiving the STD. In the present study, the quail receiving the HFD composed of palm oil actually displayed significantly reduced serum cholesterol concentrations compared to that of the STD group. Thus, further suggesting a possible variation in the handling and subsequent proportioning of lipids between different avian species; with HFD-feeding likely having different effects on cholesterol biosynthesis pathways in different avian species. Previous studies in pigs (Qureshi et al., 1991) and chickens (Ong et al., 1988) have observed significantly reduced serum cholesterol

concentrations following dietary supplementation with the tocotrienol-rich portion of palm oil or palm oil, respectively. The tocotrienols contained within palm oil have been shown to reduce cholesterol biosynthesis by decreasing the amount of hydroxy-methyl-glutaryl-CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis, within the liver (Qureshi et al., 1991). This could be the case in the present study.

In addition to the serum lipid profile, other serum metabolic markers of both kidney and liver health, including serum uric acid, total protein, albumin, AST, total bilirubin and calcium concentrations, following the HFD feeding, were also investigated. Similarly to that observed in the Guinea fowl and Muscovy ducks, the various HFDs had no significant influence on any of the above mentioned serum parameters in the Japanese quail. Jalali et al. (2015) also observed no significant differences in serum albumin and total protein concentrations in male, broiler chickens fed diets supplemented with 3.8 % (starter feed- 2 weeks) and 5.43 % (grower feed- 2 weeks) of either sunflower oil or soyabean oil. Also in agreement with the results of the present study, Febel et al. (2008) observed no significant differences in serum concentrations of uric acid and AST in male broiler chickens receiving diets supplemented with 6 % lard, sunflower oil or soyabean oil, for a period of 5 weeks.

The lack of HFD-induced effects on the general health/clinical biochemistry profile of the quail confirm that consumption of the HFDs of varying fatty acid compositions, used in the present study, produced no apparent deleterious effects with regards to the health status of the birds.

*Organ masses and small and large intestine lengths*

Overall, the lack of HFD-induced effects on the health status of the birds was paralleled by the lack of HFD-induced effects on the relative organ masses and small and large intestine lengths, with no significant differences observed between the majority of the dietary groups. The birds in the SuO group displayed significantly lower relative liver masses compared to that of the STD group and the birds in the PO group displayed significantly longer small intestines than those in the STD group.

Al Daraji et al. (2011) observed significantly reduced relative liver, heart and gizzard masses in Japanese quail supplemented with 3 % sunflower oil or corn oil, both rich in omega-6 polyunsaturated fatty acids, compared to those receiving 3 % fish oil or flax seed oil, both rich in omega-3 polyunsaturated fatty acids. The differences observed were attributed to the reduced omega-6: omega-3 fatty acid ratios of the fish oil and flax seed oil, which have previously been shown to improve productive performance in quails (Al Daraji et al., 2011; Dalton 2000). Likewise, Wang et al. (2016) observed significantly reduced relative liver masses with increasing omega-6: omega-3 fatty acid ratios in Yangzhou goslings fed diets with varying omega-6: omega-3 fatty acid ratios, ranging from between 3:1 and 12:1. Despite the large variations in omega-6: omega-3 fatty acid ratios of the added dietary lipids used in the present study, only the relative liver mass of the quail receiving SuO was significantly different from that of the STD group. The reduced relative liver mass observed in the SuO group could be due to the significantly greater percentage body mass gain observed in these birds compared to that of the STD group, thus resulting in a significantly lower relative liver mass compared to that of the STD group.

As previously mentioned in section 2.4 of this thesis, results from previous studies on the effects of HFD feeding on organ mass and intestine length in avian species are inconsistent. Viveros et al. (2009) observed no significant influence of dietary lipid source on relative liver and pancreas mass, as well as on intestinal length, in broiler chickens fed diets supplemented with 8 % of various lipid sources, two of which were palm oil and sunflower oil. Similarly, El-Katcha et al. (2014) observed no significant differences in relative liver and heart mass in broiler chickens fed diets with varying omega-6: omega-3 fatty acid ratios, ranging from 1:1 to 11:1, which was achieved by mixing different proportions of linseed oil and sunflower oil. Poorghasemi et al. (2013) observed no significant differences in relative liver, gizzard, heart and kidney mass in male, broiler chicks receiving either 4 % tallow, 4 % canola oil, 4 % sunflower oil, 2 % tallow + 2 % canola oil or 2 % tallow + 2 % sunflower oil. However, significantly increased relative pancreas masses were observed in birds receiving 4 % tallow and significantly reduced relative proventriculus masses were observed in birds receiving 2 % tallow + 2 % sunflower oil, in comparison to the remaining dietary groups. Additionally, the birds receiving 4 % sunflower oil and those receiving 2 % tallow + 2 % canola oil displayed

significantly reduced duodenum and ileum lengths compared to that observed in the remaining dietary groups. No explanation for the reduction in intestinal length was alluded to in the study.

Ahmad et al. (2006) observed significantly reduced intestine length in broilers receiving a lipid supplemented (3 %) ration compared to those receiving no extra lipids (0 %). The dietary lipid supplementation was thought to reduce the rate of passage of food within the digestive tract, resulting in increased digestive efficiency (Ahmad et al., 2006). Thus, the intestines of the birds not receiving supplemental lipids were thought to be enlarged in order to increase total passage time of digesta through the tract and ensure adequate uptake of food from the intestines (Ahmad et al., 2006). This is in contrast to that observed in the present study where an increase in small intestine length was observed following dietary supplementation with PO, compared to the STD group, and no significant differences were observed in any of the other HFD groups. Thus, the effects of HFD-feeding, of varying fatty acid profiles, on organ mass and intestine lengths needs to be further investigated. However, since no adverse changes in organ size or intestine length were observed following ingestion of the majority of the HFDs used in the present study, it seems they were well-tolerated by the birds.

*Liver lipid content and fatty acid profile and liver histology*

Liver lipid content of the birds was not significantly affected by the various HFDs used in the present study, with no significant differences observed between the various dietary groups. As previously mentioned in section 2.4 of this thesis, dietary supplementation with saturated and monounsaturated fatty acids generally results in increased tissue lipid accumulation, whereas supplementation with polyunsaturated fatty acids usually reduces lipid deposition (Crespo and Esteve-Garcia, 2002b; Smink et al., 2010; Velasco et al., 2010). Thus, based on the differing fatty acid profiles and saturation levels of the added dietary lipids used to make up the HFDs in the present study, we were expecting to observe differences with regards to total liver lipid yield between the dietary groups, which was not the case. Previous studies involving dietary lipid supplementation and the resulting liver lipid yield have produced contradictory results.

As previously mentioned in section 2.4 of this thesis, Pinchasov and Nir (1992), An et al. (1997), Smink et al. (2010) and Velasco et al. (2010) observed a reduced liver lipid yield in chickens supplemented with lipids rich in polyunsaturated fatty acids compared to those receiving lipids rich in saturated fatty acids. Saturated fatty acids have been shown to up-regulate the expression and synthesis of genes which code for key enzymes involved in lipogenesis, whereas unsaturated fatty acids have been shown to inhibit lipogenic genes (Clarke, 1993; Velasco et al., 2010). Thus, the reduction in liver lipid yield following polyunsaturated fatty acid consumption, in the studies mentioned above, was attributed to a reduction in hepatic lipogenesis or an increase in fatty acid oxidation, or a combination of both (Pinchasov and Nir, 1992; Smink et al., 2010; Velasco et al., 2010). Crespo and Esteve-Garcia (2002b) also observed a significantly reduced liver lipid yield in broiler chickens receiving the basal diet and in those receiving the basal diet supplemented with 10 % linseed oil (rich in polyunsaturated fatty acids), compared to those receiving the basal diet supplemented with 10 % tallow (rich in saturated and monounsaturated fatty acids). Contrary to suggestions that the lipid-lowering effects of polyunsaturated fatty acid consumption is due to a reduction in hepatic lipogenesis, Crespo and Esteve-Garcia (2002b) observed enhanced hepatic lipogenesis in the chickens receiving linseed oil. Thus suggesting that increased hepatic lipogenesis may not always be associated with increased liver lipid deposition (Crespo and Esteve-Garcia, 2002b). The reduced liver lipid deposition observed in the chickens receiving linseed oil was attributed to the fact that the higher levels of hepatic lipogenesis may serve as a mechanism by which to dissipate energy, thus contributing to the reduced liver lipid deposition observed (Crespo and Esteve-Garcia, 2002b). The authors attributed the increased liver lipid yield in the chickens receiving the diet supplemented with tallow to the direct transfer of dietary lipids to be deposited in the liver, rather than an increase in hepatic lipogenesis (Crespo and Esteve-Garcia, 2002b).

In contrast to the findings discussed above, Gonzalez-Ortiz et al. (2013) observed no significant differences in liver lipid content between female broilers receiving diets supplemented with either 10 % of a blend of fish oil and linseed oil (rich in polyunsaturated fatty acids) or 10 % tallow (rich in saturated and monounsaturated fatty acids). Magubane et al. (2013) also observed no significant differences between Japanese quail receiving 10 % canola oil (rich in monounsaturated fatty acids) and those receiving the STD with no added lipids. Our results are in agreement with that of Gonzalez-Ortiz et al. (2013) and Magubane et

al. (2013). The lack of diet-induced differences with regards to liver lipid deposition in the Japanese quail used in the present study further supports the possibility of genetic differences in lipid metabolism between different avian species and between different breeds of the same avian species, as discussed in section 2.4 of this thesis. These possible genetic differences need to be further investigated.

With regards to the lipid profiles of the livers from birds in the various dietary groups, the lipid profiles mirrored those of the added dietary lipids which were used to compile the HFDs. The livers of the birds receiving CO had the highest percentage of saturated fatty acids, with palmitic acid being the dominant saturated fatty acid. This was expected since the CO used was composed almost entirely of saturated fatty acids. The livers of the birds receiving SO had the highest percentage of polyunsaturated fatty acids, with linoleic acid being the dominant polyunsaturated fatty acid. This was also expected since the SO used was composed predominantly of polyunsaturated fatty acids. The highest saturated to unsaturated fatty acid ratio was observed in the livers of the birds consuming CO and the lowest ratio was observed in the livers of the birds receiving SO, which further echoes the lipid composition of the oils used to compose the respective HFDs. The birds receiving the HFDs based on L, PO and SO displayed increased omega-3 fatty acid content within their livers, which was mainly as a result of an increase in docosahexaenoic acid (DHA) content. The increased liver omega-3 fatty acid content in the L, PO and SO groups was accompanied by a concomitant reduction in the omega-6: omega-3 polyunsaturated fatty acid ratios compared to the STD group. The largest decrease in omega-6: omega-3 polyunsaturated fatty acid ratio was observed in the livers of the birds in the SO group.

The results of the present study are in agreement with previous studies involving HFD-feeding in birds, albeit mostly in chickens, and the consequent liver lipid profiles. In general, the liver lipid profile of most avian species studied to date has been shown to reflect that of the dietary lipid profile, following HFD feeding (An et al., 1997; Pierce et al., 2005; Smink et al., 2010; Ben-Hamo et al., 2011).

*Muscle lipid content and fatty acid profile*

The breast and thigh muscles examined in the present study serve as popular poultry meat portions for human consumption. The majority of studies which have focussed on the transfer of beneficial dietary polyunsaturated fatty acids into the edible tissues of poultry birds have made use of chickens. To our knowledge, the current study is unique in the fact that we investigated the effects of different HFDs, with varying fatty acid profiles, on the resulting lipid content and fatty acid profiles of the edible bird tissues of Japanese quail, which are becoming popular as table birds for human consumption.

In the present study, since the muscle lipid content and fatty acid profile analyses were performed on a single composite (representative) sample per dietary group, I was unable to perform statistical analyses on the data with a sample size of n = 1. Thus, I can merely describe the trends observed. Following the twelve-week feeding period, both the breast and thigh muscles of the Japanese quail in the various HFD groups had an average (± SD) lipid yield of 2.34 ± 0.15 % and 2.62 ± 0.80 %, respectively. These values are in agreement with previous studies in which the lipid yield of quail breast and thigh muscles ranged between 1.0 % and 3.4 %, in birds that were fed a much lower percentage of dietary lipids (Ribarski and Genchev, 2013). Thus, significantly increasing the level of lipid inclusion in the diets of the quail used in the present study was unsuccessful in increasing the overall lipid content of the edible bird tissues and cannot serve as a possible means of increasing the overall amount of lipids ingested by humans. The birds receiving the HFDs in the present study had a higher percentage lipid yield in their breast muscles compared to that observed in the STD group. Of the HFD groups, the breast muscles of the birds receiving CO had the highest percentage lipid yield, whereas the breast muscles of the birds receiving PO had the lowest percentage lipid yield. Only the birds in the PO and SO dietary groups had a higher percentage lipid yield in their thigh muscles compared to that observed in the STD group. The thigh muscles from the birds in the remaining HFD groups had a lower percentage lipid yield compared to that observed in the STD group. Crespo and Esteve-Garcia, (2001) observed no significant effect of dietary fat type or level of inclusion on the overall lipid content of the breast and thigh muscles of broiler chickens supplemented with 6 % or 10 % of either tallow, olive oil, sunflower oil or linseed oil. Similarly, Uchewa (2013) also observed no significant differences in overall lipid content in both the breast and thigh muscles of broiler chickens fed diets containing 5 % palm oil, 5 % groundnut oil (rich in unsaturated fatty acids) or no

additional lipids. Despite the inconsistent results obtained with regards to the effects of the various HFDs on the overall lipid content of the breast and thigh muscles of the Japanese quail in the present study, the lipid profiles of both the breast and thigh muscle samples of the birds receiving the HFDs were reflective of the dietary lipid profiles.

The results of the current study are in agreement with previous studies, which have shown that the tissue lipid profile of poultry birds is highly dependent on the lipid/fatty acid profile of the diet consumed by the birds during production, despite variations in levels of inclusion of dietary lipids, as well as feeding duration (Dvorin et al., 1998; Crespo and Esteve-Garcia, 2001; Duraisamy et al., 2013). The breast and thigh muscles of the birds consuming the HFD based on CO had the highest percentage of saturated fatty acids, with palmitic acid and lauric acid being the dominant fatty acids, respectively. The breast and thigh muscles of the birds receiving the HFD composed of PO had the highest percentage of monounsaturated fatty acids with oleic acid being the dominant fatty acid in both the breast and thigh muscles. The highest percentage of polyunsaturated fatty acids was observed in the breast and thigh muscles of the birds consuming the HFD based on SuO, with linoleic acid being the dominant fatty acid in both the breast and thigh muscles. The highest saturated to unsaturated fatty acid ratio was observed in both the breast and thigh muscles from the birds receiving CO, whilst the lowest ratio was observed in the breast and thigh muscles from the birds receiving SuO. The above results were expected, based on the lipid profiles of the various dietary lipids used in the present study.

With regards to the proportions of various fatty acid classes and individual fatty acids within the breast and thigh muscles of the Japanese quail, similar results have been obtained in previous studies making use of some of the same lipid sources as those used in the present study. Abdulla et al. (2015) observed a significant increase in oleic acid (C18:1), palmitic acid (C16:0) and total saturated fatty acid content in the breast muscle of broilers fed 6 % palm oil compared to those fed 6 % soyabean oil. The broilers receiving the soyabean oil had significantly increased breast muscle content of α-linolenic acid (C18:3), linoleic acid (C18:2) and total polyunsaturated fatty acids compared to those receiving the palm oil. In the current study, both the breast and thigh muscles of the Japanese quail fed the HFD containing PO had a higher percentage of oleic and palmitic acid compared to those receiving SO. The birds

receiving SO had a higher percentage of α-linolenic and linoleic acid compared to those receiving PO. Nyquist et al. (2013) also observed a significantly increased percentage of linoleic acid and total polyunsaturated fatty acids in the breast muscles of broiler chickens fed diets containing various levels of soyabean oil compared to those containing various combinations of rendered animal fat, palm oil and linseed oil. Similarly, Crespo and Esteve-Garcia (2001) observed a significantly increased percentage of saturated fatty acids in the breast and thigh muscles of broilers receiving tallow (rich in saturated fatty acids) compared to those receiving sunflower oil. The breast and thigh muscles of the broilers receiving sunflower oil had significantly higher polyunsaturated fatty acid content compared to those receiving tallow (Crespo and Esteve-Garcia, 2001). Oleic acid was the dominant fatty acid in the tissue of the broilers receiving tallow, whilst linoleic acid was the dominant fatty acid in the tissues of the broilers receiving sunflower oil (Crespo and Esteve-Garcia, 2001). Smink et al. (2008) observed an increased concentration of total saturated fatty acids, with palmitic acid being the dominant fatty acid, in the breast muscle of broiler chickens fed diets containing 4 % and 8 % of palm oil versus those receiving 4 % or 8 % of sunflower oil. An increased concentration of total unsaturated fatty acids, with linoleic acid being the dominant fatty acid, was observed in the breast muscle of the broiler chickens fed diets containing sunflower oil compared to those fed diets containing palm oil (Smink et al., 2008). Ben-Hamo et al. (2011) also observed that the degree of fatty acid unsaturation in the breast muscle of Japanese quail was reflective of the dietary lipids consumed, when quail were gavaged daily with either coconut oil (high in saturated fatty acids) or canola oil (high in unsaturated fatty acids).

The total lipid deposition, as well as the deposition of total saturated and polyunsaturated fatty acids in the breast muscles versus the thigh muscles of the Japanese quail in the present study, was inconsistent amongst the various dietary groups. Avian breast muscles are generally characterised by a low overall lipid content and high crude protein content compared to thigh muscles. In the present study, birds fed the STD diet, as well those fed the HFDs composed of PO and SO had a lower percentage total lipid yield in their breast muscles versus their thigh muscles. Genchev et al. (2005) also observed a similar trend in both Faraon and White English breeds of Japanese quail, where both breeds displayed lower lipid content in their breast muscles (3.27 % and 1.58 %, respectively) compared to their thigh muscles (5.43 % and 3.86 %, respectively). The quail were fed standardised starter and finisher diets which had

slightly lower energy content and higher protein content (Genchev et al., 2005) compared to the STD used in the present study. Vitula et al. (2011) also observed lower lipid content in the breast muscles (130.64 ± 50.41 g/kg) versus thigh muscles (299.60 ± 69.46 g/kg) of Japanese quail. The diet fed to the birds and the breed of Japanese quail which was assessed however, was not specified. In contrast, the birds in the present study fed the HFDs composed of CO, L and SuO, displayed a higher percentage lipid yield in their breast muscles compared to that in their thigh muscles.

In addition to the differences observed in the deposition of total lipid content within the breast and thigh muscles of the Japanese quail following consumption of the various HFDs, differences in the preferential deposition of specific classes of fatty acids within the muscles of the birds were also observed. The digestibility and thus the content of metabolisable energy within saturated and polyunsaturated fatty acids is different (De Witt et al., 2012), which ultimately affects the fate of the dietary fat once ingested, in terms of being stored or hydrolysed for energy (Dalton 2000). In the birds fed the HFDs composed of CO and L, both high in saturated fatty acids, saturated fatty acids were preferentially deposited in the thigh muscle whereas polyunsaturated fatty acids were preferentially deposited in the breast muscle. In contrast, in the birds receiving the HFDs composed of SO and SuO, both high in polyunsaturated fatty acids, saturated fatty acids were preferentially deposited in the breast muscle and polyunsaturated fatty acids in the thigh muscle. The breast muscle of the birds receiving PO, comprised predominantly of monounsaturated fatty acids, had a higher percentage of both saturated and polyunsaturated fatty acids compared to that observed in the thigh muscle. The mechanisms involved in the preferential deposition of total lipid content as well as specific fatty acid classes either in the breast or thigh muscles of Japanese quail needs to be further investigated.

As previously mentioned, the breast and thigh muscles of quail are popular cuts of meat consumed by humans. Despite quail meat being a valuable source of protein for human consumption, with an amino acid profile of high biological value, its omega-6: omega-3 polyunsaturated fatty acid ratio is somewhat unfavourable in terms of human health (Genchev et al., 2008). Recent studies recommend an optimal dietary omega-6: omega-3 polyunsaturated ratio of between 1:1 and 5:1. The present Western diet has an omega-6:

omega-3 ratio ranging between 15:1 and 20:1 (Duan et al., 2014). In the present study, an omega-6: omega-3 polyunsaturated fatty acid ratio of 37:1 in the breast muscles and 34.55:1 in the thigh muscles were observed for birds fed the STD. In the breast muscles of the birds receiving coconut oil, lard, palm oil and soyabean oil, increased omega-3 fatty acid content was observed, with an associated decrease in the omega-6: omega-3 polyunsaturated fatty acid ratio in these dietary groups compared to that observed in the STD group. The largest decrease was observed following lard supplementation. In the thigh muscles of the birds receiving lard and soybean oil, an increased omega-3 fatty acid content was observed, with an associated decrease in omega-6: omega-3 polyunsaturated fatty acid ratios, compared to that observed in the STD group. The largest decrease was observed following soybean oil supplementation. The omega-6: omega-3 polyunsaturated fatty acid ratios of both the breast and thigh muscles mirrored the ratios of the added dietary fats themselves. However, despite the observed decreases in omega-6: omega-3 polyunsaturated fatty acid ratios, following supplementation with the various dietary fats, as mentioned above, the omega-6: omega-3 polyunsaturated fatty acid ratios were still extremely high compared to the recommended optimal omega-6: omega-3 polyunsaturated fatty acid ratio.

*Femur mass, length and density*

Previous studies performed in birds have indicated that the ingestion of dietary lipids can improve or impair the growth and development of bone tissue as well as bone mineral content, depending on the type and amount of dietary lipids ingested (Wohl et al., 1998; Watkins et al., 2001; Liu et al., 2003). In the current study, no significant HFD-induced effects were observed with respect to the mass, length or density (Seedor index) of the femora obtained from the Japanese quail in the various dietary groups. The calculation of the Seedor index, using the bone mass and length measurements, was used as an indirect measure of bone mineral content.

A substantial number of bone fractures are commonly observed in caged, laying hens and fast-growing broilers, which are thought to be brought about by the development of osteoporosis due to structural bone loss at the onset of sexual maturity (hens), dietary deficiencies and the relative inactivity of caged birds (Whitehead and Fleming, 2000; Tarlton et al., 2013; Zhong et al., 2014). Thus, the skeletal health of poultry has become a major

welfare and economic problem in terms of poultry production (Whitehead and Fleming, 2000; Tarlton et al., 2013). In many production animals, such as poultry, which are conventionally fed grains which are rich in omega-6 polyunsaturated fatty acids, a dietary deficiency of omega-3 polyunsaturated fatty acids exists (Tarlton et al., 2013). Previous animal studies have shown that a balanced intake of omega-6 and omega-3 polyunsaturated fatty acids may in fact reduce the signs of osteoporosis, an effect which is thought to involve various mediators generated by polyunsaturated fatty acids, such as prostaglandins (Tarlton et al., 2013). Previous studies involving the effects of dietary lipid supplementation on various bone characteristics have yielded conflicting results.

Liu et al. (2003) observed no significant differences in tibial mass, length or diameter in Japanese quail fed HFDs composed of soyabean oil, hydrogenated soyabean oil, chicken fat or fish oil at 50g/kg of diet, for a period of 7 months. Bone mineral content, expressed as bone ash weight (mg) per unit of tibial length, was significantly increased in the birds receiving the diets containing hydrogenated soyabean oil and fish oil, compared to those receiving soyabean oil and chicken fat (Liu et al., 2003). These results are somewhat confusing, since the omega-6: omega-3 ratio of the hydrogenated soyabean oil was 17.85:1 and that of the fish oil was 0.66:1 (Liu et al., 2003), both of which had positive effects with respect to bone mineral content. Jiang et al. (2014) also observed no significant, direct HFD-induced effects on the femoral and tibial bone mass, length and diameter in laying chicken hens receiving a moderate-fat diet (7 % soybean oil) or HFD (10 % soybean oil) for a period of 90 days. The relative femoral and tibial mass, as well as femoral stiffness and tibial mixed force however, were significantly reduced in the hens in the moderate and HFD groups (Jiang et al., 2014). The soyabean oil used to compile the HFDs had an omega-6: omega-3 ratio of approximately 6.10:1 (Jiang et al., 2014), which is substantially lower than that of the soyabean oil used in the study by Liu et al. (2003) and yet no beneficial effects were observed. Dietary supplementation with the soyabean oil was in fact found to be harmful with regards to bone strength and mineralisation (Jiang et al., 2014). Zhong et al. (2014) observed no significant influence of dietary supplementation with lard (rich in saturated and monounsaturated fatty acids), linseed oil (rich in polyunsaturated fatty acids) or palm oil (rich in saturated and monounsaturated fatty acids) on tibial growth and various bone characteristics in broiler chickens. Broilers which were fed a mixture of the palm oil and linseed oil had significantly increased tibial mass and length, as well as bone mineral content, compared to the broilers

receiving linseed oil alone (Zhong et al., 2014). The beneficial effect of the mixture of linseed and palm oil on tibial growth was attributed to a change in lipid stability of the polyunsaturated fatty acids in the linseed oil, by the saturated fatty acids in palm oil (Zhong et al., 2014). The inconsistent results obtained from previous studies involving the beneficial effects of different omega-3 polyunsaturated fatty acid sources, on bone growth and various bone characteristics, have been attributed to conflicting properties and physiological effects of the various dominant omega-3 polyunsaturated fatty acids (Zhong et al., 2014).

However, some of the previous studies have made use of the same oils, rich in omega-3 polyunsaturated fatty acids, and still yielded conflicting results. Thus, it seems that different blends or brands of oils may have slightly different compositions with regards to the fatty acids present, which could influence their activity with regards to alterations in bone characteristics. The effect of dietary lipids on bone health needs to be clarified.

### 3.5 Conclusion

In conclusion, the results of the present study suggest that the HFDs of varying fatty acid profiles, fed to the Japanese quail, were well-tolerated by the birds with no apparent adverse effects observed with respect to their health status. The fatty acid profiles of the edible bird tissues were reflective of the dietary lipid profile of the various HFDs consumed by the birds, thus confirming successful transfer of the dietary fatty acids into the edible bird tissues. The use of HFDs such as those investigated in the present study, in a poultry production setting, would thus be effective in modifying tissue lipid profiles of alternative poultry species, such as the Japanese quail, if necessary, without any adverse consequences with regards to the health status of the birds during production. Despite the significantly increased level of dietary lipid inclusion used in the formulation of the HFDs in the present study, the diets failed to affect the total lipid content of the edible bird tissues.

Similarly to that observed in the previous study involving the Guinea fowl and Muscovy ducks, the lack of significant improvements in the growth performance of the Japanese quail following HFD feeding, compared to that of the birds in the STD group, also suggests that diets of alternative poultry species such as the Japanese quail may not need to be

supplemented with extra lipids as an energy source, with the aim of improving the growth performance of the birds.

As mentioned in section 2.5 of this thesis, further studies into the use of HFDs in alternative poultry species are required in order to establish the physiological mechanisms behind the resistance of these alternative poultry species to the metabolic effects of the HFDs. Additionally, the mechanisms involved in the regulation of the overall content of lipid deposition within the edible bird tissues should be further investigated.

# CHAPTER FOUR: OVERALL DISCUSSION AND CONCLUSIONS

In this thesis I have investigated the physiological responses of Guinea fowl, Muscovy ducks and Japanese quail to HFDs, of varying fatty acid profiles, as a means of assessing the overall health status of the birds following HFD-feeding. The research discussed in this thesis is unique in that, to our knowledge, it is the first study to assess the overall health status of these alternative poultry species following HFD-feeding of this nature. In addition to the routine growth performance and serum biochemistry analyses performed following feeding trials, the present studies also assessed the HFD-induced changes in parameters such as glucose tolerance and erythrocyte osmotic fragility. Thus, allowing us to perform a thorough investigation into any possible HFD-induced metabolic alterations within the birds, following the HFD-feeding.

I hypothesized that the HFDs used in the present studies would improve the growth performance of the birds, confirmed by a significant increase in percentage body mass gain in the HFD groups compared to that of the STD group. This was not the case. Overall, there were no significant differences in percentage body mass gain between the HFD and STD groups. Thus, as mentioned previously in sections 2.5 and 3.5 of this thesis, if the primary goal for adding additional lipids to the diets of these alternative poultry species is to improve their growth performance, it might not be necessary to do so. Since feed constitutes one of the largest expenses incurred by poultry farmers during the production process (Afolayan and Afolayan, 2008; Arango, 2009; Oladokun and Johnson, 2012; Begli et al., 2016), one could possibly eliminate part of the feed costs by foregoing the addition of added dietary lipids in the form of animal fats and vegetable oils to feed formulated for these alternative poultry species.

Besides the improvement in growth performance observed in some previous studies, the addition of lipids to poultry diets has been shown to have a number of other advantages. These advantages include reduced feed intake and improved feed efficiency; improved palatability of the feed; reduced dustiness of the feed and thus reduced feed loss; reduced rate of passage of the feed through the digestive tract and thus improved digestion and absorption of nutrients (Firman et al., 2008; Mahesar et al., 2011; Ravindran et al., 2016). As previously mentioned, I was unable to accurately measure feed intake of the birds during the experimental period. Thus, I was unable to comment on the feed conversion efficiency of

these birds following consumption of the HFDs. Despite the lack of improvement in growth performance of the Guinea fowl, Muscovy ducks and Japanese quail, following the HFD-feeding in the current studies, taking into consideration the advantages with regards to reducing feed loss as a result of reduced dustiness of the feed, following the addition of lipids to poultry diets, one might consider formulating the diets for these birds with the additional lipids nonetheless. The issues of cost versus benefit would have to be deliberated.

I also hypothesized that the HFDs used in the present studies would adversely affect the overall health profile of the birds, confirmed by alterations in glucose tolerance, erythrocyte osmotic fragility, bone health and the serum biochemistry profile, following HFD-feeding. Overall, no adverse effects with regards to the health status of the birds were observed following consumption of the various HFDs. Despite the significantly increased serum cholesterol levels observed in the Guinea fowl and Muscovy ducks following consumption of the HFD based on palm oil and lard, the HFDs were generally well-tolerated by the birds throughout the feeding periods. Thus, if HFDs of this nature were used in the production of alternative poultry species, with the intention to modify the lipid profiles of the edible bird tissues or to maximise on some of the other advantages the addition of lipids to poultry diets offers, as discussed above, the birds should remain relatively healthy throughout the production period. The maintenance of bird health and well-being during the production period often results in improved productivity and thus is also associated with economic benefits in terms of reducing production costs (Meissner et al., 2013b).

The resilience of these alternative poultry species to the HFD-feeding, in terms of the lack of HFD-induced disturbances in their overall health profile, is an area of interest for future studies. The regulatory role of different dietary fatty acids on glucose and lipid metabolism in avian species, as well as the possible inter-avian species differences with regards to the handling and subsequent proportioning of dietary lipids needs to be further investigated. The molecular mechanisms underlying these species differences with regards to lipid metabolism, including possible differences in the regulation and rates of lipid synthesis and oxidation, as well as the expression and activities of various enzymes involved in lipid handling processes, need to be further investigated.

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# APPENDICES

## Appendix 1

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

FACULTY OF HEALTH SCIENCES

**PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS**

SENATE PLAGIARISM POLICY: APPENDIX ONE

I Janine Donaldson (Student number: 0202199 N) am a student registered for the degree of Doctor of Philosophy in the academic year 2016.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature: [signature] Date: 22/06/2016

1

# Appendix 2

AESC3

**STRICTLY CONFIDENTIAL**

**ANIMAL ETHICS SCREENING COMMITTEE (AESC)**

**CLEARANCE CERTIFICATE NO.** 2011/08/2A

**APPLICANT:** **Ms J Donaldson**

**DEPARTMENT:** **School of Physiology**

**PROJECT TITLE:** ***Avian models for high-fat diet induced non-alcoholic fatty liver disease***

**Number and Species**

36 Guinea fowl
36 Muscovy duck

Approval was given for to the use of animals for the project described above at an AESC meeting held on **06 September 2011**. This approval remains valid until **30 September 2013**.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

Condition 1:- A pilot study must be carried out, using 6 animals in the first group and 9 in the second. The result should be reported to the Committee. If they justify a larger study, it will be necessary to clarify the numbers to be involved in each group.

Signed: _____ (Chairperson, AESC) Date: 12/10/2011

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 25/10/2011

cc: Supervisor:
Director: CAS

AESC 2012

*Please note that only typewritten applications will be accepted. Should additional space be required for section "I" and/or "j", please use the back of this form.*

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Janine Donaldson

b. Department: School of Physiology

c. Experiment to be modified / extended **AESC NO: 2011/08/2A**

| | | | |
|---|---|---|---|
| Original AESC number | 2011 | 08 | 2A |
| Other M&E's : N/A | | | |

d. Project Title: Avian models for high-fat diet induced non-alcoholic fatty liver disease.

| | | |
|---|---|---|
| e. Number and species of animals **originally approved**: | | |
| f. Number of additional animals **previously allocated** on M&Es: | | |
| g. Total number of animals **allocated** to the experiment to date: | | |
| h. Number of animals **used** to date: | | |

i. Specific modification / extension requested:

I would like to add additional co-workers to my study that were not originally listed on my ethics application.

Names and Surnames of co-workers to be added:
- Rachael Dangarembizi
- Busisani Lembede

j. Motivation for modification / extension:

Rachael and Busisani will be assisting me with the handling and feeding of the birds and thus are required to have access to the CAS unit for the duration of my study.

Date: 27/01 2012 Signature: .....

**AESC 2012**

***Please note that only typewritten applications will be accepted. Should additional space be required for section "I" and/or "j", please use the back of this form.***

## ANIMAL ETHICS SCREENING COMMITTEE
## MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Janine Donaldson

b. Department: School of Physiology

c. Experiment to be modified / extended **AESC NO: 2011/08/2A**

| Original AESC number | 2011 | 08 | 2A |
|---|---|---|---|
| Other M&E's: 1 previous M & E was approved on the 8th March 2012 in order to add additional co-workers to the study that were not originally listed on the ethics application. | | | |

d. Project Title: Avian models for high-fat diet induced non-alcoholic fatty liver disease.

| | | |
|---|---|---|
| e. Number and species of animals **originally approved**: | | 36 Guinea fowl<br>36 Muscovy ducks<br>6 pilot study birds |
| f. Number of additional animals **previously allocated on M&Es**: | | N/A |
| g. Total number of animals **allocated** to the experiment to date: | | 78 |
| h. Number of animals **used** to date: | | 84 |

i. Specific modification / extension requested:

I would like to request an additional 6 birds for my study (3 Guinea fowl and 3 Muscovy ducks).

j. Motivation for modification / extension:

I was requested by the Animal Ethics Screening Committee to perform a pilot study using 6 birds (which was interpreted as 6 of each bird species). In order to comply with the methods set out in my study protocol, I performed the pilot study using 12 birds (6 Guinea fowl and 6 Muscovy ducks), to enable me to compare the findings between the two different birds species (the growth performance data of the pilot study birds is attached). The pilot study was successful and the birds fared comparably on the high fat diet and without ill effect. The tissue samples collected from the pilot study birds are being used to refine the techniques I will be using for the main study (ie-molecular methods etc).

Unfortunately the birds that I received for the pilot study were from a farmer who was no longer able to provide me with additional birds for the study as originally promised. I have thus had to resort to a different farmer to keep uniformity of the strain of birds used. Thus in order to complete my study and to avoid impacting on my total sample size (to make the main study statistically significant, keeping with the n=6 at each time point), in addition to the 12 birds used for the pilot study, I require 36 Guinea fowl and 36 Muscovy ducks (a total of 84 birds). Only 78 birds were originally approved for the study and thus in order to make up the numbers, since I am not making use of the pilot birds for the final sample size, I still require 6 extra birds (3 Guinea fowl and 3 Muscovy ducks).

Signature: Date: 10/05/2012

Janine Donaldson

**RECOMMENDATIONS**:

Approved: additional animals (6 Guinea fowl and 6 Muscovy ducks).

Date:10 May. 12 Signature: .

Chairman, AESC

**Appendix 3**

Thesis2016Turnitinpdf1.pdf

ORIGINALITY REPORT

| 8% | 0% | 8% | 0% |
|---|---|---|---|
| SIMILARITY INDEX | INTERNET SOURCES | PUBLICATIONS | STUDENT PAPERS |

PRIMARY SOURCES

| | | |
|---|---|---|
| 1 | Donaldson, J., K. Pillay, M. T. Madziva, and K. H. Erlwanger. "The effect of different high-fat diets on erythrocyte osmotic fragility, growth performance and serum lipid concentrations in male, Japanese quail (Coturnix coturnix japonica )", Journal of Animal Physiology and Animal Nutrition, 2014. Publication | 4% |
| 2 | Donaldson, J., R. Dangarembizi, B. Mtetwa, M. T. Madziva, and K. H. Erlwanger. "The progressive effects of a high-fat diet on erythrocyte osmotic fragility, growth performance and serum triglyceride and cholesterol levels in Guinea fowl (Numida meleagris ) and Muscovy duck (Cairina moschata )", Journal of Animal Physiology and Animal Nutrition, 2013. Publication | 3% |

KENNEDY H. ERLWANGER MICHAEL T. MADZIVA

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EXCLUDE MATCHES < 3%