USE OF FLAXSEED OIL FOR SUSTAINABLE ENRICHMENT OF CHICKEN MEAT WITH OMEGA-3 FATTY ACIDS FOR HUMAN CONSUMPTION

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MSc (Nutrition and Food Science)

A thesis submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

FOOD*plus* Research Centre

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The University of Adelaide

April 2018

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Abstract

Chickens naturally possess the required enzymes to convert the omega-3 polyunsaturated fatty acids (n-3 PUFA) precursor α-linolenic acid (ALA, found at a high percentage in flaxseed oil) to the long chain n-3 PUFA (or n-3 LCPUFA). However, flaxseed oil is relatively expensive to be used in poultry feed. Therefore, a number of strategies have previously been studied to reduce the additional cost of the enriched n-3 LCPUFA chicken meat. Nevertheless further investigation is still needed before this approach can be commercially applied.

This research aimed to: i. investigate the ability of a flaxseed oil based diet to enrich broiler tissues with n-3 LCPUFA and/or enhance growth performance, ii. clarify the relationship between the fatty acid profile (with emphasis on n-3 PUFA) of diet and excreta in broilers, iii. investigate the shortest required period for feeding broilers the flaxseed oil diet while maintaining an acceptable level of n-3 LCPUFA in meat tissues and iv. determine whether exposing the developing chicken embryo to higher levels of n-3 PUFA will subsequently modify the broilers ability to utilize ALA and/or deposit n-3 LCPUFA in meat.

The dietary effect of six very different fats (including flaxseed oil) on changing the fatty acid profile of seven tissues in broilers was investigated. The strong correlations and regressions between diet and tissue (except brain) fatty acid levels (highest for the n-3 PUFA) validate the ability to predict the tissue fatty acid profile of broilers based on their dietary fat composition. However, unexpectedly none of the growth performance parameters or crude fat percentages of the tissues were influenced by changing dietary fat type suggesting that such effects may only become detectable when birds are under stress.

Furthermore, the relationship between excreta fatty acid composition and dietary fatty acid intake showed positive linear correlations for all fatty acid groups. Comparing the fatty acid content of diet and excreta suggested that the broilers most preferentially utilized n-3 PUFA group. Oppositely, n-6 PUFA and some saturated fatty acids (C16:0 and C18:0) were under-utilized.

Feeding broilers the flaxseed oil diet for different periods prior to slaughter showed a comparable level of n-3 LCPUFA in the meat by feeding broilers from week 3 as for the entire six week growth period. This strategy would result in a reduction of the required flaxseed oil amount by >9.4%. Thus, this is an economically-viable step towards commercializing n-3 LCPUFA-enriched chicken meat from using flaxseed oil diet.

Exposing broiler embryos to high ALA or n-3 LCPUFA via the maternal diet did not enhance the ability of the progeny to deposit n-3 LCPUFA in their tissues when they were subsequently fed a flaxseed oil diet. Thus, this strategy was not effective in further elevating n-3 LCPUFA levels in the chicken meat.

In summary, the outcomes of this research support the efficient use of a flaxseed oil-based diet to produce n-3 LCPUFA enriched chicken meat to offer a practical pathway to increase n-3 LCPUFA intake by a wide range of consumers without relying on further unsustainable exploitation of marine resources.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree. I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Khaled Adnan Khaled Kanakri

December 2017

Work arising from candidature

1.1 Publications

1. **Khaled Kanakri**, John Carragher, Robert Hughes, Beverly Muhlhausler, Robert Gibson. The effect of different dietary fats on the fatty acid composition of several tissues in broiler chickens. *European Journal of Lipid Science and Technology*, **2018**; 120(1), 1700237. (DOI: 10.1002/ejlt.201700237).

2. **Khaled Kanakri**, John Carragher, Robert Hughes, Beverly Muhlhausler, Carolyn de Koning, Robert Gibson. The fatty acid composition of excreta of broiler chickens fed different dietary fatty acids. *International Journal of Poultry Science*, **2017**; 16:424-434. (DOI: 10.3923/ijps.2017.424.433).

3. **K. Kanakri**, J. Carragher, R. Hughes, B. Muhlhausler, R. Gibson. A reduced cost strategy for enriching chicken meat with omega-3 long chain polyunsaturated fatty acids using flaxseed oil. *British Poultry Science*, **2017**; 58, 3: 283–289. (DOI: 10.1080/00071668.2017.1293798).

4. **K. Kanakri**, J. Carragher, B. Muhlhausler, R. Hughes, R. Gibson. In *ovo* exposure to omega-3 fatty acids does not enhance omega-3 long chain polyunsaturated fatty acid metabolism in broiler chickens. *Journal of Developmental Origins of Health and Disease*, **2017**; 8(5), 520–528. (DOI: 10.1017/S2040174417000216).

5. **Khaled Kanakri**, Beverly Muhlhausler, John Carragher, Robert Gibson, Reza Barekatain, Carolyn Dekoning, Kelly Drake , Robert Hughes. Relationship between the fatty acid composition of uropygial gland secretion and blood of meat chickens receiving different dietary fats. *Animal Production Science*, **2018**; 58, 828–833 (DOI: 10.1071/AN16268).

1.2 Presentations

Event	Type	Title	Authors
Australian Society for Medical Research (ASMR) meeting, Adelaide, Australia, June, 2015	Oral	Developing A Cost Effective Approach To Omega-3 Enrichment Of Chicken Meat For Human Health	**K. Kanakri**, J. Carragher, B. Muhlhausler, R. Hughes, R. Gibson *(selected for media release)*
Postgraduate Symposium, School of AFW, University of Adelaide, Urrbrae, Australia, September, 2015	Oral	Omega-3 Enrichment of Chicken Meat for Human Consumption	**K. Kanakri**, J. Carragher, B. Muhlhausler, R. Hughes, R. Gibson *(Max Tate prize-runner up)*
Australian Poultry Science Symposium (APSS), Sydney, Australia, February, 2016	Oral	Towards Commercialization of Omega-3 Enriched Chicken Meat	**K. Kanakri**, B. Muhlhausler, J. Carragher, B. Muhlhausler, R. Hughes, R. Gibson
Australian Society for Medical Research (ASMR) meeting, Adelaide, Australia, June, 2016	Oral	Enrichment of chicken meat with n-3 LCPUFA for human health: potential for inter-generational improvements	**K. Kanakri**, J. Carragher, R. Hughes, R. Gibson
The DOHaD society of Australia and New Zealand ANZ annual conference Adelaide, Australia, June, 2016	Poster	Enrichment of chicken meat with n-3 LCPUFA for human health: potential for inter-generational improvements	**K. Kanakri**, J. Carragher, B. Muhlhausler, R. Hughes, R. Gibson (*Best Poster prize*)

Australian Animal Production Society, Adelaide, Australia, July, 2016	Poster	The effect of different dietary fats on tissue fatty acid composition and growth performance of broilers	**K. Kanakri**, J. Carragher, B. Muhlhausler, R. Hughes, R. Gibson
XXV World Poultry Congress, Beijing, China, September, 2016	Poster	The effect of six dietary fats on tissue fatty acid composition and growth parameters of broiler chickens	**K. Kanakri**, J. Carragher, B. Muhlhausler, R. Hughes, R. Gibson
XXV World Poultry Congress, Beijing, China, September, 2016	Oral	Influence of high omega-3 dietary intake on the fatty acid composition of uropygial gland secretion and blood in broilers	**K. Kanakri**, J. Carragher, B. Muhlhausler, R. Hughes, R. Gibson
The 18th biannual ISSFAL, Stellenbosch, South Africa, September, 2016	Poster	Variability in the fatty acid composition of excreta in broilers	**K. Kanakri**, J. Carragher, B. Muhlhausler, R. Hughes, R. Gibson *(presented by B. Muhlhausler)*
The Southern Star Poultry Alliance Conference, The University of Adelaide, Roseworthy, Australia, 2017	Oral	Enrichment of chicken meat with n-3 LCPUFA for human health: potential for inter-generational improvements	**K. Kanakri**, J. Carragher, B. Muhlhausler, R. Hughes, R. Gibson

Acknowledgements

I would first and foremost like to express my sincere gratitude to my principal supervisor Prof Robert Gibson, whose initial positive recommendation in the PhD Scholarship application was crucial in putting me on the track to join this journey. Thank you Bob for your trust in granting me the opportunity to be one of your team, for your continuous guidance, motivation and immense supervision. Indeed, I could not have imagined having a better group director and scientific environment as I found in FOODplus research centre under your supervision.

For the previous four years, it has been a great honour to work on daily basis with Dr John Carragher, our centre commercial manager. I appreciate your magnificent contribution to every aspect of my candidature; starting from project ideas, running experiments, overcoming technical problems and research issues, data analysis, designing of posters, preparation of presentations, participation in conferences, writing manuscripts and even your health and social advice to help me cope with unforeseen life difficulties. Your great personality always inspired me to be an effective member in FOODplus for this period. It would not be possible to achieve any of the prizes, grants and scholarships that I was awarded without your involvement and I will be forever grateful.

I would like to acknowledge my co-advisor, Assoc. Prof Beverly Muhlhausler for her constructive criticism and review of my manuscripts. Bev, you provided invaluable improvements that have greatly enhanced the quality of my published papers. Our joint participation in the scientific TV show for Channel 10 was a unique experience. I must also express my sincere thanks to my second co-advisor Dr Bob Hughes from Roseworthy campus, SARDI and The University of Adelaide. You always been so generous with your time and

replying my e-mails, especially those related to poultry science. Your encouragement always stimulated me to be a more productive researcher in this field.

I acknowledge the Australian Government Research Training Program Scholarship (RTP) and PhD scholarships from the University of Adelaide. My project work was financed by a grant from the South Australian Department of Further Education, Employment, Science and Technology (DFEEST), Australia. I am more than grateful for this generous funding.

I greatly appreciate every assistance I have received from staff in Roseworthy campus, in conducting the animal part of my project. My time at FOODplus was made enjoyable in large part due to the awesome group. My sincere acknowledgments also goes to all of my lecturers, trainers and school teachers whenever/wherever I have studied. I extend my sincere thanks to all of my wonderful friends and classmates in Australia and all over the world, our memories are unforgettable. Without the knowledge that you passed to me I would not be here today.

I am so fortunate to have the greatest parents in the world, they are certainly the real unknown soldiers. Your love, confidence in me and prayers made my dream eventually comes true. I am so proud and glad to have had my lovely wife, Hajar beside me during my studies. Honestly, without your patience and support, I would not have got through this. To my little princes, Mohannad, you have provided me with hope and resoluteness to deal with stress and tribulation since you came to my life in the middle of my PhD candidature and Adnan just before finalising the final version of this thesis. To my wonderful brothers and sisters: Ashraf, Mohammad, Anas, Rawan, Razan and Ahmad and your families, sadly my PhD commitment kept me away from joining your weddings parties, but you were always in my thoughts, for all of you I dedicate this thesis. Last but not least, praise and gratitude belongs to almighty Allah multiplied by his endless bounty upon me…

Acronyms

ALA	alpha-linolenic acid
ALA2	birds fed high ALA diet for 2 weeks
ALA4	birds fed high ALA diet for 4 weeks
ALA6	birds fed high ALA diet for 6 weeks
ANOVA	analysis of variance
BW	body weight
DBS	dried blood spot
DFEEST	South Australian Department of Further Education, Employment, Science and Technology
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
EPA	eicosapentaenoic acid
en	energy
F	finisher (basal diet)
FAME	fatty acid methyl esters
FCR	feed conversion ratio
FI	Feed intake
FID	flame ionisation detector
GC	gas chromatography
HIS	hepatosomatic index
LA	linoleic acid
ME	metabolizable energy
MUFA	monounsaturated fatty acids

n-3 LCPUFA	omega-3 long chain polyunsaturated fatty acid
n-3 PUFA	omega-3 polyunsaturated fatty acid
n-6 PUFA	omega 6 polyunsaturated fatty acid
n-7 MUFA	omega-7 monounsaturated fatty acid
n-9 MUFA	omega-9 monounsaturated fatty acid
NHMRC	National Health and Medical Research Council of Australia
NRC	National Research Council
PUFA	polyunsaturated fatty acids
RTP	Research Training Program
S	starter (basal diet)
SDA	stearidonic acid
SDS	sudden death syndrome
SFA	saturated fatty acid
TPA	tetracosapentaenoic acid
x	the explanatory variable
y	the dependent variable

CHAPTER 1: INTRODUCTION AND CONTEXT

1.1 Preface

Omega-3 polyunsaturated fatty acids (n-3 PUFA), in particular the long chain type (n-3 LCPUFA) such as eicosapentaenoic acid (EPA C20:5n-3), docosapentaenoic acid; (DPA, C22:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) are necessary for normal human development and well-being. The nutritional guidelines of many national agencies and international health organizations include recommended intake levels of these fatty acids, particularly EPA and DHA. Marine resources are the main dietary source of n-3 LCPUFA, however, for various reasons, many populations (especially in Western countries) consume inadequate amounts of seafood. Therefore, the majority of people do not meet the dietary recommendation for n-3 LCPUFA intake.

To create practical, sustainable and feasible strategies to compensate for the shortfall in population intakes of n-3 LCPUFA, there have been increasing efforts to enrich popular food products with EPA and DHA (1). Since chicken meat is widely acceptable and its consumption is steadily increasing worldwide, n-3 LCPUFA enriched chicken meat has been proposed as a suitable vehicle to effectively contribute towards meeting recommended EPA and DHA intakes (2, 3). Feeding chickens a diet containing marine oil can produce n-3 LCPUFA enriched chicken meat, but this approach has some restraints as it is expensive, relies on unsustainable marine resources and has been associated with negative effects on the sensory properties of the meat (4-6).

Chickens naturally possess the hepatic enzymes required to convert the n-3 PUFA precursor α-linolenic acid (ALA, found in some plant oils) to n-3 LCPUFA (7-9). Therefore, an alternative approach to enhance the level of n-3 LCPUFA in chicken products may be to feed diets containing high levels of ALA. Flaxseed oil is one of the richest sources of ALA, and

feeding broilers a diet in which commercial dietary fats are replaced by flaxseed oil, has been found to be a successful way to increase meat n-3 LCPUFA while avoiding the disadvantages of marine oils. However, the relatively higher price of flaxseed oil compared to other fats used to formulate commercial poultry feed makes the modified high ALA diet more expensive (10). This economic barrier remains one of the main challenges to commercial uptake of this approach.

To make this strategy more economically viable, a number of approaches have been investigated. These have included manipulating the dietary flaxseed oil concentration and optimizing the dietary ratio of omega-6 PUFA (mainly as linoleic acid, LA) to n-3 PUFA, both of which were successful in reducing the amount of flaxseed oil needed in the feed, thereby reducing the cost of the enrichment process (2, 3, 7). Furthermore, some studies suggested that feeding flaxseed oil diets to broiler chickens can improve some of the growth and health parameters of the birds (9, 11, 12), thereby helping to off-set the increased cost associated with feeding birds these diets. Further effective strategies to minimize the required amount of flaxseed oil, increase the broilers efficiency in converting ALA to EPA and DHA and/or improve their capacity for n-3 LCPUFA deposition in the edible tissues are necessary to ensure the feasibility of the enrichment approach and thus adoption by the poultry industry.

This thesis aims to confirm that the dietary approach of feeding broiler chickens a flaxseed oil based diet is successful at enriching their tissues with n-3 LCPUFA. It also aims to identify enrichment strategies that can be achieved in a cost-effective way, thereby making it possible for the approach to be taken up widely in the poultry industry and offer n-3 LCPUFA enriched chicken meat to consumers at an affordable price. The commercialization of this premium food

product would increase the public intake of the beneficial n-3 LCPUFA without changing their purchasing or dietary practices.

1.2 Overview of broiler chickens

Chickens (*Gallus gallus* or *Gallus domesticus*) are grown on a commercial basis in almost every country, primarily to produce meat from broilers (13) or eggs from layer hens (14). Chicken meat and eggs have been consumed by humans for millennia as they are culturally accepted by most societies (15, 16). Chicken broilers are classified into slow-, medium- and fast-growing (or commercial) lines or genotypes. Modern broiler strains (such as Cobb 500 and Ross 308) are very fast growing due to genetic selection, efficient production systems, improved nutrition, and regular veterinary attention and vaccinations (17). The flock may be harvested for meat at 30 to 35 days or as late as 55 to 60 days of growth (16), although they typically reach acceptable market weight between 36 and 49 days (18). Broilers can be reared indoors or outdoors, and the environment in which they are grown can affect meat quality and have implications for biosecurity (17, 19). Currently, the broiler industry is a remarkable sector that employs a large number of feed producers in addition to broiler farmers, manufacturing and marketing workers (13).

1.2.1 Chicken meat

In birds, including chickens, muscle development mainly occurs in two distinct stages. During the pre-hatch phase (stage 1), the myofiber number is established when precursor cells are committed to the expression of muscle-specific genes (20). During the post-hatch phase (stage 2), the size of muscles increases by accretion of protein and nuclei originating from the proliferation and fusion of satellite cells (21, 22). Chicken meat is defined as the part of the carcass intended for human consumption (15). Chicken meat is one of least

expensive sources of animal protein and is recognized as being a low-fat protein source that provides essential vitamins and minerals, making it key to food security and a popular healthy menu item (23).

Typically, 70% of fat in the modern Western diet is of animal origin (24). As consumers become more aware of the nutritional attributes of chicken meat and its potential benefits for human health, their interest in premium meat types e.g. organic, free-range or supplemented with specific nutrients (functional food) is rapidly growing. These nutrients include bioactive peptides, minerals, vitamins, conjugated linoleic acid and n-3 LCPUFA (24-26).

Forecasts uniformly predict continued steady growth in both production and consumption of chicken meat (15, 27). In 2000, the annual production of broilers exceeded 800 million in the United Kingdom and 20 billion birds worldwide (28, 29). In Australia, the production of chicken meat has increased by more than 160% over the past two decades to reach 934 thousand tonnes in 2010, making it the most consumed type of meat in the country (23). The Australian annual per capita consumption of chicken meat is also increasing, it was only 4.2 kg in 1963 but is now expected to reach ~50 kg by 2021 (23, 30). Figure 1 shows the increase of chicken meat and consumption at expense of consumption of other types of meat, in Australia (1945 – 2021).

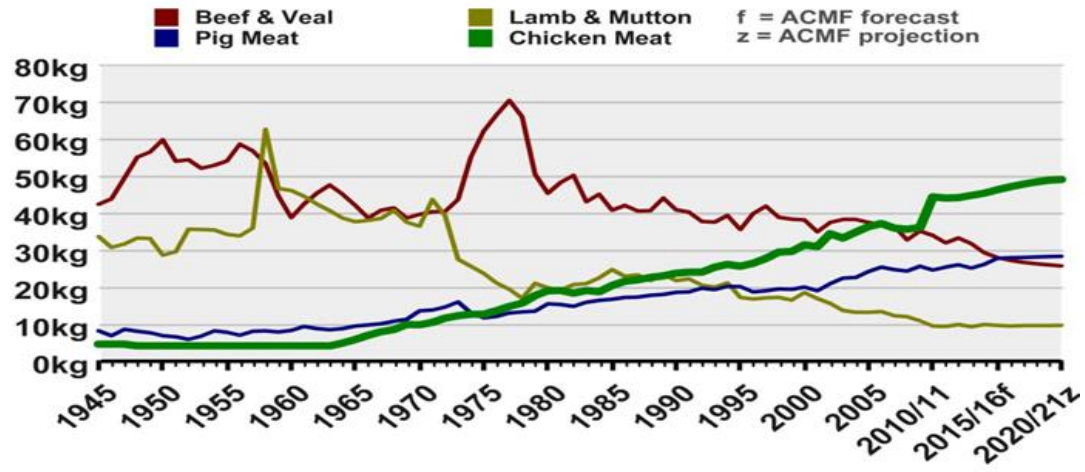

Figure 1. Australian consumption of meat between 1945 and 2021 [18].

1.2.2 Broiler nutrition

The increased demand for chicken meat by customers had led to a parallel growth in the broiler production industry. The growth in the chicken meat industry over the previous few decades has been driven by continuous research and development and involves utilizing modern technology from all over the world and adapting these for local conditions (23). The advances in the broiler industry have led to significant improvements in growth and feed efficiency of chickens, and changes to the composition of poultry feed has been a significant contributing factor. In 2015, the global broiler industry required 160 million tonnes of feed (31). In Australia, the broiler industry utilizes 3 million tonnes of feed per annum (32), of which 85-90% is comprised of grains, such as wheat, sorghum, barley, oats, lupins, soybean, canola and other oilseed meals and grain legumes (19). This amounts to over 5% of all grains produced in the country (32). Compared to 4.7 kg feed in nine weeks in 1975, the modern commercial broiler consumes only 3.4 kg feed in just five weeks to achieve market weight (23) representing a growth rate of 70-75 g per day to the age of 40-days (31). The reduction of the growth period of commercial broiler strains demonstrates the significant importance of nutrition/feed composition in the poultry industry. Feed is also the greatest contributor to

production cost of chicken meat (~60–70%), and requires large amounts of land and water to grow feed crops (27). Feed cost can be even higher if organic ingredients are utilized to produce organic broiler meat (33). Therefore, there has been a large amount of research aimed at reducing feed costs (34, 35).

Avian embryonic development occurs outside the mother, therefore, the embryo is completely dependent on the nutrients deposited in the egg prior to lay (36). The quality and quantity of these nutrients (including fatty acids) are related to the dietary intake by the broiler breeder hens (36, 37). Pre-hatch nutrition has consequences for the health, growth and quality parameters of the hatched offspring, a phenomenon referred to an early life programming. Furthermore, it has been shown that dietary fatty acids fed to roosters can also influence the fatty acid profile of the sperm (38). Fat is present in the yolk of the egg. It consists mainly of triglycerides (65%, and the main source of energy) and phospholipids (28%, having more structural and functional roles in the cell wall) which are absorbed by the embryo during the second and third weeks of incubation (18, 39). However, despite its apparent importance, the amount of research on the transgenerational effects of pre-hatch nutrition on broilers is still relatively sparse in comparison to other species, and the results to the date have been inconsistent (18, 24, 36, 40, 41).

Many broiler feeding programmes rely on the traditional nutrient requirements established by the outdated NRC recommendations (42) which are based on birds' age and fixed growing phases. However, there are also many broiler feeding programmes that have been optimized using advanced simulation models that allow growers to precisely predict broilers responses to nutrients and therefore determine the associated health outcomes (9) and growth performance (43, 44). This has resulted in adoption of superior genotypes, high-quality nutritional formulations and effective husbandry practices. The ultimate aim of

nutrition research is to design nutritional approaches to minimize the production costs and thus maximize the profit margin for broiler producers (45, 46). This can be achieved by improving broiler performance in terms of lower feed intake (FI), higher body weight (BW) gain hence a lower feed conversion ratio (FCR), and lower mortality rate.

Broiler chicken performance is often constrained by the rearing environment (physical, social, welfare and non-infectious and infectious health factors) providing a challenge to modellers attempting to predict actual performance (43). The environmental factors such as temperature, humidity, litter and air quality and stocking density (number of birds per area) differ between commercial growers and can affect both growth performance and mortality rate of the birds (29). For instance, broiler performance is closely linked with skeletal development. Bone quality disorders (e.g. lameness) compromises welfare of broilers since lame broilers have difficulty accessing feed and water causing mortalities and therefore an economic loss (47, 48). Leg disorders are also associated with mortalities during the period between third and sixth weeks of broiler growth (47, 49).

1.2.3 Fat digestion and absorption

The main lipid classes from a nutritional standpoint are triglycerides, phospholipids and sterols (35). Supplemental dietary lipid in broiler diets is typically provided as an animal-vegetable blend made from animal tallow and feed-grade hydrogenated vegetable oils. The fats from these sources are rich in saturated fatty acids (SFA), *trans* fatty acids, n-6 PUFA and low in n-3 PUFA (9, 19). Commercially, the inclusion of dietary fat is usually 1-5% w/w depending on the prices of fat and cereal grains. This level ensures enhance pellet quality (34) and an adequate supply of the essential fatty acids and avoids slow growth, increased water consumption and reduced resistance to diseases (35, 50). The supplemented fats also play a role in production, growth, health, product quality aspects and sensory characteristics

(45, 51). Besides providing essential macro and micro nutrients, the optimal feed provides about 13 MJ/kg of energy for growth (19). Dietary fat is an essential macronutrient that also provides fat-soluble vitamins (most importantly vitamin E). In the chicken meat industry there is significant interest in maximizing the utilization of supplemental fats, however this requires a better understanding of the physiological basis of and factors affecting fat digestion (35).

In comparison to carbohydrates and protein, the digestion and absorption of fats is a more complex process of physiochemical stages including breakdown of fat droplets, emulsification, lipolysis and micelle formation. In addition, the age of birds and the characteristics of dietary fatty acids, such as the degree of saturation and chain length, percentage of free fatty acids and the inclusion level of fat in the diet also influence the efficiency with which they are absorbed (32, 35, 52-54). The physiological functions required for lipid digestion and the utilization of fatty acids as an energy source are still immature in the first two weeks post-hatch (due to lack of lipase activity) but steadily develop over the subsequent four to five weeks of broiler growth (age at harvest) (24, 45, 52, 55).

The digestion of dietary fats is initiated by the grinding action of the gizzard (35). Bile salts secreted from hepatocytes and stored in the gall bladder empty into the duodenal segment of the intestine as needed, and have a significant role in fat digestion in the initial few weeks following hatch (35, 55). The main functions of bile salts are assisting in emulsification of fat, breaking coagulated fat masses into fine stable droplets to increase surface area, and activating and preventing denaturation of the pancreatic lipase (35, 56). Lipase becomes active when it appears on the surface of emulsified fat droplets along with bile salts and the co-lipase. Once the digesta enter the duodenum, it stimulates secretion of

cholecystokinin. This upregulates the pancreatic and bile secretion and therefore accelerates fat digestion (35, 57). The digestion and absorption of fatty acids differs between intestinal segments, with the jejunum being the segment where the majority of fat is digested and absorbed (58). In addition, long chain unsaturated fatty acids (≥13 carbon atoms) are relatively more susceptible to the actions of the lipases than other fatty acids (59).

Once hydrolyzed, short chain fatty acids and monoglycerides are absorbed passively from the gastrointestinal lumen via the enterocytes (60), while medium- and long chain SFA and diglycerides are dissolved in the hydrophobic cores of micelles. Fatty acids incorporated into micelles are then transported to the intestinal cells. Monoglycerides and long chain fatty acids are re-esterified, combined with other lipid classes (free and esterified cholesterol, lipoprotein and phospholipids) in the enterocytes to form chylomicrons and secreted into lymphatic vessels (35).

Chylomicrons are secreted directly into the portal vein which carries blood from the gastrointestinal tract to the liver as portomicrons. In the liver, fats are metabolized to functional compounds, used as a source of energy or exported for storage in adipose and other tissue (35, 61). In chickens, hepatic lipogenic activity is much greater compared to that in adipose tissue, and most of the fat deposited in adipose tissue is in the form of triglycerides that are either synthesized *de novo* in the liver or provided by the diet (62). Compared to the diets that are higher in n-6 PUFA and SFA, diets containing higher levels of n-3 PUFA reduce lipid synthesis and increase fatty acid oxidation and diet-mediated thermogenesis (63).

1.3 Overview of fatty acids

Fatty acids are aliphatic monocarboxylic acids derived from or contained in esterified form in an animal or vegetable fat, oil or wax. They vary in their carbon numbers (commonly have a chain length of 4 to 28) and structure (usually unbranched and even-numbered). They contain two functional groups, a methyl group (CH_3) on one end of the molecule and a carboxyl group (R–COOH) at the other. Fatty acids are typically classified into three groups depending on the number of double bonds between their carbon atoms; 0 = SFA, 1 = monounsaturated fatty acids (MUFA) and >1 = PUFA. The position of the first double bond from CH_3 group of the molecule, classifies the unsaturated fatty acids into n-7 MUFA, n-9 MUFA, n-6 PUFA and n-3 PUFA, depending on where it occurs at the 7th, 9th, 6th or 3rd carbon atom. With the same molecular formula and structure, MUFA can also be classified as having either *cis* or *trans* configuration, depending on which their geometric arrangement. In *cis* isomers, both functional groups are on the same side of the carbon chain whereas in *trans* isomers, the functional groups are on opposing sides of the carbon chain fatty acid (64-66).

1.3.1 Polyunsaturated fatty acids (PUFA)

The n-3 PUFA fatty acid group is commonly classified into short chain n-3 PUFA, the main one being ALA, (C18:3 n-3) with 18 carbon atoms, and the n-3 LCPUFA (EPA and DHA) which contain 20 or 22 carbon atoms. The molecular formula of ALA is $C_{18}H_{30}O_2$, formally designated as all-*cis*-9,12,15-octadecatrienoic acid or (9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid (IUPAC name) and its biochemical structure is shown in Figure 2.

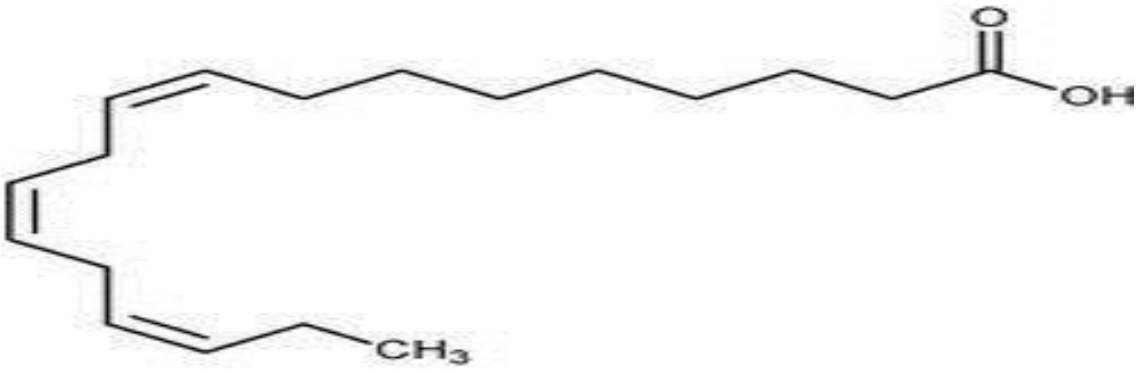

Figure 2. Structural formula of ALA (67)

ALA is a precursor of n-3 LCPUFA as is the short chain n-6 PUFA; linoleic acid (LA, C18:2 n-6) for the n-6 LCPUFA (mainly arachidonic acid, AA). Some animals (including humans) require ALA and LA for growth and physiological functions but because they lack the biochemical pathway for inserting double bonds in the n-6 PUFA and n-3 PUFA positions (*de novo* synthesis) (68), they both have to be supplied through the diet, and are thus referred to as essential fatty acids (36). The essentiality of these fatty acids was first identified in rats in the 1930s (69) and five decades later in humans (70). The dietary ALA can be elongated and desaturated to n-3 LCPUFA using a number of hepatic enzymes (elongases and desaturases) as shown in Figure 3 (71).

The same set of enzymes used for conversion of ALA to n-3 LCPUFA are also utilized for the conversion LA to n-6 LCPUFA (Figure 3), and this creates a level of competition between the conversion n-3 PUFA and n-6 PUFA substrates to their long-chain derivatives. There are still significant gaps in our understanding of exactly how PUFA metabolism is regulated, including the effect of substrate concentration, interactions among the respective enzymes, how their activity is controlled and identification of endogenous and exogenous regulatory factors (71-74). Improved understanding of these regulatory factors could allow for an increase in production of the beneficial n-3 LCPUFA at the expense of n-6 LCPUFA

(73). Due to the competition between n-3 PUFA and n-6 PUFA for metabolic conversion, reducing the ratio of LA:ALA in the diet increases the efficiency of ALA conversion to n-3 LCPUFA (75). However, higher concentration of dietary ALA above the optimal level will not further increase n-3 LCPUFA or may adversely inhibit the conversion (7, 76).

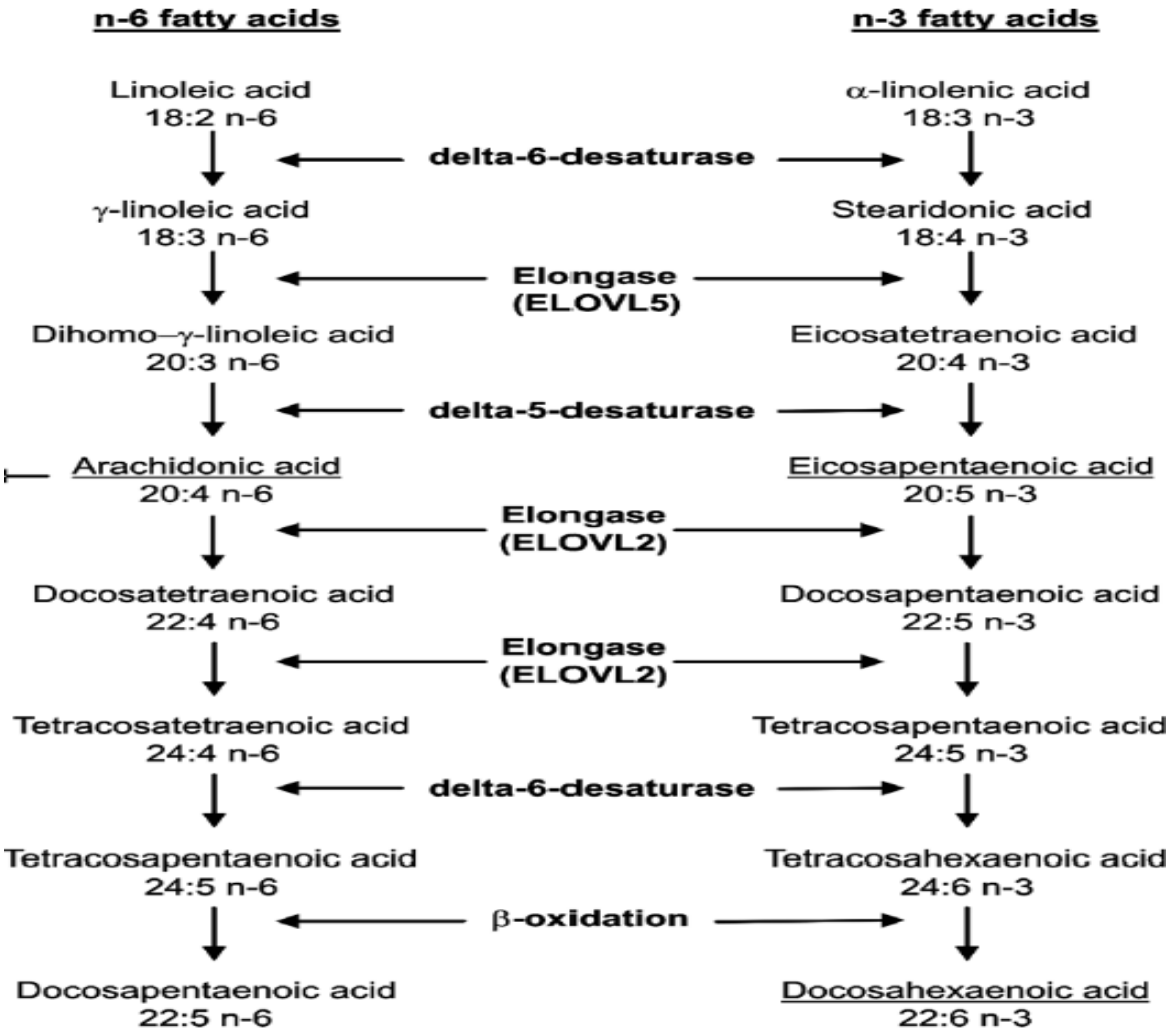

Figure 3. The interaction between n-3 and n-6 PUFA metabolism pathways (71).

It has been confirmed that PUFA, as universal cellular regulators, can affect gene transcription and modulate the metabolic state in the cell. This is essential for understanding of responses to dietary manipulation (77). In broiler chickens, dietary fat type affects gene

expression and has consequences for the total fat content in meat tissues (63). Lipogenesis occurs in the liver from dietary carbohydrates and because there is a negative correlation between PUFA concentration and enzyme activity of both acetyl-coA-carboxylase and fatty acid synthase (78), dietary n-3 PUFA can suppress hepatic *de novo* lipogenesis (79).

1.3.2 Importance of n-3 LCPUFA for humans

Role of n-3 LCPUFA in morbidity

There are several health benefits associated with n-3 LCPUFA intake in humans. The majority of these benefits are related to inflammatory and immune conditions including rheumatoid arthritis, and support of the neural and visual development (65, 80-84). Both EPA and DHA are also associated with reduction of plasma triacylglycerol concentrations, platelet aggregation, blood pressure (systolic and diastolic) and heart rate and therefore beneficial for cardiovascular health (85, 86). In a recent comprehensive meta-analysis that included 171 randomised clinical trials, AbuMweis et al. (86) demonstrated that supplementation with EPA and DHA may serve as a low cost option to manage hypertriglyceridemia and to improve several markers and/or risk factors for cardiovascular disease. Most of these beneficial effects rely on the incorporation of n-3 LCPUFA into the phospholipid fraction of the cell membrane, and subsequent release in the free fatty acid pool to give rise to bio-active mediators (87). Many of the derived messengers (prostaglandins, thromboxanes, eicosanoids and leukotrienes) of the n-3 PUFA and n-6 PUFA pathways have opposing physiological effects. Most of the EPA and DHA derived mediators have anti-inflammatory properties or are immune resolving, while most of the n-6 LCPUFA or AA-eicosanoids are pro-inflammatory. Consequently, diets high in n-6 PUFA are found to be associated with high inflammation and rise of cardiovascular disease, while the opposite is true for n-3 PUFA (71, 88). On the other hand, sources of isolated DPA are not readily

available and there is inadequate information relating to the assessment of its effects on human health (89).

Recommended intake of n-3 PUFA

Many national and international health administrations and agencies acknowledge the importance of achieving adequate n-3 LCPUFA intakes for optimal health outcomes, and confirm the safety of dietary exposure to ALA as a food ingredient even at high doses (67, 90). In addition, given the potential beneficial effects of n-3 LCPUFA particularly EPA and DHA (91) in human health and depending on their regional information and standards, many of these administrations and agencies also provide recommended n-3 LCPUFA intakes. These levels target maintaining health of the general public, vulnerable people or treating patients' with specific diseases (80, 92-95).

While most countries recognise the importance of n-3 LCPUFA in human health, the recommended daily intake (RDI) of n-3 LCPUFA varies between health authorities. The usual RDI for ALA in adults is between 0.6-1.2%en (96-98) or 2.2 g (80). In Australia, the RDI for EPA+DHA is 430 mg and 610 mg for females and males, respectively (95). Simopoulos (80), suggested a RDI of n-3 LCPUFA for adults that is slightly higher (650 mg) with minimum 220 mg each for EPA and DHA. The International Society for the Study of Fatty Acids and Lipids (96, 99) recommends that the RDI for EPA+DHA is not below 500 mg. The Food and Agricultural Organisation of the UN recommends a lower daily dose of EPA+DHA that differs depending on the gender, age and physiological status of consumers. For adults: 250 mg, for pregnant and lactating women: 300 mg (with DHA >200 mg), for 6-10, 4-6 and 2-4 years old children: 100-150, 150-200 and 200-250 mg respectively (100). A daily dose of 250 mg of EPA+DHA is recommended for adults by the European

Food Safety Authority (85). In addition, the latter authority recommends a daily dose of DHA 350-400 mg for pregnant women and 100 mg for infants (0.5-2 years old).

Natural sources of n-3 LCPUFA

Seafood, especially fatty fish (e.g. mackerel, herring and salmon), is the main dietary source of n-3 LCPUFA (97, 101, 102) and contains much higher levels compared to other sources of meat (Table 1). However, for various reasons, including high price, availability, sustainability concerns, allergy and consumer preference, many populations consume inadequate amounts of seafood (<2 serves/week) to meet n-3 LCPUFA requirements (85, 103). For instance, only around 25% of adult consumers in the UK eat fatty fish regularly (103). The daily intake of n-3 PUFA in the USA is approximately 1.6 g (~0.7% of energy intake), with the majority (~1.4 g) as ALA and only 0.1-0.2 g accounted for by EPA+DHA (104). Nevertheless, some species of fish contain methylmercury which associated with an adversely consequence of human health (especially in individuals with weekly consumption ≥ 5 servings), which may modestly decrease the cardiovascular benefits of fish intake, (105) and concerns about methylmercury content have led to avoidance of fish intake by some populations, in particular pregnant women (106).

Table 1. Typical content of n-3 LCPUFA (g/100 g food) in selected variety of seafood and livestock meat[1] (84, 101, 107)

Food	EPA	DPA	DHA	Typical adult portion size (g)	n-3 LCPUFA g/portion
Mackerel	0.71	0.12	1.10	160	3.09
Salmon	0.50	0.40	1.30	100	2.20
Herring	0.51	0.11	0.69	120	1.56
Canned sardines	0.89	0.10	0.68	100	1.67
Crab	0.47	0.08	0.45	85	0.85
Mussels	0.41	0.02	0.16	40	0.24
Canned tuna	0.02	0.02	0.14	45	0.08
Prawns	0.06	0.00	0.04	60	0.06
Lamb	0.03	0.04	0.02	90	0.08
Chicken	0.01	0.02	0.03	100	0.06
Beef	0.02	0.02	0.00	90	0.04
Pork	0.01	0.02	0.01	90	0.04

[1] These values differ between references and highly affected by external and eternal conditions.

Consumption patterns of n-3 PUFA

Both n-3 PUFA, ALA (from plants) and n-3 LCPUFA (from seafood) have always been a part of the human diet. However, the levels of consumption of ALA and n-3 LCPUFA has changed significantly over time, in conjunction with steady change in agri-food production practices (108). This has resulted in substantial variation across countries in intakes of ALA and n-3 LCPUFA as shown in Figure 4 (109), as well as n-6 PUFA. Modern Western-style diets are typically rich in plant oils and animal fats, which are rich sources of LA (~15g g/day), moderate sources of ALA (~1.5 g/day) but contain very little EPA and DHA (~0.2-0.3 g/day) (90). The dietary intake of n-3 LCPUFA in most populations world-wide has not increased significantly over the last decade (110). Therefore, the dietary ratio a n-6:n-3 PUFA in many countries (e.g. USA, Greece, Italy and Spain is ~11:1, 15:1, 18:1 and 27:1, respectively (104, 111)) is much greater than is optimal for human health (~1:1) (85, 112)

and that estimated to have been consumed in traditional human diets (113). In Australia, only one fifth of consumers meet the local RDI for n-3 LCPUFA and only one tenth of women meet the recommended DHA intake (114). Interestingly, the median n-3 LCPUFA intakes are less than 50% of the mean n-3 LCPUFA intakes. In other words, the distribution of dietary intakes of n-3 LCPUFA by Australians is highly skewed, a low percentage consume adequate or greater than adequate amounts of n-3 LCPUFA whilst the vast majority of the public consume far below these levels (114).

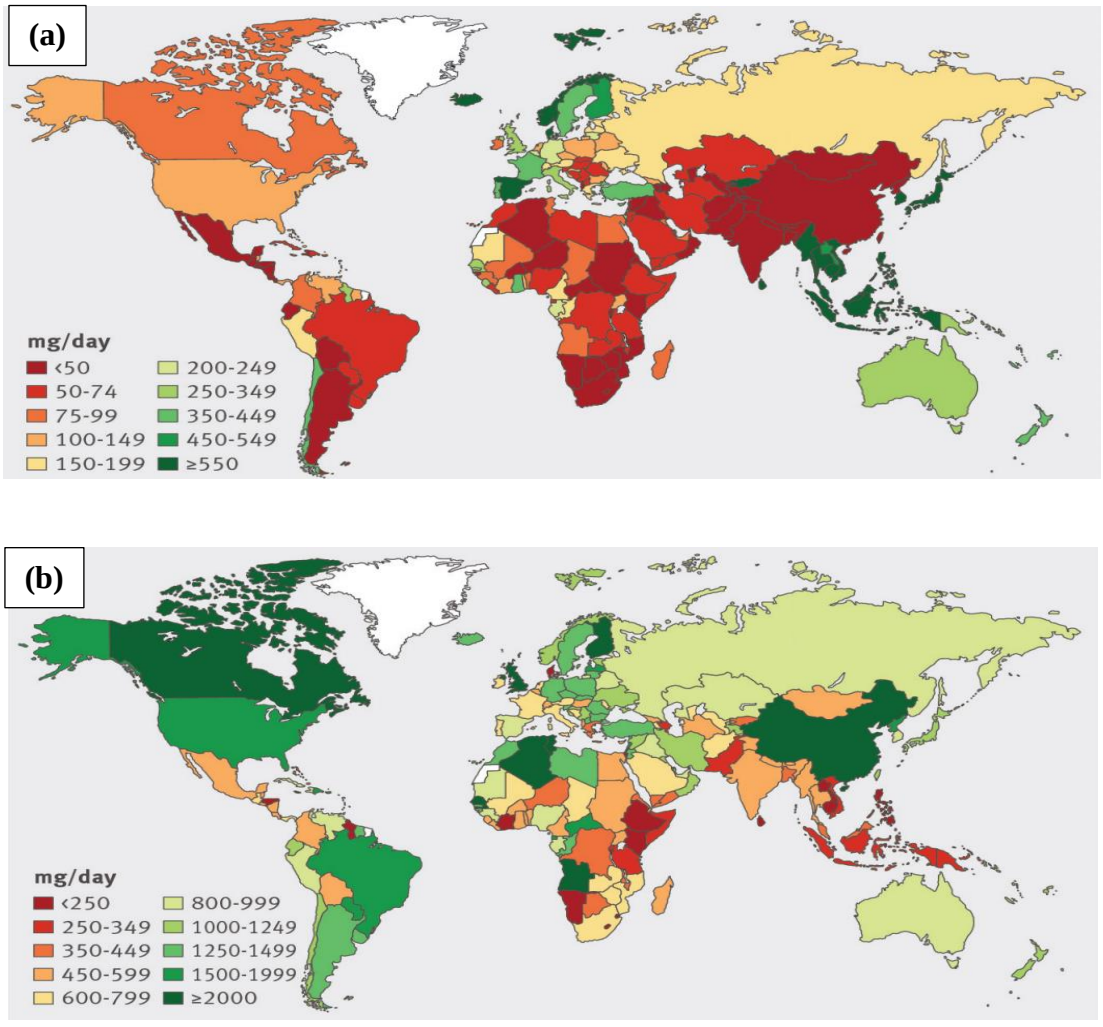

Figure 4. Global and regional mean consumption of dietary n-3 PUFA from (a) seafood and (b) plants in 2010 for adults ≥20 years of age (109)

Strategies to increase n-3 LCPUFA intake

To overcome the inadequate intake of n-3 LCPUFA from foods, many individuals take oral supplements of encapsulated marine oils (e.g. fish, krill, calamari and cod liver) that are rich in EPA and DHA (115-117). This approach typically provides the daily recommended dose of n-3 LCPUFA. However, there are some disadvantages associated with this approach; consumers have to follow a daily administration program and capsule content may be adversely affected by conditions and length of storage thus losing a considerable amount of the active ingredients (102, 118, 119). Moreover, this approach relies on unsustainable marine resources, which have already reached the maximum usage rate (5, 120). Production of marine oils strongly relies on the availability of wild fisheries and the gap between the demand and the sustainable supply of fish oil is expected to reach 40 million tonnes by 2030 (121). Thus, there is an urgent need for an alternative and more sustainable option.

Another strategy to modify customers' intake of fatty acids without requiring them to change their dietary habits is by producing functional food (specifically animal-derived products) that are enriched with n-3 PUFA and contain a low n-6:n-3 PUFA ratio. The existing literature suggests it is possible to increase EPA and DHA availability by either reducing the intake of LA or increasing the intake of ALA but more effective outcomes are via combining of both approaches (71, 90, 111). However, the level of these beneficial changes is still far less than those achieved with direct dietary supplementation of n-3 LCPUFA (75).

Over half of the total fatty acids in leafy green vegetables is ALA, but as the total fat is <2% on dry basis, and thus these products cannot serve as a significant source of n-3 PUFA to human (45). In general, humans have limited metabolic capacity for elongation and desaturation of ALA to EPA and DHA, with far less DHA being produced than EPA.

Supplementing the human diet with high amounts of ALA leads to limited increases in EPA and DPA while supplementation with preformed EPA is ~15-fold more efficacious (122). Therefore, the benefits of ALA intake on n-3 LCPUFA status and thus human health are smaller than consumption of preformed EPA and DHA (104, 111).

Enhancing the functional value of sustainable and popular food products appears to be an efficient way to achieve an increase in n-3 LCPUFA intakes without changing consumer purchasing or consumption behaviour (24, 25, 33). In this regard, producing n-3 LCPUFA enriched eggs in the USA is a very successful example, based on its steadily growing market-share, which reached 12.5% in 2017 (123). Similarly, n-3 LCPUFA enriched chicken meat is another promising functional food product for increasing population intake of n-3 LCPUFA.

1.4 Enrichment of chicken meat with n-3 LCPUFA

Since the world-wide consumption of chicken meat is increasing, (e.g. it exceeds 46 kg/person in Australia (23)) coupled with a rapid increase in global population, the production of n-3 LCPUFA enriched chicken meat could effectively contribute towards compensating for the shortfall of n-3 LCPUFA intake without relying on increasing consumption of unsustainable marine products (25, 33). Chicken meat is naturally a poor source of n-3 LCPUFA (84), nevertheless, numerous studies showed that the EPA and DHA content of chicken meat (particularly in the high growth rate genotypes) can be easily modified by dietary means (4, 7, 124, 125). Since1963 it has been known that the fatty acid compositions of breast, thigh and skin tissues of broilers reflect the fatty acid composition of the diet (126). Since that time significant research has been undertaken to test dietary strategies to elevate n-3 LCPUFA in the edible portions of broiler chickens (25, 33, 112). The traditional strategy of enriching chicken meat with n-3 LCPUFA basically involves

feeding broilers a diet in which conventional commercial fats are replaced with oils containing a higher percentage of ALA or n-3 LCPUFA.

1.4.1 Enrichment using fish oil

Feeding broilers with a fish meal or fish oil-based diet has been used successfully to increase n-3 LCPUFA levels (especially EPA and DHA) in their breast and leg meat tissues (4, 6, 124). However, the optimal inclusion level of the dietary fish oil for optimizing meat n-3 LCPUFA accumulation is unclear. Farhoomand and Checaniazer (127) reported that EPA, DHA and total n-3 PUFA levels in the breast meat (up to 10.5%, 3.8% and 20.0%, respectively) were positively and significantly related to the ratio of fish oil to poultry oil in the diet. Rymer and Givens (128) demonstrated that replacing soya oil with fish oil in broiler feed (at 4% inclusion level) led to alterations in the fatty acid profile of the breast meat (particularly increasing n-3 LCPUFA content). However, increasing the amount of fish oil from 4% to 8% did not result in further increases in n-3 LCPUFA content in the meat in the same study.

While increasing n-3 LCPUFA intake from fish oil is effective in increasing n-3 LCPUFA content in the meat tissue, this approach resulted in negative effects on the organoleptic quality in the meat products from broilers, and was associated with fishy off-flavor and off-taste (4, 6, 129). In fact, this observation is not new as it was first reported in 1926 in trials aiming to enhance the overall nutritional value (rather than n-3 LCPUFA levels specifically) of chicken meat as well as the productivity of broilers by feeding them a fish meal or oil based diet (130). This deterioration in sensory quality is likely to reduce consumer acceptability, thus it is the main limitation to industry uptake of this approach (131).

There is inconsistency in the literature in regard to the effect of supplemental fish oil on the performance parameters of broilers. In general, fish oil provided in fish meal based diets has been shown to decrease productivity in broilers compared to a similar level of fat provided by pure fish oil, possibly due to the presence of non-lipid ingredients in the meal (132). Moreover, lower inclusion levels of dietary fish oil enhanced broiler performance more than higher levels (127, 132). For instance, doubling the dietary inclusion level of the red fish meal from 7.5% up to 4-fold (30%) or doubling the dietary inclusion level of the red fish oil percentage from 2.1% to 4.2% in the feed were accompanied by a linear reduction in different performance parameters (lower BW and FI and higher FCR) in broilers (133). In contrast, other studies have reported that production parameters were not affected by the level of fish oil included in the diet (134, 135).

To avoid or minimize negative effects on sensory properties, it has been suggested that the inclusion levels of fish oil should not exceed 2% of feed weight or that it be mixed with other oils (4, 127, 128, 136-138). Another suggestion has been to add functional micronutrients (e.g. α-tocopherol acetate) along with the fish oil which can help to maintain acceptable sensory characteristics of the n-3 LCPUFA enriched chicken meat when it is reheated. The addition of 100 mg α-tocopherol acetate/kg diet is recommended when the inclusion rate of fish oil is 4%, which is the amount required to supply 50% of the UK minimum RDI of n-3 LCPUFA in a 100 g serving of breast meat (128). Similarly, Bou et al. (135) demonstrated that using 2.5% dietary fish oil together with α-tocopherol acetate supplementation of 140 mg/kg feed enabled reasonable consumer acceptability of n-3 LCPUFA enriched chicken meat after five months of frozen storage.

While fish and other seafoods are rich dietary sources of n-3 LCPUFA, it is worth noting that the n-3 LCPUFA they contain ultimately come from algae consumed by marine animals

at the lower end of the food chain (139). The sustainable algal species and oils (rich in n-3 LCPUFA) are used in many different products including infant formulas, food additives, cosmetic and pharmaceutical products, biodiesel and animal feeds (120). Dietary fish oil can be replaced with algal oils to maintain the sensory quality (140). Algal sources have been found to be comparable to fish oil at elevating n-3 LCPUFA content to nutritionally desirable level in broilers without affecting the oxidative stability of the meat (141). However, there are a number of economic and technical barriers (cost, extraction and purification) that are limiting the use of micro algal oils on a wider-scale (142). Therefore, further studies into algal species, growth and fat synthesis are necessary for further expanding this promising market.

1.4.2 Enrichment using terrestrial oils

An alternative to adding n-3 LCPUFA to feed is to add precursor ALA. Plant oils (containing a considerable level of ALA) are extensively studied and widely accepted as more practical alternatives to fish oil.

A number of vegetables, nuts (e.g. walnuts) and beans (e.g. soybean) contain n-3 PUFA (all as ALA) at levels that allow them to be classified as natural sources of n-3 PUFA (90). The consumption of some others plant sources of n-3 PUFA is still regionally limited such as camelina in Nordic countries and Perilla in Asia (90). The ALA content of the main crops that have a commercial use in poultry feed are listed in Table 2. The capacity of different species for converting ALA to n-3 LCPUFA is highly variable. Fish depend on consuming preformed n-3 LCPUFA from eating algae sources, and have a very low capacity for converting plant-sources of n-3 PUFA (ALA) (120). At the other end of the spectrum, broilers appear to have a much greater capacity for converting ALA to n-3 LCPUFA and promising results have been reported using these types of feed ingredients (7, 24, 143, 144).

Broilers fed flaxseed oil convert 5-8% of the net ALA intake to n-3 LCPUFA, (55) hence, this process is more efficient than in humans (122).

Camelina

The high oil content (~40%) in the camelina or *Camelina sativa* and its content of the plant type n-3 PUFA (ALA, ~30% oil), classify it as a nutritionally promising oilseed crop (145). This attracted the poultry industry to investigate the seed meal and its oil as an ingredient in poultry feed (146-151). Feeding diets enriched with 8%, 16% or 24% camelina cake (containing 29% ALA and 21% LA) to broilers for six weeks successfully increased the breast and thigh meat content of ALA (linear correlation) and DPA, but not EPA and DHA. At an inclusion level of 16% of camelina cake resulted in breast and thigh meat exceeding the n-3 PUFA content required for a labelling claim (300 mg/100 g meat) (147)., In another study, unfortunately, increasing levels of camelina meal from 0 to 15% (replacing canola meal) had a negative influence on growth performance of the 22 day old broilers. The 15% camelina meal significantly increased n-3 PUFA content and reduced the n-6:n-3 PUFA ratio of the meat compared to the 15% canola meal, however the apparent total tract digestibility of dry matter, energy, nitrogen retention, weight gain, FI, and FCR were all adversely affected (149). A similar adverse effect was found from inclusion of 10% camelina meal on the BW of 3-week-old broilers, however this effect could be alleviated by supplementing the feed with copper sulphate (150 mg/kg), which resulted in improved performance and carcass characteristics (148).

Canola

The effect of canola (rapeseed) meal and/or oil (alone or in combination with other nutrients) on the n-3 PUFA status in broiler tissues, particularly meat, and its beneficial consequence on birds' health has been widely reported (149, 152-158). Comparing the

dietary effect of replacing sunflower oil with canola oil (at 5% inclusion level) on the leg and breast meat n-3 PUFA status, showed the ability of canola oil to increase n-3 PUFA and lower the n-6:n-3 PUFA ratio and crude fat percentage in both muscle tissues. Moreover, this effect could be enhanced in the leg meat by the simultaneous addition of a combination of the probiotic strain *Enterococcus faecium* and prebiotic fructo-oligosaccharides, derived from chicory rich in inulin, to the diet. The canola oil did not change productivity parameters of the birds, including BW and FCR, but did adversely affect the susceptibility of the meat to oxidation (153). In another study, tallow, sunflower and canola oil individually or in combination (4% inclusion level) were compared. A positive effect of canola oil on the BW, FCR and meat yield was found, that was higher when the oil was mixed with tallow (158). Canola meal can also be used up to 20% dietary inclusion level without negatively affecting the carcass yield or the composition and sensory attributes of the meat of the broilers (154). In another study, however, canola meal was not as effective as camelina meal in raising the n-3 PUFA and reducing n-6:n-3 PUFA ratio, although it did improve BW and FCR in the 22 day-old broilers (149). One of the main criticisms of using canola oil as an alternative source of n-3 PUFA is the fact that it contains relatively high amount of n-6 PUFA in comparison to other plant oils, which increases the dietary n-6:n-3 PUFA ratio and therefore limits the capacity of the birds for converting ALA to n-3 LCPUFA.

Other plant oils

There are a limited number of studies that have investigated the influence of other plant sources of n-3 PUFA on enriching chicken meat with n-3 LCPUFA. Inclusion of hemp oil in the diet of males and females broiler chickens has been shown as an effective way to enrich meat tissues with n-3 PUFA without negatively affecting broilers' performance (159). By feeding Ross 308 broilers a hemp oil-based diet up to 6% for 3 weeks post-hatch the

contents of n-3 PUFA, ALA and n-3 LCPUFA were significantly increased in the thigh and breast meat tissues to a different extent. Feeding broilers the same diet at lower hemp oil concentration (3%) had no significant effect on n-3 PUFA level in both tissues. No effect of the gender or the interaction of gender by time on the total or individual n-3 PUFA was detected and the sensory quality of enriched n-3 PUFA meat was not evaluated (159). Inclusion of whole chia seed at 10% in the diet from week 3 to week 7 of broiler growth significantly increased ALA content in breast and leg meat. However, further increasing the dietary level to 20% (week 4 to week 7) did not result in further increases in ALA in meat tissues in comparison to 10% (160). This n-3 PUFA enrichment did not affect the cholesterol content nor the sensory quality of the meat. On the other hand, chia seed negatively influenced the BW (lowered by 6.2% at the 20% inclusion level) and FCR of the birds (160). Echium seed oil contains stearidonic acid (SDA, C18:4n-3), which is an intermediate fatty acid in n-3 PUFA conversion pathway (161). Broilers fed a diet supplemented with 3.0% echium oil had higher levels of ALA, SDA, EPA and DPA and 2-fold higher total n-3 PUFA in their meat tissues than those fed a canola oil-based diet at the same inclusion level. However, the level of DHA incorporated in the meat tissues was the same for both oils (156). There is a report of native Korean chickens fed various dietary sources of n-3 PUFA containing 10% perilla meal, 10% perilla meal + 5% flaxseed or 10% perilla meal + 5% flaxseed + 1% fish oil for 20 days (162). Compared to the birds fed a commercial diet (control), n-3 PUFA level was significantly increased in the meat tissues of the birds given all of the experimental diets, but the n-3 LCPUFA was highest in the group given the diet containing the fish oil. The inclusion of perilla meal in the diet did not affect the performance of birds in this study (162).

Table 2. Main natural sources of ALA (adapted from the reference (90))

ALA source	Percentage ALA of the total lipid
Flaxseed (linseed) oil	53.3
Flaxseed	22.8
Perilla oil	58.0
Camelina oil	38.0
Hemp oil	19.3 (163)
Chia seed	17.6
Canola (rapeseed) oil	9.1 (3-11 (164))
Soybean oil	6.8

1.4.3 Enrichment using flaxseed oil

Flaxseed or linseed *Linum usitatissimum* is a commercial oilseed with different cultivars grown in many countries including Poland, Canada and the USA. It is comprised of protein (20-30%), water-soluble phenols, fibre and 38%-45% oil (165, 166). Approximately 96% of the flaxseed oil is comprised of neutral lipids (acylglycerol and fatty acids) while only ~1.4% are polar lipids such as phospholipids and glycolipids (166, 167). Between 50% and 62% of total fatty acids are in the form of ALA (165), as shown in the fatty acid profile of flaxseed oil presented in Table 3. This oilseed (whole or ground) and in particular its oil, has been the focus of many poultry studies over the last few decades.

Ground flaxseed

The relative ALA content in the whole flaxseed (22.8 g/100g) is lower than its concentration in the pressed oil (53.3 g/100g) (90). In addition, numerous antinutritional factors naturally exist in flaxseeds such as: cyanogenic glycosides (such as linamarin and linustatin), trypsin inhibitor, phytic acid, mucilage or gum, and antipyridoxine (166, 168-170) and they are suspected to interfere with absorption and metabolism of nutrients (171-

174). The phytic acid reduces availability of nutrients (e.g. calcium, iron, magnesium, and zinc), protein digestibility (by forming electrostatic linkages with lysine, histidine and arginine) and proteolytic enzyme activity (143, 175, 176) and this eventually results in negative effects on the production parameters of broilers. This was shown in a study in which increasing dietary supplementation level of ground full-fat flaxseed at 0%, 2.5%, 5.0%, 7.5% or 10% was negatively correlated with breast meat yield and BW (up to 10% reduction), increased FCR and reduced energy efficiency ratio and lower protein efficiency ratio (177).

Feeding broilers a diet with equal proportions of full-fat flaxseed and lard (6.1%) as a source of fat increased n-3 PUFA deposition at a comparable level to the flaxseed oil only treatment (to 11.9%, 0.5%, 0.1% and 0.6% for ALA, EPA, DPA and DHA, respectively) and decreased the n-6:n-3 PUFA ratio from 10 (control) to ~ 1.3. The increases induced by adding the full-fat flaxseed were ~4-fold greater than a canola based diet. However, the flaxseed diet also increased FI and consequently there was a deterioration in FCR (172). It has also been reported that the flaxseed diet adversely increased the viscosity of the jejunal digesta and reduced protein and organic matter retention and meat yield (172). Both the dietary inclusion level of ground full-fat flaxseed and length of time it is fed to broilers influence growth performance of the birds (178). Feeding at higher levels and for longer periods were associated with the most negative effects. After 34 days of growth, BW was reduced by ~11 g/day in the birds that received the 10% flaxseed diet compared to a loss of 24 g/day that birds receiving the 17% flaxseed diet. In the same study, FCR increased from 1.8 in broilers that did not receive dietary flaxseed to 2.2 and 3.1 in broilers that received 10% and 17% dietary flaxseed diets for the same rearing period, respectively (178). Using ground flaxseed can also result in negative effects on meat quality such as changing the color and increasing susceptibility to oxidation (179). To minimize these adverse effects in broilers

it has been suggested to reduce the duration of feeding flaxseed based diets at 10% dietary concentration for only 24 days prior to slaughter at 35 days of age. This represents a balanced approach, accounting for the additional value of n-3 PUFA enrichment (increasing the breast meat content of n-3 PUFA to 7.65/100g) and mitigating the dietary adverse effects on growth performance of broilers (178).

Table 3. Fatty acid profile of crude flaxseed oil (adapted from (168))

Fatty acid	(% of total fatty acids)
C16:0 (palmitic)	7.0 ± 0.0
C18:0 (stearic)	2.9 ± 0.0
C18:1n-9 (oleic)	16.1 ± 0.2
C18:2n-6 (LA)	14.7 ± 0.0
C18:3n-3 (ALA)	58.5 ± 0.1

Flaxseed oil

Flaxseed oil is one of the most commonly used terrestrial plant fats rich in ALA (~53% of the total fatty acids) (90, 180). Over the previous decades a large number of studies have investigated strategies to improve broiler efficiency to utilize ALA from flaxseed oil in order to increase n-3 LCPUFA in the meat, improve birds' health and performance and to minimize cost. Enriching edible chicken products, including eggs, meat and edible organs such as liver and heart with n-3 LCPUFA by using a flaxseed oil-based diet is well-researched. Feeding broilers feed-grade flaxseed oil allows poultry farmers to overcome growth and sensory issues associated with using the flaxseed meal. Table 4 summarizes some of the key research carried out since 2000 which has evaluated the effects of feeding broilers a flaxseed oil based diet on tissue n-3 PUFA composition and other outcomes related to growth performance. The majority of these studies have reported positive effects i.e. show that this dietary strategy successfully increased the n-3 LCPUFA content in different tissues

to nutritionally desirable levels without affecting the sensory quality of the meat or decreasing productivity of broilers (173, 177, 178, 181-186).

The studies to date indicate that feeding chicken diets containing flaxseed oil increases both ALA and n-3 LCPUFA levels in the meat. The majority of ALA deposition tends to be in the triglyceride (the major lipid class to use as sources of energy) whereas the vast majority of n-3 LCPUFA selectively accumulate in the phospholipid class (which comprise a low percentage of meat tissue fat) for specific functional roles, and only a low percentage are deposited in the triglyceride fraction (3, 7, 12, 183, 187). Thus, changing regulation of n-3 LCPUFA deposition to increase n-3 LCPUFA accumulation in the triglyceride fraction would be an effective approach to increase total n-3 LCPUFA in the meat of broilers fed on high ALA diets.

Despite the fact that ALA conversion in broilers is relatively high compared to other animals including humans, the rate (estimated to be 0.20%, 0.13% and 0.05% for ALA conversion to EPA, DPA and DHA, respectively) (188-190) is considered low in relation to the available ALA from diet. In one study, for example increasing the dietary content of ALA successfully increased EPA and DHA in meat tissue of 28 days old broilers. However, this increase followed a curvilinear pattern, i.e. more dietary ALA over the optimal level (estimated to be ~3.2%en) raised n-3 LCPUFA level less efficiently (2, 7). A similar curvilinear pattern found in liver tissue of layer hens fed flaxseed oil diet with dietary ALA at 0%, 0.2%, 0.4% and 0.6% (% of diet) (8) suggesting that ~0.4% is the optimal dietary level when n-6 PUFA was kept constant at 1.3%.

Studying gene expression involved in n-3 PUFA desaturation and elongation (e.g. ELOVL2 and ELOVL5) is important to control the key enzymes involved in n-3 PUFA

conversion. ELOVL2 can sequentially elongate EPA to DPA and then onto tetracosapentaenoic acid (TPA, C24:5n-3, limited substrates from C20–C22 PUFA) where ELOVL5 elongation activity is wider in term of substrates (from C18–C22 PUFA) (191). It has been demonstrated that there is the competition between n-6 PUFA and n-3 PUFA pathways for Δ6-desaturase enzyme which is required for elongation of 18 carbon atoms substrates (LA and ALA) to 20 and 22 carbons (AA, EPA and DHA) (7, 143, 192).

In comparison to other species such as rats, the expression of ELOVL2 and ELOVL5 in broilers are higher and uniquely they are able to efficiently elongate DPA to TPA, the precursor of DHA. However, the expression of these genes in chickens is not upregulated by increasing dietary ALA from 0.6% to 1.3% (191). The comparison of different rates of ALA conversion to the subsequent longer chain metabolites (lowest from ALA to EPA), suggesting that the initial Δ6desaturase (FADS2) involved in the desaturation of ALA to SDA is the rate-limiting reaction in regulation of n-3 PUFA conversion (72). One suggestion to increase expression of the hepatic gene FADS2, is to feed flaxseed oil to broilers for limited duration (17 days) prior to slaughter, therefore increase production of EPA and DHA (143). Furthermore, the impact of dietary levels of ALA from flaxseed oil on the gene expression of key hepatic enzymes involved in regulating n-3 LCPUFA deposition was investigated in laying hens tissues (8). In this study, a downregulation of the elongation and desaturation enzymes in the biosynthetic pathway was associated with higher levels of dietary ALA compared to the control hens (very low n-3 PUFA).

Limited studies have compared efficiencies of ALA conversion between sexes or strains of broilers. Most of these studies have demonstrated there is no (or marginal) difference between strains or sexes of broilers in term of their response to dietary PUFA (25, 55, 193). However, this is contradicted by a recent study which showed a variation between broiler

strains fed flaxseed oil in their efficiency in depositing n-3 PUFA into their tissues. Ross 308 accumulated lower levels of total n-3 PUFA and mostly ALA in comparison Cobb 500 (14.4% vs 18.4%, respectively) as a percentage of total lipid of breast meat (3). This study also found that Cobb 500 broilers were more effective in converting ALA to n-3 LCPUFA and consequently accumulating more EPA in their meat (3). This finding was supported by Rymer and Givens (194) who also reported variation between strains in their capacity for ALA conversion to n-3 LCPUFA. However, these authors found that Ross 308 broilers were modestly but significantly more responsive than Cobb 500 to the high ALA dietary treatment. Similarly, the slow growth broilers (JA 657, genotype or Label Rouge chickens are slaughtered no earlier than 81 day of age) were reported to be less efficient in n-3 PUFA conversion than the fast growth broilers (Ross 708) from an economical point of view. However, n-3 LCPUFA deposition in breast muscle was higher in slow growth broilers, and this was accompanied by lower breast meat yield and lower lipid content but a longer growth period (84 days comparing to 56 days in Ross 708) (125).

Sex differences have also been reported in some studies. In one study, male broilers fed a 2.5% flaxseed oil diet had a reduced capacity for n-3 PUFA conversion, so their meat contained more LA and ALA but lower levels of EPA, DPA and DHA compared with females fed the same diet (12). Therefore, chicken meat producers who aim to produce n-3 LCPUFA enriched meat from flaxseed oil diet need to pay attention to factors such as the breed and sex of their birds due to possible differences in crude fat percentage, total meat yield and n-3 PUFA conversion efficiency.

Most poultry studies which have investigated broiler chicken responses to dietary flaxseed oil have also measured the production performance. This is largely for economic reasons, as poultry farmers aim to increase growth rate to reach BW of market age faster,

reduce FI and thus reduce FCR and to reduce mortality rate to minimize the overall cost. The majority of trials that have studied the effect of a flaxseed oil diet reported no significant change in performance due to the dietary manipulation. However, a semi-commercial study with a large number of males and females broilers (3,840) fed 2.5% flaxseed oil showed a positive growth performance. In this study, female birds fed the flaxseed oil diet were 77 g or 2.8% heavier and males 87 g or 2.0% heavier, with 10% lower FCR, compared to the control birds fed tallow (12). In another study, feeding broilers a diet containing flaxseed oil in conjunction with alpha-tocopheryl acetate from 21 to 42 days of growth was demonstrated to reduce FCR (~2.2 compared to ~2.4 in soy oil) (195).

Table 4. Summary of selected recent studies involved feeding broilers diets containing flaxseed oil

Author/s Year	Broilers	Experimental Diets	Period	Performance	Findings and conclusion
Kalakuntla et al., (196) 2017	300 Krishibro (sex not mentioned)	Control (sunflower oil), diets containing soybean, mustard, flaxseed or fish oil at 2% (starter diet) and 3% (finisher diet)	0-21d (2%) and 22-43d (3%)	Not significant	Mustard, flaxseed and fish oil significantly increased n-3 PUFA without affecting sensory quality of breast and leg meat. Total n-3 PUFA in both enriched tissues was ~10.5% in the flaxseed oil treatment.
Carragher et al., (12) 2016	3840 male and female Cobb 500 broilers	Control (2.5% tallow) or 2.5% flaxseed oil	0-42d	Flaxseed oil increased BW by: 77 g (females) and 87 g (males), reduced FCR by 10% compared to control	Flaxseed oil diet has the potential to enrich chicken breast meat with EPA and DHA without effects on growth rate or feed efficiency or increase in fat content of breast meat.
Konieczka et al., (197) 2016	123 female Ross 308	Control (lard 3.2%), experimental diets: fish oil 1%+ either 5.8% rapeseed oil, 6% flaxseed oil or lard 2.2%	Control for 36d or 14, 21 or 28d and the experimental diets for the rest till the 36d	Lard fed birds were heaviest	There were substantial increases in ALA levels and a lower PUFAn-6/n-3 ratio in birds fed the flaxseed + fish oil diet. 7d and 14d of feeding this diet were sufficient to enrich breast and thigh meat, respectively with n-3 LCPUFA. By increasing feeding duration prior to slaughter, n-3 PUFA accumulation in meat reached the target level to label meat as a source of n-3 PUFA.
Rostami et al., (195) 2015	192 male Ross 308. Birds reared at 2100 m altitude above sea level	5% soybean oil and 5% flaxseed oil	0-42d	Flaxseed oil reduced FCR 21-42d	Flaxseed oil increased the concentration of nitric oxide and reduced malondialdehyde in the serum. Also it reduced liver and abdominal fat, the right-to-total ventricle weight ratio (RV/TV) and mortality from pulmonary arterial hypertension (PAH) by increasing circulatory level of NO and suppressing hepatic lipogenesis.
Mandal et al., (198) 2014	300 (sex not mentioned) Cobb 500	Control (100% rice bran oil), experimental diets; combination of rice bran oil + flaxseed oil as 0.7%+0.3% and 0.3%+0.7% (0-	0-39d. Dietary fat inclusion	Not significant	The 3 dietary n-6/n-3 ratios (high-medium-low) did not affect serum glucose, cholesterol and triglyceride concentrations. Total and individual n-3 PUFA contents

		21d) and 1.6+0.4 and 1%+1% (22-39d), respectively	level 1% 0-21d and 2% 22-39d		were increased in breast and thigh tissue by lowering dietary n-6/n-3 ratio.
Baeza et al. (125) 2013	696 male Ross 708 high growth strain chicken + 750 male JA 657 low growth strain chickens	Similar starter and grower diets. Then Control, flaxseed oil (~1.3%) or extruded flaxseed diets (21-57d)	0-56d (Ross 708) or to 84d (JA 657)	Not affected	The slow growth broilers were less efficient at accumulating n–3 PUFA in breast meat than standard chickens.
Kartikasari, (3) 2013	233 male Cobb 500 and Ross 308	Experimental diets contained 6% macadamia oil, 2.4% flaxseed oil + 3.6% canola oil or 6% flaxseed oil	0-41d	Not significant	By increasing dietary ALA, n-3 PUFA increased by up to 11-fold, mostly in the form of ALA. ALA preferentially deposited in the triglycerides and n-3 LCPUFA preferentially deposited in the phospholipid fraction. Cobb 500 more efficient in n-3 PUFA conversion. 6% dietary ALA can increase n-3 LCPUFA content in breast meat without affecting the sensory properties.
Lopes et al., (199) 2013	448 (sex not mentioned) Cobb 500	Control (100% soybean oil) and experimental diets (0%, 25%, 50% or 75% soybean oil and the rest flaxseed oil) at 3%, 4% or 5% inclusion level	0-35d. Fat inclusion level 3% (0-7d), 4% (8-21d) and 5% (28-35d)	Not significant	The partial or total substitution of soybean oil with flaxseed oil did not affect carcass traits, meat chemical composition or serum fatty acid profile.
Mašek et al., (200) 2013	48 male Ross 308	Control (sunflower oil 5% without castration); experimental diets control+ castration and flaxseed oil 5% with or without castration	0-55d. Same diet 0-25d and experimental diets 25-55d. Castration on day 18.	Not significant	Castration increased in n-3 PUFA of abdominal fat and breast meat (particularly ALA) but not liver. Flaxseed oil significantly increased total and individual n-3 PUFA and decreased Δ5 and Δ4 desaturation indexes in the thighs and Δ5 and Δ5, 6 in abdominal fat and liver.
Mirshekar et al., (143) 2015	336 unsexed Cobb 500	Control (2.5% 0-21d and 5% 22-42d) soybean oil.	0-42d. Experimental diets fed from	Not affected	Increasing n-3 LCPUFA content in breast and thigh tissues with longer period of feeding the flaxseed oil diet. Flaxseed oil intake for 17d and 7d (before processing) for breast and

		Experimental diets; flaxseed oil (2.5% 0-21d and 5% 22-42d)	either 8, 15, 22, 29 or 36-42d		thigh, respectively, is enough to produce n-3 PUFA enriched meat. Replacement of soybean oil with flaxseed oil increased expression of FADS2 gene.
Kartikasari et al., (7) 2012	60 unsexed Cobb 500	Control (2.5% macadamia oil), experimental diets: 2.5% canola +0.25% flaxseed, 0.83% macadamia + 1.67% flaxseed, 2.5%, 5% or 7.5% only flaxseed oil	0-28d	Not significant	The levels of n-3 LCPUFA in breast and thigh meat increased in a curvilinear manner as dietary ALA increased, reaching 4- to 9-fold compared to the control.
Poureslami et al., (55) 2010	400 both sexes Ross 308	Control (8% palm oil); experimental diets 3% palm oil + 5% of either flaxseed or soybean or fish oil	0-42d. Experimental diets fed to broilers 0-21d or 042d	Not observed	Feeding flaxseed oil diet increased individual and total n-3 PUFA in whole carcass compared to soya and palm oil, but only ALA compared to fish oil n-3 LCPUFA were lower than fish oil broilers. Feeding period (21-42d) also increased n-3 PUFA compared to (7-21d). No difference in n-3 LCPUFA and n-3 PUFA content of meat between sexes. Elongation, desaturation and β-oxidation affected by dietary fatty acid source rather than broiler factors.
Rymer and Givens, (194) 2006	120 male (50% Ross 308 and 50% Cobb 500)	Control (5% vegetable oils mixture); experimental diets: 1% or 3% of vegetable oils mixture + 4% or 2% of flaxseed and fish oil, respectively.	0-42d. 1-21d similar diet then experimental diets 22-42d	Not observed	Ross 308 accumulated more ALA in breast and thigh meat than Cobb 500. There was no relationship between dietary ALA content and meat EPA and DHA contents. If broilers convert more ALA to n-3 LCPUFA, the latter are not then deposited in the edible tissues.
Nguyen et al., (172) 2003	134 female Cobb 500	Control (6.1% lard), experimental diets; 3% lard + either 3.1% flaxseed oil or 8%flaxseed meal and 2% lard + 4.1% rapeseed oil or 2.8% lard + 10% rapeseed meal	0-42d. Control and experimental diet fed 8-42d	Flaxseed meal had increased FI and FCR	Flaxseed oil increased n-3 PUFA in breast meat and abdominal fat by 8-fold compared to control and 3-4-fold compared to rapeseed oil fed broilers.
Crespo and Esteve-Garcia (201) 2002	60 female Ross 308	Control (basal diet), tallow, olive sunflower and flaxseed oil at 10%	28-48d	Not significant	Reduction of abdominal fat in broilers fed flaxseed oil is a consequence of higher lipid oxidation despite the higher synthesis of endogenous fatty acids.

Crespo and Esteve-Garcia (202) 2002	100 female Ross 308	Experiment 1: Control (basal diet), tallow, olive, sunflower and flaxseed oil at 10% Experiment 2: Basal diet supplemented with 10% tallow, sunflower or flaxseed oil.	28-48d (Exp. 1) and 28-53d (Exp. 2)	Not significant	Flaxseed oil broilers had higher rates of *de novo* fatty acid synthesis and relatively lower abdominal and body fat deposition due to differences in lipid oxidation rates and the higher *in vivo* lipogenesis.
Lopez-Ferrer et al., (144) 2001	250 (sex not mentioned) Cobb 500	Control (8% tallow), experimental diets; combination of flaxseed oil + tallow as 2%+6% or 4%+4%, respectively.	0-38d for all treatments. The (4%+4%) birds also processed at 24d and 52d	Not significant	While flaxseed oil diets increased tissue n-3 PUFA, the increase did not clearly reflected the respective dietary level. The n-3 LCPUFA content of (4%+4%) thighs was slightly higher than in control. Longer feeding time of flaxseed oil diets (38-52d) did not increase accumulation of n-3 LCPUFA in the thigh, although chickens could convert ALA to n-3 LCPUFA in liver.
Gonzalez-Esquerra and Leeson (136) 2000	330 male (commercial strain/not mentioned)	Control (commercial diet), diets containing 0% or 10% flaxseed with 0%, 0.75% or 1.5% fish (menhaden) oil	Commercial diet 0-35d or 0-42d and experimental diets 36-49d or 42-49d	Not significant	ALA preferentially deposited in thigh and n-3 LCPUFA in breast meat. Sensory quality of breast not affected at up to 10% flaxseed for 14d treatment. In contrast, thigh meat sensory quality adversely affected. Feeding combination of both oils to birds for just 7 days prior to slaughter resulted in significant n-3 enrichment related to their dietary levels.

1.5 Summary of strategies to enhance n-3 LCPUFA accumulation

In summary, the papers listed in the previous section confirm the beneficial effects of feeding flaxseed oil diet to chicken broilers through metabolic conversion of dietary ALA to produce a nutritious meat that may increase human intake of the beneficial n-3 LCPUFA. Collectively, and based on previous reports, feeding flaxseed oil to broilers aligns with the 6 cornerstone requirements (Figure 5) to achieve the goal of elevating n-3 LCPUFA in the chicken meat in environmentally sustainable, culturally and ethically acceptable way.

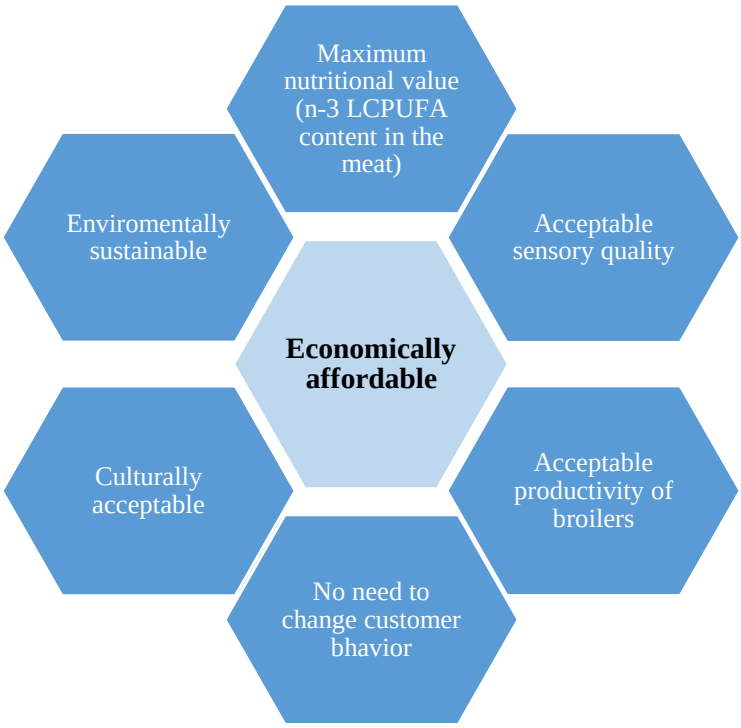

Figure 5. The six primary elements for successful economically affordable strategy for enrichment with n-3 LCPUFA

On the other hand, the previous research work showed some limitations and inconsistency of n-3 LCPUFA accumulation. Despite the promising potential of flaxseed oil to produce n-3 LCPUFA enriched chicken meat as a functional food for human health, a major challenge to widespread usage of this approach on commercial scale is the high cost

of the flaxseed oil compared to standard commercial oils. In one study, using flaxseed oil instead of commercial fats increased the cost of feed by 37% (33). Therefore, researchers need to be aware of the factors and approaches that can effectively associated with minimizing n-3 LCPUFA enrichment cost using dietary flaxseed oil (3, 144).

In order to acquire a better understanding of the relationship between dietary ALA (from flaxseed oil) and n-3 LCPUFA accumulation in chicken meat, ALA utilization by the broilers and minimizing the cost of providing flaxseed oil to chicken, the following questions were addressed in this thesis:

i. Whether the type/source of dietary fat was responsible for the improvement in some growth performance parameters that were identified in a previous study?

ii. What types of fatty acid are the most/less preferably utilized by the broilers, with emphasis on n-3 PUFA?

iii. What is the minimal required duration of feeding birds the flaxseed-oil based diet while still retaining the benefits on n-3 LCPUFA accumulation in the edible meat portions?

iv. Whether the capacity of broilers to: (i) convert ALA to n-3 LCPUFA and (ii) incorporate n-3 LCPUFA into meat tissues can be enhanced by increased *in ovo* exposure to high levels of different n-3 PUFA from feeding the broiler breeder hens a maternal diet rich in ALA (flaxseed oil) or n-3 LCPUFA (fish oil)?

The experiments in this project were intended to provide insights into possible ways to reduce the cost of providing a high ALA diet, while still maintaining the production performance improvements and retaining the increased nutritional attributes of the chicken

products. This would substantially enhance the feasibility to uptake of the n-3 LCPUFA enrichment by the poultry industry.

1.6 Structure of the thesis

1.6.1 Thesis body

This thesis is submitted as a Thesis by Publication in accordance with the University of Adelaide, Adelaide Graduate Center regulations. Khaled Kanakri, was the first and primary author on all publications included within this thesis. The published Chapters (2-4) are included in the text (formatting and minor changes in wording have been made to conform to the remainder of this thesis) and in the published form in Appendix 4.

The thesis commences with an Introduction and Context chapter containing a literature review of the importance of the n-3 LCPUFA to human health. The literature review also presents the economical and nutritional importance of chicken meat to human and the research trials carried out to manipulate its fatty acid profile in order to increase the content of the beneficial n-3 LCPUFA. The main focus for the author was on summarizing the studies that used dietary flaxseed oil for n-3 LCPUFA enrichment, highlighting the main limitations to uptake of dietary flaxseed oil by the poultry industry and proposing four experiments to better understand the mechanism of this strategy and to lower its cost.

In order to achieve the increased dietary ALA content (and higher ALA:LA ratio) in the chicken feed, the standard commercial types of fat such as tallow (from beef, solid at room temperature) which is widely used in Australia was replaced with flaxseed oil (liquid at room temperature). The inclusion of 2.5% flaxseed oil in the chicken feed was shown to results in improvements in growth performance of the broilers, and hence economic benefits, in a previous study conducted by colleagues at The University of Adelaide (12). Given the

critical importance of BW and FCR parameters for the chicken production industry, these data provide valuable and novel insights into the nutritional factors for achieving optimal economic outcomes. Any improvements in the growth performance of the n-3 PUFA enriched broilers has the potential to off-set the increased cost of the flaxseed oil diet, and therefore needs to be taken into account when assessing the potential commercial applications of flaxseed oil based poultry feeds. At present, however, it is unclear whether increasing tissue content of ALA and n-3 LCPUFA (from flaxseed oil) were associated with growth and productivity enhancement of chicken broilers. Thus, Chapter 2 aimed to confirm that the beneficial effects of the flaxseed oil diet were due specifically to the impact of ALA, rather than the absence of tallow in broilers' feed. Furthermore, a variety of plant-based oils which are very different in their fatty acid composition and physical state at room temperature were used to replace tallow in four feeds. This was to study the effects of the dietary fatty acid profile on a range of tissues and to compare the relationships between different fatty acid groups among different dietary treatments.

The non-invasive collection of excreta has potential to provide useful data on the capacity of broilers to utilize fatty acid and therefore indicate a preference for particular dietary fat sources. Apart from the digestion and metabolism process, the excreted fatty acids reflects the net utilization of dietary fatty acids. By using the same six fats in the previous experiment, Chapter 3 aimed to establish the relationship between fatty acid intake and excretion with focus on n-3 PUFA. Therefore, this information can benefit feed manufacturers about the type of fatty acids (using different lipid sources) to select when formulating chicken diets.

In earlier studies, broilers fed on the flaxseed oil diet from hatch to the age of processing had elevated EPA and DHA in the breast and thigh meat to nutritionally desirable levels at

market age. While effective, these prior studies fed broilers on the flaxseed oil based diet for the whole grow-out period, which would not be economically viable (from a cost perspective) on a commercial scale. Therefore, the aim of Chapter 4, was to determine if it was possible to achieve the same modification in the fatty acid composition of meat by feeding the flaxseed oil diet for a shorter duration prior to slaughter, when birds are putting on most of the muscle mass. This would make the usage of such a diet much more attractive to industry from an economic standpoint.

Chapter 5 consisted of two separate but interrelated phases. In a previous study my colleagues demonstrated that n-3 fatty acids content of eggs was increased by 10-fold in laying hens consuming a flaxseed diet compared to eggs from hens consuming commercial diets. This implies that chicks born to these layer hens would be exposed to an increased supply of ALA, EPA and DHA during embryonic development. As the researchers were interested in modifying the composition of eggs that were to be consumed by humans, their work was carried out with layer hens. However, broiler chickens are derived from breeding lines that over decades have been heavily genetically selected for very different phenotypic traits (BW, growth rate, muscle size, rate of lay and fertility of eggs). For this reason, it was not clear whether feeding broiler breeding hens a high ALA flaxseed diet would effectively increase n-3 LCPUFA content in the fertilized egg, as previously reported in the egg yolks of layer hens fed this diet. Phase I of Chapter 5 aimed to investigate the hypothesis that feeding broiler breeder hens (Cobb 500 strain) a maternal diet containing flaxseed (high ALA) or fish oil (high in preformed n-3 LCPUFA) would produce significant increases in ALA and/or ALA-derived EPA and DHA. Thus, the developing embryos from fertile n-3 PUFA eggs would be exposed to an increased supply of different types of n-3 PUFA.

A world-wide series of epidemiological and experimental animal studies has demonstrated that the nutritional environment experienced during fetal and early postnatal development has life-long effects on growth patterns and their risk of metabolic and cardiovascular disease. These effects are thought to be due to the ability of different nutrients to influence the development of physiological systems which regulate important metabolic processes, including insulin signaling, appetite regulation and fat oxidation. Thus, the objective of the experiment in Chapter 5 was to establish whether the capacity of chicken broilers for n-3 PUFA conversion and/or incorporation of ALA-derived EPA and DHA into their meat tissues are enhanced by exposure to increased levels of high ALA or preformed n-3 LCPUFA before hatch.

In summary, the overarching objective of the dissertation is to identify a simple and cost-effective strategy for n-3 LCPUFA enrichment of chicken meat based on a flaxseed oil diet, with the ultimate aim of developing functional food products for enhancement of human health. The current thesis is strongly aligned with the government of South Australia's priority – The South Australia Strategic Plan – of production of premium food in an environmentally responsible fashion.

1.6.2 Appendices

Appendix 1

Lipid extraction and analyses methods:

Appendix 1 shows in a Table the amounts of each type of sample (chicken tissues, oils, feeds and excreta) and the lipid extraction and transmethylation protocols used in the five experiments of this thesis to determine crude fat content and total fatty acid composition in qualitative terms.

Appendix 2

An additional publication arising from this research entitled:

Relationship between the fatty acid composition of the uropygial gland secretion and blood of meat chickens receiving different dietary fats, has been published in Animal Production Science journal, 2018, 58, 828–833. This study aimed to examine the relationship between fatty acid composition of blood and a non-invasively collected excretion (preen oil) from broilers. Appendix 2 included this manuscript and prefaced by a Statement of Authorship.

Appendix 3

Animal Ethics Approvals

Two Animal Ethics approvals were needed to perform the experiments of this thesis. The number of each approvals were included in the Materials and Methods section of the related chapters.

Appendix 4

Published manuscripts

In regard to the thesis specification of The University of Adelaide, each manuscript has to be prefaced by Statement of Authorship to demonstrate the contributions of all co-authors. In Appendix 4, four statements of authorship were prefaced the four main published manuscripts.

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CHAPTER 2: (Manuscript 1): THE EFFECT OF DIFFERENT DIETARY FATS ON THE FATTY ACID COMPOSITION OF SEVERAL TISSUES IN BROILER CHICKENS

Chapter 2

Khaled Kanakri, John Carragher, Robert Hughes, Beverly Muhlhausler, Robert Gibson. The effect of different dietary fats on the fatty acid composition of several tissues in broiler chickens. European Journal of Lipid Science and Technology, 2018; 120(1), 1700237. (DOI: 10.1002/ejlt.201700237).

Statement of Authorship is located in Appendix 4 (4.1).

Abstract The type of fat used in formulating broiler chicken diets can affect growth performance, influence the fatty acid composition of different tissues and has consequences for bird health and nutritional value for the consumer. This study aims to address the hypothesis of whether these effects are specifically due to the variation in the fatty acid composition of the diets, i.e. the proportion of different saturates, monounsaturates (n-7 and n-9) or polyunsaturates (n-3 or n-6), or other factors (physical properties, solid/liquid and source, plant/animal). A total of 480 male Cobb 500 broilers were fed *ad libitum* on one of six diets containing 4% w/w of either: beef tallow, flaxseed, corn, canola, macadamia or coconut oil (8 replicates/treatment) for six weeks. At harvest, there were no significant differences in productivity parameters nor in the crude lipid content of different tissues between dietary treatments. There were, however, substantial qualitative differences in the fatty acid profiles of all tissues. The levels of specific fatty acids in all tissues except the brain, were positively correlated with the levels of the same fatty acids in the diet however, the strength of the correlations varied between different fatty acids.

Practical applications: The results of the current study demonstrate that the dietary fatty acids types and proportions largely determines the fatty acid profile in edible tissues (meat, adipose, liver and heart). The strong correlations and regressions between diet and tissue fatty acid levels validate the ability to predict the tissue fatty acid profile of broilers based on their dietary fat composition. Contrary to our hypothesis, dietary fat type had no influence on the growth parameters which makes us speculate whether such differences in similar studies only become apparent in situations where the birds are also under some level of environmental or social stress. This information will assist poultry feed manufacturers and broiler producers in making decisions about selection of fats with known nutritional and health benefits for inclusion in chicken feed.

2.1 Introduction

The worldwide growth in the poultry meat sector (4.3% p.a., mostly chicken) is much faster than that of any other type of meat [1]. Dietary lipid is a major source of energy, a source of essential macronutrients and fat-soluble micronutrients [2,3]. The principal external factors determining carcass composition in monogastrics are arguably the quantitative and qualitative properties of the feed. Indeed, the influence of dietary fat type on the fatty acid composition of broiler chicken tissues (in particular meat) has been investigated for decades [4,5]. In addition, broiler chickens of different strains [6,7] respond differently to dietary fats and the fatty acid profile of chicken tissues reflects both the ingested and the in vivo synthesised fatty acids [8]. Many trials have associated a significant improvement in broiler performance with dietary fats that differ in fatty acid composition [9-14].

Commercially, poultry feed manufacturers typically add different animal/plant fats individually or in combinations of 2 or more (usually at 2-5% of basal diet weight) to other ingredients to formulate diets that deliver the recommended levels of energy, macro- and micronutrients for the health and growth of the birds [3,15,16]. In the chicken industry, the decision about which fat type(s) to use is mainly based on the cost per unit of energy. Other factors that are taken into consideration include the local availability of the fat source, suitability for the birds determined by digestibility [3,17], effect on growth performance [16,10,18] and the health parameters [19], impact on sensory traits of the meat [20] or delivering health benefits for consumers (e.g. enriching meat with omega-3 polyunsaturated fatty acids, n-3 PUFA) [21,22]. Clearly, whatever the reasons, the choice of a particular fat source needs to be cost-effective and increase the profit margin to the producer [16].

Different fatty acids have differential health effects on chickens. Consumption of unsaturated fatty acids (in particular n-3 PUFA) has been associated with some beneficial health effects to chickens [23,24]. However, there is a need for caution to be taken when using fats rich in MUFA and PUFA as they are more susceptible to oxidation. Thereby, there is the risk of the broilers being exposed to oxidative products which can adversely affect their health and productivity, as well as the sensory quality and shelf-life of their products [3,25-27].

In most industrialized countries, a high meat intake contributes to a higher than recommended SFA:MUFA/PUFA intake [28]. In addition, there are suggestions that increased intake of omega-6 polyunsaturated fatty acids (n-6 PUFA) relative to n-3 PUFA may be associated with adverse health effects, including an increase in chronic inflammatory disorders [22,26,29]. Simopoulos [22] reviewed the role of the n-6:n-3 PUFA ratio in human diseases and the importance of rectifying the imbalance in this ratio in human diet for health benefits. Indeed, it has been shown that n-6:n-3 PUFA long chain fatty acid balance in the phospholipid fraction of human plasma can be altered by consuming a relatively moderate amount (160g/day) of n-3 enriched chicken meat for 4 weeks [21]. In this regard, a large number of research studies have been concerned with trying to enrich chicken meat with n-3 PUFA [9,20,30-32]. Many of these studies have focused on feeding broilers high levels of a sustainable source of ALA (often from flaxseed oil) and lower levels of n-6 (especially, LA). This because these precursors use the same metabolic pathway and compete with each other in incorporation of their derivatives into broiler tissues [33].

Some organs of broilers, such as liver and heart, are classified as by-products in the poultry industry, but these organs are nevertheless edible and are consumed on a large scale worldwide. Seong et al. [34] analysed these tissues and recommended consumption of liver

and heart for their nutritional value citing the high ratio of PUFA and MUFA to the SFA in comparison with muscle. From a physiological point of view, the liver is a vital organ because this is where the majority of fat is metabolised. Markers of liver functional status include liver weight (hepatosomatic index, HSI, as a proportion of body weight), neutral lipid and fatty acid composition [35].

In general, the biochemical characteristics of dietary fatty acids, such as length (molecular weight), saturation, isomerism, polarity and position of double bond(s), as well as their proportion within the diet, affect intestinal digestion, absorption and hepatic metabolism, thereby determining their bioavailability in the serum. Furthermore, their deposition into the main lipid fractions (phospholipids and triglycerides) in different tissues can be regarded as functional substances or energy stores [3,36,37]. The dietary influence of fatty acid intake can be tracked by analysing the internal tissues (including blood) [38,39] or non-invasively (e.g. the excreta) [40] of broilers. The importance of using a regression equation (to predict the tissue fatty acid concentration from a particular combination of dietary fatty acids) in addition to a correlation coefficient (to measure the strength of the prediction) has been reviewed by Puvača et al. [41]. Using this approach, these authors proposed a predictive mathematical equation to estimate the incorporation of individual fatty acids into chicken tissues based on the dietary level of fatty acids. There has indeed been a number of studies focused on the dietary fat effects on meat chicken in different aspects, including previous studies by our research group, nonetheless there is still no consensus on what levels or types of dietary fats are most appropriate for broiler chickens, largely because the studies have all differed in the way they have been carried out. The current study sought to address the hypotheses that differences in growth parameters sometimes observed when broilers are fed different dietary fats is due to (a) differences in the fatty acid profile, (b)

whether the fat is solid or liquid at room temperature, or (c) whether the fat is from an animal or plant source.

2.2 Materials and Methods

All procedures contributing to this work complied with the ethical standards of the relevant national guides on the care and use of animals. This study was approved by the Animal Ethics Committee of the University of Adelaide (approval S-2013-152) and the Department of Primary Industries and Regions South Australia, Australia (approval 15/13).

2.2.1 Housing the Broilers

A total of 480 day-old male chicks (Cobb 500 strain) were obtained from the Baiada Hatchery (Willaston, SA, Australia) and transferred to South Australian Research and Development Institute (SARDI) facility (Roseworthy, SA, Australia). A complete randomised block design (8 pens/treatment) was implemented. Birds were randomly distributed into 48 groups of 10 and each was allocated to a raised rearing floor pen (1.2 × 0.9 m each) in 2 sheds (24 pens in each). Broilers were reared on sawdust and shavings and had continuous free access to feed and water during the entire experimental period. Pens were heated by infrared brooder lamps (175 W) in the first 3 weeks. Temperature in the rooms was 27°C for the first 4 days, gradually decreased to 20°C and then maintained at this temperature until the end of experiment.

2.2.2 Experimental diets

The study included 6 experimental diets which were produced by mixing 4% (w/w) of a selected fat (Table 1) with a basal (starter/finisher) diet (Ridley Agriproducts, Australia). The fats were: beef tallow (Ridley Agriproducts, Australia), flaxseed oil (Four Leaf Oils, Australia), corn oil (Daisy, Malaysia), canola oil (Foodland, Australia), macadamia oil (Macoils, Australia)

and coconut oil (Banaban, Fiji). The composition and nutritional profiles of the basal diets are shown in Table 2. Apart from the variation in the fatty acid composition (Table 3), all experimental diets were isocaloric, nutritionally identical and met requirements for efficient growth [42]. Batches of each feeds were prepared weekly and samples (20 g) were taken from each batch and stored at -20°C. A representative homogenised sample (n=6 per treatment) of the weekly batches were analysed for determination the crude fat content and fatty acid profile of each diet. Broilers were fed the experimental diets for the entire 42 day growth period; the starter diet (crumble form) was fed in the first 3 weeks and then the finisher diet (pellet form) in the final 3 weeks of the experiment.

Table 1. The main differences between the experimental fats used in the experiment

	Fat properties		
Fat type	Fatty acid content	Physical status[1]	Source
Beef tallow	Moderate SFA[2] and MUFA[3]	Solid	Animal
Flaxseed oil	High n-3 PUFA[4]	Liquid	Plant
Corn oil	High n-6 PUFA	Liquid	Plant
Canola oil	High n-9 MUFA	Liquid	Plant
Macadamia oil	High n-7 MUFA	Liquid	Plant
Coconut oil	High SFA	Solid	Plant

[1]At room temperature. [2]SFA: Saturated fatty acids. [3]MUFA: Monounsaturated fatty acids. [4]PUFA: Polyunsaturated fatty acids.

Table 2. Ingredient composition and nutrient content of the basal diets[1]

Ingredients (gkg^{-1})	Basal diet		Nutrient content (gkg^{-1})	Basal diet	
	Starter	Finisher		Starter	Finisher
Wheat fine	614.2	707.7	Crude protein	225.1	186.7
Barley fine	50.0	50.0	Crude fat	23.5	30.0
Tallow mixer	03.7	10.0	Crude fibre	32.3	29.2
Blood meal (91% CP)	10.0	00.0	Calcium	09.8	08.5
Soybean meal	251.7	166.7	Available phosphorus	04.5	04.2
Limestone small	08.6	06.4	Na	01.8	01.7
Monocalcium phosphate	01.4	00.0	K	08.0	06.5
Sodium chloride	02.1	01.8	CL	02.0	01.8
Sodium bicarbonate	01.6	01.8	Lysine	12.3	09.1
Choline chloride 75%	00.3	00.4	Methionine	05.7	04.7
DL-methionine 58.1	02.9	02.1	Cystine	03.9	02.8
L-threonine 73.7	01.3	02.5	Threonine	07.9	07.6
Rovabio Excel LC	00.2	00.2	Leucine	14.3	11.2
Meat meal	37.3	37.0	Isoleucine	07.8	06.4
Ronozyme NP CT	00.2	00.2	Tryptophan	02.3	01.8
Mineral/vitamin premix[1]	10.0	10.0	Arginine	12.4	09.8
L-lysine sulphate 70	04.3	03.3	Valine	09.3	07.2

[1]All vitamins/minerals met or exceeded the recommendations by the National Research Council [2], the Metabolisable Energy ($MJkg^{-1}$) was 12.14 and 12.56 for starter and finisher basal diets, respectively.

Table 3. Fat content and fatty acid composition (% of total fatty acid) of different basal and experimental[1] diets

Diet[2]	Basal diets		Tallow diets		Flaxseed diets		Corn diets		Canola diets		Macadamia diets		Coconut diets	
	Starter[3]	Finisher[4]	Starter	Finisher	Starter	Finisher	Starter	Finisher	Starter	Finisher	Starter	Finisher	Starter	Finisher
Fat%	3.9	3.9	6.9	7.1	6.9	7.6	7.2	7.3	7.3	7.0	6.9	7.2	7.0	7.2
SFA[5]	26.1	29.2	35.6	36.1	18.1	19.1	20.0	20.5	16.5	17.3	20.3	20.8	64.8	63.5
n-9 MUFA[6]	31.9	28.7	34.1	34.0	24.8	25.3	29.0	29.6	44.4	44.4	45.6	45.9	14.9	16.0
n-7 MUFA[7]	3.8	4.0	4.6	4.7	2.0	2.2	1.9	2.2	3.4	3.7	14.7	14.8	1.5	1.8
n-3 PUFA[8]	5.2	3.7	3.1	2.7	29.9	29.0	2.8	2.5	7.0	6.6	2.1	1.8	2.0	1.7
n-6 PUFA[9]	32.4	32.5	20.4	20.1	24.6	23.6	45.5	44.4	27.8	27.0	16.5	15.9	16.1	16.2
Trans	0.6	1.3	1.6	1.6	0.5	0.6	0.5	0.5	0.5	0.6	0.4	0.5	0.5	0.5
n-6:n-3	6.23	8.78	6.58	7.44	0.82	0.81	16.25	17.76	3.97	4.09	7.86	8.83	8.05	9.53
MUFA:SFA	1.35	1.14	1.10	1.09	1.49	1.45	1.56	1.57	2.92	2.80	2.99	2.93	0.26	0.29
MUFA:PUFA	0.94	0.92	1.67	1.73	0.49	0.53	0.65	0.68	1.39	1.44	3.26	3.45	0.92	1.01
PUFA:SFA	1.44	1.24	0.66	0.63	3.01	2.75	2.42	2.29	2.11	1.94	0.92	0.85	0.28	0.28

[1]The experimental diets were made by adding 4% w/w of the relevant fat (tallow, flaxseed, corn, canola, macadamia and coconut oil) to the basal diets. [2]Values represent the mean (n=2 basal diets, n=6 experimental diets) based on the wet weight. [3]Starter diet was fed to the broilers in the first 3 weeks. [4]Finisher diet was fed to the boilers in the last 3 weeks of the entire 6 weeks growth period. [5]SFA = Saturated fatty acid; [6] n-9 MUFA= Omega 9 monounsaturated fatty acid; [7]n-7 MUFA= Omega 7 monounsaturated fatty acid; [8]n-3PUFA = Omega 3 polyunsaturated fatty acid; [9]n-6 = Omega 6 polyunsaturated fatty acid.

2.2.3 Production performance parameters

Group body weight (BW) of chicks (n=10, minus the number of mortality) for each pen was taken on days 1, 7, 14 and 21 and used to estimate the individual BW of birds (group weight/number of birds in pen). The BW of each bird was then recorded separately on days 28, 35 and 40. The feed intake (FI) of birds in each pen was also recorded at the same timepoints and used to estimate the individual FI per bird (feed eaten by pen/number of birds in pen) and to calculate the feed conversion rate (FCR); kg feed/kg body weight gain. The number of deaths and culls was recorded on a daily basis and it was taken into account in calculating the FI and FCR and used to calculate mortality rate in each dietary treatment.

2.2.4 Sampling of tissues

On the day of slaughter (day 42), 1 bird/pen was randomly selected and euthanized by cervical dislocation. For each sampled bird the body and liver weights were recorded and HSI was calculated (liver weight as % of live weight). Whole blood was obtained by cardiac puncture and 2 drops were applied to a PUFAcoat™ dry blood spot (DBS) card to stabilise the long chain polyunsaturated fatty acids [43]. Approximately 30 g of lean skinless breast and leg meat, 5 g of liver and heart and 2 g of the adipose tissue from the abdominal fat pad were collected into clean plastic vials and immediately placed on dry ice. The head was removed from the carcass, placed in a sealed plastic bag and frozen in dry ice for transfer to the laboratory where the brain was removed. All tissue samples were stored at -20°C pending subsequent determination of crude fat content and fatty acid composition.

2.2.5 Fatty acid analysis

Crude lipid was extracted from 0.1-0.5 g of representative homogenised feed and tissue samples using chloroform/methanol (2:1, v/v). The gravimetric approach was utilised to

estimate total lipid content as previously described [44]. A volume of the chloroform/methanol solution containing 2-3mg of the extracted crude lipids was used for fatty acid analysis. Transmethylation of the fatty acids was performed with 1% H_2SO_4 in methanol at 70°C for 3 hours. After cooling to room temperature, the resulting fatty acid methyl esters (FAME) were extracted with *n*-heptane (2 mL) and transferred into gas chromatography (GC) vials containing approximately 30 mg of anhydrous sodium sulphate as the dehydrating agent and stored at -20°C until GC analysis. The FAME were separated using a Hewlett-Packard 6890 GC (Hewlett-Packard, CA, USA) equipped with a 50 m capillary column (0.32 mm ID) coated with 70% cyanopropyl polysilphenylene-siloxane with a film thickness of 0.25 µm (BPX-70, SGE Pty Ltd, Melbourne, VIC, Australia). The initial oven temperature was 140°C and was programmed to reach 220°C (rise rate=5°C/min.) and held for 2 min then programmed to reach 260°C (rise rate=20°C/minute) and held for 8 min. Helium (He) was used as an inert carrier gas at a velocity of 33cm/sec. and the inlet split ratio was 20:1. The injector temperature was set at 250°C and the detector (flame ionisation) temperature at 300°C. The FAME peaks were identified based on the retention time relative to authentic lipid standard (GLC 463) from NuChek Prep Inc. (Elysian, MN, USA) using the software package Agilent GC Chemstation (Agilent Technologies Inc., Palo Alto, CA, USA). Each peak from a trace was expressed as the relative percentage of the total FAME in the sample. The detection limit of each fatty acid was 0.05% of the total fatty acids.

2.2.6 Statistical analyses

The experimental unit for all parameters was the pen, with data from either all of the birds (performance parameters) being averaged for the pen, or 1 randomly selected bird from each pen (tissue parameters). The effects of dietary treatment on growth performance parameters (BW, FI and FCR) and HSI were determined by one-way ANOVA using SAS 9.3 for Windows.

The mortality rate was compared using non-parametric analysis (Kruskal-Wallis test). All reported data from fatty acid analysis of tissues are the means of 8 replicates with the standard error of the mean (SEM). The effects of dietary treatment on tissue fat content and fatty acid profile were determined by one-way analysis of variance (ANOVA). A Tukey's multiple comparison test was implemented when the ANOVA revealed significant differences between dietary treatments ($P<0.05$). Pearson correlation coefficients were used to assess the relationship between dietary and tissue fatty acid levels (r). Correlation coefficients and linear regressions were assessed by Pearson correlation using SPSS version 22 for Windows (IBM Crop., NY, USA), and a P value of <0.05 was considered statistically significant. Microsoft® Excel 2013 software was utilised to plot the linear regression.

2.3 Results and Discussion

2.3.1 Total fat and fatty acid content of experimental diets

The crude fat percentage of the basal diet was 3.9% and by adding 4% w/w fat the 6 experimental diets attained crude fat levels between 6.9-7.6%, with no significant difference between the different diets. The fatty acid profile of the 6 experimental diets reflected the wide range in composition of the added fats, as intended (Table 3). The n-6:n-3 ratio of the diet was highest in the finisher corn oil-based diet (~18:1), and lowest in the flaxseed oil-based diet (~1:1).

2.3.2 Growth performance

None of the growth performance parameters of broilers were significantly different between the 6 dietary groups (Table 4). At the end of the study the average live BW was 3367 g/bird, FI was 5311 g/bird, and FCR was 1.58 g feed : g gain when data from all birds were combined. The overall mortality rate was 4.4% and not different between treatments. These outcomes are

in agreement with our previous studies using similar dietary treatments [30,45,46] and many others [16,42,47-51] and within the commercial recommendations for male broilers of this strain [52]. However, in a previous experiment (n=3,840 birds) [9], which was specifically designed to determine the influence of the dietary flaxseed oil on growth performance, we found that the high n-3 diet improved the BW without increasing the FI and therefore reduced the FCR by 10% in comparison to the tallow based diet. This finding was particularly interesting and important because beef tallow is widely used in making commercial poultry feed. In another study, Balevi and Coskun [10] fed broilers diets containing 9 types of plant and animal fats and also demonstrated that BW and FCR were affected by dietary fat type, with the corn oil diet being the best performer. A number of other studies have that feeding high MUFA/PUFA diets benefits productivity of the birds by reducing FCR [11-13]. In contrast, Scaife et al. [14] found that a tallow diet increased BW, however this was accompanied by an increase in FI resulting in a rise in FCR compared to 3 single and 6 combined plant and marine oil-based diets. We speculate that the lack of consistency in such experiments may be due to different experimental conditions (e.g. broilers' age, breed, length and timepoints of feeding, fat types and fat inclusion level) and/or aspects of the housing environment (e.g. stocking density, air quality, litter vs cage floor) that might stress the birds and accentuate the influence of some fat types on performance.

In the current study, the HSI% (data not shown) ranged from 1.98 (coconut oil treatment) to 2.16 (flaxseed oil treatment) but was not significantly different between all treatments, in agreement with number of similar studies [47,53].

Table 4. The dietary effect on the growth performance of broilers at different experimental timepoints

	Growth performance parameter[1]			
Growth days	BW[2]	FI[3]	FCR[4]	Mortality rate
0	46	0	0	0
7	191	149	1.03	0
14	525	616	1.29	1.67
21	1085	1389	1.35	2.08
28	1815	2517	1.42	2.92
35	2711	3991	1.50	3.54
40	3367	5311	1.60	4.38

[1]The effects of dietary treatment on the accumulative pooled BW, FI and FCR parameters were determined by one-way ANOVA where the pooled mortalities and culls rate statistically analysed by Kruskal-Wallis nonparametric method. This effect was not significant P>0.5 for all parameters at all timepoints. [2]BW: The average body weight g/live bird. [3]FI: Feed intake g/live bird. [4]FCR: Feed conversion ratio = g feed: g gain

2.3.3 Total fat content of the tissues

The average crude fat percentages of the adipose, liver, heart, leg and breast meat tissues were 89.4%, 5.7%, 3.5%, 2.4% and 1.9%, respectively, and did not differ significantly between diet groups. However, the brain tissue of corn oil diet fed broilers had significantly ($P<0.05$) lower crude fat content (5.8% vs 6.7% as mean of other dietary treatments). This finding aligns with that of a previous study, in which feeding broilers diets supplemented with beef tallow or flaxseed oil did not affect the crude fat percentage of the meat tissues [16]. Consistent with the results of the current study, Mašek et al. [50] also found that the crude fat content in liver (5.6%) was not different between broilers fed either 5% sunflower or flaxseed oil diets. In contrast, however, other studies have found that increasing the dietary PUFA:SFA ratio can lower fat deposition in various tissues [54]. For instance, González-Ortiz et al. [47] found that feeding broilers with a high n-3 diet (10% crude fat from flaxseed and fish oil) reduced the crude fat content in the thigh meat and the size of the abdominal fat pad, but there was no effect on liver fat content in comparison to birds that were fed a tallow based diet. The latter results may be complicated by the high dietary fat inclusion level in their study, which can act to suppress fat utilisation [55]. Similarly, Scaife et al. [14], using 4 plant and animal fats either alone or in blends reported that the crude fat percentage of adipose tissue was highest in the birds fed the

soybean oil diet, whereas liver tissue had the lowest fat content in the birds fed the rapeseed (canola) oil diet. Thus, there is disagreement between studies regarding the impact of dietary fatty type/concentration on crude fat percentage in different tissues. Again, this may be due to physiological or environmental variations between the different studies.

2.3.4 Fatty acid composition of tissues

Rather than reporting the data on individual fatty acids, we chose to present the total for each fatty acid class measured in the diets and tissues (e.g. total saturates, total n-9 MUFA etc). The reasons for this are that all of the fatty acids measured in the tissues were also found in the diets, and there was limited amounts of *de novo* synthesis (with the exception of ALA conversion to eicosapentaenoic acid (EPA), docosapentaenoic acid and docosahexaenoic acid (DHA), particularly in flaxseed oil group [30-31] and LA conversion to arachidonic acid in all groups [20]). The fatty acid profiles of the 7 tissues sampled in this study are shown in Table 5, and the relationship between the diet and tissue content are described by regression variables and quantified by r values as in Table 6 and Fig 1. The role of the dietary fatty acid classes, the interaction of different types within the same diet treatment on different tissues and the comparison between these correlations are discussed below.

2.3.4.1 Blood

The predominant fatty acid groups in the whole blood were similar to those of the diet (Table 5). The SFA were the most abundant fatty acid group in blood across all treatments (35-46%), and highest in broilers fed the coconut oil diet, followed by the tallow treatment broilers. The second most abundant fatty acid group in whole blood was n-6 PUFA, but interestingly there was less variance in n-6 levels in the blood between the treatments (range 24% to 38%) than was present in the diets (range 16% to 44%). As expected, the highest percentage of blood

n-6 PUFA was in the corn oil group where the lowest was in the flaxseed oil group. The blood levels of both n-9 and n-7 MUFA were highest in the macadamia oil group, in line with their predominance in these diets. The level of n-3 PUFA in blood (highest in the flaxseed oil group, 15%) was approximately half of its level in the corresponding diets. The *trans* fatty acids were the least abundant fatty acid group in the blood (0.4-0.7%), and were at their highest level in the broilers fed the tallow diet (Table 5). Strong positive correlations ($r>0.9$, $P< 0.01$-0.05) were found between the diet and blood levels for all fatty acid groups, except the *trans* fatty acids being at moderate level (Table 6, Fig 1).

In the present study, the fatty acid profile of the whole blood was determined rather than the serum, plasma or red blood cells (the fractions which are more frequently analysed) [43,56]. However, Liu et al. [43] demonstrated that the whole blood fatty acid profile was closely correlated with red blood cells phospholipids. We have previously determined the effects of dietary (tallow and flaxseed oil-based diets) and sex of the broiler on the whole blood fatty acid profile [39]. The fatty acid profile of the male broilers used in the current study was very consistent with our earlier study, and also closely reflected the dietary fatty acid composition. In addition, Burlikowska et al. [57] similarly reported that the relative abundance of different fatty acid classes in serum is highly correlated to their respective levels in the diets.

2.3.4.2 Liver

Compared to the other tissues sampled, there was greater variability in the fatty acid profile (especially in total n-9 MUFA and total n-6 PUFA) of liver tissue between individual birds within each dietary treatment, as indicated by the higher SEM. It is noteworthy that those birds with the highest levels of hepatic crude fat had the heaviest livers and highest HSI% (data not

shown). These birds also had higher hepatic levels of total n-9 MUFA, at the expense of total n-6 PUFA.

The main contributor to the liver fatty acid profile were SFA (41%-45%; highest in the coconut oil group, $P<0.05$). The n-9 MUFA made up the second largest proportion of the total fatty acids in the liver but, interestingly, the levels of this fatty acid in the liver was not significantly different between diet groups (range 27%-31%). Interestingly, birds in the tallow, coconut and macadamia oil groups had the highest n-7 MUFA contents, which was not in line with the composition of these respective diets. The birds in flaxseed oil treatment contained the greatest proportion of total n-3 PUFA, consistent with the highest n-3 PUFA content of this diet, however, the percentage of n-3 PUFA in the liver (9%) was less than 30% of its contribution level in that diet. The n-6 PUFA in the liver was not greatly influenced by treatment type, except that the highest n-6 PUFA group (corn oil diet) had a higher hepatic n-6 PUFA than the flaxseed oil diet group ($P<0.001$, Table 5). It is worth noting that liver also contained the greatest levels of *trans* fatty acids of all the tissues analysed, and the *trans* fatty acid content was highest (1.6%) in the tallow treatment group, similar to its respective dietary percentage. The World Health Organization recommends *trans* fats should not exceed 1% of our daily energy intake [58]. Thus, high levels of consumption of livers from birds fed the tallow diet could contribute to exceeding the allowed threshold and adversely affect human health. In general, the levels of the respective fatty acids in the liver did not correlate particularly closely to the level in the diets. The strongest positive linear correlation was for total n-3 PUFA ($r=0.957$, $P<0.01$), followed by total SFA ($r=0.844$, $P<0.05$) and the correlations were of moderate strength for all other fatty acid groups (Table 6, Fig 1).

The level of SFA measured in liver in the present study is comparable to that reported in previous similar studies (~41%-45%) [33,47,50,59]. However, this was not the case with the other fatty acid types, with the MUFA levels being higher and PUFA levels lower than those reported elsewhere. Phetteplace and Watkins [38] for example, demonstrated that feeding broilers up to 56 days of age with a flaxseed oil diet increased the liver PUFA content to ~58% (comprised of ~32% n-3 and ~26% n-6) of total fatty acids, which was approximately 3.5- and 2-fold the levels measured in the present study, respectively. In agreement with the present study, González-Ortiz et al. [47] demonstrated that the fatty acid composition of the liver is less influenced by changes in dietary fatty acid composition in comparison with the muscle and adipose tissue.

2.3.4.3 Breast meat

The SFA and n-9 MUFA were the major fatty acid groups in the breast meat tissue, together making up more than two thirds of the total fatty acid content. Interestingly, SFA was the most abundant fatty acid in the breast meat of coconut oil fed birds, while for the other treatments n-9 MUFA were present in the highest amounts with the amount corresponding to the relative n-9 MUFA content of the diet (Table 5, $P<0.001$). The flaxseed oil treatment resulted in a breast meat total n-3 PUFA content which was at least 3.4-fold higher than the other treatments, whereas birds fed the corn oil diet had the highest proportion of n-6 PUFA. As a consequence, the n-6:n-3 ratio of the breast meat ranged from ~1.6:1 (in the birds fed flaxseed oil diet) to ~22:1 (in the birds fed corn oil diet). Perhaps surprisingly, breast meat had the second highest *trans* fatty acid content of the tissues assessed, and this was again highest in the tallow treatment group (1.3%). However, according to the American Heart Association, there are insufficient data to determine if consumption of naturally occurring *trans* fats have negative health effects

[60]. Breast meat fatty acid composition was more strongly correlated with the dietary fatty acid profile than all other sampled tissues ($P<0.01$, Table 6 and Fig 1).

Breast meat is the most important edible tissue in broiler chickens, comprising ~75% of the meat yield in a broiler carcass. As a result, the vast majority of studies focused on the nutritional aspects of chicken meat has involved sampling and assessment of breast tissue. Crespo and Esteve-Garcia [13] reported that feeding broilers a 10% tallow diet (between 21-42 growth days) resulted in a higher SFA percentage in breast meat (34%, similar to the present study, and others [47]) compared to 3 diets rich in either PUFA or MUFA. In the same study, SFA was reduced to 28% in breast meat from birds fed on a flaxseed oil based diet, which is again similar to our findings. These results suggest a very similar effect of feeding tallow and flaxseed oil diets on meat SFA content even with different inclusion levels of fat (10% vs 7% in our study). In another similar study Hrdinka et al. [61] reported that feeding broilers a diet contain 3.5% rapeseed (canola) oil, resulted in a higher content of MUFA (~50%) and SFA (38%) in the breast meat (with skin included). These levels are higher, by 5% and 8% respectively, than the levels in the current study. These differences are probably related to the type of fat in the skin since this was not included in the breast meat samples that were analysed in the present study.

2.3.4.4 Leg meat

In general, the patterns observed in the fatty acid composition of the leg meat across dietary treatments were largely in line with those seen in breast meat. However, the total n-6 PUFA levels were higher in the leg meat than in the breast meat (by up to 4.5% in the corn oil treatment), the level of *trans* fatty acids was about half of that in breast meat and the correlations of tissue fatty acid levels to the dietary levels were weaker (Table 6, Fig 1). Crespo and Esteve-Garcia [13] also reported similar patterns of fatty acids in breast and leg meat. In contrast,

however, Hrdinka et al. [61] reported that fatty acid composition of leg meat (with skin on) reflected the differences in dietary fatty acid intake more closely than did breast meat (again with skin on), and they suggested that might be related to the higher crude fat content of the leg meat. Nevertheless, these differences may not relate to the results of the deskinned meat in the present study, as the type of fat in the skin differs from that in meat.

2.3.4.5 Heart

Similar to the meat tissues, the fatty acid profile in the heart was significantly different across dietary treatments and closely related to diet composition ($P<0.001$, Table 3). The cardiac tissue was dominated by SFA in the majority of the dietary groups (tallow, flaxseed oil, macadamia oil and coconut oil) and by n-6 PUFA in the canola oil and corn oil treatments, with total SFA + n-6 PUFA contributing up to 85% of total cardiac fatty acids in the latter treatment. The range of SFA levels in the hearts of birds in the different diet treatments (3.4%) was slightly lower than measured in other tissues. The relatively high levels of n-6 PUFA (up to 45.0% in corn oil diet) made the heart the richest tissue in this group of fatty acids. The third major fatty acid group was n-9 MUFA (comprising 12.7% to 19.2% of all fatty acids), however the heart contained the lowest levels of this fatty acid group of all the tissues examined. The levels of the *trans* fatty acids were lowest in the heart than all other tissues measured in this study, ranging from 0.3% to 0.5%, but were still highest in the tallow diet group (as for the other tissues) (Table 5). The levels of all the fatty acid groups in the heart, with the exception of the n-9 PUFA, were positively correlated to their levels in the diet (Table 6 and Fig 1).

Crude fat content and fatty acid composition of cardiac tissue in fast-growing broilers has been associated with potential roles in health conditions including sudden death syndrome (SDS) that can cause significant economic loss to the chicken industry. Chung et al. [62] found

that feeding chickens a diet containing sunflower oil was associated with a lower incidence of SDS mortality than feeding a tallow based diet. The content of n-3 PUFA in the cardiac tissue seems not to be only determined by its respective dietary percentage, but also the presence of n-6 PUFA in the diet (the dietary n-6:n-3). Therefore, although the canola oil diet provided a considerable proportion of n-3 PUFA (6.6%), this diet did not elevate the n-3 PUFA content in the cardiac tissue because it was still accompanied by a high dietary n-6:n-3 ratio (4.09) in agreement with Kartikasari, et al. [63] finding. Further, Gregory et al. [51], demonstrated that a canola diet, but not a tallow based diet, enriched the phospholipid class of the cardiac lipids with certain n-3 PUFA, specifically the total of EPA and DHA. However, this elevation was not significant for the major n-3 PUFA (ALA) similarly to cardiac total n-3 PUFA content between the equivalent dietary treatments in the present study.

Phetteplace and Watkins [38] reported that the association between n-6 PUFA levels in the cardiac and hepatic tissues in broilers fed a linseed (flaxseed) oil diet was close to the pattern that observed in the flaxseed oil group in the current study. However, the cardiac n-3 PUFA level which they reported for the same treatment is higher (by ~13%) than of its respective level in the flaxseed oil group in the current study. Consistent to our finding, Kartikasari [56] reported that feeding broilers flaxseed oil diet was increased n-3 PUFA content in the cardiac membrane by 4.8-fold comparing to broilers fed on tallow diet.

2.3.4.6 Brain

The results of brain tissue fatty acid analysis were substantially different from the patterns we measured in all the other tissues. Almost half of the total fatty acids in brain were SFA, making this tissue the richest in this group of fatty acids. With regard to the variation between dietary treatments, the brain of birds fed the corn oil diet had the highest content of SFA

(50.3%), however this was only ~5.0% higher than the flaxseed oil group (the lowest); despite the very large difference in their dietary SFA levels (19%, flaxseed oil diet to 64%, coconut oil diet). Similarly, the large range in dietary n-9 MUFA (16%, coconut oil diet to 46%, macadamia oil diet) had no effect on the percentage of n-9 PUFA in the brain (17% on average). The n-7 MUFA was the second lowest group of fatty acids in the brain, and was higher in the macadamia oil, coconut oil and tallow treatments than in the other dietary groups. In 4 groups (tallow, canola oil, macadamia oil and coconut oil), where PUFA were not the most abundant fatty acid group, the n-6:n-3 in brain were similar (0.92-1.22). In the flaxseed oil group, however, the n-3 PUFA content (20.1%) was more than double the content of n-6 PUFA. In contrast, the corn oil group had a substantially higher n-6 PUFA content (16%) compared to n-3 PUFA (10%). It is also important to note, however, that the brain contained the lowest proportion of n-6 PUFA compared to all other tissues analysed in this study. The *trans* group of fatty acids contributed a very low percentage (<0.5%, Table 5) of the total fatty acids in the brain in all groups. In terms of the relationships between fatty acid content in the tissues and those in the diet, only brain n-3 PUFA content was strongly and positively ($r=0.927$, $P<0.01$) correlated to the n-3 PUFA content of the diet. The n-7 PUFA and n-6 PUFA showed weaker positive relationships ($r=0.667$ and 0.544, respectively), while the levels of all other fatty acids in the brains were not significantly related to their content in the diets. Indeed, the levels of n-9 MUFA and *trans* fatty acids in the brain were actually inversely correlated with their levels in the diet (Table 6, Fig 1).

Relatively few studies have investigated the fatty acid content of chicken brain tissue [64, 65] and to our knowledge none have compared the effect of manipulation of the dietary fatty acid on brain fatty acid composition. It is particularly noteworthy that even when broilers were fed on diets that were low in n-3 PUFA, they nevertheless maintained a relatively high level

(>11.0%) of this functionally pivotal fatty acid group in their brains. This finding is in agreement with the results from Phetteplace and Watkins [38], who reported that broilers accumulated 11%-15% of n-3 PUFA in the brain even when fed a poor dietary source of these fatty acids. However, those authors reported that their linseed (flaxseed) oil treatment resulted in an even higher (by ~8%) n-3 PUFA level than measured in the birds fed on the flaxseed oil based diet in the present study. We also found that high levels of n-3 PUFA in brain were accompanied by low levels of n-6 PUFA, in contrast to the findings by Phetteplace and Watkins [38] of higher levels of n-6 PUFA in treatment with low dietary n-3 PUFA. As in the liver, we found that feeding birds a diet with a high n-7 MUFA content (the macadamia oil treatment) did not elevate the level of this fatty acid in the brain in comparison to the poor dietary providers of n-7 MUFA, such as tallow and coconut oil diets.

The poor correlation with this diet for most of fatty acid groups in brain support the suggestion that the incorporation of fatty acids in this tissue is heavily influenced by more complicated mechanisms than merely passive transfer from the diet.

2.3.4.7 Adipose Tissue

Adipose tissue was dominated by n-9 PUFA (35% - 47%) in all treatments except for the coconut oil group where SFA was the predominant group (48%). Otherwise, with the exception of *trans* fatty acids, the fatty acid profile of this tissue typically followed the dietary fatty acid composition very closely (r>0.9, Table 6). Feeding broilers the macadamia oil diet resulted in the highest n-9 and n-7 MUFA content in the adipose tissue, corn oil and canola oil diets resulted in the highest level of adipose n-6 PUFA, whereas the flaxseed oil diet was associated with the highest (16.7%) levels of n-3 PUFA. The levels of all MUFA and PUFA in the adipose tissue correlated almost perfectly (r=0.999 to 1.00, P<0.01, Table 6 and Fig 1) to dietary fatty

acid content. The *trans* fatty acids contributed up to 0.4% of tissue fatty acid content (in the coconut oil diet, Table 5). Our results are supported by a number of other published observations showing that adipose tissue fatty acid content is strongly correlated to dietary fatty acid composition [10,11,13,48, ,61,66]. Crespo and Esteve-Garcia [13], for example, reported that MUFA tend to accumulate in the adipose tissue. Similarly, Ortiz et al. [66] found that MUFA was the predominant fatty acid class comprising 43% n-6:n-3 - 56% of the total fatty, with 38% - 49% reported by Hrdinka et al. [61]. However, the MUFA:SFA and MUFA:PUFA ratios in adipose tissue were different to the dietary ratios as reflected by variation in the values for slopes in the regression equations (Table 6). Carmona et al. [48] found that n-6 PUFA in the adipose tissue was the fatty acid group most susceptible to modifications to dietary fatty acid composition, which was consistent with the current study. Higher levels of both n-3 (by 8%) and n-6 (by 10%) PUFA were reported in the adipose tissue of broilers fed 5% w/w flaxseed oil diet for a period 2 weeks longer than the current experiment [38]. While we only analysed the abdominal adipose tissue in the current study, Hrdinka et al. [61] have previously reported that there were no differences in the effect of diet on fatty acid composition of the abdominal and subcutaneous depots.

Table 5. Fatty acid profile[1] of seven tissues of broilers fed six experimental diets2 for 42 days

Tissue	Treatment	SFA[3]	n-9 MUFA[4]	n-7 MUFA[5]	n-3 PUFA[6]	n-6 PUFA[7]	Trans
Blood	Tallow	39.5^{a}±0.4	25.4^{a}±1.2	4.3^{a}±0.3	3.3^{a}±0.2	26.2^{ab}±1.1	0.7^{a}±0.0
	Flaxseed oil	36.8^{bc}±0.2	20.8^{b}±0.6	2.6^{b}±0.2	14.9^{b}±0.3	24.3^{a}±0.5	0.3^{b}±0.1
	Corn oil	37.2^{c}±0.5	20.1^{b}±1.2	2.6^{b}±0.2	1.7^{c}±0.1	37.6^{c}±0.9	0.4^{ab}±0.0
	Canola oil	35.8^{bc}±0.4	25.6^{a}±0.6	3.2^{bc}±0.1	4.9^{d}±0.3	29.8^{d}±0.5	0.4^{ab}±0.1
	Macadamia oil	35.0^{b}±0.3	30.2^{c}±0.8	7.0^{d}±0.4	2.3^{c}±0.1	24.5^{a}±0.5	0.6^{ab}±0.2
	Coconut oil	46.0^{d}±0.6	18.9^{b}±0.5	4.0^{ac}±0.2	2.4^{ac}±0.1	27.6^{bd}±0.4	0.5^{ab}±0.0
	P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Liver	Tallow	42.2 ^{ab}±1.3	29.1±2.5	4.4^{abc}±0.4	3.4^{ac}±0.5	18.8^{ab}±2.3	1.6^{a}±0.2
	Flaxseed oil	41.6^{a} ±0.7	30.1±2.7	3.8^{ab}±0.4	9.1^{b}±1.2	14.0^{a}±1.8	1.2^{bc}±0.1
	Corn oil	43.4^{ab}±0.8	28.2±1.6	3.3^{a}±0.3	1.3^{a}±0.2	22.4^{b}±2.0	1.1^{bc}±0.0
	Canola oil	41.7^{a}±0.3	28.5±1.5	3.1^{a}±0.2	4.6^{c}±0.5	20.4^{ab}±1.2	1.4^{ab}±0.1
	Macadamia oil	41.3^{a}±0.7	31.1±2.2	5.4^{c}±0.2	2.4^{ac}±0.5	18.0^{ab}±2.2	1.4^{ab}±0.1
	Coconut oil	45.4^{b} ±0.7	27.2±1.9	5.3^{bc}±0.6	2.0^{ac}±0.3	18.1^{ab}±1.9	1.2^{bc}±0.0
	P value	<0.05	>0.05	<0.001	<0.001	<0.001	<0.01
Breast	Tallow	33.7^{a}±0.4	36.9^{acd}±0.7	8.5^{a}±0.1	3.0^{a}±0.1	16.4^{ab}±0.4	1.3^{a}±0.0
	Flaxseed oil	28.1^{b}±0.6	32.0^{ab}±0.8	6.2^{b}±0.2	16.8^{b}±0.3	15.8^{ab}±0.4	0.8^{b}±0.0
	Corn oil	30.9^{ab}±1.1	33.9^{ac}±1.4	6.6^{b}±0.4	2.0^{c}±0.1	25.6^{c}±2.8	0.8^{b}±0.1
	Canola oil	28.4^{b}±0.9	38.2^{cd}±2.1	7.2^{b}±0.2	4.9^{d}±0.3	20.1^{bc}±1.2	0.9^{b}±0.0
	Macadamia oil	29.2^{b}±0.4	40.6^{d}±1.5	12.7^{d}±0.3	2.2^{ac}±0.2	14.1^{a}±1.1	0.8^{b}±0.1
	Coconut oil	43.2^{c}±0.6	28.3^{b}±0.7	8.3^{a}±0.2	2.2^{ac}±0.2	15.8^{ab}±0.8	0.8^{b}±0.0
	P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Leg	Tallow	34.5^{a}±0.3	36.4 ^{a}±0.7	7.9^{a}±0.2	2.7^{a}±0.1	16.9^{a}±0.5	0.8^{a}±0.0
	Flaxseed oil	29.3^{b}±0.6	30.4^{b}±0.8	5.7^{b}±0.2	16.3^{b}±0.5	17.4^{a}±0.5	0.4^{b}±0.0
	Corn oil	31.0^{b}±0.5	30.6^{b}±1.0	5.8^{b}±0.1	1.8^{a}±0.1	30.1^{b}±0.8	0.4^{bc}±0.0
	Canola oil	29.8^{b}±0.7	35.3^{a}±1.3	6.5^{b}±0.2	5.0^{c}±0.2	22.6^{c}±0.8	0.4^{bc}±0.0
	Macadamia oil	30.1^{b}±0.5	39.2^{a}±1.6	12.1^{c}±0.5	2.0^{a}±0.2	15.9^{a}±1.4	0.4^{b}±0.0
	Coconut oil	43.1^{c}±0.4	27.5^{b}±0.8	7.6^{a}±0.1	2.1^{a}±0.1	18.1^{a}±1.1	0.8^{c}±0.0
	P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Heart	Tallow	40.8^{a}±0.3	13.5^{ab}±0.7	4.1^{a}±0.1	2.1^{ac}±0.1	38.9^{a}±0.5	0.5^{a}±0.0
	Flaxseed oil	38.8^{b}±0.6	12.7^{ab}±0.5	3.3^{b}±0.1	9.3^{b}±0.9	35.5^{b}±0.4	0.3^{b}±0.0
	Corn oil	40.1^{ab}±0.3	10.5^{a}±0.4	3.1^{b}±0.1	0.9^{a}±0.1	45.0^{c}±0.3	0.3^{bc}±0.0
	Canola oil	38.6^{b}±0.5	14.5^{bc}±1.2	4.2^{a}±0.1	2.8^{c}±0.2	39.4^{a}±0.8	0.3^{bc}±0.0
	Macadamia oil	38.3^{b}±0.4	19.2^{c}±1.1	5.9^{c}±0.2	1.4^{ac}±0.1	34.8^{b}±0.7	0.3^{c}±0.0

	Coconut oil	41.7^{a}±0.6	13.4^{b}±0.6	3.9^{a}±0.1	1.2^{ac}±0.1	38.7^{a}±1.1	0.4^{d}±0.0
	P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Brain	Tallow	47.9^{ab}±0.9	17.6±0.8	7.6^{ac}±0.2	12.9^{ad}±0.2	13.3^{a}±0.3	0.3^{acd}±0.0
	Flaxseed oil	45.5^{a}±0.8	17.8±1.1	6.7^{b}±0.2	20.1^{b}±0.7	9.5^{b}±0.2	0.2^{b}±0.0
	Corn oil	50.3^{b}±1.1	15.8±1.0	7.0^{ab}±0.2	9.7^{c}±0.3	16.6^{c}±0.4	0.2^{ab}±0.0
	Canola oil	47.2^{ab}±0.9	17.1±0.7	7.0^{ab}±0.1	14.6^{d}±0.5	13.5^{a}±0.4	0.3^{abc}±0.0
	Macadamia oil	49.3^{b}±0.7	17.3±0.5	8.2^{c}±0.2	11.0^{ac}±0.2	13.4^{a}±0.3	0.4^{cd}±0.0
	Coconut oil	48.3^{ab}±0.8	18.1±1.0	7.9^{c}±0.3	11.3^{ac}±0.8	13.7^{a}±0.2	0.4^{d}±0.0
	P value	<0.01	>0.05	<0.001	<0.001	<0.001	<0.001
Adipose	Tallow	34.0^{a}±0.4	42.1^{a}±0.5	9.5^{a}±0.5	1.5^{a}±0.0	11.3^{a}±0.3	0.3^{ab}±0.1
	Flaxseed oil	27.0^{b}±0.4	35.4^{b}±0.4	6.7^{b}±0.4	16.7^{b}±0.2	13.5^{b}±0.2	0.2^{a}±0.0
	Corn oil	29.5^{c}±0.3	36.6^{b}±0.4	6.6^{b}±0.4	1.6^{a}±0.0	24.9^{c}±0.6	0.2^{ab}±0.1
	Canola oil	27.0^{b}±0.2	46.0^{c}±0.3	7.2^{b}±0.2	3.5^{c}±0.5	15.7^{d}±0.3	0.2^{ab}±0.0
	Macadamia oil	27.9^{b}±0.3	47.1^{c}±0.3	14.3^{c}±0.3	1.2^{a}±0.0	8.8^{e}±0.2	0.2^{ab}±0.0
	Coconut oil	48.1^{d}±0.5	31.0^{d}±0.4	9.5^{a}±0.4	1.1^{a}±0.0	9.0^{e}±0.1	0.4^{b}±0.1
	P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1] Values are the pooled mean (n=8) ± SEM of fatty acid group percentage of the total fatty acids. Values in the same column with no common superscript are significantly different. [2]Supplemented with 6 fats at 4% w/w inclusion level. [3]SFA = Saturated fatty acid; [4] n-9 MUFA= Omega 9 monounsaturated fatty acid; [5]n-7 MUFA= Omega 7 monounsaturated fatty acid; [6]n-3PUFA = Omega 3 polyunsaturated fatty acid, [7]n-6 PUFA = Omega 6 polyunsaturated fatty acid

Table 6. Pearson correlation (r) and linear regression coefficient (y=a+bx) between fatty acid levels in diets and tissue (n=8) of six different experimental dietary treatments in 42-days-old broiler chickens

Tissue		SFA	n-9 MUFA	n-7 MUFA	n-3 PUFA	n-6 PUFA	Trans
Blood	a	31.892	12.448	2.438	1.488	17.911	0.323
	b	0.220	0.340	0.309	0.464	0.425	0.223
	r	0.979**	0.903*	0.929**	0.995**	0.907*	0.660
Liver	a	40.438	26.730	3.691	1.931	14.594	1.084
	b	0.073	0.071	0.107	0.253	0.164	0.325
	r	0.844*	0.581	0.540	0.957**	0.620	0.772
Breast	a	22.896	22.518	6.067	1.208	8.535	0.575
	b	0.317	0.383	0.446	0.538	0.384	0.453
	r	0.989**	0.980**	0.938**	0.999**	0.966**	0.987**
Leg	a	24.283	21.729	5.389	1.096	8.255	0.332
	b	0.294	0.354	0.451	0.527	0.486	0.281
	r	0.995**	0.916*	0.942**	0.998**	0.957**	0.593
Heart	a	37.764	8.794	3.165	0.776	31.657	0.233
	b	0.066	0.159	0.187	0.295	0.288	0.163
	r	0.868*	0.630	0.939**	0.993**	0.844*	0.851*
Brain	a	47.840	17.996	7.013	11.963	10.495	0.308
	b	0.008	-0.022	0.079	0.287	0.116	-0.011
	r	0.088	-0.311	0.667	0.927**	0.544	-0.051
Adipose	a	18.847	21.783	6.365	0.039	-0.010	0.216
	b	0.454	0.551	0.531	0.573	0.566	0.047
	r	0.994**	0.988*	0.902*	1.000**	0.999**	0.247

*Correlation is significant at $P<0.05$ level (2-tailed). **Correlation is significant at $P< 0.01$ level (2-tailed).

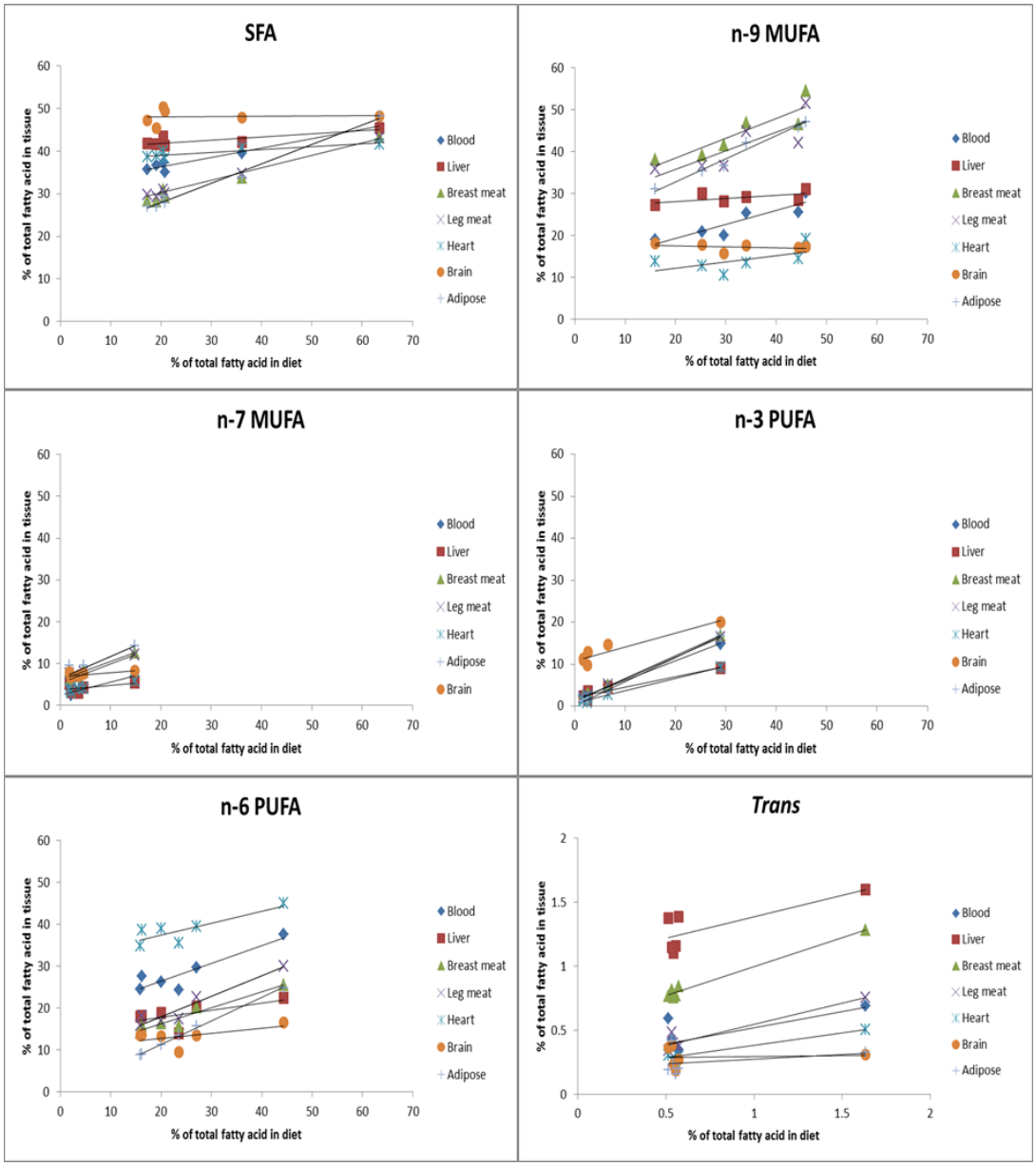

Figure 1. Correlations between contents of six fatty acid groups (% of total fatty acids) in diet (independent variable) and tissue (dependent variable) of seven tissues in 42-days-old broiler chickens receiving six different dietary fats at 4% w/w inclusion level.

2.4 Conclusion

None of the growth parameters measured in this study were influenced by the different fats used in the diets. We speculate that differences in growth performance related to dietary fat type may only become evident if the birds are also stressed by some aspect of the environmental or experimental conditions.

On the other hand, different fatty acids contribute in varying amounts to the composition of different tissues. Except for the brain, the fatty acid composition of the major tissues largely reflected the fatty acid composition of the diet. In particular, all tissues were correlated in a strong linear positive relationship to the dietary n-3 PUFA content. Thus, chicken products from broilers fed a flaxseed oil-based diet have a potential health benefits to human. Conversely, feeding broilers a corn oil-based diet was associated with a lower n-3:n-6 ratio in different tissues and feeding them a tallow-based diet was associated with a higher trans percentage, particularly in liver and breast meat. Therefore, the chicken broiler industry needs to be more aware that different dietary fats will have significant effects on the fatty acid composition of chicken tissues, and thereby on the nutritional benefits or health issues conferred by those chicken tissues to consumers.

2.5 References

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CHAPTER 3: (Manuscript 2): THE FATTY ACID COMPOSITION OF EXCRETA OF BROILER CHICKENS FED DIFFERENT DIETARY FATTY ACIDS

Khaled Kanakri, John Carragher, Robert Hughes, Beverly Muhlhausler, Carolyn de Koning, Robert Gibson. The fatty acid composition of excreta of broiler chickens fed different dietary fatty acids. International Journal of Poultry Science, 2017; 16:424-434. (DOI: 10.3923/ijps.2017.424.433).

Statement of Authorship is located in Appendix 4 (4.2).

Abstract

Excreted fatty acids represent the net result of fat digestion, absorbtion and bioconversion by chickens or their intestinal microbiome and thus provide information on the capacity of the birds to utilize different fat types. This study aimed to clarify the relationship between the fatty acid profile of diet and excreta in broiler chickens. Male Cobb 500 broilers (n=240) were fed (ad libitum) one of six different diets supplemented with 4% (w/w) beef tallow, flaxseed, corn, macadamia, canola or coconut oils (4 replicate pens per treatment) from hatching day. At Day-40 post-hatch, excreta samples were collected for fatty acids analysis. Significant positive linear correlations (R=0.82 to 0.99) were found in the fatty acid content of diets and excreta for all fatty acid groups in all treatments. Comparing the individual fatty acid content of diet and excreta suggested that the broilers preferentially utilized (in decending order, if present) omega-3 polyunsaturated fatty acids, omega-9 and omega-7 monounsaturated fatty acids and most saturated fatty acids (except C16:0 and C18:0), but the omega-6 polyunsaturated fatty acids were under-utilized even when they were the most abundant. This suggests that fat sources which are high in the C16:0, C18:0 and omega-6 fatty acids may not be ideal for broiler feed formulations for nutritional and economical reasons.

Key words: Oils, fatty acid, diet, excreta, chicken broiler.

3.1 Introduction

Fats from animal and/or plant origins are added to commercial chicken feed as a source of essential fatty acids and a source of energy. Manipulation of the fat composition (e.g. the type/s and inclusion level) is commonly implemented for economic and nutritional purposes and results in altered dietary fatty acids composition (e.g. chain length, degree of saturation and molecular structure). Previous studies in chickens have shown different associations between the fatty acid content of diets and tissues depending on tissue type (Balevi and Coskun, 2000; Kanakri et al., 2017b; Kanakri et al., 2016). There is considerable evidence that dietary fatty acid composition is related to growth performance (Carragher et al., 2016) and health status (Cherian, 2007; Kartikasari et al., 2017) in chickens, but less is known about whether dietary fat composition influences the utilization of different fat types.

The utilization of fatty acids as an energy source in broilers is limited in the first two weeks post-hatch but it improves as birds get older and physiological functions develop (Tancharoenrat et al., 2013). Fatty acid analysis of digesta sampled from along the gastrointestinal tract is commonly used to evaluate the digestion of nutrients and shows variation in the fat digestion coefficient between different intestinal segments (Ajuyah et al., 1996; Boguslawska-Tryk et al., 2016) with jejunum being the segment where the majority of fat is digested and absorbed (Tancharoenrat et al., 2014). To complicate matters further, variation in the microbial content between different intestinal sections, such as ileum and cecum, has been reported in broilers (Bjerrum et al., 2006) which suggests intestinal microbiota could play a role in fat metabolism. In support of this, changes in gut microbiota are known to affect the performance parameters of broiler chickens (Rubio et al., 2015).

Many aspects of dietary fatty acids, including inclusion level (Leek et al., 2004), source (Tancharoenrat et al., 2015), re-esterification (Vilarrasa et al., 2015) as well as their interactions with other dietary macronutrients (Boguslawska-Tryk et al., 2016), micronutrients (Atteh and Leeson, 1984; Azman and Seven, 2005) and enzymes (Danicke et al., 1997) have been reported to affect their intestinal absorption and excretion in broilers.

Number of studies have investigated different aspects of broiler excreta. These studies have led to better understanding of lipid utilization and losses (Fuhrmann and Kamphues, 2016), fatty acid influence on excreta microbiota (Lee et al., 2016), estimating metabolizable energy (Leek et al., 2004; Wiseman et al., 1998), feed conversion efficiency (Metzler-Zebeli et al., 2016), using excreta as a dietary component itself (Bansal et al., 2011; Nambi et al., 1992), influencing manure mineral content (Azman and Seven, 2005) and even how excreta affects the foot pad health of the birds (Fuhrmann and Kamphues, 2016). However, it is important to acknowledge that the fatty acid content of excreta is a consequence of several factors, including: the lipid composition of the diet, the activities of lipase and bile salts, the efficacy of absorptive, metabolic and excretive processes throughout the length of the gut, and the utilization and potential modification of fatty acids by the microbiota (Blanch et al., 1996; Firman et al., 2008; Ravindran et al., 2016; Zumbado et al., 1999).

To our knowledge, no published literature has compared the dietary effect of a wide variety of dietary fats which are very different in their fatty acid composition on the fatty acid composition in the excreta (as opposed to the digesta) of broilers. Therefore, the present study aimed to examine this relationship in broilers at harvest age fed diets supplemented with a range of different types of fats. A better understanding of this relationship is potentially useful to

provide advice to poultry feed producers regarding the best choice of available fats to use when formulating broiler diets.

3.2 Materials and Methods

3.2.1 Broilers and Experimental Design

The Animal Ethics Committees of the University of Adelaide and Primary Industries and Regions South Australia approved this study. A total of 240 day-old male chicks of the Cobb 500 strain were obtained from the Baiada Hatchery (Willaston, SA, Australia) and transferred to South Australian Research and Development Institute (SARDI) facility (Roseworthy, SA, Australia). A complete randomized block design (4 pens/treatment) was implemented. Birds were randomly distributed into 24 groups of 10 and allocated to 24 raised rearing floor pens (1.2 × 0.9 m each) in one shed. Chickens were reared on sawdust and shavings in a temperature controlled room and had free access to both feed and water at all times. Pens were heated by infrared brooder lamps (175 W) during the first 3 weeks post-hatch. Temperature in the room was 27ºC for the first 4 days, gradually decreased to 20ºC, and then maintained for the 40-day experimental period.

3.2.2 Experimental Diets

The study included 6 dietary treatments. In each, broilers were fed *ad libitum* 1 of 6 experimental diets by adding a different fat at 4% w/w to starter (crumble form, first 3 weeks) and finisher (pellet form, last 3 weeks) basal diets. The basal diets (containing ~3% crude fat) were obtained from a poultry feed manufacturer (Ridley Agriproducts, Australia). The added fat types were; flaxseed oil (high omega-3 polyunsaturated fatty acids (n-3 PUFA); Four Leaf Oils, Australia), corn oil (high omega-6 polyunsaturated fatty acids (n-6 PUFA); Daisy, Malaysia), canola oil (high omega-9 monounsaturated fatty acids (n-9 MUFA); Foodland,

Australia), macadamia oil (high omega-7 monounsaturated fatty acids (n-7 MUFA); Macoils, Australia), coconut oil (high saturated fatty acids (SFA); Banaban, Fiji) or beef tallow (moderate SFA and MUFA, Ridley Agriproducts, Australia). The composition and nutritional profiles of the two basal diets are shown in Table 1. Apart from the variation in the fatty acid composition of the experimental diets (Table 2), they all were nutritionally identical and met requirements for healthy growth (NRC, 1994).

Table 1. Ingredient composition and nutrient content of the basal diets

Ingredients (%)	Basal diet[1]	
	Starter[2]	Finisher[3]
Wheat fine	61.42	70.77
Barley fine	5.00	5.00
Tallow mixer	0.37	1.00
Blood meal (91% CP)	1.00	0.00
Soybean meal	25.17	16.67
Limestone small	0.86	0.64
Monocalcium phosphate	0.14	0.00
Sodium chloride	0.21	0.18
Sodium bicarbonate	0.16	0.18
Choline chloride 75%	0.03	0.04
DL-methionine 58.1	0.29	0.21
L-threonine 73.7	0.13	0.25
Rovabio Excel LC	0.02	0.02
Meat meal	3.73	3.70
Ronozyme NP CT	0.02	0.02
Mineral/vitamin premix[1]	1.00	1.00
L-lysine sulphate 70	0.43	0.33

[1]The starter and finisher basal diets Metabolisable Energy = 2899.59 and 2999.90 Kcal/kg, the nutrient contents (g/kg) were: Crude protein 225.1 and 186.7, Crude fat 23.5 and 30.0, Crude fibre 32.3 and 29.2, Ca 9.8 and 8.5, Available phosphorus 4.5 and 4.2, Na 1.8 and 1.7, K 0.8 and 6.5, Cl 0.2 and 1.8, Lysine 12.3 and 9.1, Methionine 5.7 and 4.7, Cystine 3.9 and 2.8, Threonine 7.9 and 7.6, Leucine 14.3 and 11.2, Isoleucine 7.8 and 6.4, Tryptophan 2.3 and 1.8, Arginine 12.4 and 9.8 and Valine 9.3 and 7.2, respectively. [2]Used to formulate 2 experimental diets to feed broilers up to 3 weeks old. [3]Used to formulate 2 experimental diets to feed broilers from 4 to 6 weeks old.

Table 2. Crude fat content and fatty acid composition (as a percentage of total fatty acids) of the six experimental diets[1]

	Crude fat%[2]	Total SFA[3]	Total *trans*	Total n-9[4]	Total n-7[5]	Total n-3[6]	Total n-6[7]
Diet							
Tallow	7.0	35.8	1.6	34.1	4.6	2.9	20.2
Flaxseed oil	7.2	18.6	0.5	25.0	2.1	29.5	24.1
Corn oil	7.2	20.3	0.5	29.3	2.0	2.7	45.0
Canola oil	7.2	16.9	0.5	44.4	3.5	6.9	27.4
Macadamia oil	7.0	20.6	0.5	45.7	14.8	2.0	16.2
Coconut oil	7.1	64.3	0.5	15.3	1.7	1.9	16.2
Excreta[8]							
Tallow	1.2±0.1	52.3^{a}±1.1	1.7^{a}±0.1	20.5^{ab}±1.0	2.7^{a}±0.2	2.1^{a}±0.1	20.4^{a}±0.3
Flaxseed oil	0.9±0.1	22.4^{b}±0.4	0.3^{bd}±0.1	22.8^{ab}±0.3	2.2^{a}±0.1	16.0^{e}±0.5	36.1^{b}±0.5
Corn oil	1.0±0.1	23.3^{b}±0.3	0.2^{d}±0.1	25.7^{b}±0.6	2.4^{a}±0.1	3.2^{b}±0.1	45.1^{c}±0.4
Canola oil	1.0±0.0	21.0^{b}±0.5	0.3^{bd}±0.1	33.6^{c}±0.7	3.0^{a}±0.1	5.3^{c}±0.2	36.6^{b}±0.8
Macadamia oil	1.1±0.2	27.1^{c}±1.1	0.6^{c}±0.0	39.0^{c}±4.4	9.5^{b}±1.4	2.6^{ab}±0.0	21.0^{a}±6.6
Coconut oil	1.0±0.1	48.2^{d}±2.7	0.5^{bc}±0.1	17.3^{a}±0.4	2.0^{a}±0.1	2.7^{ab}±0.1	29.2^{b}±0.8

[1]Finisher basal diet + added fat, fed to broilers between 22 and 40 days of age. [2]Values are means of 4 replicates and the percentages are based on the wet weight. [3]SFA = Saturated fatty acid; [4] n-9 = Omega 9 monounsaturated fatty acid; [5]n-7 = Omega 7 monounsaturated fatty acid; [6]n-3 = Omega 3 polyunsaturated fatty acid; [7]n-6 = Omega 6 polyunsaturated fatty acid, [8]Values are mean of 4 replicates±SEM and different superscript letters within the same column are significantly different (P<0.001).

3.2.3 Production Parameters

Total body weight (BW) of the birds in each pen was taken on the day of hatch and then on a weekly basis for the first 3 weeks. Feed intake (FI) of birds in each pen was also recorded on a weekly basis and used to calculate the feed conversion ratio (FCR; kg feed/kg body weight gain). Number of deaths and culls was used to calculate mortality rate in all treatments on a weekly basis.

3.2.4 Sample Collection

On Day-40, paper drop-sheets were placed in each pen to collect excreta samples. Approximately 10-12 fresh droppings (deposited within 3 hours) were randomly transferred into clean plastic containers. Samples were immediately frozen on dry ice before they were

transferred to the laboratory to be stored at -18°C until subsequent determination of crude fat content and fatty acid analysis.

3.2.5 Fatty Acid Analysis

Crude lipid was extracted from homogenized feed and excreta samples (Folch et al., 1957). The gravimetric approach was utilized to estimate total lipid percentages. Fatty acid profiling was performed after transmethylation of the extracted crude lipids with 1% H_2SO_4 in methanol at 70°C for 3 h (Gregory et al., 2010; Tu et al., 2010). After cooling to room temperature, the resulting fatty acid methyl esters (FAME) were extracted with n-heptane (2 mL) and transferred into gas chromatography (GC) vials containing about 30 mg of anhydrous sodium sulphate. Vials were stored at -18°C until GC analysis.

3.2.6 Gas Chromatography Analysis of FAME

The FAME were separated using a Hewlett-Packard 6890 GC (Hewlett-Packard, CA, USA) equipped with a flame ionization detector (FID), a split injector and a BPX-70 capillary column (50 m x 0.32 mm internal diameter) with a 0.25 µm film thickness (SGE, Victoria, Australia). The operating conditions of the GC, identification of fatty acids using the GLC 463 external standard (Nu-Chek Prep Inc, MN, USA) and qualitative analysis was as described previously (Kartikasari et al., 2012; Tu et al., 2010).

3.2.7 Statistical Analysis

The effects of dietary treatment on the excreta fatty acid profile were tested by one-way analysis of variance (ANOVA) and Duncan's multiple comparison test was implemented when the ANOVA indicated the differences between dietary treatment effects were significant ($P<0.05$) using SPSS version 22 for Windows (IBM Crop., NY, USA). The effects of dietary

treatment on BW, FI and FCR were determined by one-way ANOVA ($P<0.05$) using SAS 9.3 for Windows.

3.3 Results

3.3.1 Broiler productivity

There were no significant differences in any of the production parameters between different treatments. The average BW of 40-day-old broilers was 3367g, the FI was 5414 g/bird, the FCR was 1.63 g feed/g weight gain and the overall mortality rate was 5.4 %.

3.3.2 The Diets

The crude fat content of the different experimental diets ranged from 7.0% to 7.2% (Table 2). In the tallow diet, the two prevalent fatty acid groups were SFA and n-9 MUFA in almost equal portions, and these together made up more than two thirds of total fatty acids. The major fatty acid group in the flaxseed oil diet was n-3 PUFA (at ~30% of the total fatty acids) with n-9 MUFA and n-6 PUFA also present at lower proportions. The main fatty acid group in the corn oil diet was the n-6 PUFA (45%) followed by n-9 MUFA (~29%). The n-9 MUFA was the predominant fatty acid group in the canola and macadamia oil-based diets (~44 - 46%). The canola oil diet also contained, in descending order, n-6 PUFA, SFA and n-3 PUFA; whereas the macadamia oil diet contained more SFA than either n-6 or n-3 PUFA, and while the n-7 MUFA was only the third most abundant fatty acid group in the macadamia oil diet (at 15% total fatty acids), this level was still 3 to 9 times higher than in the other 5 diets. The coconut oil diet contained a higher percentage of SFA (64%) compared to all other diets. The contribution of trans fatty acids was the lowest of all the fatty acid groups, and highest percentage was in the tallow diet with 1.6% (Table 2).

3.3.3 The Excreta

The colour, shape, size or viscosity of droppings between different pens (dietary treatments) were not different; however, droppings from individual birds within the same group did differ in appearance (data not shown). The overall percentage of the crude fat in the wet excreta ranged from 0.9% to 1.2% with overall average approximately 1.1% (Table 2). The tallow treatment resulted in excreta with the highest SFA percentage (52%) followed by n-9 MUFA and n-6 PUFA with equal contributions (~20% each). The excreta of flaxseed oil treatment contained higher n-3 PUFA content (16%) than any other treatment. However, this excreta was dominated by n-6 PUFA, and n-3 PUFA was only the fourth main contributor. Similar to its respective dietary level (45%), n-6 PUFA was the main fatty acid group in excreta of the corn oil treatment, followed by n-9 MUFA and SFA. Likewise, but at a lower level, n-6 PUFA was the dominant (37%) fatty acid group in the excreta of the canola oil treatment followed by n-9 MUFA and SFA. Noteworthy, and reflecting the dietary composition, canola oil excreta contained the second highest percentage of n-3 PUFA (5.3%). The excreta of broilers fed the macadamia oil diet was dominated by n-9 PUFA (39%) and contained the highest n-7 MUFA (~10%) content of all treatments. Excreta of birds in the coconut oil treatment group was dominated by SFA (48%), however this was 16% lower than its respective dietary level. The *trans* fatty acid group was the lowest contributor to the fatty acid composition of excreta of all treatment groups, reflecting the level in the diets (Table 2). The clear majority of the PUFA in the excreta were as alpha-linolenic acid (ALA, 92-99% of n-3 PUFA) and linoleic acid (LA, 98-99% of n-6 PUFA).

In general, the correlations between the content of the different groups of fatty acid in the diet and excreta were positive, strong and significant. Levels of two fatty acid groups (n-3 PUFA and n-7 MUFA) were particularly closely related between the diet and excreta, with R

values ~1.00. The linear regression equations (y=ax+b) reflect the differences in the slopes (a = 0.490 for n-3 PUFA to 0.762 for n-6 PUFA) of the relationships between the diet and excreta for the different fatty acid groups (Table 3 and Fig. 1).

To provide an indication of the relative utilization of each of the fatty acid groups, and individual fatty acids, we compared the proportion of each in the diet and in the excreta. We inferred that fatty acids that were proportionally higher in the feed than in the excreta were preferentially utilized by the bird (and its microbiome). On the other hand, fatty acids that were proportionally higher in the excreta than in the diet, were preferentially under-utilized. We considered that fatty acids that qualitatively differed between diet and excreta by <1% were neither preferentially utilized nor under-utilized. Comparing the relative abundancy of the main fatty acid groups between diets and excreta there was a preference in utilization of n-3 PUFA and n-9 MUFA and a relative selection against the utilization of n-6 PUFA (Fig. 2A). A detailed comparison of the qualitative relative abundance of individual fatty acids in the diet and excreta showed that of the 29 fatty acids measured in the diet and excreta, 19 of them (C9:0, C10:0, C15:0, C17:0, C22:0, C24:0, C13:1, C18:1n-7, C22:1n-9, C20:3n-3, C18:3n-6, C20:2n-6, C20:3n-6, C22:2n-6, C22:4n-6, C22:5n-6, *trans* C18:1n-9, *trans* C18:1n-7 and *trans* C18:2n-6) differed by less than 1% (data not shown), and 10 differed by more than 1% (range -13% to +16%, Fig. 2B). Seven fatty acids from 4 fatty acid groups: C18:3n-3, C16:1n-7, C18:1n-9 and the medium chain length SFA (C8:0 to C14:0) were present at relatively lower levels in the excreta compared to the diet, indicating they were preferentially utilized by the broilers. Conversely, 3 fatty acids from 2 fatty acid groups: C18:2n-6, C16:0 and C18:0 were found at relatively higher levels in the excreta, indicating these fatty acids were somewhat under-utilized by the broilers (Fig. 2B).

Table 3. The relationship between the levels of the main fatty acid groups in the diets and excreta[1]

Correlation term	Fatty acid group				
	Total SFA[1]	Total n-9[2]	Total n-7[3]	Total n-3[4]	Total n-6[5]
R value	0.82	0.89	0.99	1.00	0.85
P value	0.046	0.018	<0.001	<0.001	0.032
y[7]	0.627x[8] + 13.981	0.629x + 6.191	0.574x + 0.905	0.490x + 1.552	0.762x + 12.455

[1]Values are the means of 4 replicates. [2]SFA = Saturated fatty acid; [3]n-9 = Omega 9 monounsaturated fatty acid; [4]n-7 = Omega 7 monounsaturated fatty acid; [5]n-3 = Omega 3 polyunsaturated fatty acid; [6]n-6 = Omega 6 polyunsaturated fatty acid, [7]The dependent variable (fatty acid level in excreta), [8]The explanatory variable (dietary fatty acid level).

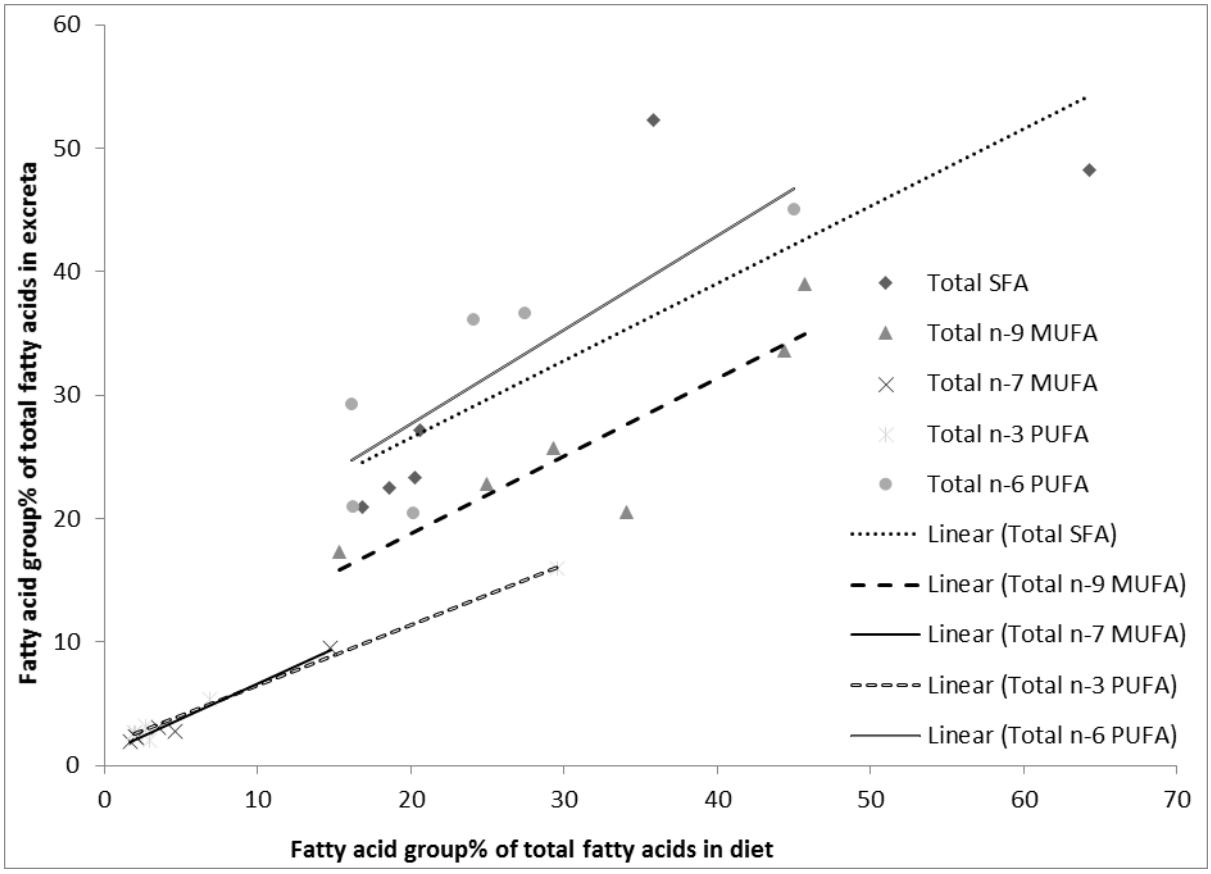

Figure 1. The relationship between diet and excreta for different fatty acid groups (total saturates, total n-9, total n-7, total n-3 and total n-6) in broilers grown for 40 days on one of six different dietary treatments containing 4% w/w tallow, flaxseed, corn, canola, macadamia or coconut oil. The mean values for excreta are based on 4 replicates.

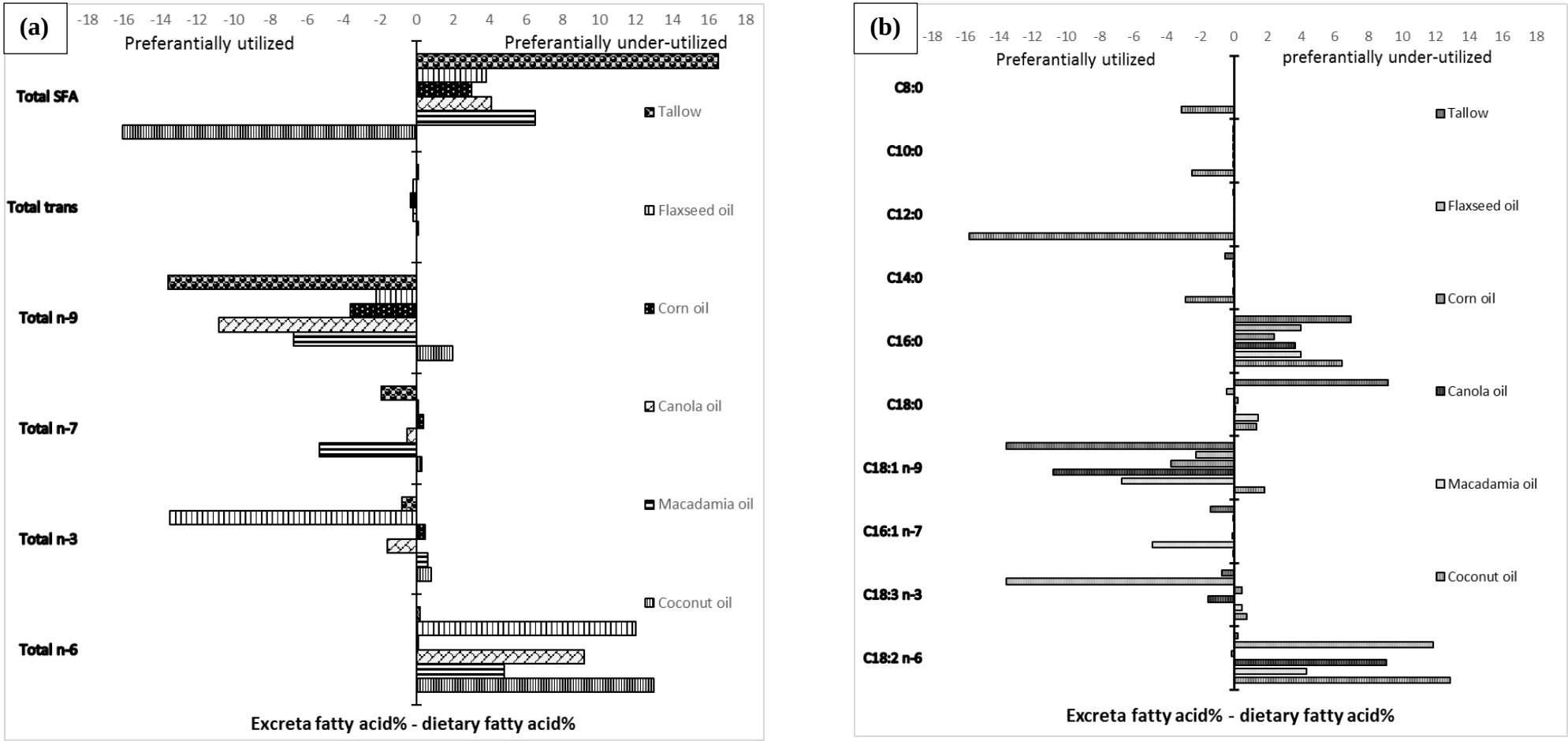

Figure 2. (a-b). Effect of six different experimental diets containing tallow, flaxseed, corn, canola, macadamia or coconut oil on altering the proportions of the (a) fatty acid groups and (b) individual fatty acids between diet and excreta.

Presented values are for fatty acids with percentage difference >1% based on the means of 4 replicates for excreta.

3.4 Discussion

The productivity parameters measured in this study were similar to our previous observations (Kanakri et al., 2017a; Kanakri et al., 2017b; Kanakri et al., 2016) and better than the recommended commercial values for the Cobb 500 strain (Cobb 500, 2015). Again, in agreement with most previous studies, there were no significant differences in production parameters between treatment groups. This suggests that all the fats trialled in this study are appropriate sources of macro- and micro-nutrients for broilers and their microbiome without affecting feed palatability. On the other hand, this leads us to speculate whether the differential effects of dietary fats on broiler performance only become evident if there is an interaction with external factors (e.g. environmental or social stress). The subjective visual observation of the excreta did not indicate any obvious difference between different dietary treatments; however consistent with a previous report (Pauwels et al., 2015), the difference in appearance of droppings from individual birds likely reflects their caecal and faecal origins and excreta moisture content.

There is a limited number of publications which have correlated the type and percentage of excreted fatty acids to the dietary intake. Most of studies were focused on the digestion/absorption efficiency of different fatty acids along the length of the intestinal tract (Ajuyah et al., 1996; Hurwitz et al., 1973; Sklan et al., 1973; Tancharoenrat et al., 2014). Like the present study, those studies found that the main fatty acids detected in the digesta (C16:0 (palmitic acid), C18:0 (stearic acid) and C18:2n-6, LA) were also the main fatty acids present in the dietary fats (soybean oil and tallow). In addition, to the latter 3 fatty acids, C18:1n-9 (oleic acid) was also detected at high levels in the excreta in the present study.

It is acknowledged that these interpretations are general observations only, as the preferential/non-preferential utilization of most fatty acids is obviously affected by the presence or absence of other fatty acids in the diet. Thus, C18:1n-9 was preferentially utilized in all diets except when the fat was provided by coconut oil, suggesting that the medium length SFA in coconut oil were preferentially utilized instead. Similarly, C18:3n-3 (ALA) and C16:1n-7 (palmitoleic acid) were utilized when they were present at relatively high levels. At the other end of the spectrum, C18:2n-6 (LA) was preferentially under-utilized, except when it was the most abundant or at high level relative to other fatty acids (e.g. in the corn oil and tallow diets). The main exception to the rule that degree of utilization was influenced by the other types of fats in the diet was in the case of C16:0, which was always under-utilized by the broilers, irrespective of the level it was in the diet either overall or relative to other dietary fats. These observations agree with the performance data that showed that the birds grew well with good feed intake and feed conversion ratios on all diets. This suggests that whilst there may be a qualitative preference for utilization of different fatty acids, each of these fats can be used successfully to support growth.

While the correlation coefficient is beneficial in showing the type and strength of the relationship between dietary and excreta fatty acid levels, the regression analysis is also valuable. The latter create a mathematical model that allows the researcher or feed manufacturer to estimate from the level of different fatty acids in the chicken diet (independent variable) the proportion that would be excreted (dependent variable) and thereby the amount that would be retained by the birds (Puvača et al., 2014). Although all fatty acid groups were strongly and positively correlated between the diet and excreta, there was some variation between them. Thus, the relatively low R values for SFA and n-6 PUFA compared to the other fatty acid groups

was evidence of their relative under-utilization even when they were the most available fatty acids.

The flaxseed oil treatment has a particular significance as many studies have used this oil to enrich chicken products with beneficial n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) (Baeza et al., 2013; Konieczka et al., 2017; Lopez-Ferrer et al., 2001). Although the flaxseed oil diet was dominated by n-3 PUFA, the main fatty acid in the excreta of this treatment was n-6 PUFA, and this resulted in increasing the n-6:n-3 ratio in the excreta to 2.25 compared to 0.82 in the diet similar to our previous findings in blood (Kanakri et al., 2016) and meat (Kanakri et al., 2017a). Previously it has been demonstrated that by feeding broilers a 2.5% and 5% flaxseed oil-based diet, a considerable percentage of the dietary n-3 PUFA substrate ALA can be elongated and desaturated to n-3 LCPUFA and deposited in various tissues (e.g. 30%-40% of total n-3 PUFA in breast meat) (Carragher et al., 2016; Kartikasari et al., 2012). In the present study we found that 99% of excreted n-3 PUFA was in the form of ALA, indicating the bio-significance of the n-3 LCPUFA in many cellular and health mechanisms (Cherian, 2007).

It was interesting that all fatty acids identified in excreta samples were also detected in the diets. Therefore, we speculate there was no modification (e.g. elongation, saturation/desaturation) of excreted fatty acids by the broiler gut and/or microbiome, despite this being reported by others (Clement, 1980; van der Hoeven-Hangoor et al., 2013). The only evidence of fatty acid modification was by the chicken itself (Kanakri et al., accepted), with hepatic elongases and desaturases converting linoleic acid to arachidonic acid (n-6 PUFA) and ALA to n-3 LCPUFA (n-3 PUFA), with none of these end products being excreted. Thus, the effect of the dietary fat composition (especially the level of PUFA) on the net endogenous fat

synthesis in broilers remains unclear (Crespo and Esteve-Garcia, 2002; Sanz et al., 2000), and further studies will be required resolve the underlying mechanism.

3.5 Conclusion

Broilers were fed 1 of 6 diets that had different sources of fat, and therefore different fatty acid compositions, produced excreta that generally reflected the dietary fat composition. However, there were some subtle differences in excreta fatty acid composition which suggested variation in preferences for fatty acid utilization by the bird and/or its microbiota. The relative utilization of fatty acids was dependent on the dietary level and on the composition of other fatty acids in the feed. Several fatty acids, particularly the medium SFA (C8:0 (caprylic acid), C10:0 (capric acid), C12:0 (lauric acid) and C14:0 (myristic acid)), C18:1n-9 (oleic acid), C18:3n-3 (alpha-linolenic acid) and C16:1n-7 (palmitoleic acid) were preferentially utilized when they were present. In contrast, C18:2n-6 (linoleic acid) and C16:0 (palmitic acid) were always under-utilized. Therefore, the non-invasive collection of excreta may provide useful data to feed manufacturers about the utilization of fatty acids in diets made using different lipid sources.

3.6 References

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CHAPTER 4:(Manuscript 3): A REDUCED COST STRATEGY FOR ENRICHING CHICKEN MEAT WITH OMEGA-3 LONG CHAIN POLYUNSATURATED FATTY ACIDS USING FLAXSEED OIL

K. Kanakri, J. Carragher, R. Hughes, B. Muhlhausler, R. Gibson. A reduced cost strategy for enriching chicken meat with omega-3 long chain polyunsaturated fatty acids using flaxseed oil. British Poultry Science, 2017; 58, 3: 283–289. (DOI: 10.1080/00071668.2017.1293798).

Statement of Authorship is located in Appendix 4 (4.3.).

Abstract 1. This study aimed to determine the minimal duration required for feeding male broilers (Cobb 500) with a flaxseed oil diet while still retaining long chain omega-3 polyunsaturated fatty acid (n-3 LCPUFA) accumulation in the meat at a desirable level.

2. Three groups of broilers (60 each) were fed on a 3% flaxseed oil (high α-linolenic acid (ALA)) diet for either 6, 4 or 2 weeks prior to slaughter. During the remaining time they were maintained on a 3% macadamia oil (low ALA) diet. A fourth group (Control, n=60) was fed a commercial diet for 6 weeks.

3. No significant difference was observed in growth performance of broilers between groups. The amounts of total n-3 and n-3 LCPUFA in breast and thigh meat were not different between broilers fed the flaxseed oil diet for 4 and 6 weeks, but they were lower ($P<0.001$) in those fed the flaxseed diet for only 2 weeks.

4. These results suggest comparable levels of n-3 LCPUFA in the meat can be achieved by only feeding the flaxseed oil diet in the last 3-4 weeks of the growth period; this would result in a ≥9.4% reduction in the use of flaxseed oil compared to 6 weeks feeding; thereby reducing the cost of the enrichment process.

Key words: Broiler, ALA, n-3 LCPUFA, feeding duration.

4.1 Introduction

Dietary omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) are essential for normal development and maintenance of optimal health and may play a role in helping to reduce the risk of cardiovascular and allergic disease (van den Elsen et al., 2012), diabetes (Oliver et al., 2012), arthritis (Estevão-Silva et al., 2016) and other inflammatory conditions (Honda et al., 2015; Connor, 2000). A number of national and global health agencies, including the World Health Organisation, recommend increasing n-3 LCPUFA intake for cardiovascular health. The amounts vary depending on factors including; region, age, gender, health status and physiological conditions (FAO/WHO, 2014). In Australia, the current recommended intakes of n-3 LCPUFA for adults is 430-610 mg/day (National Health and Medical Research Council, 2006) or 500 mg/day of eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) to reduce the risk of cardiovascular disease (Heart Foundation, 2008). In order to achieve this, health agencies recommend an intake of 2-3 servings of oily fish per week, since this is the richest dietary source of these fatty acids. However, recent data indicates that the vast majority of westerners, including Australians, fail to meet this recommendation, and the average Australian consumes less than one fish meal per fortnight (Dickinson et al., 2015; Meyer, 2016). As a result, the use of fish oil supplements has increased dramatically (Meyer, 2016), but this could negatively impact on the sustainability of marine resources.

Annual consumption of chicken meat in Australia has steadily increased over the last few decades to reach ~46 kg/person in 2016 (Australian Chicken Meat Federation, 2013). Chicken meat is generally classified as a poor source of n-3 LCPUFA; however, the content can be increased by manipulating the broilers' diet e.g. replacing the usual fat source with fish oil. However, this practice is accompanied by an adverse effect on sensory quality (fishy taint) of

the chicken meat at a 2% level of the dietary fish oil (Edwards and May, 1965), a negative effect on growth performance and/or higher drip loss of meat (Zhang et al., 2016). An alternative approach is to substitute the fish oil with sustainably grown plant oil which is rich in the short chain n-3 fatty acid, alpha linolenic acid (ALA), such as flaxseed oil. Chickens can use hepatic elongase and desaturase enzymes to produce n-3 LCPUFA from ALA (Lopez-Ferrer et al., 2001) which can then be incorporated into the chicken tissues to increase the nutritional quality of the meat (or eggs) without detrimental organoleptic effects (Baeza et al., 2013). By feeding broilers a high ALA diet, Kanakri et al. (2016) increased the total omega-3 polyunsaturated fatty acids (n-3 PUFA) in whole blood by 6-fold and Kartikasari et al. (2012) reported that n-3 PUFA content in meat was elevated up to 9-fold without affecting the growth parameters of the birds or the sensory properties of the meat.

From an economic point of view, flaxseed oil is more expensive than other plant oils or animal fats used to make chicken feed. This increases the cost of production and thereby the price of the n-3 LCPUFA enriched chicken meat. Optimising an approach to minimise the required amount of the expensive flaxseed oil whilst maintaining enhanced levels of n-3 LCPUFA in meat is essential for commercial uptake. To do this, one of the key parameters is to determine the minimal time the high ALA diet needs to be fed to the birds prior to slaughter, and this has been addressed to some extent in previous research using whole ground flaxseed (Betti et al., 2009; Zuidhof et al., 2009) and a combination of flaxseed oil with plant oil (Gonzalez-Esquerra and Leeson, 2000) or animal fat (Lopez-Ferrer et al., 2001). Those studies concluded that it is possible to reduce the feeding period on the relatively expensive diets containing flaxseed or flaxseed oil.

Previous research by the authors identified the minimal sufficient level of flaxseed oil (3%) and optimal n-6:n-3 ratio (~1:1) in the diet to reduce the cost of enriching chicken meat with n-3 LCPUFA (Kartikasari et al., 2012). The present study aimed to investigate a further potential approach of reducing the additional cost of flaxseed-induced enrichment of chicken meat with n-3 LCPUFA by shortening the duration of feeding the flaxseed oil supplemented diet. Thereby, making this strategy more economically viable for the industry to produce n-3 enriched chicken meat products.

4.2 Materials and Methods

4.2.1 Birds and experimental design

The Animal Ethics Committees of the University of Adelaide and the Department of Primary Industries and Resources South Australia approved the study (S-2013-152). A total of 240 male one-day-old Cobb 500 broilers were purchased from a local hatchery. Birds were randomly distributed into groups of 10 and allocated to 24 raised rearing floor pens (1.2 × 0.9 m each) in one shed. A complete randomised block design (6 pens/treatment) was implemented. Broilers were reared under strictly controlled environmental conditions with free access to both feed and water at all times. The room temperature was 27ºC for the first 4 days then gradually decreased to 20ºC and maintained at this temperature for the 6 week experimental period. Individual pens were heated by infrared lamps (175 W) during the first 3 weeks.

4.2.2 Experimental treatments

Three types of diets were utilised in this study. The first diet was a commercial broiler formulation (Ridley Agriproducts Pty Ltd, Murray Bridge, SA, Australia). The other 2 diets were prepared by adding 3% (w/w) of a selected fat type; macadamia oil (low ALA; Macoils,

Alstonville, NSW, Australia) or flaxseed oil (high ALA; Four Leaf Oils, SA, Australia) to basal starter and finisher broiler diet formulations. Table 1 shows the composition of the basal diets. Starter diets were fed in the first 3 weeks and the finisher diets were fed in the last 3 weeks of the experiment. Apart from the variation in the fatty acid composition, both of the experimental diets were nutritionally identical and met requirements for efficient broiler performance.

The study included 4 feeding treatments. In the control treatment, birds were fed on the commercial diet for the entire 6 week rearing period. In the second experimental feeding treatment, birds were fed on the low ALA, macadamia oil based diet for the first 2 weeks then they were maintained on the high ALA, flaxseed oil based diet for the remaining period of 4 weeks (ALA4). Birds in the third group were fed the low ALA diet in the first 4 weeks, then they were maintained on the high ALA diet for the remaining period of 2 weeks (ALA2). In the fourth group (ALA6), birds were fed the high ALA diet from day 1 post-hatch until the end of the growing period of 6 weeks.

Table 1. Ingredient composition and nutrient content of the basal diets

Ingredients (gkg^{-1})	Basal diet[1]	
	Starter[2]	Finisher[3]
Wheat fine	614.2	707.7
Barley fine	50.0	50.0
Tallow mixer	3.7	10.0
Blood meal (91% CP)	10.0	0.0
Soybean meal	251.7	166.7
Limestone small	8.6	6.4
Monocalcium phosphate	1.4	0.0
Sodium chloride	2.1	1.8
Sodium bicarbonate	1.6	1.8
Choline chloride 75%	0.3	0.4
DL-methionine 58.1	2.9	2.1
L-threonine 73.7	1.3	2.5
Rovabio Excel LC	0.2	0.2
Meat meal	37.3	37.0
Ronozyme NP CT	0.2	0.2
Mineral/vitamin premix[1]	10.0	10.0
L-lysine sulphate 70	4.3	3.3

[1]The starter and finisher basal diets ME = 12.14 and 12.56 $MJkg^{-1}$, the nutrient contents ($g.kg^{-1}$) were: Crude protein 225.1 and 186.7, Crude fat 23.5 and 30.0, Crude fibre 32.3 and 29.2, Ca 9.8 and 8.5, Available phosphorus 4.5 and 4.2, Na 1.8 and 1.7, K 0.8 and 6.5, Cl 0.2 and 1.8, Lysine 12.3 and 9.1, Methionine 5.7 and 4.7, Cystine 3.9 and 2.8, Threonine 7.9 and 7.6, Leucine 14.3 and 11.2, Isoleucine 7.8 and 6.4, Tryptophan 2.3 and 1.8, Arginine 12.4 and 9.8 and Valine 9.3 and 7.2, respectively. [2]Used to formulate 2 experimental diets to feed broilers up to 3 weeks old. [3]Used to formulate 2 experimental diets to feed broilers from 4 to 6 weeks old.

4.2.3 Growth performance

Group weight of the birds in each pen was taken on day 1 and then on a weekly basis for the first 3 weeks. In the final 3 weeks of the experiment the body weight (BW) of birds was recorded individually. Feed intake (FI) of birds in each pen was also recorded on a weekly basis and used to calculate the feed conversion ratio (FCR; kg feed/kg body weight gain) taking into account the number of deaths and culls. The latter was recorded on a daily basis to calculate the mortality rate.

4.2.4 Tissue collection

At 6 weeks of age, 2 birds per pen were randomly selected and euthanised by cervical dislocation. Approximately 30 g of lean skinless thigh and breast meat tissue were collected into clean plastic containers and immediately placed on dry ice before transfer to the laboratory.

Samples were stored in freezer at -18°C till subsequent determination of crude fat content and fatty acid composition.

4.2.5 Fatty acid analysis

Total lipid was extracted from a representative feed and tissues samples as described by Folch et al. (1957) with chloroform/methanol (2:1, v/v) solution. The gravimetric approach was utilised to estimate total lipid content. Fatty acid profiling was performed after transmethylation of the extracted crude lipids with 1% H2SO4 in methanol at 70°C for 3 h (Gregory et al., 2010; Tu et al., 2010). After cooling to room temperature, the resulting fatty acid methyl esters (FAME) were extracted with n-heptane (2 mL) and transferred into gas chromatography (GC) vials containing about 30 mg of anhydrous sodium sulphate. Vials were stored at -18°C until GC analysis.

4.2.6 Gas chromatography analysis of FAME

The FAME were separated using a Hewlett-Packard 6890 GC (Hewlett-Packard, CA, USA) equipped with a flame ionisation detector (FID), a split injector and a BPX-70 capillary column (50 m x 0.32 mm internal diameter) with a 0.25 µm film thickness (SGE, Victoria, Australia). The operating conditions of the GC, fatty acid identification using the GLC 463 external standard (Nu-Chek Prep Inc, MN, USA) and qualitative analysis were as described by Kartikasari et al. (2012).

4.2.7 Statistical analysis

The effects of dietary treatment on tissue fatty acid profile were tested by one-way analysis of variance (ANOVA) using SPSS version 22 for Windows (IBM Corp., NY, USA). Tukey multiple comparison test was implemented when treatment effects were significantly different

($P < 0.05$). The effects of dietary treatment on the production parameters; BW, FI and FCR were determined by one-way ANOVA using SAS 9.3 for Windows. The mortality rate was examined by non-parametric analysis (Kruskal-Wallis test) in SPSS version 21 for Windows (IBM Corp., NY, USA).

4.3 Results

4.3.1 Diets

On average, the commercial diet (Control) contained less crude fat (4.5%) than the other 2 experimental diets; 6.2% and 6.3% in the macadamia oil and flaxseed oil diets, respectively. The fatty acid composition of the diets is shown in Table 2. The total saturated fatty acids (SFA) of the different diets comprised approximately 19-28% of total fatty acids and trans fatty acids were only found in low levels (<1.0%). As intended, the level of total n-3 was highest (27.6%) in the flaxseed oil diet and lowest in the macadamia oil diet (2.0%). The macadamia oil diet had a total monounsaturated fatty acids (MUFA) level of 46.9%, most of which was n-9 fatty acids. Total omega-6 polyunsaturated fatty acids (n-6 PUFA) were the predominant group (40%) in the control diet. The n-3 and n-6 fatty acids in the diets were ALA (Table 2) and linoleic acid (data not shown), respectively. There was no more than 0.1% n-3 LCPUFA detected in any diet. The dietary n-6:n-3 ratio decreased from about 15:1 and 9:1 in the macadamia oil and control diets, respectively, to approximately 1:1 in the flaxseed oil diet.

Table 2. Fatty acid profile (% of total fatty acid) of the basal and experimental diets[1]

	Basal diets[2]		Experimental diets[3]					
			Commercial		High ALA		Low ALA	
	S	F	S	F	S	F	S	F
Crude fat%[4]	3.0	3.8	4.6	4.3	6.1	6.4	6.3	6.1
Fatty acid (%)[5]								
Total SFA	27.5	29.0	26.1	27.7	18.6	19.9	20.8	21.0
Total *trans*	1.0	1.2	0.8	1.0	0.5	0.6	0.5	0.5
Total n-9	20.4	22.9	25.1	24.1	21.2	22.7	37.5	37.2
Total n-7	2.1	2.6	2.5	2.6	1.5	1.7	9.6	9.5
Total n-6	45.0	40.3	40.3	40.0	29.6	28.0	29.3	29.7
Total n-3	3.9	3.5	4.9	4.2	28.4	26.8	2.0	1.9
ALA	3.8	3.4	4.8	4.0	28.3	26.7	2.0	1.8
EPA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
DPA	0.1	0.1	0.0	0.1	0.0	0.0	0.0	0.0
DHA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
n-6:n-3	11.5	11.5	8.2	9.6	1.0	1.0	14.6	15.6

[1]Values are means of basal diet n=2, commercial diet n=3, high ALA diet n=9 and low ALA diet n=5. [2]S= Starter; F = Finisher. [3]Commercial = a standard commercial diet. High ALA = Flaxseed oil based diet (3% w/w); Low ALA = Macadamia oil based diet (3% w/w). [4] Percentages are based on the wet weight. [5]SFA = Saturated fatty acid; n-9 = omega 9 monounsaturated fatty acid; n-7 = omega 7 monounsaturated fatty acid; n-6 = omega 6 polyunsaturated fatty acid; n-3 = omega 3 polyunsaturated fatty acid; ALA = α-linolenic acid; EPA = Eicosapentaenoic acid; DPA = Docosapentaenoic acid; DHA = Docosahexaenoic acid.

4.3.2 Growth performance

No growth performance parameter was found to be significantly different between the 4 dietary treatments either during any individual week or at the end of the experiment (Table 3). By the final day of the study the average live BW was 3689 g, FI was 5941 g/bird, and FCR was 1.62 g feed/g gain across all treatments. Overall mortality was 7.1% and not statistically different between treatments.

Table 3. Growth performance parameters and flaxseed oil consumption by broilers for six weeks[1]

Week	Growth performance parameters[1]			Flaxseed oil consumption[5] g/bird (% of total)
	BW[2] (g)	FI[3] (g)	FCR[4]	
1	173.2	143	1.13	4.3 (2.4%)
2	496.4	557	1.26	16.7 (9.4%)
3	1055	1317	1.33	39.5 (22.2%)
4	1797	2491	1.43	74.7 (41.9%)
5	2661	4002	1.52	120.1 (67.4%)
6	3689	5941	1.62	178.2 (100%)

[1]Values are the means of 240 broilers from four groups receiving different dietary treatments; accumulatively on weekly basis. [2]Body weight. [3]Feed intake. [4]Feed conversion ratio (feed intake (g): body weight gain (g)). [5]Based on 3% (w/w).

4.3.3 Fatty acid composition of meat tissues

The crude fat percentage and fatty acid analysis of broiler breast and thigh meat are presented in Table 4. Crude fat determination of lean meat tissues showed no significant difference between dietary groups, with thigh meat containing 2.0% and breast meat 1.9% fat on average, across all treatments. The broilers in the control (commercial diet) group had the same low level (2.5%) of total n-3 fatty acids in both types of meat. This percentage increased to 10.3%, 13.0% and 13.4% ($P<0.001$) in breast meat, and 9.5%, 12.1% and 12.8% in thigh meat for the ALA2, ALA4 and ALA6 groups, respectively. The percentage increase in total n-3 PUFA content between the control and ALA2, ALA4 and ALA6 groups was greater in breast meat than thigh meat ($P<0.001$). There was no significant difference in tissue total n-3 PUFA content between the ALA4 and ALA6 diet treatments, but both were significantly higher than ALA2 (Table 4). The increase in total n-3 PUFA content was at the expense of most other groups of fatty acids, particularly n-6 PUFA and n-9 and n-7 MUFA, whereas the percentage of SFA was not different between treatments.

A detailed breakdown of the individual n-3 PUFA in both meat tissues indicated that the majority of the increase in total n-3 PUFA content (approximately two thirds) was due to an increase in ALA, with the remainder being n-3 LCPUFA (docosapentaenoic acid (DPA), EPA

and DHA). The pattern of increase of ALA, DPA, EPA and DHA was similar to that seen for total n-3, in that the levels of all individual n-3 fatty acids were not significantly different between the ALA4 and ALA6 groups, but were higher in both these groups than in the ALA2 group (except that of ALA in breast meat). In addition, all ALA groups had higher levels of all n-3 fatty acids compared to the control group ($P<0.001$).

Another nutritional benefit of feeding flaxseed oil diet to birds was a reduction in the n-6:n-3 ratio in the thigh and breast meat from 8.4 and 7.6 in the control group, to 1.3 (thigh meat) and 1.1 (breast meat) in the ALA4 and ALA6 birds (Table 4).

Table 4. Fatty acid profiles[1] (as % of total fatty acids) of meat tissues from male broiler chickens fed with different dietary treatments and sampled at 42 days of age

Dietary treatment	Control[2]	ALA2[3]	ALA4[4]	ALA6[5]	
Breast meat					*P*-value
Crude fat%[6]	1.8±0.10	1.9±0.17	1.8±0.11	2.0±0.15	0.584
Fatty acids (%)[7]					
Total SFA	34.5^{a}±0.24	32.6^{b}±0.45	32.9^{ab}±0.52	33.3^{ab}±0.65	0.046
Total n-9	34.2^{a}±0.55	32.1^{ab}±0.83	30.6^{b}±0.59	30.4^{b}±1.06	0.005
Total n-7	8.9^{a}±0.15	8.2^{b}±0.14	7.3^{c}±0.17	7.1^{c}±0.22	0.000
Total n-6	19.1^{a}±0.38	16.0^{b}±0.43	15.3^{b}±0.23	15.0^{b}±0.44	0.000
Total n-3	2.5^{a}±0.07	10.3^{b}±0.23	13.0^{c}±0.23	13.4^{c}±0.27	0.000
ALA	1.2^{a}±0.03	7.0^{b}±0.32	8.0^{b}±0.49	7.9^{b}±0.54	0.000
n-3 LCPUFA	1.2^{a}±0.02	3.0^{b}±0.11	4.7^{c}±0.17	5.1^{c}±0.24	0.000
EPA	0.3^{a}±0.01	1.0^{b}±0.09	1.3^{bc}±0.11	1.6^{c}±0.22	0.000
DPA	0.6^{a}±0.03	1.5^{b}±0.16	2.3^{c}±0.24	2.4^{c}±0.32	0.000
DHA	0.4^{a}±0.02	0.6^{a}±0.08	1.0^{b}±0.17	1.1^{b}±0.18	0.000
n-6:n-3	7.60	1.54	1.17	1.12	0.000
Thigh meat					
Crude fat%[6]	1.84±0.19	2.19±0.17	1.79±0.18	2.23±0.11	0.124
Fatty acids (%)[7]					
Total SFA	35.3^{a}±0.40	32.9^{b}±0.47	32.9^{b}±0.33	33.3^{b}±0.44	0.000
Total n-9	31.6±0.47	31.2±0.66	30.5±0.87	29.5±0.55	0.101
Total n-7	8.4^{a}±0.15	8.0^{a}±0.26	7.1^{b}±0.20	6.9^{b}±0.18	0.000
Total n-6	21.4^{a}±0.30	17.6^{b}±0.70	17.0^{b}±0.60	16.8^{b}±0.35	0.000
Total n-3	2.5^{a}±0.16	9.5^{b}±1.20	12.1^{c}±0.32	12.8^{c}±0.20	0.000
ALA	1.1^{a}±0.40	6.6^{b}±0.89	8.1^{c}±0.33	8.2^{c}±0.31	0.000
n-3 LCPUFA	1.3^{a}±0.09	2.7^{b}±0.11	3.7^{c}±0.07	4.3^{c}±0.06	0.000
EPA	0.3^{a}±0.08	0.8^{b}±0.12	1.1^{c}±0.08	1.2^{c}±0.06	0.000
DPA	0.7^{a}±0.18	1.4^{b}±0.21	1.9^{c}±0.16	2.2^{c}±0.10	0.000
DHA	0.4^{a}±0.09	0.5^{a}±0.09	0.8^{b}±0.06	0.9^{b}±0.07	0.000
n-6:n-3	8.43	1.84	1.41	1.32	0.000

[1]Values are means of 12 birds per treatment and their pooled standard error of the mean (SEM). Values in the same row without a common superscript are significantly different. [2]A standard commercial broiler diet (Ridley Agriproducts Pty Ltd, Murray Bridge, Australia) was fed for the entire 6 week rearing period. [3]Broilers were fed a macadamia oil diet in the first 4 weeks then a flaxseed oil diet in the last 2 weeks of the 6 week rearing period. [4]Broilers were fed a macadamia oil diet in the first 2 weeks then a flaxseed oil diet in the last 4 weeks of the 6 week rearing period. [5]Broilers fed the flaxseed oil diet for all of the 6 week rearing period. [6]Based on the wet weight. [7]SFA = Saturated fatty acid; n-9 = omega 9 monounsaturated fatty acid; n-7 = omega 7 monounsaturated fatty acid; n-6 = omega 6 polyunsaturated fatty acid; n-3 = omega 3 polyunsaturated fatty acid; ALA = α-linolenic acid; n-3 LCPUFA = omega 3 long chain polyunsaturated fatty acids; EPA = Eicosapentaenoic acid; DPA = Docosapentaenoic acid; DHA = Docosahexaenoic acid.

4.4 Discussion

The present study shows that feeding broilers a high ALA diet for any interval during the growing period does not significantly affect their growth performance (BW, FI, FCR or mortality rate). Indeed, broilers of all treatments performed better than the published Cobb 500™, (2015) performance objectives at the same age (42 days), with a higher BW (by 645 g/bird), a lower daily FI (by 91 g/bird) and lower FCR by 0.06. The observation that growth performance of broilers fed n-3 PUFA enriched diet was not different from those fed a control diet is consistent with the outcomes of several previous studies (Fota et al., 2010; Lopes et al., 2013; Mandal et. al., 2014; Mirshekar et. al., 2015; Kanakri et. al., 2016), while other studies have reported that a high ALA diet was associated with improved growth and/or FCR (Carragher et al., 2015; Duarte et al., 2014; Zelenka et al., 2006). Thus, the vast majority of studies conducted to date suggest that dietary ALA is not deleterious to broiler production, and may even have potential advantages.

In this study, the duration of feeding broilers on the flaxseed oil diet did not change crude fat percentages in either breast or thigh meat. This indicates that the influence of the high ALA dietary treatment did not change the total fat content of the meat, but only altered the fatty acid composition of the lipid fraction. This finding agrees with a similar study in this field (Gonzalez-Esquerra and Leeson, 2000).

In general, the effect of feeding the high ALA diet in the present study was consistent with the published literature and with our previous findings, such that the broilers converted some of the dietary ALA to n-3 LCPUFA and deposited these beneficial fatty acids in the meat tissues (Carragher et al., 2015; Kartikasari et al., 2012). However, the main aim of the present study

was to determine whether feeding broilers on the high ALA diet for reduced periods prior to slaughter adversely affected the tissue accumulation of n-3 LCPUFA. There was no difference in the accumulation of n-3 LCPUFA between broilers that were fed the ALA diet for 4 or 6 weeks prior to slaughter. However the n-3 LCPUFA content of broilers fed the diet for only 2 weeks was about 30% lower than ALA4, but still 2.3-fold higher than the control. This indicates that feeding 3% w/w flaxseed oil diet for only 2 weeks prior to slaughter is not sufficient for the birds to accumulate similar amounts of n-3 LCPUFA as feeding the same diet for 4 or 6 weeks. However, it appears that a feeding duration of between 2 and 4 weeks may be sufficient for the broilers to accumulate the same amount of total n-3 PUFA/LCPUFA as feeding the diet for 6 weeks.

A recent study by Mirshekar et al. (2015) reported the effect of various durations of feeding flaxseed oil based diet on the n-3 LCPUFA levels in broiler meat. However, in their study, the percentage of dietary flaxseed oil was 2.5 (w/w) in the starter diet and 5% (w/w) in the finisher diet. In the experimental substitute diet, soybean oil (48.8% n-6 PUFA and 9.0% n-3 PUFA) was used as a replacement lipid source and sex of the birds was not taken into account. These experimental differences mean it is not possible to extrapolate their findings to our experimental model. Nevertheless, on the basis of the results of their study, they recommended that a feeding 5% flaxseed oil diet to mixed sex birds for 2.5 weeks prior to slaughter was sufficient for optimising n-3 LCPUFA accumulation in breast meat, but thigh meat required a shorter time (1 week). Our results do not, however, indicate that there was a difference between these tissues in the time taken to accumulate n-3 LCPUFA, although there were differences in the magnitude of the effect between tissues (discussed further below).

Gonzalez-Esquerra and Leeson (2000), found that feeding broilers a high (10% w/w) flaxseed oil diet for only 1 week before slaughter increased ALA and total n-3 content of the chicken meat, but did not increase in n-3 LCPUFA. This is similar to the findings of the current study, since ALA levels in the breast tissue were not significantly different between birds fed the ALA diet for only 2 weeks and those fed it for 6 weeks. This therefore suggests that short-term feeding with a flaxseed oil diet will allow a similar amount of ALA to be deposited in breast meat tissue in comparison to a longer feeding duration, but that longer feeding periods are required for this to increase n-3 LCPUFA synthesis and tissue deposition. Furthermore, greater than two thirds of the total accumulated n-3 in meat tissues in ALA2 birds was ALA. In breast meat, this contribution decreased from 68.0% (ALA2) to 61.5% and 59.0% in ALA4 and ALA6, respectively. This may suggest that it takes some time for the elongase and desaturase enzymes to respond to the change in dietary fatty acids in order to synthesize the n-3 LCPUFA or deposit it in the phospholipid fraction. Previous studies have reported that increases in ALA consumption do not alter the mRNA expression of elongases and desaturases in the breast meat (Haug et al., 2014), and longer durations of ALA feeding have even been associated with reduced expression of these enzymes (Mirshekar et al., 2015). However, this is unlikely to affect the capacity for n-3 LCPUFA synthesis, since this process is regulated to a greater extent by substrate availablility, rather than gene expression of the regulatory enzymes (Tu et al., 2010).

Interestingly, although the same changes in ALA and n-3 LCPUFA content were observed in breast and thigh meat, the magnitude of ALA contribution of the total n-3 PUFA was less (by 2% and 5% in ALA4 and ALA6, respectively). This may be because thigh meat has more extracellular fat, which is mostly triglycerides; and this fraction contains most of ALA (Betti et al., 2009). These data also suggest that increasing feeding time on a high ALA diet could be a

reason to improve the ALA conversion efficiency to n-3 LCPUFA, and the deposition of n-3 LCPUFA in meat tissues, particularly in breast meat. A similar recommendation was reported by Zuidhof et al. (2009) using a diet containing ground full-fat flaxseed.

The trends of changes in the individual n-3 LCPUFA in both meat tissues demonstrated a similar pattern to that of the total n-3 PUFA content, as discussed previously. In general, EPA, DPA and DHA percentages were slightly higher in breast meat than in thigh meat, with DPA being the most prevalent n-3 LCPUFA (50% of all n-3 LCPUFA) in all treatments. In relative terms, EPA increased to the greatest extent, by 5.3- and 4.0-fold in breast and thigh meat, while DHA accumulated least, with a 2.8- and 2.3-fold increase in breast and thigh meat, respectively in the broilers fed the high ALA diet for 6 weeks.

In terms of the potential of n-3 LCPUFA enriched broiler meat to improve daily n-3 LCPUFA intake of consumers who follow a western-style diet: based on the n-3 LCPUFA percentages, the total amount of fat in the chicken meat, and the average daily consumption of chicken meat in Australia, the ALA6 treatment group would provide an additional 28.6, 41.2 and 15.5 mg/person/day of EPA, DPA and DHA, respectively and a total n-3 LCPUFA intake of 117 mg/day compared to 31.7 mg/day from the control (commercial) chicken meat. The n-3 PUFA enriched chicken meat product could therefore contribute 14% and 18.5% of the recommended daily intakes for men and women, respectively (National Health and Medical Research Council, 2006). Moreover, since the increase in total n-3 PUFA content was coupled with a decrease in total n-6 PUFA content, the n-6: n-3 PUFA ratio was lower, by 77.9% and 85.5% in the breast and thigh meat respectively, in comparison to the control meat. This is likely to provide additional nutritional and health benefits for the consumer, given recent

evidence implicating the high n-6 PUFA intakes in modern western diets in a range of chronic diseases (Wijendran and Hayes, 2004; Wall et al., 2010).

Our results confirm that it is possible to implement a time-reduction strategy to lower the production cost of enriching broiler meat with n-3 LCPUFA from a flaxseed oil based diet. The estimated benefit of excluding the first 2-3 weeks of feeding broilers the relatively expensive high ALA diet is a saving of 9.4-22.2% (16.7-39.5 g, Table 3) of the required amount of flaxseed oil. Based on the difference in price between flaxseed oil and other cheaper dietary fats, the additional cost of the enrichment process can be reduced by a similar percentage. Being able to feed broilers the high ALA diet for a shorter period will support commercialisation of n-3 LCPUFA enriched broiler meat at an affordable price for the industry and consumers.

4.5 References

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CHAPTER 5: (Manuscript 4): IN OVO EXPOSURE TO OMEGA-3 FATTY ACIDS DOES NOT ENHANCE OMEGA-3 LONG CHAIN POLYUNSATURATED FATTY ACID METABOLISM IN BROILER CHICKEN

K. Kanakri, J. Carragher, B. Muhlhausler, R. Hughes, R. Gibson. In ovo exposure to omega-3 fatty acids does not enhance omega-3 long chain polyunsaturated fatty acid metabolism in broiler chickens. Journal of Developmental Origins of Health and Disease, 2017; 8(5), 520–528. (DOI: 10.1017/S2040174417000216).

Statement of Authorship is located in Appendix 4 (4.4.).

Abstract

The content of omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) in chicken meat can be boosted by feeding broilers a diet containing α-linolenic acid (ALA, from flaxseed oil), some of which is converted by hepatic enzymes to n-3 LCPUFA. However, most of the accumulated n-3 polyunsaturated fatty acid (PUFA) in meat tissues is still in the form of ALA. Despite this, the levels of chicken diets are being enhanced by the inclusion of vegetable and marine sources of omega-3 fats. This study investigated whether the capacity of chicken for n-3 LCPUFA accumulation could be enhanced or inhibited by exposure to an increased supply of ALA or n-3 LCPUFA *in ovo*. Breeder hens were fed either flaxseed oil (High-ALA), fish oil (high n-3 LCPUFA) or tallow (low n-3 PUFA, Control) based diets. The newly-hatched chicks in each group were fed either the High-ALA or the Control diets until harvest at 42 days' post-hatch. The n-3 PUFA content of egg yolk and day-old chick meat closely matched the n-3 PUFA composition of the maternal diet. In contrast, the n-3 PUFA composition of breast and leg meat tissues of the 42 day old offspring closely matched the diet fed post-hatch, with no significant effect of maternal diet. Indeed, there was an inhibition of n-3 LCPUFA accumulation in meat of the broilers from the maternal Fish-Oil diet group when fed the post-hatch High-ALA diet. Therefore this approach is not valid to elevate n-3 LCPUFA in chicken meat.

Key words: Maternal diet, flaxseed oil, fish oil, chicks, meat, n-3.

5.1 Introduction

The omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) have a number of important health benefits in humans, in particular in relation to inflammatory conditions such as rheumatoid arthritis and protection against cardiovascular diseases[1, 2]. These effects rely on the incorporation of n-3 LCPUFA into the phospholipid fraction of the cell membrane, and subsequent release in the free fatty acid pool to give rise to bio-active mediators[3]. This has led to recommendations from a number of health agencies for humans to increase their consumption of fish and seafood, the richest dietary sources of these fatty acids. Despite this, consumption of these sources remains low in western countries[1, 4], and fish and seafood are also not environmentally sustainable sources of these fats[5]. In contrast to seafood, the global consumption of poultry, especially chicken, is steadily increasing and this is now the most popular type of meat in many societies[6]. This has led to suggestion that one strategy to increase dietary n-3 LCPUFA intake in western countries, and one which would avoid placing additional pressure on global marine resources, is to increase the n-3 LCPUFA content of chicken meat[7, 8].

Chicken meat is naturally low in fat (~2.0%), and a poor source of n-3 polyunsaturated fatty acids (n-3 PUFA) at only ~2.5% of total fatty acids including n-3 LCPUFA at ~1.3%[9]. However, we and other researchers have demonstrated that increasing the amount of n-3 PUFA in the diet, by feeding chickens diets supplemented with flaxseed (*Linum usitatissimum*) oil (high in the short-chain n-3 PUFA, α-linolenic acid (ALA)), results in substantial increases in the ALA content of the meat, without increasing the overall fat content[10]. Importantly, chickens also possess the hepatic enzymes required to synthesize the n-3 LCPUFA; eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), from ALA[7, 11]. Consequently, replacing standard fat sources in formulated chicken diets such as corn oil, canola oil, soybean oil (all rich in n-6

polyunsaturated fatty acids (PUFA)), animal fat or their blends, with flaxseed oil reduces the dietary n-6: n-3 PUFA ratio and increases the n-3 LCPUFA content of chicken tissues[9, 10, 12-14], since n-6 and n-3 PUFA precursors compete for the same hepatic enzymes in their elongation and desaturation pathways[10, 15]. Despite the success of High-ALA diets for increasing the level of n-3 LCPUFA in chicken meat, the actual levels of n-3 LCPUFA which are achieved (76.5-108 mg/100g meat)[9] are still substantially lower than those in oily fish (e.g. herring, salmon and mackerel), which contains >1500 mg in the similar amount of meat[16].

Improving the efficiency through which high-ALA diets improve chicken meat n-3 LCPUFA content relies on altering the processes which regulate ALA conversion to n-3 LCPUFA and/or deposition of fatty acids into different lipid fractions[17, 18]. The egg yolk is the main reserve of energy and the sole source of essential fatty acids during embryogenesis[19], and previous studies have demonstrated that feeding layer hens a diet enriched in ALA increases the n-3 LCPUFA content of the eggs[20-23] and of the chicks at hatch[22]. In addition, feeding hens a diet enriched with fish oil results in higher n-3 PUFA levels in cardiac tissues in their chicks[24]. However, no previous studies have determined the effect of *in ovo* exposure to an increased supply of n-3 PUFA on the chicken's subsequent capacity for ALA-derived n-3 LCPUFA accumulation in meat tissues. Therefore, the aim of the present study was to determine the effect of maternal dietary treatments that would expose developing chicks *in ovo* to an elevated level of n-3 LCPUFA, or its precursor (ALA) on the capacity of chickens to accumulate n-3 LCPUFA when fed a High-ALA diet post-hatch.

5.2 Methods

5.2.1 Maternal Dietary Treatments

A total of 324 of broiler breeder hens of the Cobb 500 strain were housed in the HiChick Breeding Company facility (Bethyl, SA, Australia). Prior to the study all the hens received the same commercial breeder diet. Hens were allocated to one of 3 dietary groups (n=108/group): Control (basal diet mixed with 4% w/w beef tallow), High-ALA (basal diet mixed with 4% w/w flaxseed oil) or Fish-Oil (basal diet mixed with 4% w/w fish-oil) (Fig. 1.). The breeder basal diet used for all feeds was purchased from Lauke Mills, Australia. The 3 diets contained the same proportions of fat, carbohydrate and protein and differed only in their fatty acid composition (Table 1). All 3 experimental diets contained the same levels of vitamins and minerals and these either met or exceeded the recommended levels [25]. Each group of hens was housed separately with 8 roosters from the same strain, and fed the diets for the duration of the experiment (Fig. 1.).

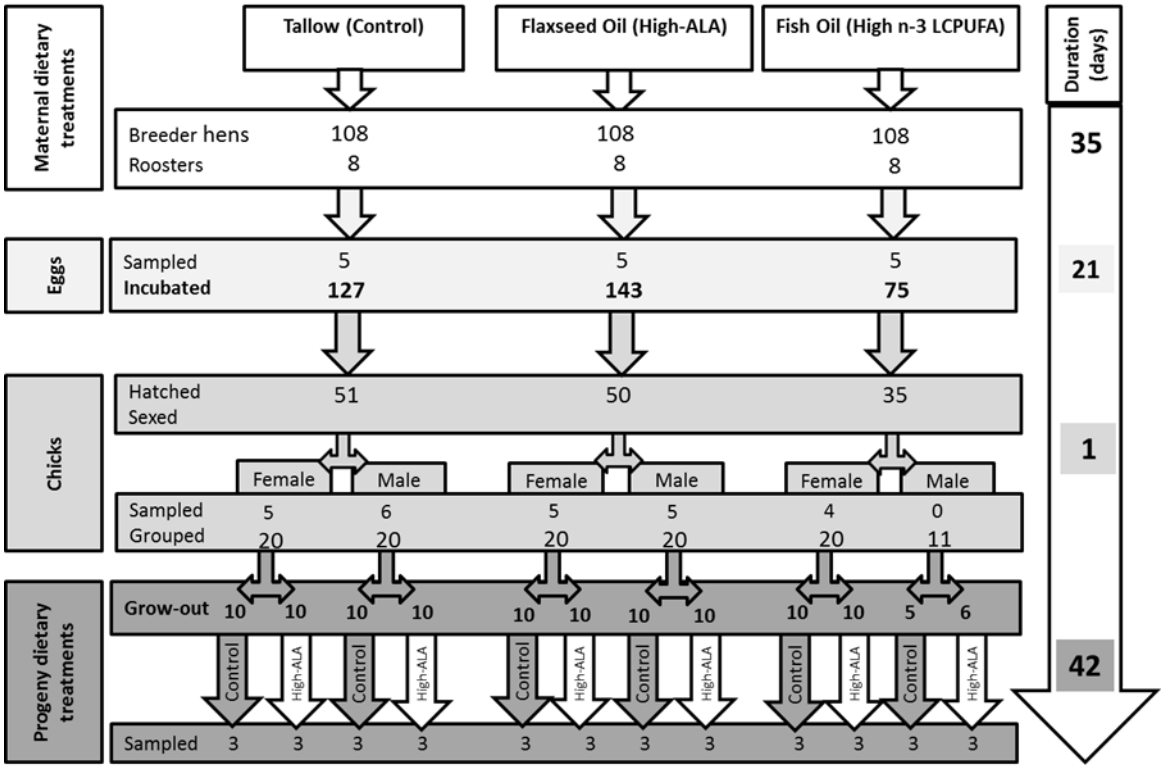

Figure 1. The methodology and numbers of birds used during different experimental phases.

Table 1. Fatty acid composition of the experimental diets[a]

Fatty acid group[b]	Maternal diets[c]			Progeny diets[d]	
	Control	Flaxseed oil	Fish oil	Control	Flaxseed oil
Crude fat %[e]	7.5	7.7	7.8	6.3	6.6
Total SFA	33.0	15.3	25.4	36.7	19.3
Total *trans*	0.6	0.1	0.3	1.7	0.3
Total n-9 MUFA	37.1	25.9	27.1	30.5	18.2
Total n-7 MUFA	4.2	2.0	7.4	3.7	1.6
Total n-6 PUFA	19.8	25.2	18.9	24.4	30.5
Total n-3 PUFA	3.4	30.6	19.4	2.7	29.9
ALA	3.2	30.6	3.3	2.4	29.8
Total n-3 LCPUFA	0.2	0.1	16.1	0.2	0.0
n-6:n-3	5.8	0.8	1.0	9.0	1.0

[a] Values are fatty acid group percentage of the total fatty acid. [b] SFA = Saturated fatty acid; n-9 MUFA = Omega 9 monounsaturated fatty acid; n-7 MUFA = Omega 7 monounsaturated fatty acid; n-6 PUFA = Omega 6 polyunsaturated fatty acid; n-3 PUFA = Omega 3 polyunsaturated fatty acid; ALA = α-linolenic acid ALA, n-3 LCPUFA = Omega 3 long chain polyunsaturated fatty acid. [c] By mixing basal breeder hens diet with (4% w/w) beef tallow (Control), flaxseed oil based diet or fish oil (n=3). [d] By mixing finisher basal diet with (4% w/w) beef tallow (Control), flaxseed oil (n=6). [e] Percentages are based on the wet weight.

5.2.2 Egg Sampling

All eggs that were laid in the 5th week of the maternal diet regimen (Control, n=132 eggs; High-ALA, n=148 eggs; Fish-Oil, n=80 eggs) were collected and stored at holding temperature to temporarily prevent initiation of embryonic development. The yolk from 5 eggs selected at random from each maternal diet group were collected and stored at -18ºC for subsequent fatty acid analysis. The remaining eggs were transferred to the South Australian Research and Development Institute (SARDI) (Roseworthy, SA, Australia) and immediately placed in incubators under standardized conditions (38ºC and 55% humidity, increasing to 60% in the last 4 days of incubation). After 1 week of incubation, the fertility of each egg was assessed, and non-fertile eggs and eggs with dead embryos were discarded.

5.2.3 Chick Hatch and Sampling

Chicks were hatched at ~21 days after the start of the incubation, and at one-day old were feather-sexed into pullets and cockerels[26] (Control: n=26 females, n=25 males; High-ALA: n=25 females, n=25 males; Fish-Oil: n=24 females, n=11 males). Twenty male and 20 female chicks in each maternal dietary group were then allocated to 2 separate raised floor pens (1.2 × 0.9 m each; n=10 chicks per pen), except for the Fish-Oil group males where the pens held 6 and 5 chicks due to the smaller number of hatchlings (Fig. 1.).

The unallocated 1 day old chicks (Control: 6 females and 5 males; High-ALA: 5 of each sex; and Fish-Oil: 4 females only) were euthanized by cervical dislocation and approximately 1-2 g of breast and leg meat were collected in plastic vials and immediately placed on dry ice. Samples then were transferred to the laboratory where they stored at -18°C until subsequent determination of crude fat content and fatty acid composition.

5.2.4 Housing of Broilers

A complete factorial randomized block design (3x2x2) was implemented such that 1 pen of male and 1 pen of female chicks in each of the 3 maternal dietary treatment were fed either the control (4% w/w beef tallow) or the High-ALA (4% w/w flaxseed oil) progeny diets for the entire 6 weeks of grow-out. The 2 experimental progeny diets were nutritionally identical, met requirements for healthy growth and all vitamins and minerals met or exceeded the recommended levels[25]. Broilers were reared under controlled environmental conditions with free access to feed from hoppers and water from a nipple drinker line. The room temperature was 27ºC for the first 4 days then gradually decreased to 20ºC and maintained until harvest, with pens heated by infrared lamps (175 W) during the first 3 weeks. Feed intake and final body weights (BW) of broilers were recorded and feed conversion rate (FCR) during the final week

prior to slaughter was calculated. The numbers of birds that were culled or died was recorded on daily basis.

5.2.5 Tissue Sampling

On day 42 of grow-out, 3 birds from each pen (n=36) were randomly selected and euthanized by cervical dislocation. Breast and leg meat tissues were sampled, frozen on dry ice and stored at -18˚C for subsequent fatty acid profiling.

5.2.6 Fatty Acid Analysis

Crude lipid was extracted from a representative sample of homogenized feed, egg yolk and lean meat samples[27]. The gravimetric approach was utilized to estimate total crude lipid (% of wet weight). Fatty acid profiling was performed after transmethylation of the extracted crude lipids with 1% H_2SO_4 in methanol at 70°C for 3 hours. Briefly, after cooling to room temperature, the resulting fatty acid methyl esters (FAME) were extracted with *n*-heptane (2 mL) and transferred into gas chromatography (GC) vials containing about 30 mg of anhydrous sodium sulphate and stored at -18°C until GC analysis. The FAMEs were separated using a Hewlett-Packard 6890 GC (Hewlett-Packard, CA, USA) equipped with a flame ionization detector (FID), a split injector and a BPX-70 capillary column (50 m x 0.32 mm internal diameter) with a 0.25 µm film thickness (SGE, Victoria, Australia). The operating conditions of the GC, fatty acid identification using the GLC 463 external standard (Nu-Chek Prep Inc., MN, USA) and qualitative analysis were as described previously [28].

5.2.7 Statistical Analyses

The effects of dietary treatment on the fatty acid profile were tested by 1-way, 2-way and 3-way ANOVA for egg, chick and broiler tissues, respectively, using SPSS version 21 for Windows (IBM Corp., NY, USA). Duncan's multiple comparison test was implemented where

the ANOVA showed significant differences between groups ($P<0.05$). Due to the uneven number of broilers in each pen, it was not possible to reliably assess the impact of the dietary treatments on growth performance.

5.3 Results

5.3.1 Fatty Acid Composition of the Experimental Diets

There was no difference in the crude fat percentages between the 3 breeder diets or the 2 progeny diets. The crude fat percentage of the progeny finisher diet (fed in the last 3 weeks of broilers grow-out) was lower than the breeder hens' diets by ~1.2%, due to the different nutritional requirements of birds, however the fatty acid profiles of both the breeder and progeny diets similarly reflected the type of lipid added to the basal feed. Thus, the Control (beef tallow) diet comprised predominately of SFA and n-9 MUFA, the High-ALA diet comprised predominately of n-3 PUFA as ALA, while the Fish-Oil diet was the only one which contained n-3 LCPUFA (Table 1). The ratio of n-6: n-3 PUFA in the diets decreased from 5.8 in the maternal control diet to 0.8- 1.0 in the flaxseed and fish oil diets (Table 1).

5.3.2 Productivity of Breeder Hens

The laying rate of the breeder hens (number of eggs/breeder) appeared to be lower in hens fed the Fish-Oil diet compared to those fed either the Control or high-ALA diets (Control, 1.22; High-ALA, 1.37; Fish-Oil, 0.74). The ratio of female:male chicks hatched, on the other hand, appeared to be higher in the Fish-Oil group compared to the other dietary treatments (Control, 0.96; High-ALA,1.00 and Fish-Oil, 2.18). The hatchability of the eggs (number of chicks hatched/egg laid) was similar between groups (Control, 0.39; High-ALA, 0.33; Fish-Oil, 0.44).

5.3.3 Fatty Acid Composition of Egg Yolks

The crude fat content of egg yolks ranged from 32-38% of the yolk weight (data not shown), and did not differ between dietary treatments. The fatty acid composition of the yolk reflected that of the maternal diet (Fig 2*a*). Thus, the ALA content of the yolk was higher in the High-ALA dietary group compared to the Control and Fish-Oil groups (Fig 2*b*; $P<0.0001$), while the n-3 LCPUFA level in the yolk was the highest in the Fish-Oil group compared to the other treatments (Fig 2*b*, $P<0.0001$). The yolk from hens in the Fish-Oil treatment group also contained less n-6 PUFA than both other groups ($P<0.01$). The ratio of n-6: n-3 PUFA of the egg yolk was 4-5 fold lower in both the High-ALA (1.3) and Fish-Oil (1.2) groups compared to the Control (5.6) group ($P<0.01$).

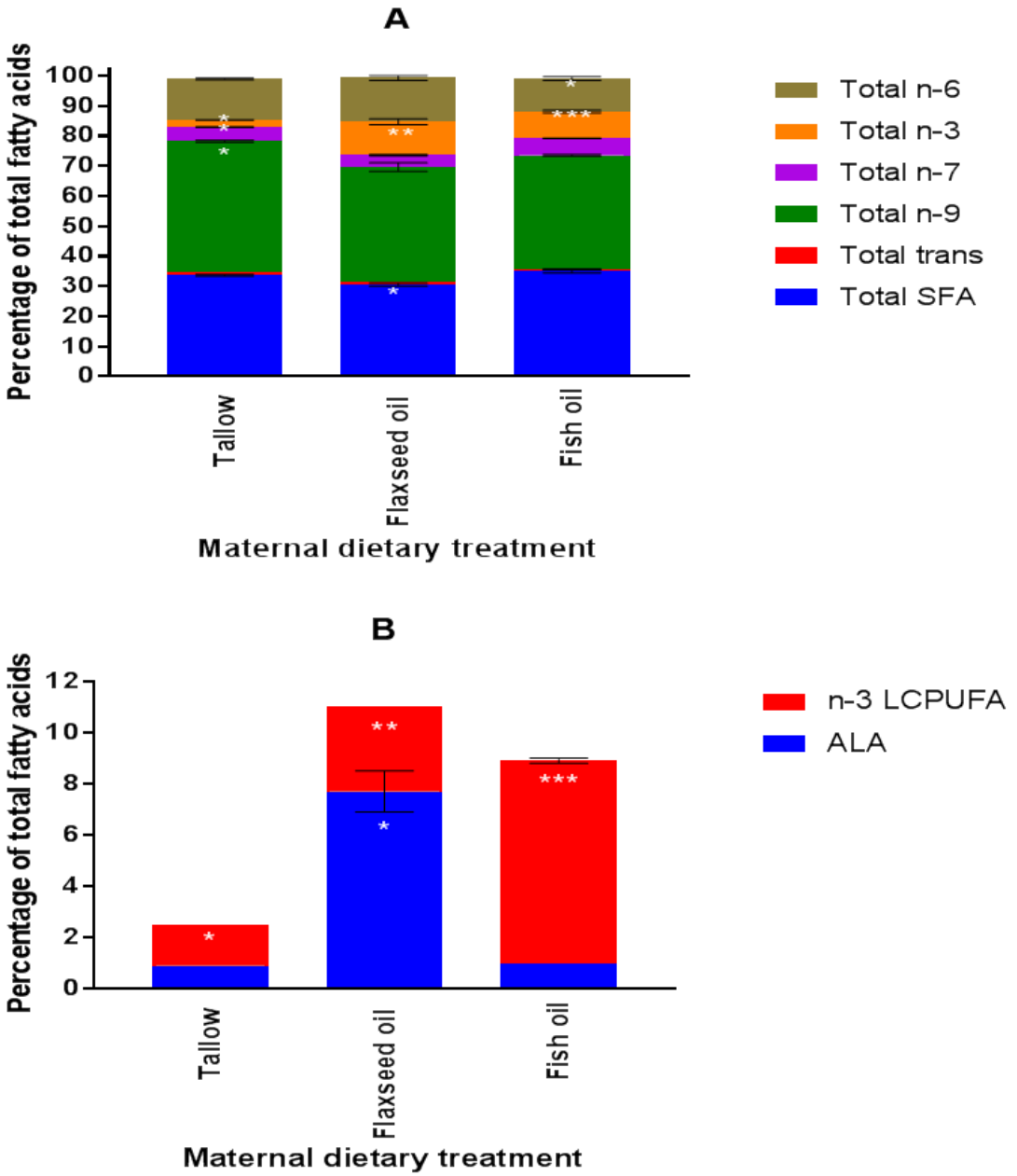

Figure 2. Fatty acid profile (a) and omega-3 distribution (b) in egg yolk of breeder hens fed 3 different diets (n=5±SEM). Different stars within the same fatty acid indicate significant difference ($P<0.01$ for n-9 and n-6) or ($P<0.001$ for other fatty acids) between maternal treatments.

5.3.4 Fatty Acid Composition of Day-Old Chicks

There were significant differences (P<0.0001) in the levels of all major fatty acids in both the breast and leg tissues of the day-old chicks between dietary treatment groups, with the exception of the SFA content, which was similar between treatments. In the breast meat, the MUFA content was lower in the chicks in the High-ALA treatment group compared to both the Fish-Oil and Control treatments. The total n-3 PUFA content (ALA + n-3 LCPUFA) was highest (13.7%) in chicks in the Fish-Oil group, slightly lower (13.4%) in High-ALA and lowest (5.4%) in the Control group (Fig 3a; P<0.0001). A similar effect was observed for the n-3 LCPUFA content, which was 11.6%, 9.0% and 4.9% in the Fish-Oil, High-ALA and Control groups, respectively. The n-3 LCPUFA made up a higher proportion of total n-3 PUFA in the Fish-Oil group compared to the High-ALA group (Fig 3b; P<0.0001). The ALA content of the chicks was also different between treatments, and was higher in the High-ALA (4.4%) and Fish-Oil (2.1%) groups compared to the Control group (0.5%) (Fig 3b; P<0.0001). The n-6 PUFA content was ~5% higher in the Control and High-ALA chicks compared to chicks in the Fish-Oil group (Fig 3a; P<0.0001). However, the n-6: n-3 PUFA ratio was reduced by 2.5 fold and 3.2 fold (P<0.0001) in the High-ALA and Fish-Oil groups compared to the Control group. Similar effects were observed in the leg meat, and there were no differences between sexes (Control and High-ALA groups) or interactions between chick sex and maternal dietary treatment (Males of Fish-Oil group excluded) in either tissue.

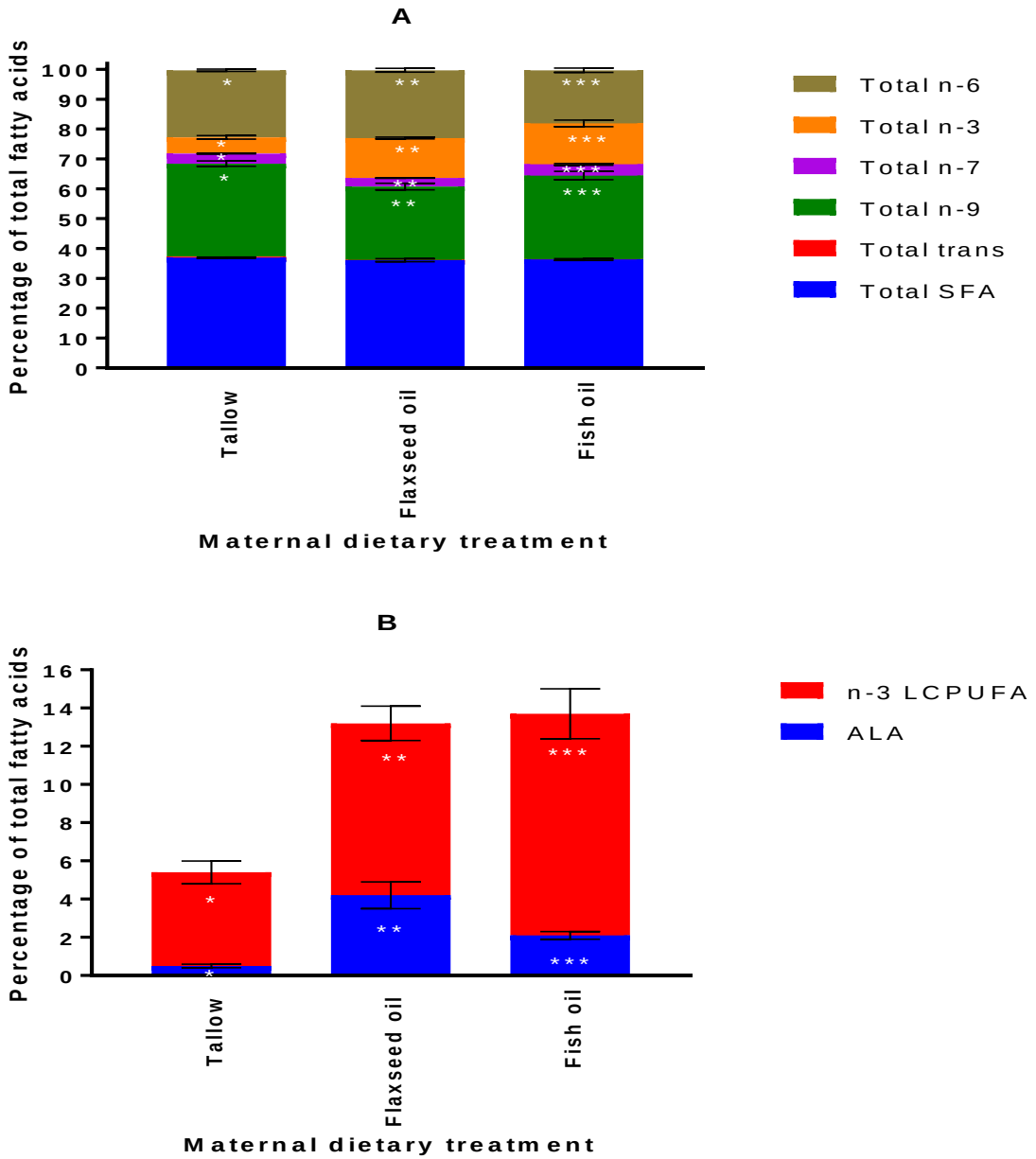

Figure 3. Fatty acid profile (a) and omega-3 distribution (b) in breast meat of female day-old chicks from 3 different maternal dietary treatments (n=6, 5 and 4±SEM for control, flaxseed and fish oil treatments, respectively). Different stars within the same fatty acid indicate significant difference (P<0.0001) between maternal treatments.

5.3.5 Growth of Broilers

The final BW of the broilers at 42 days' post-hatch was 2.98kg in females and 3.64 kg in males and the FCR in the final week prior to tissue collection were 1.92 and 1.81 for females and males, respectively. Only two broilers were culled or died prior to tissue collection throughout the entire 6-week grow-out period.

5.3.6 Fatty Acid Composition of Broilers

Overall, the fatty acid composition of the leg and breast meat tissues was broadly reflective of the diets that the offspring were fed post-hatch, independent of the diet to which they had been exposed *in ovo* (Table 2). Therefore, tissues from broilers fed on the High-ALA diet post-hatch had higher levels of total n-3 PUFA, ALA and n-3 LCPUFA and lower levels of MUFA and SFA compared to those fed on the control diet, independent of the treatment the chicks had been exposed to *in ovo* (Figs 4 and 5). The percentage of SFA in the tissues ranged from ~28% to ~38% across the different treatments, and was higher in progeny fed the Control diet compared to those fed the High-ALA diet ($P<0.0001$). Tissue levels of n-9 and n-7 MUFA were lower in broilers fed the High-ALA diet post-hatch compared to broilers fed a control diet, independent of *in ovo* diet exposure ($P<0.0001$). The n-6 PUFA content of the meat was not different between broilers fed the Control and High-ALA diets. The n-6: n-3 ratio therefore reflected the variation in n-3 PUFA content, and was lower in broilers fed the High-ALA diet (0.8-1.4) compared to those fed the Control diet (4.7-6.4) (Table 2).

Broilers that were exposed to the Fish-Oil treatment *in ovo* had higher ALA levels in the breast meat compared to those exposed *in ovo* to the Control and High-ALA treatments, independent of the diet fed post-hatch, however the magnitude of this difference was small (Table 2 and Fig 5, $P<0.05$). This was accompanied by a reduction in the EPA content of the

meat from these broilers. There were no other differences in fatty acid composition of either the breast or leg meat between *in ovo* treatment groups (Table 2 and Fig 5). There was, however, an interaction between *in ovo* dietary exposure and post-hatch diet on tissue n-3 PUFA concentrations (Table 2, $P<0.05$), such progeny exposed *in ovo* to the Fish-Oil diet that were fed the High-ALA diet post-hatch had significantly higher levels of ALA in breast meat compared to other two maternal treatments fed this same diet (Fig 5).

The fatty acid profiles of the leg meat tissue of progeny at 42 days of age were largely consistent with the breast meat with the exception that males had higher level of n-3 PUFA in their leg meat compared to females (Table 2, $P<0.01$) and maternal High-ALA diet reduced ALA content only in breast meat (Table 2 and Fig 5, $P<0.05$).

Table 2. *P values* at different levels of interaction of 3 experimental factors (maternal by progeny diets by gender) on six weeks old broilers [a]

Fatty acid	Tissue	*P*-value of Independent variable interaction (Treatment)						
		G[b]	M[c]	P[d]	G*M	G*P	M*P	G*M*P
Total SFA[e]	Breast	<0.001	NS[l]	<0.001	NS	NS	NS	NS
	Leg	<0.001	NS	<0.001	NS	0.004	NS	NS
Total *trans*	Breast	0.001	NS	<0.001	NS	0.023	NS	NS
	Leg	0.004	NS	<0.001	NS	NS	NS	NS
Total n-9 MUFA[f]	Breast	0.008	NS	<0.001	0.025	NS	NS	NS
	Leg	0.047	NS	<0.001	NS	NS	NS	NS
Total n-7 MUFA[g]	Breast	0.033	NS	<0.001	NS	NS	NS	NS
	Leg	0.024	NS	<0.001	NS	NS	0.031	NS
Total n-6 PUFA[h]	Breast	NS	NS	NS	0.022	NS	NS	NS
	Leg	NS	NS	NS	NS	NS	NS	NS
Total n-3 PUFA[i]	Breast	NS	NS	<0.001	NS	0.004	0.015	NS
	Leg	0.002	NS	<0.001	NS	0.001	0.013	NS
ALA[j]	Breast	<0.001	0.028	<0.001	NS	0.000	0.036	NS
	Leg	<0.001	NS	<0.001	NS	0.001	NS	NS
Total n-3 LCPUFA[k]	Breast	<0.001	NS	<0.001	NS	0.004	NS	NS
	Leg	NS	NS	<0.001	NS	0.028	NS	NS

[a] Mean of 3. [b] G=gender. [c] M = maternal diets (birds received 3 different diets). [d] P= progeny (birds received 2 different progeny diets). [e] SFA = Saturated fatty acid; [f] n-9 MUFA = Omega 9 monounsaturated fatty acid; [g] n-7 MUFA = Omega 7 monounsaturated fatty acid; [h]n-6 PUFA = Omega 6 polyunsaturated fatty acid; [I] n-3 PUFA = Omega 3 polyunsaturated fatty acid; [j]ALA = α-linolenic acid ALA, [k] n-3 LCPUFA = Omega 3 long chain polyunsaturated fatty acid. [l] NS=Not significant (P>0.05).

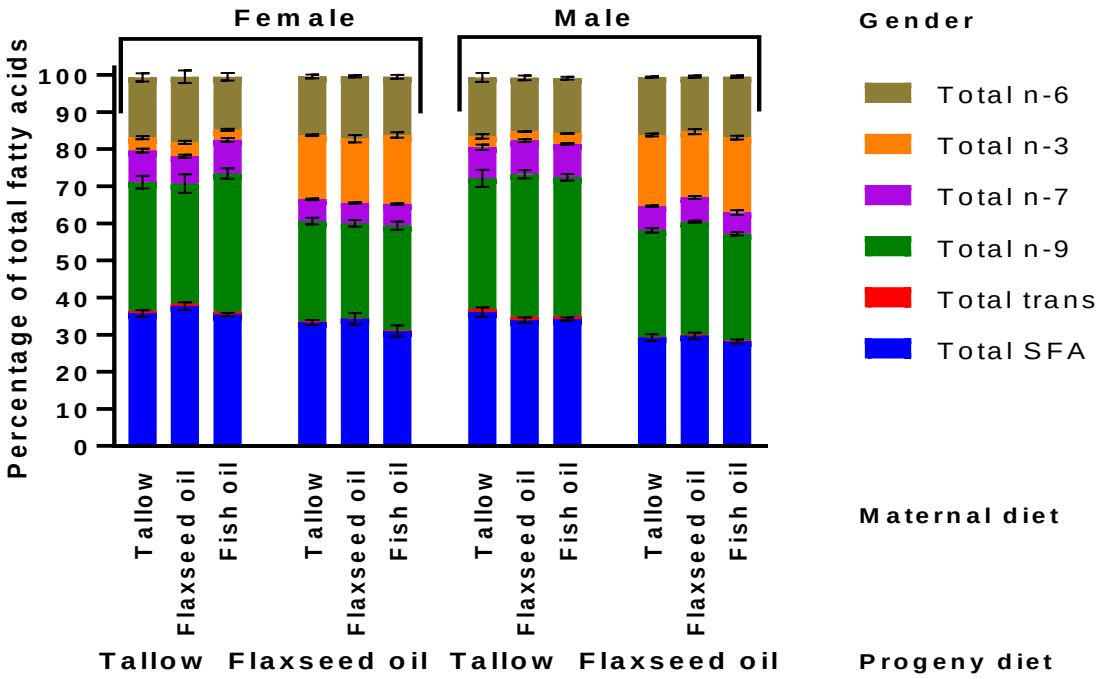

Figure 4. Fatty acid profile in breast meat of 42 days old broilers received 3 maternal by 2 progeny diets (n=3±SEM).

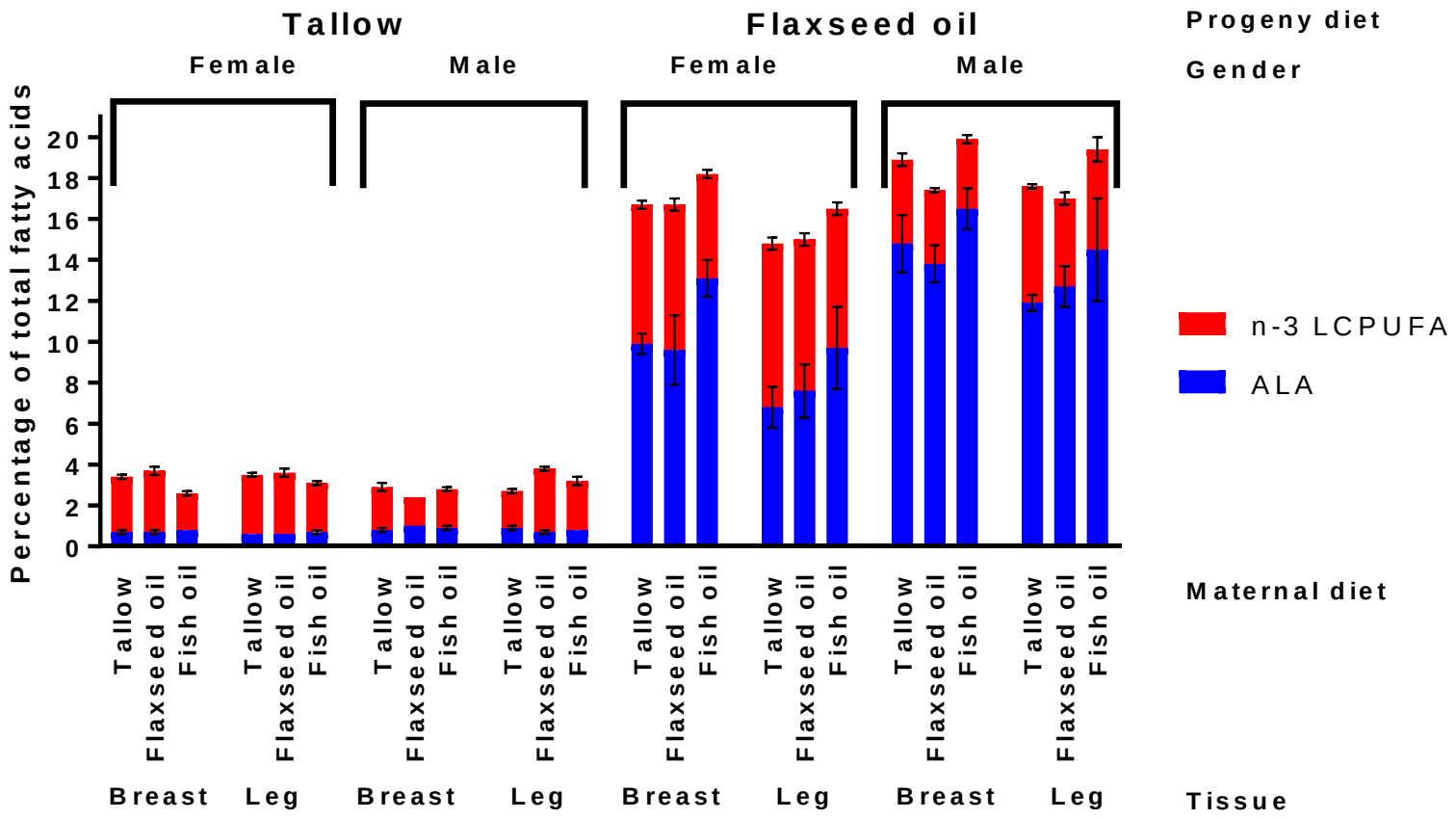

Figure 5. Omega-3 distribution in meat tissues of 42 days old broilers received 3 maternal by 2 progeny diets (n=3±SEM).

5.4 Discussion

The reduced number of chicks in the Fish-Oil group appeared to be a result of reduced laying rate in the breeder hens fed the Fish-Oil diet, while the proportion of eggs that were laid that produced live chicks did not appear to be adversely affected. It is important to note, however, that the study was not powered to investigate differences in production characteristics between treatments and thus further studies are required to confirm if there are any adverse effects of the Fish-Oil diet on laying performance.

While our study was not specifically designed to investigate differences in growth in the progeny, there are no suggestions of adverse effects of any of the diets on the productivity of the chicks. The broilers from all treatments grew at a normal rate, and BW, FCR and mortality in the progeny across the different treatments were within the normal standards for this strain[29] and in agreement with our previous findings[9].

Our finding that feeding breeder hens a high-ALA diet increased not only the ALA content, but also the n-3 LCPUFA content of the eggs, supports the capacity of chickens for ALA conversion to n-3 LCPUFA, and confirms that ALA-supplementation of feed is an effective strategy for increasing the n-3 LCPUFA content of eggs[20, 22, 30-32]. This is likely to be due to the combined effects of the higher amount of ALA, and lower amount of n-6 PUFA in the feed, since the n-3 and n-6 PUFA compete with each other for both metabolic conversion and accumulation into tissues[33].

Interestingly, while supplementing the breeder hens directly with n-3 LCPUFA, in the form of fish oil, led to the greatest increase in n-3 LCPUFA (5-fold of the control eggs), the n-3 LCPUFA content of the eggs (8%), was still only half of that of the feed (16.1%). This

observation suggests there is a maximum level to which n-3 LCPUFA can be incorporated into egg yolk, which may be due to structural limitation of triglycerides and phospholipids. In contrast, the n-3 LCPUFA percentage was relatively increased in eggs of the other 2 treatments in comparison to the levels in the diets. This observation indicates the importance of optimizing the dietary content of n-3 LCPUFA in broiler feed, since the incorporation of these fatty acids into eggs appears to follow a curvilinear, rather than linear, pattern and reach a plateau at high n-3 LCPUFA intakes.

Previous studies have established that >80% of lipids deposited in the egg yolk are consumed by the developing embryo prior to hatch, and therefore represent a major source of nutrition for supporting chick growth and development [34]. In addition, ~50% of egg total fatty acids are incorporated into the newly hatched chick with embryonic preference to incorporate PUFA at the expense of MUFA [35]. Consistent with the findings of the current study, previous studies [22, 32] have demonstrated that the relationship between fatty acid content of the egg yolk and post-hatch chick were closest for the essential fatty acids (n-3 and n-6 PUFA), compared to MUFA and SFA. Our finding that the n-3 and n-6 PUFA content in all groups were relatively higher in the meat of the newly hatched chicks than in the eggs provides evidence of continuous synthesis and accumulation of both of PUFA types in the muscle tissue *in ovo*. Hence, the ratio of n-6: n-3 PUFA in the meat tissues of chicks (especially in the high n-3 PUFA treatments) did not shift as much as the levels of the individual PUFA. This was important for the second stage of the experiment, since it ensured that the capacity of the chicks for converting ALA to the n-3 LCPUFA was unlikely to be limited by the presence of excessive amounts of n-6 PUFA in the tissues.

Interestingly, the contribution of n-3 LCPUFA to the total n-3 PUFA pool in day-old chicks exposed to the high-ALA diet *in ovo* was ~37% greater than in eggs from High-ALA hens, suggesting that these chicks had the capacity for ALA conversion to long chain PUFA during embryogenesis. As with the eggs, however, the ability of the chicks to accumulate n-3 LCPUFA appeared to be limited at higher concentrations, since the n-3 LCPUFA content of meat tissues in chicks exposed to the Fish-Oil diet *in ovo* was about half that in the yolk. Lin, Connor [35] found a linear relationship in n-3 and n-6 PUFA levels between eggs and chicks. However, these authors also suggested that the synthesising of n-3 LCPUFA from ALA was suppressed at higher levels of dietary n-3 LCPUFA.

The growth of the 42 days old broilers (data not shown) in the current study agreed with our previous study [9] and was not affected by dietary fat exposure either *in ovo* or post-hatch [24, 36]. At 6 weeks of age, the fatty acid profile of broilers was found to be mainly affected by the post-hatch diet and sex, with minimal influences of dietary exposure *in ovo*. As the post-hatch control diet contained more SFA, *trans*, n-9 and n-7 MUFA, these fatty acids were predominant in broilers fed this diet. On the other hand, n-3 and n-6 PUFA were the predominant fatty acid groups in broilers fed High-ALA diet, consistent with our previous findings [9]. Although the High-ALA diet was relatively higher than the Control diet in n-6 PUFA content, this did not affect the n-6 PUFA level in broiler meat. This is probably related to the preferential utilization of n-3 PUFA substrates (ALA) by the enzymes involved in the metabolic conversion of shorter-chain fatty acids to their long-chain derivatives and preferential incorporation of n-3 PUFA into tissues[10, 15].

The major finding of this study was that exposure to either a high n-3 PUFA (ALA) or n-3 LCPUFA (fish-oil) diet *in ovo* had very little impact on the capacity of the progeny for

converting ALA to the n-3 LCPUFA. Indeed, the only effect observed was an apparently inhibitory effect of *in ovo* exposure to maternal fish oil supplementation on ALA conversion to n-3 LCPUFA. One possibility is that *in ovo* exposure to a high dietary n-3 LCPUFA content may have acted to suppress the expression and/or activity of genes involved n-3 PUFA metabolism, specifically the desaturase and elongase enzymes required for the conversion of ALA to n-3 LCPUFA. This is supported by previous studies showing that DHA supplementation suppresses endogenous synthesis of n-3 LCPUFA from ALA in human subjects [37] and that feeding chickens a diet enriched in fish oil based diet resulted in an increase in the percentage of both of ALA and LA in the chicken meat, suggesting reduced conversion [38]. Similarly, in chicken, Ajuyah and colleauges reported no effect of a reserve of yolk n-3 LCPUFA from maternal Fish-Oil diet on the n-3 PUFA in the offspring cardiac tissue, but showed an adverse effect on the EPA percentage [24]. In contrast, *in ovo* exposure to the High-ALA diet had no effect on the subsequent capacity of the chickens for ALA conversion. Haug and colleagues reported that the concentration of dietary ALA does not affect the gene expression of the elongation and desaturation enzymes in adult chickens [39], and the results of the current study suggest that this may also be the case during the embryonic stage. Thus, exposing embryos to either High-ALA or High-n-3 LCPUFA environments do not enhance their subsequent capacity for depositing more n-3 LCPUFA after hatch.

While there were relatively few differences between the male and female chickens in their response to the diet, we did identify that male broilers fed the High-ALA diet post-hatch accumulated more total n-3 PUFA in the leg meat, independent of their *in ovo* exposure. In addition, despite no differences in fatty acid profile of male and female chicks at one day post-hatch (Control and High-ALA groups), there were sex differences in the levels of all fatty acids (except n-6 PUFA) in the 42 days old broilers. Thus, tissues from male chickens contained

relatively more n-3 PUFA and lower n-3 LCPUFA, which indicates their lower ALA conversion efficiency, a finding is consistent with our previous study [14].

In summary, we have shown that exposing broiler chickens to elevated levels of ALA or n-3 LCPUFA *in ovo* was effective in increasing n-3 LCPUFA deposition into their meat tissues during embryonic development. However, neither of these strategies were effective at increasing the subsequent capacity of the chickens for accumulating n-3 LCPUFA in their meat tissues when fed a High-ALA diet post-hatch. In fact, increased *in ovo* n-3 LCPUFA exposure appeared to be associated with an impaired capability of the broilers to convert ALA to n-3 LPCUFA.

Conclusion

In ovo n-3 LCPUFA exposure appeared to be associated with an impaired capability of the broilers to convert ALA to n-3 LPCUFA. Manipulation of dietary fatty acids can affect yolk composition, but on its own it is not an appropriate strategy for enhancing n-3 LCPUFA content in the offspring at market age.

Ethical standard

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of animals. This study was approved by the Animal Ethics Committee of the University of Adelaide (approval S-2013-152) and the Department of Primary Industries and Regions South Australia, Australia (approval 15/13).

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CHAPTER 6: GENERAL DISCUSSION

6.1 Contribution to the knowledge

This project built on the outcomes of a large number of previous studies which demonstrated that manipulation of chicken feed is an effective approach to elevate the nutritional value of chicken products. Chickens are sustainably grown and chicken meat is culturally accepted worldwide so the enhancement of chicken products could have consequences for improved health of human consumers without requiring them to change their consumption behavior. Taken together, the different chapters that form this thesis confirm that it is feasible to enrich chicken meat with nutritionally valuable n-3 LCPUFA for human consumption using a flaxseed oil diet containing high levels of ALA.

The low percentage of fat (~2%) in the deskinned chicken meat reported in this research make it a healthy meat option [1-3]. Feeding broiler chicken a flaxseed oil based diet at 3% or 4% w/w inclusion level did not change the crude fat content in the meat nor in other studied tissue. Based on the average 2% fat content in the chicken meat and the fatty acid results in the current project, the corresponding amounts of n-3 LCPUFA and n-3 PUFA in a standard serve of chicken meat (100 g) [3] can be elevated up to 86 mg and 256 mg (leg portion) or 102 mg and 268 mg (breast portion), respectively in broilers fed on 3% w/w flaxseed oil diet.

Taking into account that the average daily consumption of chicken meat exceeds 125 g per capita in Australia (2015-2016) [4], the intake of EPA+DHA from enriched chicken meat would be approximately 60 mg representing 10% and 14% of male and female RDI [5], respectively without changing their consumption behavior. According to the Australian and New Zealand food labelling standard (Clause 13 of Standard 1.2.8) a nutrition claim can be made about this enriched chicken meat as a source of n-3 PUFA as it contains more than 30 mg of EPA+DHA per serving and less than 5 g of total SFA and trans per 100 g meat.

Moreover, claims that the chicken meat is a good source of n-3 PUFA can be made if the food contained at least 60 mg of EPA+DHA per serving [6], which has been achieved in this thesis. This is an important consideration for chicken meat producers as content claims are widely understood by consumers and justify higher pricing of products due to the added value in the product. In turn, this will help the producer to justify the extra cost of feed containing 3% flaxseed oil.

Importantly, compared to a variety of dietary fats that differ in their source (plant or animal), physical status at room temperature (solid or liquid) and fatty acid composition, only flaxseed oil possessed the ability to enrich broiler tissues with n-3 PUFA and n-3 LCPUFA at a nutritionally desirable level. Within the same dietary treatment that included one fat type, there were substantial qualitative differences in the fatty acid profile between seven studied tissues (breast and leg meat, liver, heart, blood, brain and adipose). However, n-3 PUFA maintained a constant positive correlation between dietary and tissue levels for all tissues and had the strongest correlation among all fatty acid groups. These outcomes would attract nutritionists in both poultry and human sectors in regard to selection of the most efficient dietary fat type in order to meet dietary recommendations.

The fatty acids in the non-invasively collected excreta represent the net result of fat digestive and metabolic capabilities by broilers and their microbiota. Comparing the fatty acid profile of diet and excreta of broilers consuming different types and quantities of fatty acids allowed me to rank their relative rates of digestion and absorption in the broiler's gut. Selectively, n-3 PUFA was the group most preferentially-utilized in comparison to other fatty acids with nearly no n-3 LCPUFA being excreted. This finding highlights the physiological significance of n-3 LCPUFA for broilers and indicates the high efficiency of broilers in utilizing the dietary ALA-derived n-3 LCPUFA. Thus, this would further support

using flaxseed oil as a viable option for poultry feed manufacturers and premium chicken meat producers.

The current research investigated whether there was a positive effect of dietary flaxseed oil on growth performance parameters in broilers as has been reported in some previous studies, including my own research group [7]. Somewhat surprisingly, none of the experiments carried out in this project showed a significant effect of dietary fat type on the broilers performance parameters. An important difference between the current studies and the previous study by my colleagues wherein a significant difference was reported was the scale of the experiment. In the current thesis the numbers of broilers used in each experiment ranged between 109 and 480; in comparison, the previous study had 3,800 birds in a single barn. This could suggest, therefore, that significant variation in productivity parameters may only become evident when broilers are grown in commercial-like conditions and perhaps under some type of environmental or social stress, but this remains to be determined.

Previous attempts by my colleagues at the University of Adelaide to make the relatively expensive flaxseed oil diet more efficient have focused on reducing the dietary inclusion level of flaxseed oil and optimizing dietary n-6:n-3 PUFA ratio [8-10]. In the current thesis two alternative strategies were tested. These were (a) a reduction in the duration and timing of feeding flaxseed oil diet, and (b) increasing n-3 LCPUFA incorporation in meat tissues through an intergenerational-nutrigenetic approach. Of these, only the first strategy was found to be effective.

Feeding broilers a 3% w/w flaxseed oil-based diet with n-6 PUFA:n-3 PUFA ratio ~1:1 for four weeks prior to slaughter rather than for the entire growing period would reduce the required amount of flaxseed oil by 9.4% (or 16.7 g) as a minimum with statistically

comparable level of n-3 LCPUFA accumulated in the chicken meat. However, a shorter period (only two weeks prior to harvest) of feeding broilers the flaxseed oil diet would still achieve a lower but considerable level of n-3 LCPUFA incorporation into the chicken meat (59% and 63% in breast and leg tissue, respectively, compared to six weeks). Based on the overall 2% fat content, a 100 g of leg meat from this treatment contained 26 mg EPA+DHA, and more importantly, the same weight of breast meat contained 32 mg EPA+DHA (slightly exceeding the required threshold to be labelled as a source of n-3 PUFA). In this situation the reduction in the amount of flaxseed oil in the enrichment process could be up to ~75 g (or 42% compared to six weeks). Moreover and depending on the previous outcomes, the three weeks treatment has a high potential to produce n-3 PUFA labelled chicken meat. This enrichment would reduce the flaxseed oil usage by ~40 g (22% compared to the six weeks). In other words, this approach would make the enrichment process more economically viable and contribute in providing n-3 LCPUFA chicken meat at affordable price for the end users.

Although the second strategy (the intergenerational-nutrigenetic approach) was not successful, it does deserve further consideration. Broiler breeder hens fed a flaxseed oil diet showed a similar capacity as reported in layer hens to convert part of dietary ALA to n-3 LCPUFA and to deposit it into their fertilized egg-yolk. Consequently, the greater *in ovo* availability of ALA allowed embryos to successfully elongate and desaturate this precursor before hatch and to accumulate high levels of n-3 LCPUFA in their tissues at the time of hatch. Unfortunately, however, this ability was either lost or not expressed sometime after hatch and when the birds reached market weight at 6 weeks of age. The current study did not sample birds between these timepoints so the exact timeline is not known.

6.2 Research limitations

While the results of this project are in line with the literature in terms of the strong positive correlation between dietary and tissue n-3 LCPUFA content, it is worth pointing out that only one strain (Cobb 500) was used in this research work. Thus, other breeds of broiler chickens (which differ in their nutritional requirements and growth performance) may require more investigation to validate the positive results of this project. Similarly, diet formulations differ in their ingredient components and supplements, therefore the variation in feed composition may also affect the efficiency of meat enrichment with n-3 LCPUFA.

The experiments described in this thesis were designed to answer specific questions and therefore sufficient numbers of birds and replication were used to provide robust statistically valid data and conclusions in terms of n-3 LCPUFA in tissues. Whilst valid experiments, the relatively low number of broilers (maximum 480) fall well short of the scale of previous experiments. Hence, they lacked sufficient power to demonstrate statistically significant differences in commercially relevant measures such as growth rate and feed conversion that are already better than industry standards. It is always unwise to extrapolate laboratory scale outcomes to commercial-scale scenarios.

A limitation of the experiment described in Chapter 3 was that the quality and quantity of excreta, and the potential for stage of growth of the birds to have an effect on digesta were not properly addressed. Furthermore, this study did not attempt to separate and define the role of the gut microbiota from the consequences of endogenous fatty acid metabolism in the host.

6.3 Future directions

The outcome of the present thesis suggests that the n-3 LCPUFA enriched chicken meat can be labelled as a source of n-3 PUFA. However, the actual nutritional value of n-3 LCPUFA enriched chicken meat can only be judged by clinical studies. Therefore, studying the bioavailability of n-3 LCPUFA in human blood at different timepoints after consumption of one or more servings of enriched chicken meat is recommended. Also, EPA and DHA levels in blood of volunteers who consume a diet containing enriched chicken meat over a long term (e.g. 3 to 6 months) need to be carried out before claims concerning improved health value can be made.

It may be worth repeating the study where broilers were fed a variety of physically different fats but on a commercial-like scale in order to increase the statistical power to determine differences in growth rate and feed conversion, and exposing birds to realistic commercial conditions (e.g. higher stocking density, poorer air quality and a soiled litter environment). These conditions might have a bearing on the influence of some dietary fat types on performance. Hence, this might provide a better estimation of the anticipated positive relationship between elevating n-3 PUFA in chicken tissues (specifically liver, heart and brain) and growth parameters and therefore the economic consequences for chicken growers.

Fatty acid profiling of excreta could be used to inform the poultry industry about ideal fat sources for feed formulations for nutritional and economic reasons. However, the quantitative analysis of the fatty acid utilization would require collecting the total amount of excreta dropped by bird along the entire growth period which highly recommended to be undertaken in future research. The determined fat percentage in diets was ~7% and in excreta was ~1% (both on wet weight basis). Therefore, inclusion of inert markers in the feed and

considering the dry weight basis of the excreta may be useful in future studies to better evaluate total fat utilization by broilers.

Further investigation concerning the type of excreta (cecal or fecal), the digesta along the digestion tract segments (e.g. duodenum, jejunum and ileum) and the excreta composition at different timepoints of growth are needed to differentiate between the potential roles of gut microbiota and endogenous fatty acid metabolism by the host. This would present better understanding of the relationship between digested and excreted fatty acids and the ideal inclusion level of different fat types (particularly flaxseed oil). This is potentially useful to provide advice to poultry feed producers and chicken growers regarding formulation of broiler diets.

Despite the indication that ALA was highly utilized by broilers, it still existed in considerable percentage (up to 16%) of total fatty acids in excreta when it was the most abundant (flaxseed oil treatment). Higher level of PUFA in excreta will increase its susceptibility to oxidation and therefore increase the rancidity rate. Litter moisture, smell and attraction to flies are genuine issues in poultry rearing, and the effect of high ALA excreta on these factors deserves to be studied.

Similarly, the adipose tissue from birds in the flaxseed oil treatment contained ~17% n-3 PUFA (11-fold of the control). These poultry by-products have industrial applications including being components of some processed foods (manufactured meats), livestock and fish diets. Therefore, the consequences of inclusion of dietary high n-3 PUFA poultry oil in these products deserves further investigation.

It would be appropriate to repeat the experiment of feeding flaxseed oil diet for two to four weeks prior to harvest on a larger group of birds (e.g. a 3,840 group was used in a

previous study by colleagues at the University of Adelaide [7]), in order to determine whether the same outcomes are found. There is some suggestion that using higher number of birds may create levels of stress that could affect the way the birds respond. Furthermore, investigation of the feeding duration and environmental effects recommended to be undertaken on daily or bi-daily basis (15 to 28 day prior to slaughter) of the 42-days-old broilers growth. This is required to precisely determine the minimal feeding period and thus the exact required amount of flaxseed oil to achieve the target level of n-3 LCPUFA in the meat.

It has been demonstrated in the final experiment (Chapter 5), that at the baseline (freshly hatched chicks), offspring from maternal high ALA or n-3 LCPUFA dietary treatments contained higher levels of n-3 PUFA than the control but this had no effect at market age of broilers. Therefore, further investigation of the accumulated n-3 LCPUFA level in the meat and the hepatic gene expression at different timepoints (e.g. on a weekly basis), may allow a better understanding of the metabolic changes during the lifetime of the chicken. Thus, unveil the time the strategy broke down and may suggest other dietary approaches that might allow the process to continue until the broilers are harvested at the market age.

The inclusion of naturally-occurring n-3 LCPUFA (from maternal fish oil diet) affected the broilers efficiency to incorporate ALA-derived n-3 LCPUFA (from progeny flaxseed oil diet) into their tissues. Apart from that, it also seemed to negatively affect different aspects of breeder hen productivity (e.g. rates of lay, fertility and hatchability) which were parameters not specifically targeted in this study (i.e. the maternal treatment groups were not replicated). The observed decrease of these productivity parameters might be related to issues with diet palatability or perhaps a physiological response to the different fatty acid composition of the diets. Hence, designing a specific experiment to study the effects of

feeding broiler breeder hens a fish oil based diet on their productivity and egg quality parameters in future experiments is highly recommended.

6.4 Conclusion

This thesis shows that it is feasible to enrich chicken meat with nutritionally valuable n-3 LCPUFA for human consumption using a 3% w/w flaxseed oil diet (containing ~27% ALA of total fatty acids and ~1:1 ratio n-6 PUFA:n-3 PUFA) through exploitation of the metabolic ability of chickens to desaturate and elongate ALA. The enriched chicken meat would contain enough EPA and DHA to meet the requirements to be labelled as a source of n-3 PUFA. An average Australian eating 125g per day of this meat would ingest as minimum 10-14% of their RDI of n-3 LCPUFA. There is potential to reduce the amount of flaxseed oil by 22% by feeding the oil for the last 3 weeks prior to harvest and therefore reducing costs for the producer and the customer.

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APPENDICES

Appendix 1

Appendix 1. Table 1. Lipid analysis protocols

	Crude fat extraction		Dilution		Weighing fat	Transmethylation		Fatty acid GC analysis	
Sample	Wet homogenised sample weight (g)*	Weight of extracted lipid (mg)	Vol. of 9:1 chloroform/methanol (ml) added	Vol. of 9:1 chloroform/methanol (ml) transferred	Weight of lipid (mg)	Vol. of 1% H_2SO_4 in methanol (ml)	Vol. of *n*-heptane (ml)	Vol. of (heptane + FAME***) injected (µl)	Split ratio
Oil	0.10	100	2.5	0.1	4	5	2	2	40:1
Feed	0.50	35	2.5	0.2	3	5	2	2	40:1
Meat	0.25	5	1	0.5	3	5	2	2	40:1
Liver	0.20	12	1	0.2	2	5	2	2	40:1
Heart	0.25	10	1	0.2	2	5	2	2	40:1
Brain	0.10	7	1	0.3	2	5	2	2	40:1
Adipose	0.10	90	2.5	0.1	4	5	2	2	40:1
Preen oil	0.10	98	2.5	0.1	4	5	2	2	40:1****
Egg yolk	0.10	35	2.5	0.2	3	5	2	2	40:1
Excreta	0.50	10	1	0.2	2	5	2	2	40:1
Whole Blood (DBS)**	1 blood drop (14mm spot)**	-	-	-	-	2	0.6	2	40:1

*Sample weights depending on crude fat%.
** DBS: Dry Blood Spot® technique, only for fatty acid profiling.
***FAME: Fatty acid methyl esters after methylation of fat at 70℃ for 3h.
****Usual GC analysis, 50 m length column for 30 min run. Preen oil samples have been reanalysed again using 20:1 split ratio, 30 m GC column length for 18 min run to distinguish between the fatty acids C19:0 and C18:2n-6 peaks.

Appendix 2. Relationship between the fatty acid composition of the uropygial gland secretion and blood of meat chickens receiving different dietary fats

Statement of Authorship

Title of Paper	Relationship between the fatty acid composition of the uropygial gland secretion and blood of meat chickens receiving different dietary fats
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Published online 6 December 2016 in the Animal Production Science journal, http://dx.doi.org/10.1071/AN16268

Principal Author

Name of Principal Author (Candidate)	Khaled Kanakri		
Contribution to the Paper	Co-designed the experiment, performed the experimental work (field part and fatty acid analysis), statistical analysis, data interpretation, wrote the manuscript and acted as corresponding author.		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	26.07.2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Beverly Muhlhausler		
Contribution to the Paper	Assisted in data interpretation and critical revision of the manuscript.		
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Name of Co-Author	John Carragher		
Contribution to the Paper	Co-designed the experiment, supervised the analysis of samples, the statistical analysis and manuscript outlining and revision.		
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Please cut and paste additional co-author panels here as required.

Appendix 2

Name of Co-Author	Robert Gibson
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Name of Co-Author	Robert Hughes
Contribution to the Paper	Designed the experiment, contributed to the animal care and contributed to manuscript revision.
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Appendix 2

CSIRO PUBLISHING

Animal Production Science
http://dx.doi.org/10.1071/AN16268

Relationship between the fatty acid composition of uropygial gland secretion and blood of meat chickens receiving different dietary fats

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Abstract. Manipulation of the fatty acid composition of chicken feed has been shown to be effective for improving the nutritional value of chicken products. Currently, however, evaluation of the effectiveness of this approach requires invasive blood sampling or post mortem tissue sampling of the birds. Preen oil can be collected non-invasively from live birds. So this study aimed to test the hypothesis that the fatty acid composition of preen oil reflects that of the blood. Male and female meat chickens (Cobb 500) were fed a diet supplemented with 4% (w/w) flaxseed oil (high n-3 polyunsaturates) or beef tallow (mostly monounsaturates and saturates) for 6 weeks. Preen oil and whole blood samples ($n = 9$ birds per sex/diet treatment group) were collected freshly post mortem for fatty acid analysis. Preen oil analysis showed that ~97% of fatty acids were saturates, with a small percentage of n-6 polyunsaturates and traces of other types. There were negligible n-3 polyunsaturates in preen oil. Proportions of some saturated fatty acids were slightly, but significantly, affected by diet (C16:0 ($P < 0.05$) and C17:0 ($P < 0.01$)) or by gender (C10:0 and C18:0) ($P < 0.05$). Some fatty acids with odd numbers of carbon atoms (e.g. C17:0 and C19:0) were found in relatively high concentrations in preen oil, despite not being detectable in either the diet or blood. In conclusion, the fatty acid composition of preen oil does not accurately reflect the fatty acid profile of the blood; it is not, therefore, a suitable alternative for determining fatty acid status of meat chickens.

Additional keywords: gender effect, preen oil.

Received 26 April 2016, accepted 18 October 2016, published online 6 December 2016

Introduction

Preen oil secreted from the holocrine uropygial or preen gland found on the dorsal surface at the base of the tail of most avian species has been well described for centuries in relation to gland structure and chemical analysis of the secretion (Odham and Stenhagen 1971). Once birds spread this oily secretion over their plumage (preening), it has been reported to provide a diversity of functions; for example, water-proofing (Odham and Stenhagen 1971) or a preservative to maintain healthy feathers (Shawkey *et al.* 2003), to protect eggs and embryos (Martínez-García *et al.* 2015), and as a pheromone or olfactory signal in social behaviour (Hirao *et al.* 2009; Tuttle *et al.* 2014). The functional importance of this gland to domesticated chickens is still a subject of controversy (Madec *et al.* 2008; Salibian and Montalti 2009; Jawad *et al.* 2015).

The biochemical composition of preen oil has been reported to significantly vary between birds in several ways, for example, the qualitative and quantitative content of the volatile compounds (Soini *et al.* 2013), chain length of diols (Kolattukudy and Sawaya 1974), lipid concentration (Bhattacharyya and Chowdhury 1995), ester saturation (Haribal *et al.* 2009), type of fatty acids and their percentage composition (Sandilands *et al.* 2004). This variation is due to a range of factors, including species, age, sex and season (Jacob *et al.* 1979; Sandilands *et al.* 2004; Tuttle *et al.* 2014).

In previous studies in chickens, Haahti *et al.* (1964) detected several fatty acids ranging from C10:0 to C20:0 in the saponifiable fraction and four fatty acids in the range C20:0–C24:0 in the unsaponifiable fraction of the preen oil. Sandilands *et al.* (2004) identified 13 saturated fatty acids ranging from C10:0 to C24:0 and one monounsaturated fatty acid C18:1 in the wax esters derived from preen oil.

A limited number of studies have investigated the specific effect of diet on the fatty acid composition of preen oil in meat chickens. Apandi and Edwards (1964) fed chickens (White Plymouth Rocks) fat-free or corn oil-based diets. They identified saturated and unsaturated fatty acids with a wide range of chain length C8:0–C25:0 in the preen oil and concluded there were major qualitative and quantitative differences in several fatty acids detected. Pan *et al.* (1979) detected 13 fatty

Appendix 2

acids but only identified the major ones as five saturated, one monounsaturated and two polyunsaturated fatty acids ranging from C10:0 to C18:0 in the preen oil. Furthermore, they reported no influence of gender or levels of six dietary fats from plant and animal origins on the type and percentage of any of the identified and unidentified fatty acids of the preen oil.

The lack of consistency in the limited published literature regarding the effect of diet on preen oil composition justifies the need for further study. We tested the hypothesis that the fatty acid composition of preen oil could potentially provide a quick, simple and non-invasive method of assessing the fatty acid profile of blood in meat chickens. This study also aimed to determine if there were differences between male and female chickens in relation to the impact of the two diets on preen oil composition.

Materials and methods

Diets

Two experimental diets were prepared by mixing standard commercial starter and finisher basal chicken diets (Ridley Agriproducts Pty Ltd, Murray Bridge, SA, Australia) with 4% w/w of beef tallow (high in saturated and monounsaturated fatty acids) or flaxseed oil (high in n-3 fatty acid) (Table 1). Birds were fed starter/grower diet in the first 3 weeks followed by finisher/withdrawal diet for the last 3 weeks. Except for the difference in fatty acid composition; both experimental diets were nutritionally identical and both met requirements for healthy growth.

Birds and experimental environment

This study was approved by The Animal Ethics Committees of the University of Adelaide and the Department of Primary Industries and Regions, SA, Australia (S-2015–067). Fertilised eggs (Cobb 500) were obtained from the HiChick Breeding Co. (Gawler, SA, Australia) and transferred to Roseworthy Campus, University of Adelaide. A total of 111 healthy newly hatched chicks were feather sexed and allocated by sex to 12 raised floor rearing pens (1.2 × 0.9 m each) with wood shavings litter in an environmentally controlled room. A complete randomised block design (three pens per diet by gender treatment) was implemented. All groups were fed on their allocated diet for the entire rearing period. Chickens were reared for 6 weeks with free access to both feed and water at all times. The temperature was 27°C for the first 4 days then gradually decreased to 20°C before being maintained until the end of the experimental period.

Table 1. Fatty acid composition of the experimental diets
Values are total fatty acid group as a percentage of the total fatty acid

Fatty acid group	Tallow-based diet	Flaxseed oil-based diet
Total saturates	36.7	19.3
Total n-9 monounsaturates	30.5	18.2
Total n-7 monounsaturates	3.7	1.6
Total n-3 polyunsaturates	2.7	29.9
Total n-6 polyunsaturates	24.4	30.5
Total *trans*	1.7	0.3

Sample collection

At Day 42, three birds per pen ($n = 36$) were randomly selected and euthanised by cervical dislocation. Samples of blood and preen oil were collected post mortem. Fresh whole blood was obtained from the heart and two drops were collecting using the PUFAcoat dry blood spot (DBS) system (Liu *et al.* 2014). Feathers around the uropygial gland were cut away and gloved fingers were used to massage as much as possible of preen oil directly into an Eppendorf vial, which was immediately immersed in dry ice then stored at −18°C until fatty acid analysis.

Fatty acid analyses

Crude fat in homogenised feed pellets and preen oil samples was extracted by the method of Folch *et al.* (1957). Fatty acid profiling was performed by transmethylation of the whole blood DBS and crude fats from feed and preen oil with 1% H_2SO_4 in methanol at 70°C for 3 h following methodology described by Lepage and Roy (1986). After cooling to room temperature, the resulting fatty acid methyl esters (FAME) were extracted with n-heptane (2 mL) and transferred to gas chromatography (GC) vials containing ~30 mg of anhydrous sodium sulfate. Vials were stored at −18°C until GC analysis.

Gas chromatograph analyses of FAME

FAME were separated and measured using a Hewlett-Packard 6890 GC (Hewlett-Packard, Palo Alto, CA, USA) equipped with a split injector, a BPX-70 capillary column (50-m × 0.32-mm internal diameter) coated with a 0.25-μm film thickness and a flame ionisation detector. The operating conditions of the GC, fatty acid identification and qualitative analysis were as described in detail previously (Tu *et al.* 2010; Kartikasari *et al.* 2012).

Statistical analyses

The normality of the distribution of residuals for fatty acid percentages were tested by a Shapiro–Wilk test. Variables that were not normally distributed were transformed to normality for parametric analysis by the two steps fractional rank method. This experiment involved a 2 × 2 factorial design (sex and dietary treatment). The effects of gender and dietary treatment and the presence of interactions between these factors on blood and preen oil fatty acid profiles were tested by two-way ANOVA using IBM SPSS Statistics for Windows version 21 (IBM Corp., Armonk, NY, USA).

Results

Growth performance

The overall growth performance of birds was excellent in all treatments, with very low mortality (only two birds died throughout the experiment). The final average female and male weights (g/bird) were 2981 and 3638, respectively.

Diet analyses

The crude fat levels in the tallow and flaxseed oil based diets were 6.3% and 6.6%, respectively, representing the sum of original basal diet fat and the added tallow/flaxseed oil. The overall fatty acids profiles were significantly different between the two experimental diets show a broad variation in the main fatty acid

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groups (Table 1). The tallow based diet contained a higher proportion of saturates, n-9 and n-7 monounsaturates (by 90%, 67% and 130%, respectively), and lower levels of n-3 and n-6 polyunsaturates (by 91% and 20%, respectively), compared with the flaxseed oil diet. Levels of *trans* fatty acids were low (<2%) but were 5-fold higher in the tallow-based diet in comparison to the flaxseed oil diet. The detailed saturated fatty acid composition of the two diets is shown in Table 2; both diets contained mainly C16:0 and C18:0.

Blood analyses

The effect of the different dietary treatments and gender on blood fatty acid composition varied depending on the fatty acid group. The blood of chickens fed the flaxseed oil diet contained significantly ($P < 0.0001$) greater levels of total n-3 but significantly ($P < 0.0001$) lower levels of saturates, n-7 and n-9 monounsaturates than the tallow-fed birds. There were no significant differences in blood levels of n-6 polyunsaturates between dietary groups (Table 3). The effect of gender was significant in all fatty acid groups except saturates, although the extent of the difference varied between fatty acids; ($P <$ 0.0001 for n-3, <0.001 for n-9, <0.01 for n-6 and <0.05 for n-7) with females having higher n-9 and n-7 monounsaturates and males higher n-3 and n-6 polyunsaturates. There was a significant ($P < 0.05$) gender by diet interaction effect in the n-6 group, as shown in Table 3. Detailed identification of the individual saturated fatty acids in whole blood showed four medium- or long-chain fatty acids were detectable (Table 4), with C16:0 and C18:0 being the most prevalent in both dietary groups.

Table 2. Dietary content of individual saturated fatty acids
Values are the mean percentage of total fatty acid ($n = 5$) ± standard error of the mean (s.e.m.)

Saturated fatty acid	Tallow-based diet	Flaxseed oil-based diet
C10:0	0.1 ± 0.00	0.0 ± 0.00
C12:0	0.1 ± 0.00	0.0 ± 0.00
C14:0	1.6 ± 0.02	0.4 ± 0.00
C15:0	0.4 ± 0.00	0.1 ± 0.00
C16:0	22.1 ± 0.04	13.2 ± 0.17
C17:0	0.8 ± 0.01	0.2 ± 0.00
C18:0	11.2 ± 0.08	5.0 ± 0.16
C20:0	0.2 ± 0.00	0.1 ± 0.00
C22:0	0.1 ± 0.00	0.2 ± 0.01
C24:0	0.1 ± 0.00	0.1 ± 0.01

Preen oil analyses

The predominant fatty acid group in the preen oil was saturates, which comprised 96.1–97.4% of all fatty acids and this was not different between the two dietary treatments, however it was slightly but significantly ($P < 0.05$) higher in males. The remaining fatty acids were mostly n-6 (2.4–3.1%) and all other fatty acid groups were non-detectable or present in negligible (<1%) levels (Table 3). Detailed characterisation of the individual saturated fatty acids in preen oil identified 13 medium- or long-chain fatty acids ranging from C8:0 to C22:0 (Table 4). Levels of some of the saturated fatty acids were significantly affected by the experimental factors of diet or gender. Females had significantly ($P < 0.05$) lower levels of C10:0 and C18:0 than the males independent of diet. Birds fed on the flaxseed oil diet had significantly higher C16:0 ($P < 0.05$) and

Table 3. Fatty acid profiles of blood and preen oil from female and male meat chickens fed with tallow or flaxseed oil diets (% of total fatty acids)
Values are means ± s.e.m. ($n = 9$)

Fatty acids	Treatment Group						
	Female		Male		*P*-value[F]		
	Tallow	Flaxseed oil	Tallow	Flaxseed oil	Sex	Diet	S × D
			Whole blood total lipid				
Total SFA[A]	39.7 ± 0.14	37.4 ± 0.26	39.7 ± 0.19	36.5 ± 0.59	0.21	0.000	0.20
Total n-9[B]	28.3 ± 1.07	19.2 ± 0.69	24.5 ± 0.55	17.6 ± 0.65	0.001	0.000	0.15
Total n-7[C]	4.8 ± 0.27	3.1 ± 0.14	4.1 ± 0.18	2.8 ± 0.21	0.020	0.000	0.22
Total n-3[D,G]	2.4 ± 0.20	15.0 ± 0.12	2.8 ± 0.21	17.6 ± 0.45	0.000	0.000	0.10
Total n-6[E]	24.4 ± 1.13	25.3 ± 0.65	28.5 ± 0.60	25.4 ± 0.48	0.007	0.15	0.012
			Preen oil total lipid				
Total SFA[G]	96.1 ± 0.68	96.5 ± 0.57	97.0 ± 0. 37	97.4 ± 0.15	0.034	0.63	0.73
Total n-9	0.1 ± 0.02	0.2 ± 0.03	0.1 ± 0.04	0.1 ± 0.04	0.023	0.24	0.002
Total n-7[G]	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.02	0.1 ± 0.02	0.006	0.046	0.10
Total n-3[G]	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.02	0.1 ± 0.02	0.016	0.08	0.06
Total n-6	3.1 ± 0.32	2.7 ± 0.23	2.6 ± 0.36	2.4 ± 0.14	0.41	0.16	0.58

[A]Saturated fatty acids.
[B]Omega-9 monounsaturated fatty acids.
[C]Omega-7 monounsaturated fatty acids.
[D]Omega-3 polyunsaturated fatty acids.
[E]Omega-6 polyunsaturated fatty acids.
[F]Two-way ANOVA.
[G]Not normally distributed (Shapiro–Wilk test) and so transformed to normal distribution by Fractional Rank method.

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Table 4. Saturated fatty acid composition of blood and preen oil from female and male meat chickens fed with tallow or flaxseed oil diets (% of total fatty acids)
Values are means ± s.e.m. (n = 9)

	Treatment group						
	Female		Male		*P*-value[B]		
	Tallow	Flaxseed oil	Tallow	Flaxseed oil	Sex	Diet	S × D
			Whole blood SFA[A]				
C14:0[C]	0.6 ± 0.03	0.0 ± 0.00	0.4 ± 0.09	0.0 ± 0.0	0.017	0.000	0.06
C16:0	22.3 ± 0.34	20.5 ± 0.25	21.7 ± 0.15	20.2 ± 0.21	0.09	0.000	0.58
C17:0[C]	0.3 ± 0.07	0.0 ± 0.0	0.3 ± 0.08	0.0 ± 0.0	0.65	0.000	0.65
C18:0[C]	16.6 ± 0.41	16.9 ± 0.36	17.3 ± 0.45	16.2 ± 0.57	0.99	0.63	0.27
			Preen oil SFA[A]				
C8:0	0.8 ± 0.02	0.9 ± 0.05	0.9 ± 0.06	0.9 ± 0.06	0.72	0.43	0.42
C10:0	9.0 ± 0.37	9.9 ± 0.50	10.6 ± 0.55	10.3 ± 0.43	0.049	0.51	0.23
C11:0	1.1 ± 0.07	1.1 ± 0.04	1.3 ± 0.10	1.1 ± 0.09	0.10	0.17	0.29
C12:0	13.1 ± 0.43	12.0 ± 0.38	12.3 ± 0.39	11.8 ± 0.46	0.21	0.050	0.50
C13:0	1.6 ± 0.07	1.5 ± 0.07	1.7 ± 0.10	1.6 ± 0.12	0.46	0.29	0.68
C14:0	22.0 ± 0.64	21.9 ± 0.35	22.7 ± 1.17	22.9 ± 0.74	0.28	0.98	0.83
C15:0	1.2 ± 0.14	1.5 ± 0.09	1.3 ± 0.10	1.3 ± 0.13	0.71	0.10	0.32
C16:0	13.6 ± 1.47	16.1 ± 0.83	12.5 ± 0.68	15.0 ± 1.08	0.31	0.024	0.97
C17:0	2.1 ± 0.41	3.4 ± 0.34	2.7 ± 0.37	3.6 ± 0.49	0.27	0.009	0.62
C18:0	10.2 ± 0.53	11.7 ± 0.74	13.3 ± 1.12	12.7 ± 0.80	0.020	0.56	0.22
C19:0	7.1 ± 0.32	7.9 ± 0.39	7.9 ± 0.52	7.4 ± 0.27	0.63	0.70	0.10
C20:0	12.3 ± 1.66	8.0 ± 0.82	9.1 ± 1.59	8.2 ± 1.57	0.16	0.17	0.48
C22:0	1.9 ± 0.51	0.6 ± 0.20	0.7 ± 0.25	0.5 ± 0.19	0.13	0.06	0.71

[A]Saturated fatty acids.
[B]Two-way ANOVA.
[C]Not normally distributed (Shapiro–Wilk test) and so transformed to normal distribution by Fractional Rank method.

C17:0 (P < 0.01) levels compared with the tallow-fed birds. The other saturated fatty acids were not significantly affected by diet or gender, nor were there any significant gender by diet interactions (Table 4).

Discussion

The results of the diet analysis (Table 1) demonstrated that we successfully increased the level of total n-3 fatty acids (all as α-linolenic acid (ALA)) from only 3% in the finisher basal to 30% in the flaxseed oil-based diet. An increase in the level of ALA and a reduction in the n-6 to n-3 ratio from 11 : 1 in the tallow diet, to close to 1 : 1 in the flaxseed oil diet are required for the birds to convert the ALA into the long-chain n-3 fatty acids eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) (Kartikasari *et al.* 2012; Carragher *et al.* 2016).

Blood or blood serum are commonly sampled to study the bioavailability of nutrients or other compounds related to the health status in human and animals (e.g. chickens) (Newman *et al.* 2002; Lopes *et al.* 2013). Depending on the parameter, blood can also be a good indicator of the composition of other tissues (An *et al.* 1997; Aghaei *et al.* 2012). In another study (K. Kanakri, J. Carragher, B. Muhlhausler, R. Hughes and R. Gibson, unpubl. data), we found a strong positive correlation (r = 0.94–1.00) between the fatty acid composition of whole blood and meat tissues. Plasma is usually the fraction sampled to measure short-term metabolic products; however, we sampled the whole blood in this experiment as our focus was on the long-term metabolites. As expected, the difference in the fatty acids intake of the two dietary treatments affected whole blood fatty acid composition with the tallow diet resulting in higher saturates and monounsaturates. However, the flaxseed oil diet produced higher blood levels of total n-3, including n-3 long-chain polyunsaturated fatty acids e.g. EPA, DPA and DHA (data not shown) indicating that the birds were converting some of the dietary ALA to the long chain n-3, as intended (Cherian and Hayat 2009). Despite the 90% difference in total saturates between the two diets, there was only a small (but significant) change in the level of total saturates in the blood i.e. 37.0% for flaxseed oil-fed birds (both sexes combined) compared with 39.7% in the tallow-fed birds. Indeed, 98.1–100% of whole blood total saturates for different treatments consisted of only two fatty acids; C16:0 and C18:0, the rest were C14:0 and C17:0. The individual effect of dietary factor was significant (P < 0.0001) for C14:0, C16:0 and C17:0, whereas only C14:0 was significantly different between female and male birds (P < 0.05).

The purpose of this study was to compare the composition of fatty acids in preen oil and blood of meat chickens. The rapid solidification of the preen oil when it was collected in the vial at room temperature was a very good indicator of its biochemical composition. More than 96% of the identified fatty acids in preen oil were saturates, similar to the 96% reported by Sandilands *et al.* (2004) in chicken hens. Our results indicated that preen oil from meat chickens contained a total of 15 fatty acids, 13 saturated and 2 n-6 polyunsaturated, from C8:0 to C22:0. This number is slightly higher (by 1–3) than those measured by Sandilands *et al.* (2004), Pan *et al.* (1979) and Saito and Gamo (1968) but less

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than the 22 saturated and unsaturated fatty acids (with percentages ≥0.1%) that Apandi and Edwards (1964) reported. The latter authors also identified very long fatty acids, for example, C25:0 at an extremely high 42.5% of total fatty acids. Differences in the number of fatty acids reported between studies is likely to be affected by the sensitivity of the methods used, and chromatography conditions (e.g. GC column length, injector and oven temperatures) rather than differences between strains of chickens, diet or environmental conditions.

We observed that the major contributors to the fatty acid of chicken preen oil were (in decreasing order) C14:0, C16:0, C18:0, C12:0, C10:0, C20:0, C19:0 and C17:0 with other fatty acids making up to 5.6% of the total. This order was different to and more complex than the saturated fatty acid composition of either the diet or blood, where C16:0 and C18:0 dominated. The sequence of overall proportions of fatty acids was also different to that reported in hens (Sandilands *et al.* 2004), and meat chickens (Apandi and Edwards 1964; Pan *et al.* 1979).

Several saturated fatty acids with odd numbers of carbon atoms, for example, C19:0, C17:0, C13:0, C15:0 and C11:0 were detected in preen oil despite being either low or not detected in the diet or blood. This finding suggests they are not food-derived and so they are either self-synthesised by the gland and/or by resident microbiota. These findings and this conclusion is similar to those made by other researchers (Apandi and Edwards 1964; Saito and Gamo 1968; Sandilands *et al.* 2004). Fendri *et al.* (2012) studied the lipolytic enzymes of homogenised uropygial gland and demonstrated it possessed esterase activity for short-chain triacylglycerols but not for medium- and long-chain triacylglycerol substrates, suggesting that *in situ* lipolysis is unlikely to be the main pathway to synthesise odd-chain fatty acids. In another study, Biester *et al.* (2012) concluded that preen glands have both monofunctional and bifunctional wax ester and triglyceride synthesis proteins that vary in their expression patterns and synthetic activities with different substrate specificities. In contrast, the preen gland of certain species of birds, such as the hoopoe bird (*Upupa epops*), contains microorganisms of the enterococci, clostridia and many other facultative and obligatory anaerobic bacterial families. These bacteria are thought to contribute to the synthesis of the fatty acids that are found in the preen oil (Rodríguez-Ruano *et al.* 2015). It may be that a similarly diverse microbiome exists in the uropygial gland of chickens and that some of the species can facilitate lipolysis, but additional specific studies would be required to determine whether this was the case.

The low levels of n-6 and n-9 in preen oil (2.4–3.0% and 0.1–0.2%, respectively), are in clear contrast to their substantial presence in whole blood (25.3–26.5% and 18.4–26.4%, respectively). This is a further demonstration that the uropygial gland produces a highly specialised secretion. The small percentage of n-6 polyunsaturated fatty acids detected in preen oil in the present study comprised C22:5 and C20:4; again, this does not reflect the composition of n-6 fatty acids in the blood (or the diet), where C18:2 (linoleic acid) was highest. A similar finding was reported by Sandilands *et al.* (2004); however, Saito and Gamo (1968) and Pan *et al.* (1979) did find C18:2 at 25 and 9%, respectively.

The main difference of the flaxseed oil dietary manipulation was on the n-3 fatty acid composition in whole blood. This, however, was not reflected in the preen oil where n-3 was less than the limit of detection (0.1%). Of note, the flaxseed oil diet did result in significant increases of two saturated fatty acids in preen oil of female and male chickens; C16:0 ($P < 0.05$) by 18% and 21%, and C17:0 ($P < 0.01$) by 70% and 33%, respectively. The reason for this, and the mechanism underlying these changes are unclear. Importantly, the increased levels of ALA and the long-chain n-3 fatty acids in the blood of flaxseed oil fed birds was not reflected in the fatty acid composition of the preen oil.

The lack of any substantial effect of diet on preen oil composition in the present study agrees with the findings of Pan *et al.* (1979). That study used blends of animal fat, soy oil, palm oil and coconut oil but the resulting diets were not as varied in their fatty acid composition as the diets used in the present study. However, Apandi and Edwards (1964) reported a dietary effect on the fatty acid composition of preen oil in chicken fed with a fat-free diet against chickens fed with a corn oil diet. The main difference they found was that C25:0 comprised 42.5% of the preen oil in the corn oil-fed treatment but was not detected in the fat-free treatment. Not surprisingly, the appearance of C25:0 was accompanied by a proportional decrease in C18:0, C19:0 and C20:0. Contemporary analysis of corn oil does not show the presence of any C25:0 so it is unclear where this may have come from.

Throughout the study there were small but significant gender differences in the whole blood and preen oil fatty acid composition. As the birds were offered the same diets, these differences might be due to differences in intestinal absorption and/or hepatic metabolism of fat and/or fatty acids. Similar small but significant differences in the fatty composition of meat from male and female chickens eating the same diet have been reported (Carragher *et al.* 2016). In contrast, Pan *et al.* (1979) reported no significant difference in preen oil composition due to gender.

Conclusion

The preen oil of meat chickens is dominated by saturated fatty acids, which make up to 97% of the total fatty acids, with 13 different medium- to long-chain saturated fatty acids (C8:0–C22:0) detected in this study. The preen oil contained several odd-chain fatty acids, which suggests they may be derived from lipolysis by the uropygial gland and/or its microbiome. Diet and gender had small but significant effects on levels of specific saturated fatty acid in the preen oil. The fatty acid composition of the preen oil did not reflect the more diverse fatty acid compositions of the diet or whole blood. Therefore, the results of this study clearly indicate that measuring the fatty acid profile of preen oil is not a suitable alternative approach for predicting the fatty acid composition of the blood of meat chickens.

Acknowledgements

The authors are grateful to the technical support staff at the Pig and Poultry Science Program, Roseworthy Campus and FOOD*plus*, Waite Campus, University of Adelaide. We also acknowledge the financial support of The University of Adelaide, and the South Australian Department of Further Education, Employment, Science and Technology, Australia.

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Appendix 3. Animal Ethics

PIRSA ANIMAL ETHICS COMMITTEE APPROVAL FORM

APPLICANT:	**Dr Bob Hughes**
DEPARTMENT:	**School of Animal & Veterinary Sciences, Faculty of Sciences, The University of Adelaide SA 5005**
TITLE OF PROJECT:	**Omega 3 fatty acid enrichment of chicken meat**

APPLICATION NUMBER	15/13
ANIMAL ETHICS COMMITTEE APPROVAL	Approved
DATE OF COMMENCEMENT	26 September 2013
DATE OF CONCLUSION	31 December 2014
TERM OF PROTOCOL DESCRIBED	1 year & 3 months
NUMBER OF ANIMALS	720 Chickens

COMMENTS:

It would be appropriate to record the AEC approval number on the daily clinical observation record sheet. If Dr Carragher is to be involved in animal observations, the Committee requests that he undertake Animal Ethics training at the earliest opportunity. Approved subject to the foregoing being adhered to.

SIGNED: .... **Date: 5 September 2013**
Jenny Harvy, Executive Officer
PIRSA Animal Ethics Committee

Ms Jenny Harvy
Executive Officer
PIRSA Animal Ethics Committee
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Appendix 3

RESEARCH BRANCH
Office of Research Ethics, Compliance and Integrity

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email: helen.malby@adelaide.edu.au
CRICOS Provider Number 00123M

APPLICANT: Dr J Carragher
School of Agriculture, Food and Wine

PROJECT TITLE: ***Feeding broiler breeders with omega 3 fatty acids and epigenetic effects on progeny***

PROJECT NUMBER: **S-2015-067** RM No: 0000020008

APPROVED BY THE UNIVERSITY OF ADELAIDE ANIMAL ETHICS COMMITTEE SCIENCE

FOR THE PERIOD 18 MAY 2015 to 31 DECEMBER 2015

INVOLVING:	Chicken (Cobb 500 N/A)	180
	Chicken (Cobb female line Female)	60
	Chicken (Cobb male line Male)	3

The AEC has not specified additional conditions of approval

This approval is subject to notification of project commencement. Please send an email to the AEC Secretary helen.malby@adelaide.edu.au giving the date when animal work actually begins.
Refer also to the accompanying letter setting out requirements applying to approval of this project.

.....

PROFESSOR R RUSSELL, Convenor *Date:* 23.4.15

Appendix 3

THE UNIVERSITY of ADELAIDE

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Office of Research Ethics, Compliance and Integrity

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23 April 2015

Dr J Carragher
School of Agriculture, Food and Wine

Dear Dr Carragher

Ref: ANIMAL ETHICS COMMITTEE PROJECT NO: S-2015-067

Feeding broiler breeders with omega 3 fatty acids and epigenetic effects on progeny

I write to advise you that the Animal Ethics Committee has approved the above project for the period from 18 May 2015 to 31 December 2015. Please refer to the enclosed endorsement sheet which gives further details and may include particular conditions applying to this approval. All use of animals is conditional on compliance with the Australian code for the care and use of animals for scientific purposes, 8th Edition, 2013.

- *You have personal responsibility for all matters related to the welfare of the animals you use and you must act in accordance with all requirements of the Code.*
- *Any adverse or unexpected effects that impact on animal wellbeing which occur during the period of the approved project must be reported promptly to the AEC.*
- *You must ensure that records of the use and monitoring of animals used in this project are maintained. Records should include the origin and fate of issued animals, how animal welfare was assessed, any unexpected negative impact on animal wellbeing and notation of procedures.*
- *You must provide an annual report to the AEC - the continuation of all projects is subject to receipt of written annual reports that should advise on: (i) what progress has been achieved; (ii) any problems that may have interfered with progress of the project; (iii) how many animals have been used; (iv) whether the wellbeing of the animals is consistent with that anticipated in the proposal; (v) whether any changes are envisaged; and (vi) whether the project is meeting its aims. You must inform the Committee when an approved project is completed or discontinued.*

The above project number and approval expiry date must be included on the animal cage/pen label. All animals housed on University premises and subject to multiple ethics approvals must be identified by the display of the relevant approval numbers from all approving AECs.

Statistics regarding the use of animals in this project must be provided to the AEC annually for collation, usually as part of the annual report. It is necessary to apply to the AEC for approval if the project is to continue for a longer period of time, if additional animals are required or if any change to procedure is proposed. Please refer to the AEC website for further information on reporting and other matters including the *AEC Animal User's Handbook: information about your responsibility to use animals humanely and ethically*: http://www.adelaide.edu.au/ethics/animal/.

Yours sincerely

RICHARD RUSSELL
Convenor, Animal Ethics Committee Science

Appendix 3

PIRSA ANIMAL ETHICS COMMITTEE
MINOR AMENDMENT APPROVAL TO USE ANIMALS FOR RESEARCH OR TEACHING

PRINCIPAL APPLICANT: Dr John Carragher, University of Adelaide, School of Agriculture Food and Wine, Waite Campus

TITLE OF PROJECT: "Feeding broiler breeders with omega 3 fatty acids and epigenetic effects on progeny"

APPLICATION NUMBER	**# 10/15**
ANIMAL ETHICS COMMITTEE APPROVAL	**Minor Amendment Approved**
DATE OF COMMENCEMENT	18 May 2015
DATE OF CONCLUSION	31 December 2015
ANIMAL TYPE & NUMBERS	243 Chicken Cobb male and female

SPECIAL CONDITIONS

The Committee noted

- Change in animal use is approved

NOTE: The general conditions are listed on the following page.

SIGNED: ... . **Date:** 22 / 09 / 2015

Sandy Wyatt, Executive Officer

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Appendix 3

GENERAL CONDITIONS OF ANIMAL ETHICS APPROVAL

Please note the following conditions apply to all approvals from the PIRSA Animal Ethics Committee.

The project must be conducted in accordance with the protocol approved by the AEC, and with the *Animal Welfare Act 1985* and Regulations and with the *Australian Code of Practice for the care and use of animals for scientific purposes 8th edition 2013.*

Any change proposed to the project must be submitted to the AEC for approval before the change is implemented.

The minor amendment form is available through the link below.

The principal applicant must ensure that all personnel involved with the project are aware of the requirements of the Act, the Code and the protocol as approved by the AEC.

All personnel are personally responsible for the welfare of the animals they use and monitor during this project, and the principal applicant has primary responsibility for animal welfare.

The use and monitoring of animals in this project must be recorded and the records must be maintained and retained. The project, animals and records may be inspected by the AEC or its delegates during the project, and the records and site after the project.

The principal applicant must notify the AEC as soon as possible of any unexpected adverse events that may affect the wellbeing of the animals during the project. The adverse event form is available through the link below.

The principal applicant must submit an ANNUAL REPORT/STATISTICS RETURN for the project for every calendar year during the life of the project. The report must be lodged with the AEC by 28 February the following year. The form is available through the link below.

Forms and information - http://www.pir.sa.gov.au/research/pirsa_animal_ethics_committee

Appendix 4. Published manuscripts

4.1. Published manuscript 1 for Chapter 2

Statement of Authorship

Title of Paper	The effect of different dietary fats on the fatty acid composition of several tissues in broiler chickens
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	European Journal of Lipid Science and Technology

Principal Author

Name of Principal Author (Candidate)	Khaled Kanakri
Contribution to the Paper	Performed the experimental work (field part and fatty acid analysis), statistical analysis, data interpretation, wrote the manuscript and acted as corresponding author
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper
Signature	Date 3/11/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution

Name of Co-Author	John Carragher
Contribution to the Paper	Supervised the analysis of samples, the statistical analysis and manuscript outlining and revision.
Signature	Date 3/11/17

Name of Co-Author	Robert Hughes
Contribution to the Paper	Contributed to the animal care and contributed to manuscript revision.
Signature	Date 3.11.17

Please cut and paste additional co-author panels here as required

Name of Co-Author	Beverly Muhlhausler		
Contribution to the Paper	Assisted in data interpretation and critical revision of the manuscript.		
Signature		Date	6/11/2017

Name of Co-Author	Robert Gibson		
Contribution to the Paper	Principal supervisor of the project, supervised the work development and revised the manuscript.		
Signature		Date	6/11/17

RESEARCH ARTICLE
Fatty Acids
European Journal of Lipid Science and Technology
www.ejlst.com

The Effect of Different Dietary Fats on the Fatty Acid Composition of Several Tissues in Broiler Chickens

Khaled Kanakri, John Carragher, Robert Hughes, Beverly Muhlhausler, and Robert Gibson*

The type of fat used in formulating broiler chicken diets can affect growth performance, influence the fatty acid composition of different tissues and has consequences for bird health and nutritional value for the consumer. This study aimes to address the hypothesis of whether these effects are specifically due to the variation in the fatty acid composition of the diets, that is, the proportion of different saturates, monounsaturates (n-7 and n-9) or polyunsaturates (n-3 or n-6), or other factors (physical properties, solid/liquid and source, plant/animal). A total of 480 male Cobb 500 broilers are fed ad libitum on one of six diets containing 4% w/w of either: beef tallow, flaxseed, corn, canola, macadamia, or coconut oil (eight replicates/treatment) for 6 weeks. At harvest, there are no significant differences in productivity parameters nor in the crude lipid content of different tissues between dietary treatments. There are, however, substantial qualitative differences in the fatty acid profiles of all tissues. The levels of specific fatty acids in all tissues except the brain, are positively correlated with the levels of the same fatty acids in the diet however, the strength of the correlations varied between different fatty acids.
***Practical Applications*: The results of the current study demonstrate that the dietary fatty acids types and proportions largely determines the fatty acid profile in edible tissues (meat, adipose, liver, and heart). The strong correlations and regressions between diet and tissue fatty acid levels validate the ability to predict the tissue fatty acid profile of broilers based on their dietary fat composition. Contrary to our hypothesis, dietary fat type had no influence on the growth parameters which makes us speculate whether such differences in similar studies only become apparent in situations where the birds are also under some level of environmental or social stress. This information will assist poultry feed manufacturers and broiler producers in making decisions about selection of fats with known nutritional and health benefits for inclusion in chicken feed.**

1. Introduction

The worldwide growth in the poultry meat sector (4.3% p.a., mostly chicken) is much faster than that of any other type of meat.[1] Dietary lipid is a major source of energy, a source of essential macronutrients and fat-soluble micronutrients.[2,3] The principal external factors determining carcass composition in monogastrics are arguably the quantitative and qualitative properties of the feed. Indeed, the influence of dietary fat type on the fatty acid composition of broiler chicken tissues (in particular meat) has been investigated for decades.[4,5] In addition, broiler chickens of different strains[6,7] respond differently to dietary fats and the fatty acid profile of chicken tissues reflects both the ingested and the in vivo synthesised fatty acids.[8] Many trials have associated a significant improvement in broiler performance with dietary fats that differ in fatty acid composition.[9–14]

Commercially, poultry feed manufacturers typically add different animal/plant fats individually or in combinations of two or more (usually at 2–5% of basal diet weight) to other ingredients to formulate diets that deliver the recommended levels of energy, macro- and micronutrients for the health and growth of the birds.[3,15,16] In the chicken industry, the decision about which fat type(s) to use is mainly based on the cost per unit of energy. Other factors

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DOI: 10.1002/ejlt.201700237

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that are taken into consideration include the local availability of the fat source, suitability for the birds determined by digestibility,[3,17] effect on growth performance[16,10,18] and the health parameters,[19] impact on sensory traits of the meat[20] or delivering health benefits for consumers (e.g., enriching meat with omega-3 polyunsaturated fatty acids, n-3 PUFA).[21,22] Clearly, whatever the reasons, the choice of a particular fat source needs to be cost-effective and increase the profit margin to the producer.[16]

Different fatty acids have differential health effects on chickens. Consumption of unsaturated fatty acids (in particular n-3 PUFA) has been associated with some beneficial health effects to chickens.[23,24] However, there is a need for caution to be taken when using fats rich in MUFA and PUFA as they are more susceptible to oxidation. Thereby, there is the risk of the broilers being exposed to oxidative products which can adversely affect their health and productivity, as well as the sensory quality and shelf-life of their products.[3,25–27]

In most industrialized countries, a high meat intake contributes to a higher than recommended SFA:MUFA/PUFA intake.[28] In addition, there are suggestions that increased intake of omega-6 polyunsaturated fatty acids (n-6 PUFA) relative to n-3 PUFA may be associated with adverse health effects, including an increase in chronic inflammatory disorders.[22,26,29] Simopoulos[22] reviewed the role of the n-6:n-3 ratio in human diseases and the importance of rectifying the imbalance in this ratio in human diet for health benefits. Indeed, it has been shown that n-6:n-3 long chain fatty acid balance in the phospholipid fraction of human plasma can be altered by consuming a relatively moderate amount (160 g day^{-1}) of n-3 enriched chicken meat for 4 weeks.[21] In this regard, a large number of research studies have been concerned with trying to enrich chicken meat with n-3 PUFA.[9,20,30–32] Many of these studies have focused on feeding broilers high levels of a sustainable source of ALA (often from flaxseed oil) and lower levels of n-6 (especially, LA). This because these precursors use the same metabolic pathway and compete with each other in incorporation of their derivatives into broiler tissues.[33]

Some organs of broilers, such as liver and heart, are classified as by-products in the poultry industry, but these organs are nevertheless edible and are consumed on a large scale worldwide. Seong et al.[34] analysed these tissues and recommended consumption of liver and heart for their nutritional value citing the high ratio of PUFA and MUFA to the SFA in comparison with muscle. From a physiological point of view, the liver is a vital organ because this is where the majority of fat is metabolised. Markers of liver functional status include liver weight (hepatosomatic index, HSI, as a proportion of body weight), neutral lipid and fatty acid composition.[35]

In general, the biochemical characteristics of dietary fatty acids, such as length (molecular weight), saturation, isomerism, polarity and position of double bond(s), as well as their proportion within the diet, affect intestinal digestion, absorption and hepatic metabolism, thereby determining their bioavailability in the serum. Furthermore, their deposition into the main lipid fractions (phospholipids and triglycerides) in different tissues can be regarded as functional substances or energy stores.[3,36,37] The dietary influence of fatty acid intake can be tracked by analysing the internal tissues (including blood)[38,39] or non-invasively (e.g., the excreta)[40] of broilers. The importance of using a regression equation (to predict the tissue fatty acid concentration from a particular combination of dietary fatty acids) in addition to a correlation coefficient (to measure the strength of the prediction) has been reviewed by Puvača et al.[41] Using this approach, these authors proposed a predictive mathematical equation to estimate the incorporation of individual fatty acids into chicken tissues based on the dietary level of fatty acids.

There has indeed been a number of studies focused on the dietary fat effects on meat chicken in different aspects, including previous studies by our research group, nonetheless there is still no consensus on what levels or types of dietary fats are most appropriate for broiler chickens, largely because the studies have all differed in the way they have been carried out. The current study sought to address the hypotheses that differences in growth parameters sometimes observed when broilers are fed different dietary fats is due to (a) differences in the fatty acid profile, (b) whether the fat is solid or liquid at room temperature, or (c) whether the fat is from an animal or plant source.

2. Materials and Methods

All procedures contributing to this work complied with the ethical standards of the relevant national guides on the care and use of animals. This study was approved by the Animal Ethics Committee of the University of Adelaide (approval S-2013-152) and the Department of Primary Industries and Regions South Australia, Australia (approval 15/13).

2.1. Housing the Broilers

A total of 480 day-old male chicks (Cobb 500 strain) were obtained from the Baiada Hatchery (Willaston, SA, Australia) and transferred to South Australian Research and Development Institute (SARDI) facility (Roseworthy, SA, Australia). A complete randomised block design (8 pens/treatment) was implemented. Birds were randomly distributed into 48 groups of 10 and each was allocated to a raised rearing floor pen (1.2 × 0.9 m each) in two sheds (24 pens in each). Broilers were reared on sawdust and shavings and had continuous free access to feed and water during the entire experimental period. Pens were heated by infrared brooder lamps (175 W) in the first 3 weeks. Temperature in the rooms was 27 °C for the first 4 days, gradually decreased to 20 °C and then maintained at this temperature until the end of experiment.

2.2. Experimental Diets

The study included 6 experimental diets which were produced by mixing 4% (w/w) of a selected fat (**Table 1**) with a basal (starter/finisher) diet (Ridley Agriproducts, Australia). The fats were: beef tallow (Ridley Agriproducts, Australia), flaxseed oil (Four Leaf Oils, Australia), corn oil (Daisy, Malaysia), canola oil (Foodland, Australia), macadamia oil (Macoils, Australia), and coconut oil (Banaban, Fiji). The composition and nutritional

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Table 1. The main differences between the experimental fats used in the experiment.

	Fat properties		
Fat type	Fatty acid content	Physical status[a)]	Source
Beef tallow	Moderate SFA and MUFA	Solid	Animal
Flaxseed oil	High n-3 PUFA	Liquid	Plant
Corn oil	High n-6 PUFA	Liquid	Plant
Canola oil	High n-9 MUFA	Liquid	Plant
Macadamia oil	High n-7 MUFA	Liquid	Plant
Coconut oil	High SFA	Solid	Plant

SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids.
a) At room temperature.

profiles of the basal diets are shown in **Table 2**. Apart from the variation in the fatty acid composition (**Table 3**), all experimental diets were isocaloric, nutritionally identical, and met requirements for efficient growth.[42] Batches of each feeds were prepared weekly and samples (20 g) were taken from each batch and stored at −20 °C. A representative homogenised sample ($n = 6$ per treatment) of the weekly batches were analysed for determination the crude fat content and fatty acid profile of each diet. Broilers were fed the experimental diets for the entire 42 day growth period; the starter diet (crumble form) was fed in the first 3 weeks and then the finisher diet (pellet form) in the final 3 weeks of the experiment.

Table 2. Ingredient composition and nutrient content of the basal diets.[a)]

	Basal diet			Basal diet	
Ingredients (g kg^{-1})	Starter	Finisher	Nutrient content (g kg^{-1})	Starter	Finisher
Wheat fine	614.2	707.7	Crude protein	225.1	186.7
Barley fine	50.0	50.0	Crude fat	23.5	30.0
Tallow mixer	03.7	10.0	Crude fibre	32.3	29.2
Blood meal (91% CP)	10.0	00.0	Calcium	09.8	08.5
Soybean meal	251.7	166.7	Available phosphorus	04.5	04.2
Limestone small	08.6	06.4	Na	01.8	01.7
Monocalcium phosphate	01.4	00.0	K	08.0	06.5
Sodium chloride	02.1	01.8	CL	02.0	01.8
Sodium bicarbonate	01.6	01.8	Lysine	12.3	09.1
Choline chloride 75%	00.3	00.4	Methionine	05.7	04.7
DL-methionine 58.1	02.9	02.1	Cystine	03.9	02.8
L-threonine 73.7	01.3	02.5	Threonine	07.9	07.6
Rovabio Excel LC	00.2	00.2	Leucine	14.3	11.2
Meat meal	37.3	37.0	Isoleucine	07.8	06.4
Ronozyme NP CT	00.2	00.2	Tryptophan	02.3	01.8
Mineral/vitamin premix[a)]	10.0	10.0	Arginine	12.4	09.8
L-lysine sulphate 70	04.3	03.3	Valine	09.3	07.2

a) All vitamins/minerals met or exceeded the recommendations by the National Research Council,[2] the Metabolisable Energy (MJkg^{-1}) was 12.14 and 12.56 for starter and finisher basal diets, respectively.

2.3. Production Performance Parameters

Group body weight (BW) of chicks ($n = 10$, minus the number of mortality) for each pen was taken on days 1, 7, 14, and 21 and used to estimate the individual BW of birds (group weight/number of birds in pen). The BW of each bird was then recorded separately on days 28, 35, and 40. The feed intake (FI) of birds in each pen was also recorded at the same timepoints and used to estimate the individual FI per bird (feed eaten by pen/number of birds in pen) and to calculate the feed conversion rate (FCR); kg feed/kg body weight gain. The number of deaths and culls was recorded on a daily basis and it was taken into account in calculating the FI and FCR and used to calculate mortality rate in each dietary treatment.

2.4. Sampling of Tissues

On the day of slaughter (day 42), 1 bird/pen was randomly selected and euthanized by cervical dislocation. For each sampled bird the body and liver weights were recorded and HSI was calculated (liver weight as% of live weight). Whole blood was obtained by cardiac puncture and drops drops were applied to a PUFAcoat™ dry blood spot (DBS) card to stabilise the long chain polyunsaturated fatty acids.[43] Approximately 30 g of lean skinless breast and leg meat, 5 g of liver and heart, and 2 g of the adipose tissue from the abdominal fat pad were collected into clean plastic vials and immediately placed on dry ice. The head was removed from the carcass, placed in a sealed plastic bag and frozen in dry ice for transfer to the laboratory where the brain was removed. All tissue samples were stored at −20 °C pending subsequent determination of crude fat content and fatty acid composition.

2.5. Fatty acid Analysis

Crude lipid was extracted from 0.1–0.5 g of representative homogenised feed and tissue samples using chloroform/methanol (2:1, v/v). The gravimetric approach was utilised to estimate total lipid content as previously described.[44] A volume of the chloroform/methanol solution containing 2–3 mg of the extracted crude lipids was used for fatty acid analysis. Transmethylation of the fatty acids was performed with 1% H_2SO_4 in methanol at 70 °C for 3 h. After cooling to room temperature, the resulting fatty acid methyl esters (FAME) were extracted with *n*-heptane (2 mL) and transferred into gas chromatography (GC) vials containing approximately 30 mg of anhydrous sodium sulphate as the dehydrating agent and stored at −20 °C until GC analysis. The FAME were separated using a Hewlett-Packard

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Table 3. Fat content and fatty acid composition (% of total fatty acid) of different basal and experimental[a)] diets.

	Basal diets		Tallow diets		Flax seed diets		Corn diets		Canola diets		Macadamia diets		Coconut diets	
Diet[b)]	Starter[c)]	Finisher[d)]	Starter	Finisher	Starter	Finisher	Starter	Finisher	Starter	Finisher	Starter	Finisher	Starter	Finisher
Fat%	3.9	3.9	6.9	7.1	6.9	7.6	7.2	7.3	7.3	7.0	6.9	7.2	7.0	7.2
SFA[e)]	26.1	29.2	35.6	36.1	18.1	19.1	20.0	20.5	16.5	17.3	20.3	20.8	64.8	63.5
n-9 MUFA[f)]	31.9	28.7	34.1	34.0	24.8	25.3	29.0	29.6	44.4	44.4	45.6	45.9	14.9	16.0
n-7 MUFA[g)]	3.8	4.0	4.6	4.7	2.0	2.2	1.9	2.2	3.4	3.7	14.7	14.8	1.5	1.8
n-3 PUFA[h)]	5.2	3.7	3.1	2.7	29.9	29.0	2.8	2.5	7.0	6.6	2.1	1.8	2.0	1.7
n-6 PUFA[i)]	32.4	32.5	20.4	20.1	24.6	23.6	45.5	44.4	27.8	27.0	16.5	15.9	16.1	16.2
Trans	0.6	1.3	1.6	1.6	0.5	0.6	0.5	0.5	0.5	0.6	0.4	0.5	0.5	0.5
n-6:n-3	6.23	8.78	6.58	7.44	0.82	0.81	16.25	17.76	3.97	4.09	7.86	8.83	8.05	9.53
MUFA:SFA	1.35	1.14	1.10	1.09	1.49	1.45	1.56	1.57	2.92	2.80	2.99	2.93	0.26	0.29
MUFA:PUFA	0.94	0.92	1.67	1.73	0.49	0.53	0.65	0.68	1.39	1.44	3.26	3.45	0.92	1.01
PUFA:SFA	1.44	1.24	0.66	0.63	3.01	2.75	2.42	2.29	2.11	1.94	0.92	0.85	0.28	0.28

[a)] The experimental diets were made by adding 4% w/w of the relevant fat (tallow, flaxseed, corn, canola, macadamia and coconut oil) to the basal diets; [b)]values represent the mean ($n = 2$ basal diets, $n = 6$ experimental diets) based on the wet weight; [c)]starter diet was fed to the broilers in the first 3 weeks; [d)]finisher diet was fed to the boilers in the last 3 weeks of the entire 6 weeks growth period; [e)]SFA = Saturated fatty acid; [f)]n-9 MUFA = Omega 9 monounsaturated fatty acid; [g)]n-7 MUFA = Omega 7 monounsaturated fatty acid; [h)]n-3 PUFA = Omega 3 polyunsaturated fatty acid; [i)]n–6 PUFA = Omega 6 polyunsaturated fatty acid.

6890 GC (Hewlett-Packard, CA, USA) equipped with a 50 m capillary column (0.32 mm ID) coated with 70% cyanopropyl polysilphenylene-siloxane with a film thickness of 0.25 μm (BPX-70, SGE Pty Ltd, Melbourne, VIC, Australia). The initial oven temperature was 140 °C and was programmed to reach 220 °C (rise rate = 5 °C min^{-1}) and held for 2 min then programmed to reach 260 °C (rise rate = 20 °C min^{-1}) and held for 8 min. Helium (He) was used as an inert carrier gas at a velocity of 33 cm s^{-1}. and the inlet split ratio was 20:1. The injector temperature was set at 250 °C and the detector (flame ionisation) temperature at 300 °C. The FAME peaks were identified based on the retention time relative to authentic lipid standard (GLC 463) from NuChek Prep Inc. (Elysian, MN, USA) using the software package Agilent GC Chemstation (Agilent Technologies Inc., Palo Alto, CA, USA). Each peak from a trace was expressed as the relative percentage of the total FAME in the sample. The detection limit of each fatty acid was 0.05% of the total fatty acids.

2.6. Statistical Analyses

The experimental unit for all parameters was the pen, with data from either all of the birds (performance parameters) being averaged for the pen, or one randomly selected bird from each pen (tissue parameters). The effects of dietary treatment on growth performance parameters (BW, FI and FCR) and HSI were determined by one-way ANOVA using SAS 9.3 for Windows. The mortality rate was compared using non-parametric analysis (Kruskal-Wallis test). All reported data from fatty acid analysis of tissues are the means of 8 replicates with the standard error of the mean (SEM). The effects of dietary treatment on tissue fat content and fatty acid profile were determined by one-way analysis of variance (ANOVA). A Tukey's multiple comparison test was implemented when the ANOVA revealed significant differences between dietary treatments ($P < 0.05$). Pearson correlation coefficients were used to assess the relationship between dietary and tissue fatty acid levels (r). Correlation coefficients and linear regressions were assessed by Pearson correlation using SPSS version 22 for Windows (IBM Crop., NY, USA), and a P value of <0.05 was considered statistically significant. Microsoft® Excel 2013 software was utilised to plot the linear regression.

3. Results and Discussion

3.1. Total Fat and Fatty Acid Content of Experimental Diets

The crude fat percentage of the basal diet was 3.9% and by adding 4% w/w fat the 6 experimental diets attained crude fat levels between 6.9–7.6%, with no significant difference between the different diets. The fatty acid profile of the 6 experimental diets reflected the wide range in composition of the added fats, as intended (Table 3). The n-6:n-3 ratio of the diet was highest in the finisher corn oil-based diet (≈18:1), and lowest in the flaxseed oil-based diet (≈1:1).

3.2. Growth Performance

None of the growth performance parameters of broilers were significantly different between the 6 dietary groups (**Table 4**). At the end of the study the average live BW was 3367 g/bird, FI was 5311 g/bird, and FCR was 1.58 g feed: g gain when data from all birds were combined. The overall mortality rate was 4.4% and not different between treatments. These outcomes are in agreement with our previous studies using similar dietary treatments[30,45,46] and many others[16,42,47–51] and within the commercial recommendations for male broilers of this strain.[52] However, in a previous experiment ($n = 3{,}840$ birds),[9] which

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Table 4. The dietary effect on the growth performance parameters of broilers at different experimental timepoints.

	Growth performance parameter[a)]			
Growth days	BW[b)]	FI[c)]	FCR[d)]	Mortality rate
0	46	0	0	0
7	191	149	1.03	0
14	525	616	1.29	1.67
21	1085	1389	1.35	2.08
28	1815	2517	1.42	2.92
35	2711	3991	1.50	3.54
40	3367	5311	1.60	4.38

[a)] The effects of dietary treatment on the accumulative pooled BW, FI and FCR parameters were determined by one-way ANOVA where the pooled mortalities and culls rate statistically analysed by Kruskal-Wallis nonparametric method. This effect was not significant $P > 0.5$ for all parameters at all timepoints; [b)]BW: The average body weight g/live bird; [c)]FI: Feed intake g/live bird; [d)]FCR: Feed conversion ratio = g feed: g gain.

was specifically designed to determine the influence of the dietary flaxseed oil on growth performance, we found that the high n-3 diet improved the BW without increasing the FI and therefore reduced the FCR by 10% in comparison to the tallow based diet. This finding was particularly interesting and important because beef tallow is widely used in making commercial poultry feed. In another study, Balevi and Coskun[10] fed broilers diets containing 9 types of plant and animal fats and also demonstrated that BW and FCR were affected by dietary fat type, with the corn oil diet being the best performer. A number of other studies have that feeding high MUFA/PUFA diets benefits productivity of the birds by reducing FCR.[11–13] In contrast, Scaife et al.[14] found that a tallow diet increased BW, however this was accompanied by an increase in FI resulting in a rise in FCR compared to three single and six combined plant and marine oil-based diets. We speculate that the lack of consistency in such experiments may be due to different experimental conditions (e.g., broilers' age, breed, length, and timepoints of feeding, fat types and fat inclusion level) and/or aspects of the housing environment (e.g., stocking density, air quality, litter vs. cage floor) that might stress the birds and accentuate the influence of some fat types on performance.

In the current study, the HSI% (data not shown) ranged from 1.98 (coconut oil treatment) to 2.16 (flaxseed oil treatment) but was not significantly different between all treatments, in agreement with number of similar studies.[47,53]

3.3. Total Fat Content of the Tissues

The average crude fat percentages of the adipose, liver, heart, leg, and breast meat tissues were 89.4, 5.7, 3.5, 2.4, and 1.9%, respectively, and did not differ significantly between diet groups. However, the brain tissue of corn oil diet fed broilers had significantly ($P < 0.05$) lower crude fat content (5.8 vs. 6.7% as mean of other dietary treatments). This finding aligns with that of a previous study, in which feeding broilers diets supplemented with beef tallow or flaxseed oil did not affect the crude fat percentage of the meat tissues.[16] Consistent with the results of the current study, Mašek et al.[50] also found that the crude fat content in liver (5.6%) was not different between broilers fed either 5% sunflower or flaxseed oil diets. In contrast, however, other studies have found that increasing the dietary PUFA:SFA ratio can lower fat deposition in various tissues.[54] For instance, González-Ortiz et al.[47] found that feeding broilers with a high n-3 diet (10% crude fat from flaxseed and fish oil) reduced the crude fat content in the thigh meat and the size of the abdominal fat pad, but there was no effect on liver fat content in comparison to birds that were fed a tallow based diet. The latter results may be complicated by the high dietary fat inclusion level in their study, which can act to suppress fat utilisation.[55] Similarly, Scaife et al.,[14] using 4 plant and animal fats either alone or in blends reported that the crude fat percentage of adipose tissue was highest in the birds fed the soybean oil diet, whereas liver tissue had the lowest fat content in the birds fed the rapeseed (canola) oil diet. Thus, there is disagreement between studies regarding the impact of dietary fatty type/concentration on crude fat percentage in different tissues. Again, this may be due to physiological or environmental variations between the different studies.

3.4. Fatty Acid Composition of Tissues

Rather than reporting the data on individual fatty acids, we chose to present the total for each fatty acid class measured in the diets and tissues (e.g., total saturates, total n-9 MUFA etc). The reasons for this are that all of the fatty acids measured in the tissues were also found in the diets, and there was limited amounts of de novo synthesis (with the exception of ALA conversion to eicosapentaenoic acid (EPA), docosapentaenoic acid and docosahexaenoic acid (DHA), particularly in flaxseed oil group[30–31] and LA conversion to arachidonic acid in all groups[20]). The fatty acid profiles of the 7 tissues sampled in this study are shown in **Table 5**, and the relationship between the diet and tissue content are described by regression variables and quantified by r values as in **Table 6** and **Figure 1**. The role of the dietary fatty acid classes, the interaction of different types within the same diet treatment on different tissues and the comparison between these correlations are discussed below.

3.4.1. Blood

The predominant fatty acid groups in the whole blood were similar to those of the diet (Table 5). The SFA were the most abundant fatty acid group in blood across all treatments (35–46%), and highest in broilers fed the coconut oil diet, followed by the tallow treatment broilers. The second most abundant fatty acid group in whole blood was n-6 PUFA, but interestingly there was less variance in n-6 levels in the blood between the treatments (range 24–38%) than was present in the diets (range 16–44%). As expected, the highest percentage of blood n-6 PUFA was in the corn oil group where the lowest was in the flaxseed oil group. The blood levels of both n-9 and n-7 MUFA were highest in the macadamia oil group, in line with their predominance in

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Table 5. Fatty acid profile[a)] of seven tissues of broilers fed six experimental diets[b)] for 42 days.

Tissue	Treatment	SFA[c)]	n-9 MUFA[d)]	n-7 MUFA[e)]	n-3 PUFA[f)]	n-6 PUFA[g)]	*Trans*
Blood	Tallow	$39.5^{a}\pm0.4$	$25.4^{a}\pm1.2$	$4.3^{a}\pm0.3$	$3.3^{a}\pm0.2$	$26.2^{ab}\pm1.1$	$0.7^{a}\pm0.0$
	Flaxseed oil	$36.8^{bc}\pm0.2$	$20.8^{b}\pm0.6$	$2.6^{b}\pm0.2$	$14.9^{b}\pm0.3$	$24.3^{a}\pm0.5$	$0.3^{b}\pm0.1$
	Corn oil	$37.2^{c}\pm0.5$	$20.1^{b}\pm1.2$	$2.6^{b}\pm0.2$	$1.7^{c}\pm0.1$	$37.6^{c}\pm0.9$	$0.4^{ab}\pm0.0$
	Canola oil	$35.8^{bc}\pm0.4$	$25.6^{a}\pm0.6$	$3.2^{bc}\pm0.1$	$4.9^{d}\pm0.3$	$29.8^{d}\pm0.5$	$0.4^{ab}\pm0.1$
	Macadamia oil	$35.0^{b}\pm0.3$	$30.2^{c}\pm0.8$	$7.0^{d}\pm0.4$	$2.3^{c}\pm0.1$	$24.5^{a}\pm0.5$	$0.6^{ab}\pm0.2$
	Coconut oil	$46.0^{d}\pm0.6$	$18.9^{b}\pm0.5$	$4.0^{ac}\pm0.2$	$2.4^{ac}\pm0.1$	$27.6^{bd}\pm0.4$	$0.5^{ab}\pm0.0$
	P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Liver	Tallow	$42.2^{ab}\pm1.3$	29.1 ± 2.5	$4.4^{abc}\pm0.4$	$3.4^{ac}\pm0.5$	$18.8^{ab}\pm2.3$	$1.6^{a}\pm0.2$
	Flaxseed oil	$41.6^{a}\pm0.7$	30.1 ± 2.7	$3.8^{ab}\pm0.4$	$9.1^{b}\pm1.2$	$14.0^{a}\pm1.8$	$1.2^{bc}\pm0.1$
	Corn oil	$43.4^{ab}\pm0.8$	28.2 ± 1.6	$3.3^{a}\pm0.3$	$1.3^{a}\pm0.2$	$22.4^{b}\pm2.0$	$1.1^{bc}\pm0.0$
	Canola oil	$41.7^{a}\pm0.3$	28.5 ± 1.5	$3.1^{a}\pm0.2$	$4.6^{c}\pm0.5$	$20.4^{ab}\pm1.2$	$1.4^{ab}\pm0.1$
	Macadamia oil	$41.3^{a}\pm0.7$	31.1 ± 2.2	$5.4^{c}\pm0.2$	$2.4^{ac}\pm0.5$	$18.0^{ab}\pm2.2$	$1.4^{ab}\pm0.1$
	Coconut oil	$45.4^{b}\pm0.7$	27.2 ± 1.9	$5.3^{bc}\pm0.6$	$2.0^{ac}\pm0.3$	$18.1^{ab}\pm1.9$	$1.2^{bc}\pm0.0$
	P value	<0.05	>0.05	<0.001	<0.001	<0.001	<0.01
Breast	Tallow	$33.7^{a}\pm0.4$	$36.9^{acd}\pm0.7$	$8.5^{a}\pm0.1$	$3.0^{a}\pm0.1$	$16.4^{ab}\pm0.4$	$1.3^{a}\pm0.0$
	Flaxseed oil	$28.1^{b}\pm0.6$	$32.0^{ab}\pm0.8$	$6.2^{b}\pm0.2$	$16.8^{b}\pm0.3$	$15.8^{ab}\pm0.4$	$0.8^{b}\pm0.0$
	Corn oil	$30.9^{ab}\pm1.1$	$33.9^{ac}\pm1.4$	$6.6^{b}\pm0.4$	$2.0^{c}\pm0.1$	$25.6^{c}\pm2.8$	$0.8^{b}\pm0.1$
	Canola oil	$28.4^{b}\pm0.9$	$38.2^{cd}\pm2.1$	$7.2^{b}\pm0.2$	$4.9^{d}\pm0.3$	$20.1^{bc}\pm1.2$	$0.9^{b}\pm0.0$
	Macadamia oil	$29.2^{b}\pm0.4$	$40.6^{d}\pm1.5$	$12.7^{d}\pm0.3$	$2.2^{ac}\pm0.2$	$14.1^{a}\pm1.1$	$0.8^{b}\pm0.1$
	Coconut oil	$43.2^{c}\pm0.6$	$28.3^{b}\pm0.7$	$8.3^{a}\pm0.2$	$2.2^{ac}\pm0.2$	$15.8^{ab}\pm0.8$	$0.8^{b}\pm0.0$
	P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Leg	Tallow	$34.5^{a}\pm0.3$	$36.4^{a}\pm0.7$	$7.9^{a}\pm0.2$	$2.7^{a}\pm0.1$	$16.9^{a}\pm0.5$	$0.8^{a}\pm0.0$
	Flaxseed oil	$29.3^{b}\pm0.6$	$30.4^{b}\pm0.8$	$5.7^{b}\pm0.2$	$16.3^{b}\pm0.5$	$17.4^{a}\pm0.5$	$0.4^{b}\pm0.0$
	Corn oil	$31.0^{b}\pm0.5$	$30.6^{b}\pm1.0$	$5.8^{b}\pm0.1$	$1.8^{a}\pm0.1$	$30.1^{b}\pm0.8$	$0.4^{bc}\pm0.0$
	Canola oil	$29.8^{b}\pm0.7$	$35.3^{a}\pm1.3$	$6.5^{b}\pm0.2$	$5.0^{c}\pm0.2$	$22.6^{c}\pm0.8$	$0.4^{bc}\pm0.0$
	Macadamia oil	$30.1^{b}\pm0.5$	$39.2^{a}\pm1.6$	$12.1^{c}\pm0.5$	$2.0^{a}\pm0.2$	$15.9^{a}\pm1.4$	$0.4^{b}\pm0.0$
	Coconut oil	$43.1^{c}\pm0.4$	$27.5^{b}\pm0.8$	$7.6^{a}\pm0.1$	$2.1^{a}\pm0.1$	$18.1^{a}\pm1.1$	$0.8^{c}\pm0.0$
	P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Heart	Tallow	$40.8^{a}\pm0.3$	$13.5^{ab}\pm0.7$	$4.1^{a}\pm0.1$	$2.1^{ac}\pm0.1$	$38.9^{a}\pm0.5$	$0.5^{a}\pm0.0$
	Flaxseed oil	$38.8^{b}\pm0.6$	$12.7^{ab}\pm0.5$	$3.3^{b}\pm0.1$	$9.3^{b}\pm0.9$	$35.5^{b}\pm0.4$	$0.3^{b}\pm0.0$
	Corn oil	$40.1^{ab}\pm0.3$	$10.5^{a}\pm0.4$	$3.1^{b}\pm0.1$	$0.9^{a}\pm0.1$	$45.0^{c}\pm0.3$	$0.3^{bc}\pm0.0$
	Canola oil	$38.6^{b}\pm0.5$	$14.5^{bc}\pm1.2$	$4.2^{a}\pm0.1$	$2.8^{c}\pm0.2$	$39.4^{a}\pm0.8$	$0.3^{bc}\pm0.0$
	Macadamia oil	$38.3^{b}\pm0.4$	$19.2^{c}\pm1.1$	$5.9^{c}\pm0.2$	$1.4^{ac}\pm0.1$	$34.8^{b}\pm0.7$	$0.3^{c}\pm0.0$
	Coconut oil	$41.7^{a}\pm0.6$	$13.4^{b}\pm0.6$	$3.9^{a}\pm0.1$	$1.2^{ac}\pm0.1$	$38.7^{a}\pm1.1$	$0.4^{d}\pm0.0$
	P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Brain	Tallow	$47.9^{ab}\pm0.9$	17.6 ± 0.8	$7.6^{ac}\pm0.2$	$12.9^{ad}\pm0.2$	$13.3^{a}\pm0.3$	$0.3^{acd}\pm0.0$
	Flaxseed oil	$45.5^{a}\pm0.8$	17.8 ± 1.1	$6.7^{b}\pm0.2$	$20.1^{b}\pm0.7$	$9.5^{b}\pm0.2$	$0.2^{b}\pm0.0$
	Corn oil	$50.3^{b}\pm1.1$	15.8 ± 1.0	$7.0^{ab}\pm0.2$	$9.7^{c}\pm0.3$	$16.6^{c}\pm0.4$	$0.2^{ab}\pm0.0$
	Canola oil	$47.2^{ab}\pm0.9$	17.1 ± 0.7	$7.0^{ab}\pm0.1$	$14.6^{d}\pm0.5$	$13.5^{a}\pm0.4$	$0.3^{abc}\pm0.0$
	Macadamia oil	$49.3^{b}\pm0.7$	17.3 ± 0.5	$8.2^{c}\pm0.2$	$11.0^{ac}\pm0.2$	$13.4^{a}\pm0.3$	$0.4^{cd}\pm0.0$
	Coconut oil	$48.3^{ab}\pm0.8$	18.1 ± 1.0	$7.9^{c}\pm0.3$	$11.3^{ac}\pm0.8$	$13.7^{a}\pm0.2$	$0.4^{d}\pm0.0$
	P value	<0.01	>0.05	<0.001	<0.001	<0.001	<0.001
Adipose	Tallow	$34.0^{a}\pm0.4$	$42.1^{a}\pm0.5$	$9.5^{a}\pm0.5$	$1.5^{a}\pm0.0$	$11.3^{a}\pm0.3$	$0.3^{ab}\pm0.1$
	Flaxseed oil	$27.0^{b}\pm0.4$	$35.4^{b}\pm0.4$	$6.7^{b}\pm0.4$	$16.7^{b}\pm0.2$	$13.5^{b}\pm0.2$	$0.2^{a}\pm0.0$
	Corn oil	$29.5^{c}\pm0.3$	$36.6^{b}\pm0.4$	$6.6^{b}\pm0.4$	$1.6^{a}\pm0.0$	$24.9^{c}\pm0.6$	$0.2^{ab}\pm0.1$

(*Continued*)

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Table 5. (*Continued*)

Tissue	Treatment	SFA[c)]	n-9 MUFA[d)]	n-7 MUFA[e)]	n-3 PUFA[f)]	n-6 PUFA[g)]	*Trans*
	Canola oil	$27.0^{b} \pm 0.2$	$46.0^{c} \pm 0.3$	$7.2^{b} \pm 0.2$	$3.5^{c} \pm 0.5$	$15.7^{d} \pm 0.3$	$0.2^{ab} \pm 0.0$
	Macadamia oil	$27.9^{b} \pm 0.3$	$47.1^{c} \pm 0.3$	$14.3^{c} \pm 0.3$	$1.2^{a} \pm 0.0$	$8.8^{e} \pm 0.2$	$0.2^{ab} \pm 0.0$
	Coconut oil	$48.1^{d} \pm 0.5$	$31.0^{d} \pm 0.4$	$9.5^{a} \pm 0.4$	$1.1^{a} \pm 0.0$	$9.0^{e} \pm 0.1$	$0.4^{b} \pm 0.1$
	P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[a)] Values are the pooled mean (n = 8) ± SEM of fatty acid group percentage of the total fatty acids. Values in the same column (within the same tissue) with no common superscript are significantly different; [b)]supplemented with 6 fats at 4% w/w inclusion level; [c)]SFA = Saturated fatty acid; [d)]n-9 MUFA = Omega 9 monounsaturated fatty acid; [e)]n-7 MUFA = Omega 7 monounsaturated fatty acid; [f)]n-3 PUFA = Omega 3 polyunsaturated fatty acid; [g)]n-6 PUFA = Omega 6 polyunsaturated fatty acid.

these diets. The level of n-3 PUFA in blood (highest in the flaxseed oil group, 15%) was approximately half of its level in the corresponding diets. The *trans* fatty acids were the least abundant fatty acid group in the blood (0.4–0.7%), and were at their highest level in the broilers fed the tallow diet (Table 5). Strong positive correlations ($r > 0.9$, $P < 0.01$–0.05) were found between the diet and blood levels for all fatty acid groups, except the *trans* fatty acids being at moderate level (Table 6, Figure 1).

In the present study, the fatty acid profile of the whole blood was determined rather than the serum, plasma or red blood cells (the fractions which are more frequently analysed).[43,56] However, Liu et al.[43] demonstrated that the whole blood fatty acid profile was closely correlated with red blood cells phospholipids. We have previously determined the effects of dietary (tallow and flaxseed oil-based diets) and sex of the broiler on the whole blood fatty acid profile.[39] The fatty acid profile of the male broilers used in the current study was very consistent with our earlier study, and also closely reflected the dietary fatty acid composition. In addition, Burlikowska et al.[57] similarly reported that the relative abundance of different fatty acid classes in serum is highly correlated to their respective levels in the diets.

Table 6. Pearson correlation (r) and linear regression coefficient ($y = a + bx$) between fatty acid levels in diets and tissue (n = 8) of six different experimental dietary treatments in 42-days-old broiler chickens.

Tissue		SFA	n-9 MUFA	n-7 MUFA	n-3 PUFA	n-6 PUFA	*Trans*
Blood	a	31.892	12.448	2.438	1.488	17.911	0.323
	b	0.220	0.340	0.309	0.464	0.425	0.223
	r	0.979**	0.903*	0.929**	0.995**	0.907*	0.660
Liver	a	40.438	26.730	3.691	1.931	14.594	1.084
	b	0.073	0.071	0.107	0.253	0.164	0.325
	r	0.844*	0.581	0.540	0.957**	0.620	0.772
Breast	a	22.896	22.518	6.067	1.208	8.535	0.575
	b	0.317	0.383	0.446	0.538	0.384	0.453
	r	0.989**	0.980**	0.938**	0.999**	0.966**	0.987**
Leg	a	24.283	21.729	5.389	1.096	8.255	0.332
	b	0.294	0.354	0.451	0.527	0.486	0.281
	r	0.995**	0.916*	0.942**	0.998**	0.957**	0.593
Heart	a	37.764	8.794	3.165	0.776	31.657	0.233
	b	0.066	0.159	0.187	0.295	0.288	0.163
	r	0.868*	0.630	0.939**	0.993**	0.844*	0.851*
Brain	a	47.840	17.996	7.013	11.963	10.495	0.308
	b	0.008	−0.022	0.079	0.287	0.116	−0.011
	r	0.088	−0.311	0.667	0.927**	0.544	−0.051
Adipose	a	18.847	21.783	6.365	0.039	−0.010	0.216
	b	0.454	0.551	0.531	0.573	0.566	0.047
	r	0.994**	0.988*	0.902*	1.000**	0.999**	0.247

* Correlation is significant at $P < 0.05$ level (2-tailed).
** Correlation is significant at $P < 0.01$ level (2-tailed).

3.4.2. Liver

Compared to the other tissues sampled, there was greater variability in the fatty acid profile (especially in total n-9 MUFA and total n-6 PUFA) of liver tissue between individual birds within each dietary treatment, as indicated by the higher SEM. It is noteworthy that those birds with the highest levels of hepatic crude fat had the heaviest livers and highest HSI% (data not shown). These birds also had higher hepatic levels of total n-9 MUFA, at the expense of total n-6 PUFA.

The main contributor to the liver fatty acid profile were SFA (41–45%; highest in the coconut oil group, $P < 0.05$). The n-9 MUFA made up the second largest proportion of the total fatty acids in the liver but, interestingly, the levels of this fatty acid in the liver was not significantly different between diet groups (range 27–31%). Interestingly, birds in the tallow, coconut and macadamia oil groups had the highest n-7 MUFA contents, which was not in line with the composition of these respective diets. The birds in flaxseed oil treatment contained the greatest proportion of total n-3 PUFA, consistent with the highest n-3 PUFA content of this diet, however, the percentage of n-3 PUFA in the liver (9%) was less than 30% of its contribution level in that diet. The n-6 PUFA in the liver was not greatly influenced by treatment type, except that the highest n-6 PUFA group (corn oil diet) had a higher hepatic n-6 PUFA than the flaxseed oil diet group ($P < 0.001$, Table 5). It is worth noting that liver also contained the greatest levels of *trans* fatty acids of all the tissues analysed, and the *trans* fatty acid content was highest (1.6%) in the tallow treatment group, similar to its respective dietary percentage. The World Health Organization recommends *trans* fats should not exceed 1% of our daily energy intake.[58] Thus, high levels of consumption of livers from birds fed the tallow diet could contribute to exceeding the allowed threshold and

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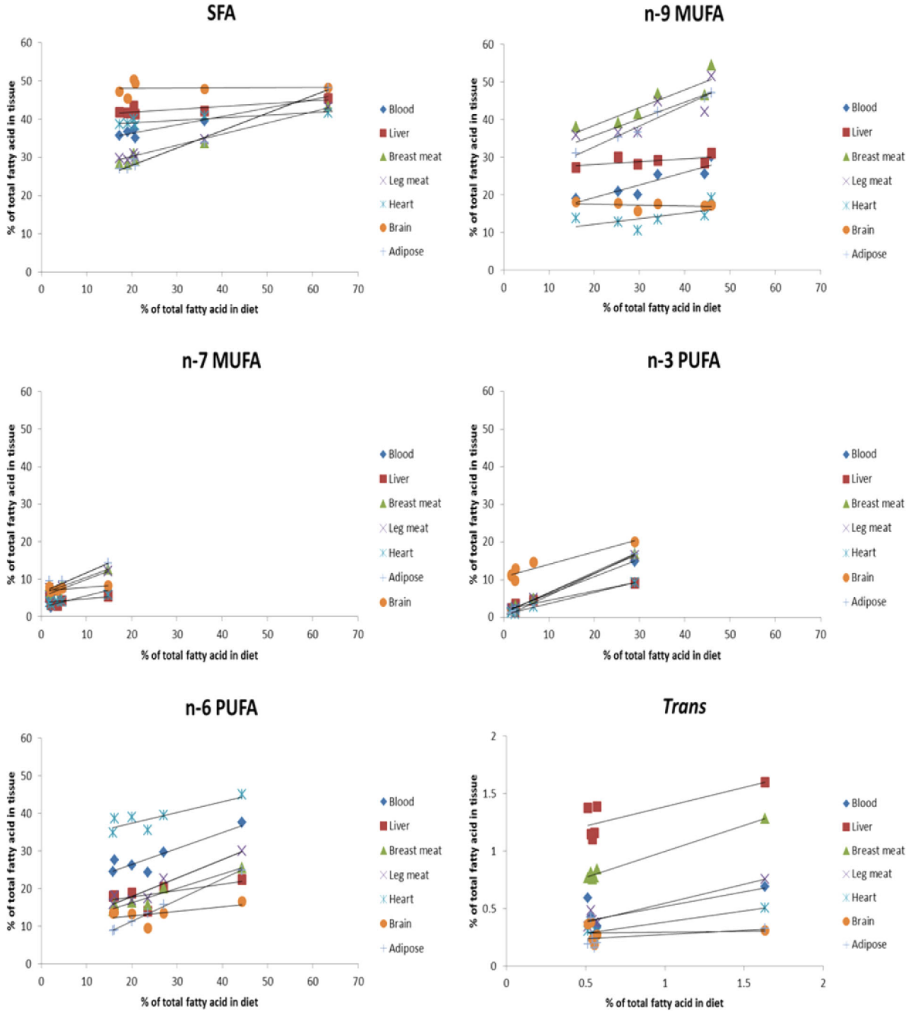

Figure 1. Correlations between contents of six fatty acid groups (% of total fatty acids) in diet (independent variable) and tissue (dependent variable) of seven tissues in 42-days old broiler chickens receiving six different dietary fats at 4% w/w inclusion level.

adversely affect human health. In general, the levels of the respective fatty acids in the liver did not correlate particularly closely to the level in the diets. The strongest positive linear correlation was for total n-3 PUFA ($r = 0.957$, $P < 0.01$), followed by total SFA ($r = 0.844$, $P < 0.05$) and the correlations were of moderate strength for all other fatty acid groups (Table 6, Figure 1).

The level of SFA measured in liver in the present study is comparable to that reported in previous similar studies (≈41–45%).[33,47,50,59] However, this was not the case with the other fatty acid types, with the MUFA levels being higher and PUFA levels lower than those reported elsewhere. Phetteplace and Watkins[38] for example, demonstrated that feeding broilers up to 56 days of age with a flaxseed oil diet increased the liver

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PUFA content to ≈58% (comprised of ≈32% n-3 and ≈26% n-6) of total fatty acids, which was approximately 3.5- and 2-fold the levels measured in the present study, respectively. In agreement with the present study, González-Ortiz et al.[47] demonstrated that the fatty acid composition of the liver is less influenced by changes in dietary fatty acid composition in comparison with the muscle and adipose tissue.

3.4.3. Breast Meat

The SFA and n-9 MUFA were the major fatty acid groups in the breast meat tissue, together making up more than two thirds of the total fatty acid content. Interestingly, SFA was the most abundant fatty acid in the breast meat of coconut oil fed birds, while for the other treatments n-9 MUFA were present in the highest amounts with the amount corresponding to the relative n-9 MUFA content of the diet (Table 5, $P < 0.001$). The flaxseed oil treatment resulted in a breast meat total n-3 PUFA content which was at least 3.4-fold higher than the other treatments, whereas birds fed the corn oil diet had the highest proportion of n-6 PUFA. As a consequence, the n-6:n-3 ratio of the breast meat ranged from ≈1.6:1 (in the birds fed flaxseed oil diet) to ≈22:1 (in the birds fed corn oil diet). Perhaps surprisingly, breast meat had the second highest *trans* fatty acid content of the tissues assessed, and this was again highest in the tallow treatment group (1.3%). However, according to the American Heart Association, there are insufficient data to determine if consumption of naturally occurring *trans* fats have negative health effects.[60] Breast meat fatty acid composition was more strongly correlated with the dietary fatty acid profile than all other sampled tissues ($P < 0.01$, Table 6 and Figure 1).

Breast meat is the most important edible tissue in broiler chickens, comprising ≈75% of the meat yield in a broiler carcass. As a result, the vast majority of studies focused on the nutritional aspects of chicken meat has involved sampling and assessment of breast tissue. Crespo and Esteve-Garcia[13] reported that feeding broilers a 10% tallow diet (between 21 and 42 growth days) resulted in a higher SFA percentage in breast meat (34%, similar to the present study, and others[47]) compared to 3 diets rich in either PUFA or MUFA. In the same study, SFA was reduced to 28% in breast meat from birds fed on a flaxseed oil based diet, which is again similar to our findings. These results suggest a very similar effect of feeding tallow and flaxseed oil diets on meat SFA content even with different inclusion levels of fat (10 vs. 7% in our study). In another similar study Hrdinka et al.[61] reported that feeding broilers a diet contain 3.5% rapeseed (canola) oil, resulted in a higher content of MUFA (≈50%) and SFA (38%) in the breast meat (with skin included). These levels are higher, by 5 and 8% respectively, than the levels in the current study. These differences are probably related to the type of fat in the skin since this was not included in the breast meat samples that were analysed in the present study.

3.4.4. Leg Meat

In general, the patterns observed in the fatty acid composition of the leg meat across dietary treatments were largely in line with those seen in breast meat. However, the total n-6 PUFA levels were higher in the leg meat than in the breast meat (by up to 4.5% in the corn oil treatment), the level of *trans* fatty acids was about half of that in breast meat and the correlations of tissue fatty acid levels to the dietary levels were weaker (Table 6, Figure 1). Crespo and Esteve-Garcia[13] also reported similar patterns of fatty acids in breast and leg meat. In contrast, however, Hrdinka et al.[61] reported that fatty acid composition of leg meat (with skin on) reflected the differences in dietary fatty acid intake more closely than did breast meat (again with skin on), and they suggested that might be related to the higher crude fat content of the leg meat. Nevertheless, these differences may not relate to the results of the deskinned meat in the present study, as the type of fat in the skin differs from that in meat.

3.4.5. Heart

Similar to the meat tissues, the fatty acid profile in the heart was significantly different across dietary treatments and closely related to diet composition ($P < 0.001$, Table 3). The cardiac tissue was dominated by SFA in the majority of the dietary groups (tallow, flaxseed oil, macadamia oil and coconut oil) and by n-6 PUFA in the canola oil and corn oil treatments, with total SFA + n-6 PUFA contributing up to 85% of total cardiac fatty acids in the latter treatment. The range of SFA levels in the hearts of birds in the different diet treatments (3.4%) was slightly lower than measured in other tissues. The relatively high levels of n-6 PUFA (up to 45.0% in corn oil diet) made the heart the richest tissue in this group of fatty acids. The third major fatty acid group was n-9 MUFA (comprising 12.7 to 19.2% of all fatty acids), however the heart contained the lowest levels of this fatty acid group of all the tissues examined. The levels of the *trans* fatty acids were lowest in the heart than all other tissues measured in this study, ranging from 0.3 to 0.5%, but were still highest in the tallow diet group (as for the other tissues) (Table 5). The levels of all the fatty acid groups in the heart, with the exception of the n-9 PUFA, were positively correlated to their levels in the diet (Table 6 and Figure 1).

Crude fat content and fatty acid composition of cardiac tissue in fast-growing broilers has been associated with potential roles in health conditions including sudden death syndrome (SDS) that can cause significant economic loss to the chicken industry. Chung et al.[62] found that feeding chickens a diet containing sunflower oil was associated with a lower incidence of SDS mortality than feeding a tallow based diet. The content of n-3 PUFA in the cardiac tissue seems not to be only determined by its respective dietary percentage, but also the presence of n-6 PUFA in the diet (the dietary n-6:n-3). Therefore, although the canola oil diet provided a considerable proportion of n-3 PUFA (6.6%), this diet did not elevate the n-3 PUFA content in the cardiac tissue because it was still accompanied by a high dietary n-6:n-3 ratio (4.09) in agreement with Kartikasari, et al.[63] finding. Further, Gregory et al.[51], demonstrated that a canola diet, but not a tallow based diet, enriched the phospholipid class of the cardiac lipids with certain n-3 PUFA, specifically the total of EPA and DHA. However, this elevation was not significant for the major n-3 PUFA (ALA) similarly to cardiac total n-3 PUFA content between the equivalent dietary treatments in the present study.

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Phetteplace and Watkins[38] reported that the association between n-6 PUFA levels in the cardiac and hepatic tissues in broilers fed a linseed (flaxseed) oil diet was close to the pattern that observed in the flaxseed oil group in the current study. However, the cardiac n-3 PUFA level which they reported for the same treatment is higher (by ≈13%) than of its respective level in the flaxseed oil group in the current study. Consistent to our finding, Kartikasari[56] reported that feeding broilers flaxseed oil diet was increased n-3 PUFA content in the cardiac membrane by 4.8-fold comparing to broilers fed on tallow diet.

3.4.6. Brain

The results of brain tissue fatty acid analysis were substantially different from the patterns we measured in all the other tissues. Almost half of the total fatty acids in brain were SFA, making this tissue the richest in this group of fatty acids. With regard to the variation between dietary treatments, the brain of birds fed the corn oil diet had the highest content of SFA (50.3%), however this was only ≈5.0% higher than the flaxseed oil group (the lowest); despite the very large difference in their dietary SFA levels (19%, flaxseed oil diet to 64%, coconut oil diet). Similarly, the large range in dietary n-9 MUFA (16%, coconut oil diet to 46%, macadamia oil diet) had no effect on the percentage of n-9 PUFA in the brain (17% on average). The n-7 MUFA was the second lowest group of fatty acids in the brain, and was higher in the macadamia oil, coconut oil and tallow treatments than in the other dietary groups. In 4 groups (tallow, canola oil, macadamia oil and coconut oil), where PUFA were not the most abundant fatty acid group, the n-6:n-3 in brain were similar (0.92–1.22). In the flaxseed oil group, however, the n-3 PUFA content (20.1%) was more than double the content of n-6 PUFA. In contrast, the corn oil group had a substantially higher n-6 PUFA content (16%) compared to n-3 PUFA (10%). It is also important to note, however, that the brain contained the lowest proportion of n-6 PUFA compared to all other tissues analysed in this study. The *trans* group of fatty acids contributed a very low percentage (<0.5%, Table 5) of the total fatty acids in the brain in all groups. In terms of the relationships between fatty acid content in the tissues and those in the diet, only brain n-3 PUFA content was strongly and positively ($r = 0.927$, $P < 0.01$) correlated to the n-3 PUFA content of the diet. The n-7 PUFA and n-6 PUFA showed weaker positive relationships ($r = 0.667$ and 0.544, respectively), while the levels of all other fatty acids in the brains were not significantly related to their content in the diets. Indeed, the levels of n-9 MUFA and *trans* fatty acids in the brain were actually inversely correlated with their levels in the diet (Table 6, Figure 1).

Relatively few studies have investigated the fatty acid content of chicken brain tissue[64,65] and to our knowledge none have compared the effect of manipulation of the dietary fatty acid on brain fatty acid composition. It is particularly noteworthy that even when broilers were fed on diets that were low in n-3 PUFA, they nevertheless maintained a relatively high level (>11.0%) of this functionally pivotal fatty acid group in their brains. This finding is in agreement with the results from Phetteplace and Watkins,[38] who reported that broilers accumulated 11–15% of n-3 PUFA in the brain even when fed a poor dietary source of these fatty acids. However, those authors reported that their linseed (flaxseed) oil treatment resulted in an even higher (by ≈8%) n-3 PUFA level than measured in the birds fed on the flaxseed oil based diet in the present study. We also found that high levels of n-3 PUFA in brain were accompanied by low levels of n-6 PUFA, in contrast to the findings by Phetteplace and Watkins[38] of higher levels of n-6 PUFA in treatment with low dietary n-3 PUFA. As in the liver, we found that feeding birds a diet with a high n-7 MUFA content (the macadamia oil treatment) did not elevate the level of this fatty acid in the brain in comparison to the poor dietary providers of n-7 MUFA, such as tallow and coconut oil diets.

The poor correlation with this diet for most of fatty acid groups in brain support the suggestion that the incorporation of fatty acids in this tissue is heavily influenced by more complicated mechanisms than merely passive transfer from the diet.

3.4.7. Adipose Tissue

Adipose tissue was dominated by n-9 PUFA (35–47%) in all treatments except for the coconut oil group where SFA was the predominant group (48%). Otherwise, with the exception of *trans* fatty acids, the fatty acid profile of this tissue typically followed the dietary fatty acid composition very closely ($r > 0.9$, Table 6). Feeding broilers the macadamia oil diet resulted in the highest n-9 and n-7 MUFA content in the adipose tissue, corn oil and canola oil diets resulted in the highest level of adipose n-6 PUFA, whereas the flaxseed oil diet was associated with the highest (16.7%) levels of n-3 PUFA. The levels of all MUFA and PUFA in the adipose tissue correlated almost perfectly ($r = 0.999$ to 1.00, $P < 0.01$, Table 6 and Figure 1) to dietary fatty acid content. The *trans* fatty acids contributed up to 0.4% of tissue fatty acid content (in the coconut oil diet, Table 5). Our results are supported by a number of other published observations showing that adipose tissue fatty acid content is strongly correlated to dietary fatty acid composition.[10,11,13,48,61,66] Crespo and Esteve-Garcia,[13] for example, reported that MUFA tend to accumulate in the adipose tissue. Similarly, Ortiz et al.[66] found that MUFA was the predominant fatty acid class comprising 43% n-6:n-3 − 56% of the total fatty, with 38–49% reported by Hrdinka et al.[61] However, the MUFA:SFA and MUFA:PUFA ratios in adipose tissue were different to the dietary ratios as reflected by variation in the values for slopes in the regression equations (Table 6). Carmona et al.[48] found that n-6 PUFA in the adipose tissue was the fatty acid group most susceptible to modifications to dietary fatty acid composition, which was consistent with the current study. Higher levels of both n-3 (by 8%) and n-6 (by 10%) PUFA were reported in the adipose tissue of broilers fed 5% w/w flaxseed oil diet for a period 2 weeks longer than the current experiment.[38] While we only analysed the abdominal adipose tissue in the current study, Hrdinka et al.[61] have previously reported that there were no differences in the effect of diet on fatty acid composition of the abdominal and subcutaneous depots.

4. Conclusion

None of the growth parameters measured in this study were influenced by the different fats used in the diets. We speculate that differences in growth performance related to dietary fat type may only become evident if the birds are also stressed by some aspect of the environmental or experimental conditions.

On the other hand, different fatty acids contribute in varying amounts to the composition of different tissues. Except for the brain, the fatty acid composition of the major tissues largely reflected the fatty acid composition of the diet. In particular, all tissues were correlated in a strong linear positive relationship to the dietary n-3 PUFA content. Thus, chicken products from broilers fed a flaxseed oil-based diet have a potential health benefits to human. Conversely, feeding broilers a corn oil-based diet was associated with a lower n-3: n-6 ratio in different tissues and feeding them a tallow-based diet was associated with a higher trans percentage, particularly in liver and breast meat. Therefore, the chicken broiler industry needs to be more aware that different dietary fats will have significant effects on the fatty acid composition of chicken tissues, and thereby on the nutritional benefits or health issues conferred by those chicken tissues to consumers.

Abbreviations

ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HSI, hepatosomatic index; LA, linoleic acid; n-9 MUFA, Omega 9 monounsaturated fatty acid; n-7 MUFA, Omega 7 monounsaturated fatty acid; n-3 PUFA, Omega 3 polyunsaturated fatty acid; n-6 PUFA, Omega 6 polyunsaturated fatty acid; PUFA, polyunsaturated fatty acid; SDS, sudden Death Syndrome; SFA, saturated fatty acid.

Acknowledgements

K. Kanakri acknowledges the generous funding received from the Australian Government Research Training Program Scholarship (RTP) and PhD scholarships (full-time and supplementary) from the Faculty of Sciences, The University of Adelaide. B. Muhlhausler is supported by a Career Development Fellowship from the National Health and Medical Research Council of Australia (NHMRC) and R. Gibson is supported by a NHMRC Senior Research Fellowship. The experimental work was financed by a grant from the South Australian Department of Further Education, Employment, Science, and Technology (DFEEST), Australia. The authors are grateful to Dr Carolyn de Koning and Mr Derek Schultz from SARDI, Roseworthy campus for their assistance in animal care in conducting the field part of the experiment.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

chicken broilers, diet, fatty acid, oils, tissue

Received: May 22, 2017
Revised: September 14, 2017
Published online:

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4.2. Published manuscript 2 for Chapter 3

Statement of Authorship

Title of Paper	The fatty acid composition of excreta of broiler chickens fed different dietary fatty acids
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	International Journal of Poultry Science

Principal Author

Name of Principal Author (Candidate)	Khaled Kanakri
Contribution to the Paper	Co-designed the experiment, performed the experimental work (field part and fatty acid analysis), statistical analysis, data interpretation, wrote the manuscript and acted as corresponding author.
Overall percentage (%)	75%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 3/11/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	John Carragher
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Name of Co-Author	Robert Hughes
Contribution to the Paper	Contributed to the animal care and contributed to manuscript revision.
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Signature		Date	03/11/2017

Name of Co-Author	Robert Gibson		
Contribution to the Paper	Principal supervisor of the project, supervised the work development and revised the manuscript.		
Signature		Date	6/11/17

OPEN ACCESS International Journal of Poultry Science

ISSN 1682-8356
DOI: 10.3923/ijps.2017.424.433

Research Article

The Fatty Acid Composition of Excreta of Broiler Chickens Fed Different Dietary Fatty Acids

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Abstract

Background and Objective: Excreted fatty acids represent the net result of fat digestion, absorbtion and bioconversion by chickens or their intestinal microbiome and thus provide information on the capacity of the birds to utilize different fat types. This study aimed to clarify the relationship between the fatty acid profile of diet and excreta in broiler chickens. **Materials and Methods:** Male Cobb 500 broilers (n = 240) were fed (*ad libitum*) one of 6 different diets supplemented with 4% (w/w) beef tallow, flaxseed, corn, macadamia, canola or coconut oils (4 replicate pens/treatment) from hatching day. At day-40 post-hatch, excreta samples were collected for fatty acids analysis. **Results:** Significant positive linear correlations (R = 0.82-0.99) were found in the fatty acid content of diets and excreta for all fatty acid groups in all treatments. Comparing the individual fatty acid content of diet and excreta suggested that the broilers preferentially utilized (in decending order, if present) omega-3 polyunsaturated fatty acids, omega-9 and omega-7 monounsaturated fatty acids and most saturated fatty acids (except C16:0 and C18:0), but the omega-6 polyunsaturated fatty acids were under utilized even when they were the most abundant. **Conclusion:** Fat sources which are high in the C16:0, C18:0 and omega-6 fatty acids may not be ideal for broiler feed formulations for nutritional and economical reasons.

Key words: Oils, fatty acid, diet, excreta, chicken broiler

Received: August 03, 2017 Accepted: September 15, 2017 Published: October 15, 2017

Citation: Khaled Kanakri, John Carragher, Robert Hughes, Beverly Muhlhausler, Carolyn de Koning and Robert Gibson, 2017. The fatty acid composition of excreta of broiler chickens fed different dietary fatty acids. Int. J. Poult. Sci., 16: 424-433.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

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INTRODUCTION

Fats from animal and/or plant origins are added to commercial chicken feed as a source of essential fatty acids and a source of energy. Manipulation of the fat composition (e.g., the types and inclusion level) is commonly implemented for economic and nutritional purposes and results in altered dietary fatty acids composition (e.g., chain length, degree of saturation and molecular structure). Previous studies in chickens have shown different associations between the fatty acid content of diets and tissues depending on tissue type[1-3]. There is considerable evidence that dietary fatty acid composition is related to growth performance[4] and health status[5,6] in chickens, but less is known about whether dietary fat composition influences the utilization of different fat types. The utilization of fatty acids as an energy source in broilers is limited in the first two weeks post-hatch but it improves as birds get older and physiological functions develop[7]. Fatty acid analysis of digesta sampled from along the gastrointestinal tract is commonly used to evaluate the digestion of nutrients and shows variation in the fat digestion coefficient between different intestinal segments[8,9] with jejunum being the segment where the majority of fat is digested and absorbed[10]. To complicate matters further, variation in the microbial content between different intestinal sections, such as ileum and cecum, has been reported in broilers[11] which suggests intestinal microbiota could play a role in fat metabolism. In support of this, changes in gut microbiota are known to affect the performance parameters of broiler chickens[12].

Many aspects of dietary fatty acids, including inclusion level[13], source[14], re-esterification[15] as well as their interactions with other dietary macronutrients[9], micronutrients[16,17] and enzymes[18] have been reported to affect their intestinal absorption and excretion in broilers.

Number of studies have investigated different aspects of broiler excreta. These studies have led to better understanding of lipid utilization and losses[19], fatty acid influence on excreta microbiota[20], estimating metabolizable energy[13,21], feed conversion efficiency[22], using excreta as a dietary component itself[23,24], influencing manure mineral content[17] and even how excreta affects the foot pad health of the birds[19]. However, it is important to acknowledge that the fatty acid content of excreta is a consequence of several factors, including: The lipid composition of the diet, the activities of lipase and bile salts, the efficacy of absorptive, metabolic and excretive processes throughout the length of the gut and the utilization and potential modification of fatty acids by the microbiota[25-28].

To our knowledge, no published literature has compared the dietary effect of a wide variety of dietary fats which are very different in their fatty acid composition on the fatty acid composition in the excreta (as opposed to the digesta) of broilers. Therefore, the present study aimed to examine this relationship in broilers at harvest age fed diets supplemented with a range of different types of fats. A better understanding of this relationship is potentially useful to provide advice to poultry feed producers regarding the best choice of available fats to use when formulating broiler diets.

MATERIALS AND METHODS

Broilers and experimental design: The Animal Ethics Committees of the University of Adelaide and Primary Industries and Regions South Australia approved this study. A total of 240 day-old male chicks of the Cobb 500 strain were obtained from the Baiada Hatchery (Willaston, SA, Australia) and transferred to South Australian Research and Development Institute (SARDI) facility (Roseworthy, SA, Australia). A complete randomized block design (4 pens/treatment) was implemented. Birds were randomly distributed into 24 groups of 10 and allocated to 24 raised rearing floor pens (1.2×0.9 m each) in one shed. Chickens were reared on sawdust and shavings in a temperature controlled room and had free access to both feed and water at all times. Pens were heated by infrared brooder lamps (175 W) during the first 3 weeks post-hatch. Temperature in the room was 27°C for the first 4 days, gradually decreased to 20°C and then maintained for the 40-day experimental period.

Experimental diets: The study included 6 dietary treatments. In each, broilers were fed *ad libitum* 1 of 6 experimental diets by adding a different fat at 4% w/w to starter (crumble form, first 3 weeks) and finisher (pellet form, last 3 weeks) basal diets. The basal diets (containing ~3% crude fat) were obtained from a poultry feed manufacturer (Ridley Agriproducts, Australia). The added fat types were: Flaxseed oil (high omega-3 polyunsaturated fatty acids (n-3 PUFA): Four Leaf Oils, Australia), corn oil (high omega-6 polyunsaturated fatty acids (n-6 PUFA): Daisy, Malaysia), canola oil (high omega-9 monounsaturated fatty acids (n-9 MUFA): Foodland, Australia), macadamia oil (high omega-7 monounsaturated fatty acids (n-7 MUFA): Macoils, Australia), coconut oil (high saturated fatty acids (SFA): Banaban, Fiji) or beef tallow (moderate SFA and MUFA, Ridley Agriproducts, Australia). The composition and nutritional profiles of the two basal diets are shown in Table 1. Apart from the variation in the fatty acid

composition of the experimental diets (Table 2), they all were nutritionally identical and met requirements for healthy growth[29].

Production parameters: Total body weight (BW) of the birds in each pen was taken on the day of hatch and then on a weekly basis for the first 3 weeks. Feed intake (FI) of birds in each pen was also recorded on a weekly basis and used to calculate the feed conversion ratio (FCR, kg FI kg^{-1} b.wt., gain). Number of deaths and culls was used to calculate mortality rate in all treatments on a weekly basis.

Sample collection: On day-40, paper drop-sheets were placed in each pen to collect excreta samples. Approximately 10-12 fresh droppings (deposited within 3 h) were randomly transferred into clean plastic containers. Samples were immediately frozen on dry ice before they were transferred to the laboratory to be stored at -18°C until subsequent determination of crude fat content and fatty acid analysis.

Fatty acid analysis: Crude lipid was extracted from homogenized feed and excreta samples[30]. The gravimetric approach was utilized to estimate total lipid percentages. Fatty acid profiling was performed after transmethylation of the extracted crude lipids with 1% H_2SO_4 in methanol at 70°C for 3 h[31,32]. After cooling to room temperature, the resulting fatty acid methyl esters (FAME) were extracted with n-heptane (2 mL) and transferred into gas chromatography (GC) vials containing about 30 mg of anhydrous sodium sulphate. Vials were stored at -18°C until GC analysis.

Gas chromatography analysis of FAME: The FAME were separated using a Hewlett-Packard 6890 GC (Hewlett-Packard, CA, USA) equipped with a flame ionization detector (FID), a split injector and a BPX-70 capillary column (50 m×0.32 mm internal diameter) with a 0.25 µm film thickness (SGE, Victoria, Australia). The operating conditions of the GC, identification of fatty acids using the GLC 463 external standard (Nu-Chek Prep Inc, MN, USA) and qualitative analysis was as described previously[33,34].

Statistical analysis: The effects of dietary treatment on the excreta fatty acid profile were tested by one-way analysis of

Table 1: Ingredient composition and nutrient content of the basal diet

	Basal diet[1]	
Ingredients (%)	Starter[2]	Finisher[3]
Wheat fine	61.42	70.77
Barley fine	5.00	5.00
Tallow mixer	0.37	1.00
Blood meal (91% CP)	1.00	0.00
Soybean meal	25.17	16.67
Limestone small	0.86	0.64
Monocalcium phosphate	0.14	0.00
Sodium chloride	0.21	0.18
Sodium bicarbonate	0.16	0.18
Choline chloride 75%	0.03	0.04
DL-methionine 58.1	0.29	0.21
L-threonine 73.7	0.13	0.25
Rovabio Excel LC	0.02	0.02
Meat meal	3.73	3.70
Ronozyme NP CT	0.02	0.02
Mineral/vitamin premix[1]	1.00	1.00
L-lysine sulphate 70	0.43	0.33

[1]The starter and finisher basal diets metabolisable energy = 2899.59 and 2999.90 Kcal kg^{-1}, the nutrient contents (g kg^{-1}) were: Crude protein 225.1 and 186.7, Crude fat 23.5 and 30.0, Crude fibre 32.3 and 29.2, Ca 9.8 and 8.5, Available phosphorus 4.5 and 4.2, Na 1.8 and 1.7, K 0.8 and 6.5, Cl 0.2 and 1.8, Lysine 12.3 and 9.1, Methionine 5.7 and 4.7, Cystine 3.9 and 2.8, Threonine 7.9 and 7.6, Leucine 14.3 and 11.2, Isoleucine 7.8 and 6.4, Tryptophan 2.3 and 1.8, Arginine 12.4 and 9.8 and Valine 9.3 and 7.2, respectively, [2]Used to formulate 2 experimental diets to feed broilers up to 3 weeks old, [3]Used to formulate 2 experimental diets to feed broilers from 4-6 weeks old

Table 2: Crude fat content and fatty acid composition (as a percentage of total fatty acids) of the six experimental diets[1]

	Crude fat (%)[2]	Total SFA[3]	Total trans	Total n-9[4]	Total n-7[5]	Total n-3[6]	Total n-6[7]
Diet							
Tallow	7.0	35.8	1.6	34.1	4.6	2.9	20.2
Flaxseed oil	7.2	18.6	0.5	25.0	2.1	29.5	24.1
Corn oil	7.2	20.3	0.5	29.3	2.0	2.7	45.0
Canola oil	7.2	16.9	0.5	44.4	3.5	6.9	27.4
Macadamia oil	7.0	20.6	0.5	45.7	14.8	2.0	16.2
Coconut oil	7.1	64.3	0.5	15.3	1.7	1.9	16.2
Excreta[8]							
Tallow	1.2±0.1	52.3±1.1^{a}	1.7^{a}±0.1^{a}	20.5±1.0^{ab}	2.7±0.2^{a}	2.1±0.1^{a}	20.4±0.3^{a}
Flaxseed oil	0.9±0.1	22.4±0.4^{b}	0.3±0.1^{bd}	22.8±0.3^{ab}	2.2±0.1^{a}	16.0±0.5^{e}	36.1±0.5^{b}
Corn oil	1.0±0.1	23.3±0.3^{b}	0.2±0.1^{d}	25.7±0.6^{b}	2.4±0.1^{a}	3.2±0.1^{b}	45.1±0.4^{c}
Canola oil	1.0±0.0	21.0±0.5^{b}	0.3±0.1^{bd}	33.6±0.7^{c}	3.0±0.1^{a}	5.3±0.2^{c}	36.6±0.8^{b}
Macadamia oil	1.1±0.2	27.1±1.1^{c}	0.6±0.0^{c}	39.0±4.4^{c}	9.5±1.4^{b}	2.6±0.0^{ab}	21.0±6.6^{a}
Coconut oil	1.0±0.1	48.2±2.7^{d}	0.5±0.1^{ab}	17.3±0.4^{a}	2.0±0.1^{a}	2.7±0.1^{ab}	29.2±0.8^{b}

[1]Finisher basal diet+added fat, fed to broilers between 22 and 40 days of age. [2]Values are means of 4 replicates and the percentages are based on the wet weight. [3]SFA = Saturated fatty acid, [4] n-9 = Omega 9 monounsaturated fatty acid, [5]n-7 = Omega 7 monounsaturated fatty acid, [6]n-3 = Omega 3 polyunsaturated fatty acid, [7]n-6 = Omega 6 polyunsaturated fatty acid, [8]Values are mean of 4 replicates±SEM and different superscript letters within the same column are significantly different ($p<0.001$)

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variance (ANOVA) and Duncan's multiple comparison test was implemented when the ANOVA indicated the differences between dietary treatment effects were significant ($p<0.05$) using SPSS version 22 for Windows (IBM Crop., NY, USA). The effects of dietary treatment on BW, FI and FCR were determined by one-way ANOVA ($p<0.05$) using SAS 9.3 for windows.

RESULTS

Broiler productivity: There were no significant differences in any of the production parameters between different treatments. The average BW of 40-day-old broilers was 3367g, the FI was 5414 g/bird, the FCR was 1.63 g feed g^{-1} weight gain and the overall mortality rate was 5.4%.

The diets: The crude fat content of the different experimental diets ranged from 7.0-7.2% (Table 2). In the tallow diet, the two prevalent fatty acid groups were SFA and n-9 MUFA in almost equal portions and these together made up more than two thirds of total fatty acids. The major fatty acid group in the flaxseed oil diet was n-3 PUFA (at ~30% of the total fatty acids) with n-9 MUFA and n-6 PUFA also present at lower proportions. The main fatty acid group in the corn oil diet was the n-6 PUFA (45%) followed by n-9 MUFA (~29%). The n-9 MUFA was the predominant fatty acid group in the canola and macadamia oil-based diets (~44-46%). The canola oil diet also contained, in descending order, n-6 PUFA, SFA and n-3 PUFA; whereas the macadamia oil diet contained more SFA than either n-6 or n-3 PUFA and while the n-7 MUFA was only the third most abundant fatty acid group in the macadamia oil diet (at 15% total fatty acids), this level was still 3-9 times higher than in the other 5 diets. The coconut oil diet contained a higher percentage of SFA (64%) compared to all other diets. The contribution of trans fatty acids was the lowest of all the fatty acid groups and highest percentage was in the tallow diet with 1.6% (Table 2).

The excreta: The colour, shape, size or viscosity of droppings between different pens (dietary treatments) were not different; however, droppings from individual birds within the same group did differ in appearance (data not shown). The overall percentage of the crude fat in the wet excreta ranged from 0.9-1.2% with overall average approximately 1.1% (Table 2). The tallow treatment resulted in excreta with the highest SFA percentage (52%) followed by n-9 MUFA and n-6 PUFA with equal contributions (~20% each). The excreta of flaxseed oil treatment contained higher n-3 PUFA content (16%) than any other treatment. However, this excreta was dominated by n-6 PUFA and n-3 PUFA was only the fourth main contributor. Similar to its respective dietary level (45%), n-6 PUFA was the main fatty acid group in excreta of the corn oil treatment, followed by n-9 MUFA and SFA. Likewise, but at a lower level, n-6 PUFA was the dominant (37%) fatty acid group in the excreta of the canola oil treatment followed by n-9 MUFA and SFA. Noteworthy and reflecting the dietary composition, canola oil excreta contained the second highest percentage of n-3 PUFA (5.3%). The excreta of broilers fed the macadamia oil diet was dominated by n-9 PUFA (39%) and contained the highest n-7 MUFA (~10%) content of all treatments. Excreta of birds in the coconut oil treatment group was dominated by SFA (48%), however this was 16% lower than its respective dietary level. The trans fatty acid group was the lowest contributor to the fatty acid composition of excreta of all treatment groups, reflecting the level in the diets (Table 2). The clear majority of the PUFA in the excreta were as alpha-linolenic acid (ALA, 92-99% of n-3 PUFA) and linoleic acid (LA, 98-99% of n-6 PUFA).

In general, the correlations between the content of the different groups of fatty acid in the diet and excreta were positive, strong and significant. Levels of two fatty acid groups (n-3 PUFA and n-7 MUFA) were particularly closely related between the diet and excreta, with R values ~1.00. The linear regression equations ($y = ax+b$) reflect the differences in the slopes ($a = 0.490$ for n-3 PUFA to 0.762 for n-6 PUFA) of the relationships between the diet and excreta for the different fatty acid groups (Table 3 and Fig. 1).

To provide an indication of the relative utilization of each of the fatty acid groups and individual fatty acids, the proportion of each in the diet and in the excreta was compared. It is inferred that fatty acids that were proportionally higher in the feed than in the excreta were preferentially utilized by the bird (and its microbiome). On the other hand, fatty acids that were proportionally higher in the excreta than in the diet, were preferentially under-utilized. In the light of the results of the present study it is considered that fatty acids that qualitatively differed between diet and excreta by <1% were neither preferentially utilized nor under-utilized. Comparing the relative abundancy of the main fatty acid groups between diets and excreta there was a preference in utilization of n-3 PUFA, n-9 MUFA and a relative selection against the utilization of n-6 PUFA (Fig. 2a). A detailed comparison of the qualitative relative abundance of individual fatty acids in the diet and excreta showed that of the 29 fatty acids measured in the diet and excreta, 19 of them (C9:0, C10:0, C15:0, C17:0, C22:0, C24:0, C13:1, C18:1n-7, C22:1n-9, C20:3n-3, C18:3n-6, C20:2n-6, C20:3n-6, C22:2n-6, C22:4n-6, C22:5n-6, trans C18:1n-9, trans C18:1n-7 and trans C18:2n-6)

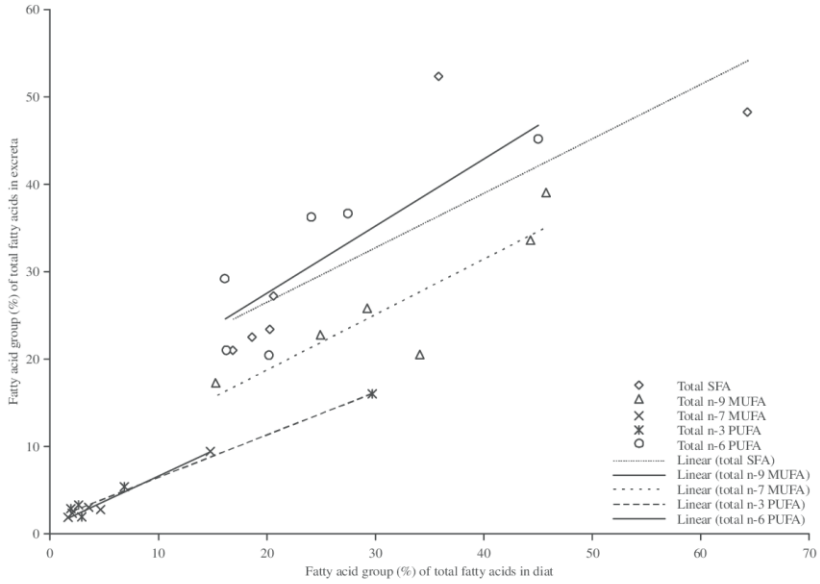

Fig. 1: Relationship between diet and excreta for different fatty acid groups (total saturates, total n-9, total n-7, total n-3 and total n-6) in broilers grown for 40 days on one of six different dietary treatments containing 4% w/w tallow, flaxseed, corn, canola, macadamia or coconut oil

Mean values for excreta are based on 4 replicates

Table 3: Relationship between the levels of the main fatty acid groups in the diets and excreta[1]

	Fatty acid group				
Correlation term	Total SFA[2]	Total n-9[3]	Total n-7[4]	Total n-3[5]	Total n-6[6]
R value	0.82	0.89	0.99	1.00	0.85
p value	0.046	0.018	<0.001	<0.001	0.032
y[7]	0.627x[8]+13.981	0.629x+6.191	0.574x+0.905	0.490x+1.552	0.762x+12.455

[1]Values are the means of 4 replicates. [2]SFA = Saturated fatty acid, [3]n-9 = Omega 9 monounsaturated fatty acid, [4]n-7 = Omega 7 monounsaturated fatty acid, [5]n-3 = Omega 3 polyunsaturated fatty acid, [6]n-6 = Omega 6 polyunsaturated fatty acid, [7]The dependent variable (fatty acid level in excreta), [8]The explanatory variable (dietary fatty acid level)

differed by less than 1% (data not shown) and 10 differed by more than 1% (range -13% to +16%, Fig. 2b). Seven fatty acids from 4 fatty acid groups: C18:3n-3, C16:1n-7, C18:1n-9 and the medium chain length SFA (C8:0 to C14:0) were present at relatively lower levels in the excreta compared to the diet, indicating they were preferentially utilized by the broilers. Conversely, 3 fatty acids from 2 fatty acid groups: C18:2n-6, C16:0 and C18:0 were found at relatively higher levels in the excreta, indicating these fatty acids were somewhat under-utilized by the broilers (Fig. 2b).

DISCUSSION

The productivity parameters measured in this study were similar to our previous observations[2,3,32,34] and better than the recommended commercial values for the Cobb 500 strain[35]. Again, in agreement with most previous studies, there were no significant differences in production parameters between treatment groups. This suggests that all the fats trialled in this study are appropriate sources of macro, micro-nutrients for broilers and their microbiome without affecting feed palatability. On the other hand, this leads us to speculate

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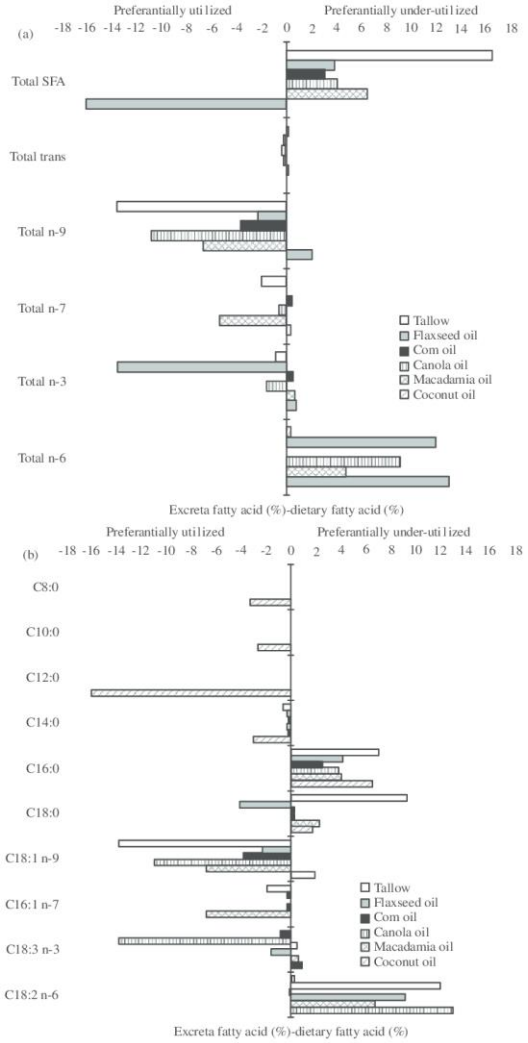

Fig. 2(a-b): Effect of six different experimental diets containing tallow, flaxseed, corn, canola, macadamia or coconut oil on altering the proportions of the (a) Fatty acid groups and (b) Individual fatty acids between diet and excreta
Presented values are for fatty acids with percentage difference >1% based on the means of 4 replicates for excreta

whether the differential effects of dietary fats on broiler performance only become evident if there is an interaction with external factors (e.g., environmental or social stress). The subjective visual observation of the excreta did not indicate any obvious difference between different dietary treatments, however consistent with a previous report[36], the difference in appearance of droppings from individual birds likely reflects their caecal and faecal origins and excreta moisture content.

There is a limited number of publications which have correlated the type and percentage of excreted fatty acids to the dietary intake. Most of studies were focused on the digestion/absorption efficiency of different fatty acids along the length of the intestinal tract[8,10,37,38]. Like the present study, those studies found that the main fatty acids detected in the digesta [(C16:0 (palmitic acid), C18:0 (stearic acid) and C18:2n-6, LA)] were also the main fatty acids present in the dietary fats (soybean oil and tallow). In addition, to the latter 3 fatty acids, C18:1n-9 (oleic acid) was also detected at high levels in the excreta in the present study.

It is acknowledged that these interpretations are general observations only, as the preferential/non-preferential utilization of most fatty acids is obviously affected by the presence or absence of other fatty acids in the diet. Thus, C18:1n-9 was preferentially utilized in all diets except when the fat was provided by coconut oil, suggesting that the medium length SFA in coconut oil were preferentially utilized instead. Similarly, C18:3n-3 (ALA) and C16:1n-7 (palmitoleic acid) were utilized when they were present at relatively high levels. At the other end of the spectrum, C18:2n-6 (LA) was preferentially under-utilized, except when it was the most abundant or at high level relative to other fatty acids (e.g., in the corn oil and tallow diets). The main exception to the rule that degree of utilization was influenced by the other types of fats in the diet was in the case of C16:0, which was always under-utilized by the broilers, irrespective of the level it was in the diet either overall or relative to other dietary fats. These observations agree with the performance data that showed that the birds grew well with good feed intake and feed conversion ratios on all diets. This suggests that whilst there may be a qualitative preference for utilization of different fatty acids, each of these fats can be used successfully to support growth.

While the correlation coefficient is beneficial in showing the type and strength of the relationship between dietary and excreta fatty acid levels, the regression analysis is also valuable. The latter create a mathematical model that allows the researcher or feed manufacturer to estimate from the level of different fatty acids in the chicken diet (independent variable) the proportion that would be excreted (dependent variable) and thereby the amount that would be retained by the birds[39]. Although all fatty acid groups were strongly and positively correlated between the diet and excreta, there was some variation between them. Thus, the relatively low R values for SFA and n-6 PUFA compared to the other fatty acid groups was evidence of their relative under-utilization even when they were the most available fatty acids.

The flaxseed oil treatment has a particular significance as many studies have used this oil to enrich chicken products with beneficial n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA)[40-42]. Although the flaxseed oil diet was dominated by n-3 PUFA, the main fatty acid in the excreta of this treatment was n-6 PUFA and this resulted in increasing the n-6:n-3 ratio in the excreta to 2.25 compared to 0.82 in the diet similar to our previous findings in blood and meat[3,32,34]. Previously it has been demonstrated that by feeding broilers a 2.5 and 5% flaxseed oil-based diet, a considerable percentage of the dietary n-3 PUFA substrate ALA can be elongated and desaturated to n-3 LCPUFA and deposited in various tissues (e.g. 30-40% of total n-3 PUFA in breast meat)[4,33]. In the present study it is observed that 99% of excreted n-3 PUFA was in the form of ALA, indicating the bio-significance of the n-3 LCPUFA in many cellular and health mechanisms[5].

It was interesting that all the fatty acids identified in excreta samples were also detected in the diets. Therefore, we speculate there was no modification (e.g. elongation, saturation/desaturation) of excreted fatty acids by the broiler gut and/or microbiome, despite this being reported by others[43,44]. The only evidence of fatty acid modification was by the chicken itself[32], with hepatic elongases and desaturases converting linoleic acid to arachidonic acid (n-6 PUFA) and ALA to n-3 LCPUFA (n-3 PUFA), with none of these end products being excreted. Thus, the effect of the dietary fat composition (especially the level of PUFA) on the net endogenous fat synthesis in broilers remains unclear[45,46] and further studies will be required resolve the underlying mechanisms.

CONCLUSION

Broilers were fed 1 of 6 diets that had different sources of fat and therefore different fatty acid compositions, produced excreta that generally reflected the dietary fat composition. However, there were some subtle differences in excreta fatty acid composition which suggested variation in preferences for fatty acid utilization by the bird and/or its microbiota. The relative utilization of fatty acids was dependent on the dietary level and on the composition of other fatty acids in the feed. Several fatty acids, particularly the medium SFA (C8:0 (caprylic acid), C10:0 (capric acid), C12:0 (lauric acid) and C14:0 (myristic acid)), C18:1n-9 (oleic acid), C18:3n-3 (alpha-linolenic acid) and C16:1n-7 (palmitoleic acid) were preferentially utilized when they were present. In contrast,

C18:2n-6 (linoleic acid) and C16:0 (palmitic acid) were always under-utilized. Therefore, the non-invasive collection of excreta may provide useful data to feed manufacturers about the utilization of fatty acids in diets made using different lipid sources.

ACKNOWLEDGMENTS

Khaled Kanakri acknowledges the Australian Government Research Training Program (RTP) and the Faculty of Sciences - The University of Adelaide (full-time and supplementary) PhD scholarships. Beverly Muhlhausler is supported by a Career Development Fellowship from the National Health and Medical Research Council of Australia (NHMRC). Robert Gibson is supported by a NHMRC Senior Research Fellowship. This work was supported by the South Australian Department of Further Education, Employment, Science and Technology (DFEEST), Australia.

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4.3. Published manuscript 3 for Chapter 4

Statement of Authorship

Title of Paper	A reduced cost strategy for enriching chicken meat with omega-3 long chain polyunsaturated fatty acids using dietary flaxseed oil.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Accepted 19.12.2016, British Poultry Science, VOL. 58, NO. 3, 283–289 Doi 10 1080/00071668.2017 1293798.

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Name of Principal Author (Candidate)	Khaled Kanakri
Contribution to the Paper	Performed the experimental work, statistical analysis, data interpretation and wrote the manuscript.
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper
Signature	Date 26.07.2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution

Name of Co-Author	John Carragher
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Contribution to the Paper	Principal supervisor of the project, supervised the work development and revised the manuscript and acted as corresponding author.		
Signature		Date	26/7/17

BRITISH POULTRY SCIENCE, 2017
VOL. 58, NO. 3, 283–289
https://doi.org/10.1080/00071668.2017.1293798

A reduced cost strategy for enriching chicken meat with omega-3 long chain polyunsaturated fatty acids using dietary flaxseed oil

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ABSTRACT

1. This study aimed to determine the minimal duration required for feeding male broilers (Cobb 500) with a flaxseed oil diet while still retaining long chain omega-3 polyunsaturated fatty acid (n-3 LCPUFA) accumulation in the meat at a desirable level.
2. Three groups of broilers (60 each) were fed on a 3% flaxseed oil (high α-linolenic acid (ALA)) diet for either 6, 4 or 2 weeks prior to slaughter. During the remaining time they were maintained on a 3% macadamia oil (low ALA) diet. A fourth group (control, $n = 60$) was fed on a commercial diet for 6 weeks.
3. No significant difference was observed in growth performance of broilers between groups. The amounts of total n-3 and n-3 LCPUFA in breast and thigh meat were not different between broilers fed the flaxseed oil diet for 4 and 6 weeks, but they were lower ($P < 0.001$) in those fed the flaxseed diet for only 2 weeks.
4. These results suggest comparable levels of n-3 LCPUFA in the meat can be achieved by only feeding the flaxseed oil diet in the last 3–4 weeks of the growth period; this would result in $a \geq 9.4\%$ reduction in the use of flaxseed oil compared to 6 weeks of feeding; thereby reducing the cost of the enrichment process.

ARTICLE HISTORY
Received 2 June 2016
Accepted 19 December 2016

KEYWORDS
ALA; broiler; feeding duration; n-3 LCPUFA

Introduction

Dietary omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) are essential for normal development and maintenance of optimal health and may play a role in helping to reduce the risk of cardiovascular and allergic disease (Van Den Elsen *et al.*, 2012), diabetes (Oliver *et al.*, 2012), arthritis (Estevão-Silva *et al.*, 2016) and other inflammatory conditions (Connor, 2000; Honda *et al.*, 2015). A number of national and global health agencies, including the World Health Organisation, recommend increasing n-3 LCPUFA intake for cardiovascular health. The amounts vary depending on factors including region, age, gender, health status and physiological conditions (FAO/WHO, 2014). In Australia, the current recommended intake of n-3 LCPUFA for adults is 430–610 mg/d (NATIONAL HEALTH AND MEDICAL RESEARCH COUNCIL (NHMRC), 2006) or 500 mg/d of eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) to reduce the risk of cardiovascular disease (HEART FOUNDATION, 2008). In order to achieve this, health agencies recommend an intake of 2–3 serves of oily fish per week, since this is the richest dietary source of these fatty acids. However, recent data indicates that the vast majority of westerners, including Australians, fail to meet this recommendation, and the average Australian consumes less than one fish meal per fortnight (Dickinson *et al.*, 2015; Meyer, 2016). As a result, the use of fish oil supplements has increased dramatically (Meyer, 2016), but this could negatively impact on the sustainability of marine resources.

Annual consumption of chicken meat in Australia has steadily increased over the last few decades to reach ~46 kg/person in 2016 (Australian Chicken Meat Federation, 2013). Chicken meat is generally classified as a poor source of n-3 LCPUFA; however, the content can be increased by manipulating the broilers' diet, e.g. replacing the usual fat source with fish oil. However, this practice is accompanied by an adverse effect on sensory quality (fishy taint) of the chicken meat at a 2% level of the dietary fish oil (Edwards and May, 1965), a negative effect on growth performance and/or higher drip loss of meat (Zhang *et al.*, 2016). An alternative approach is to substitute the fish oil with sustainably grown plant oil which is rich in the short chain n-3 fatty acid, alpha linolenic acid (ALA), such as flaxseed oil. Chickens can use hepatic elongase and desaturase enzymes to produce n-3 LCPUFA from ALA (Lopez-Ferrer *et al.*, 2001) which can then be incorporated into the chicken tissues to increase the nutritional quality of the meat (or eggs) without detrimental organoleptic effects (Baeza *et al.*, 2013). By feeding broilers a high ALA diet, Kanakri *et al.* (2016) increased the total omega-3 polyunsaturated fatty acids (n-3 PUFA) in whole blood by 6-fold, and Kartikasari *et al.* (2012) reported that n-3 PUFA content in meat was elevated up to 9-fold without affecting the growth parameters of the birds or the sensory properties of the meat.

From an economic point of view, flaxseed oil is more expensive than other plant oils or animal fats used to make chicken feed. This increases the cost of production and thereby the price of the n-3 LCPUFA enriched chicken meat. Optimising an approach to minimise the required

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amount of the expensive flaxseed oil whilst maintaining enhanced levels of n-3 LCPUFA in meat is essential for commercial uptake. To do this, one of the key parameters is to determine the minimal time the high ALA diet needs to be fed to the birds prior to slaughter, and this has been addressed to some extent in previous research using whole ground flaxseed (Betti *et al*., 2009; Zuidhof *et al*., 2009) and a combination of flaxseed oil with plant oil (Gonzalez-Esquerra and Leeson, 2000) or animal fat (Lopez-Ferrer *et al*., 2001). Those studies concluded that it is possible to reduce the feeding period on the relatively expensive diets containing flaxseed or flaxseed oil.

Previous research by the authors identified the minimal sufficient level of flaxseed oil (3%) and optimal n-6:n-3 ratio (~1:1) in the diet to reduce the cost of enriching chicken meat with n-3 LCPUFA (Kartikasari *et al*., 2012). The present study aimed to investigate a further potential approach of reducing the additional cost of flaxseed-induced enrichment of chicken meat with n-3 LCPUFA by shortening the duration of feeding the flaxseed oil supplemented diet, thereby making this strategy more economically viable for the industry to produce n-3-enriched chicken meat products.

Table 1. Ingredient composition and nutrient content of the basal diets.

	Basal diet[a]	
Ingredients (g/kg)	Starter[b]	Finisher[c]
Wheat fine	614.2	707.7
Barley fine	50.0	50.0
Tallow mixer	3.7	10.0
Blood meal (91% CP)	10.0	0.0
Soybean meal	251.7	166.7
Limestone small	8.6	6.4
Monocalcium phosphate	1.4	0.0
Sodium chloride	2.1	1.8
Sodium bicarbonate	1.6	1.8
Choline chloride 75%	0.3	0.4
DL-methionine 58.1	2.9	2.1
L-threonine 73.7	1.3	2.5
Rovabio Excel LC	0.2	0.2
Meat meal	37.3	37.0
Ronozyme NP CT	0.2	0.2
Mineral/vitamin premix[a]	10.0	10.0
L-lysine sulphate 70	4.3	3.3

[a]The starter and finisher basal diets ME = 12.14 and 12.56 MJ/kg, the nutrient contents (g/kg) were as follows: crude protein 225.1 and 186.7, crude fat 23.5 and 30.0, crude fibre 32.3 and 29.2, Ca 9.8 and 8.5, available phosphorus 4.5 and 4.2, Na 1.8 and 1.7, K 0.8 and 6.5, Cl 0.2 and 1.8, lysine 12.3 and 9.1, methionine 5.7 and 4.7, cystine 3.9 and 2.8, threonine 7.9 and 7.6, leucine 14.3 and 11.2, isoleucine 7.8 and 6.4, tryptophan 2.3 and 1.8, arginine 12.4 and 9.8 and valine 9.3 and 7.2, respectively.
[b]Used to formulate two experimental diets to feed broilers up to 3 weeks old.
[c]Used to formulate two experimental diets to feed broilers from 4 to 6 weeks old.

Materials and methods

Birds and experimental design

The Animal Ethics Committees of the University of Adelaide and the Department of Primary Industries and Resources South Australia approved the study (S-2013-152). A total of 240 male 1-d-old Cobb 500 broilers were purchased from a local hatchery. Birds were randomly distributed into groups of 10 and allocated to 24 raised rearing floor pens (1.2 × 0.9 m each) in one shed. A complete randomised block design (6 pens/treatment) was implemented. Broilers were reared under strictly controlled environmental conditions with free access to both feed and water at all times. The room temperature was 27°C for the first 4 d and then gradually decreased to 20°C and maintained at this temperature for the 6-week experimental period. Individual pens were heated by infrared lamps (175 W) during the first 3 weeks.

Experimental treatments

Three types of diets were utilised in this study. The first diet was a commercial broiler formulation (Ridley Agriproducts Pty Ltd, Murray Bridge, SA, Australia). The other two diets were prepared by adding 3% (w/w) of a selected fat type; macadamia oil (low ALA; Macoils, Alstonville, NSW, Australia) or flaxseed oil (high ALA; Four Leaf Oils, SA, Australia) to basal starter and finisher broiler diet formulations. Table 1 shows the composition of the basal diets. Starter diets were fed in the first 3 weeks and the finisher diets were fed in the last 3 weeks of the experiment. Apart from the variation in the fatty acid composition, both of the experimental diets were nutritionally identical and met requirements for efficient broiler performance.

The study included 4 feeding treatments. In the control treatment, birds were fed on the commercial diet for the entire 6-week rearing period. In the second experimental feeding treatment, birds were fed on the low ALA, macadamia-oil-based diet for the first 2 weeks and then they were maintained on the high ALA, flaxseed-oil-based diet for the remaining period of 4 weeks (ALA4). Birds in the third group were fed the low ALA diet in the first 4 weeks and then they were maintained on the high ALA diet for the remaining period of 2 weeks (ALA2). In the fourth group (ALA6), birds were fed the high ALA diet from d 1 post-hatch until the end of the growing period of 6 weeks.

Growth performance

Group weight of the birds in each pen was taken on d 1 and then on a weekly basis for the first 3 weeks. In the final 3 weeks of the experiment, the body weight (BW) of birds was recorded individually. Feed intake (FI) of birds in each pen was also recorded on a weekly basis and used to calculate the feed conversion ratio (FCR; kg feed/kg BW gain) taking into account the number of deaths and culls. The latter was recorded on a daily basis to calculate the mortality rate.

Tissue collection

At 6 weeks of age, two birds per pen were randomly selected and killed by cervical dislocation. Approximately 30 g of lean skinless thigh and breast meat tissue were collected in clean plastic containers and immediately placed on dry ice before transferring to the laboratory. Samples were stored in freezer at −18°C till subsequent determination of crude fat content and fatty acid composition.

Fatty acid analysis

Total lipid was extracted from a representative feed and tissues samples as described by Folch *et al.* (1957) with chloroform/methanol (2:1, v/v) solution. The gravimetric approach was

utilised to estimate total lipid content. Fatty acid profiling was performed after transmethylation of the extracted crude lipids with 1% H_2SO_4 in methanol at 70°C for 3 h (Gregory *et al.*, 2010; Tu *et al.*, 2010). After cooling to room temperature, the resulting fatty acid methyl esters (FAME) were extracted with *n*-heptane (2 ml) and transferred into gas chromatography (GC) vials containing about 30 mg of anhydrous sodium sulphate. Vials were stored at −18°C until GC analysis.

Gas chromatography analysis of FAME

The FAME were separated using a Hewlett-Packard 6890 GC (Hewlett-Packard, CA, USA) equipped with a flame ionisation detector, a split injector and a BPX-70 capillary column (50 m × 0.32 mm internal diameter) with a 0.25-µm film thickness (SGE, Victoria, Australia). The operating conditions of the GC, fatty acid identification using the GLC 463 external standard (Nu-Chek Prep Inc, MN, USA) and qualitative analysis were as described by Kartikasari *et al.* (2012).

Statistical analysis

The effects of dietary treatment on tissue fatty acid profile were tested by one-way analysis of variance (ANOVA) using SPSS version 22 for Windows (IBM Corp., NY, USA). Tukey multiple comparison test was implemented when treatment effects were significantly different ($P < 0.05$). The effects of dietary treatment on the production parameters, BW, FI and FCR, were determined by one-way ANOVA using SAS 9.3 for Windows. The mortality rate was examined by non-parametric analysis (Kruskal–Wallis test) in SPSS version 21 for Windows (IBM Corp., NY, USA).

Results

Diets

On average, the commercial diet (control) contained less crude fat (4.5%) than the other two experimental diets, 6.2% and 6.3% in the macadamia oil and flaxseed oil diets, respectively. The fatty acid composition of the diets is shown in Table 2. The total saturated fatty acids (SFA) of the different diets comprised approximately 19%–28% of total fatty acids and *trans* fatty acids were only found in low levels (<1.0%). As intended, the level of total n-3 was highest (27.6%) in the flaxseed oil diet and lowest in the macadamia oil diet (2.0%). The macadamia oil diet had a total monounsaturated fatty acids (MUFA) level of 46.9%, most of which was n-9 fatty acids. Total omega-6 polyunsaturated fatty acids (n-6 PUFA) were the predominant group (40%) in the control diet. The n-3 and n-6 fatty acids in the diets were ALA (Table 2) and linoleic acid (data not shown), respectively. There was no more than 0.1% n-3 LCPUFA detected in any diet. The dietary n-6:n-3 ratio decreased from about 15:1 and 9:1 in the macadamia oil and control diets, respectively, to approximately 1:1 in the flaxseed oil diet.

Growth performance

No growth performance parameter was found to be significantly different between the 4 dietary treatments either during any individual week or at the end of the experiment (Table 3). By the final day of the study, the average live BW was 3689 g, FI was 5941 g/bird and FCR was 1.62 g feed/g gain across all treatments. Overall mortality was 7.1% and not statistically different between treatments.

Table 2. Fatty acid profile (% of total fatty acid) of the basal and experimental diets[a].

	Basal diets[b]		Experimental diets[c]					
			Commercial		High ALA		Low ALA	
	S	F	S	F	S	F	S	F
Crude fat, %[d]	3.0	3.8	4.6	4.3	6.1	6.4	6.3	6.1
Fatty acid (%)[e]								
Total SFA	27.5	29.0	26.1	27.7	18.6	19.9	20.8	21.0
Total *trans*	1.0	1.2	0.8	1.0	0.5	0.6	0.5	0.5
Total n-9	20.4	22.9	25.1	24.1	21.2	22.7	37.5	37.2
Total n-7	2.1	2.6	2.5	2.6	1.5	1.7	9.6	9.5
Total n-6	45.0	40.3	40.3	40.0	29.6	28.0	29.3	29.7
Total n-3	3.9	3.5	4.9	4.2	28.4	26.8	2.0	1.9
ALA	3.8	3.4	4.8	4.0	28.3	26.7	2.0	1.8
EPA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
DPA	0.1	0.1	0.0	0.1	0.0	0.0	0.0	0.0
DHA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
n-6:n-3	11.5	11.5	8.2	9.6	1.0	1.0	14.6	15.6

[a]Values are means of basal diet $n = 2$, commercial diet $n = 3$, high ALA diet $n = 9$ and low ALA diet $n = 5$.
[b]S = starter, F = finisher.
[c]Commercial = a standard commercial diet, high ALA = flaxseed-oil-based diet (3% w/w), low ALA = macadamia-oil-based diet (3% w/w).
[d] Percentages are based on the wet weight.
[e]SFA = saturated fatty acid; n-9 = omega 9 monounsaturated fatty acid; n-7 = omega 7 monounsaturated fatty acid; n-6 = omega 6 polyunsaturated fatty acid; n-3 = omega 3 polyunsaturated fatty acid; ALA = α-linolenic acid; EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid.

Table 3. Growth performance parameters and flaxseed oil consumption by broilers for 6 weeks[a]

	Growth performance parameters[a]			Flaxseed oil consumption[e]
Week	BW[b] (g)	FI[c] (g)	FCR[d]	g/bird (% of total)
1	173.2	143	1.13	4.3 (2.4%)
2	496.4	557	1.26	16.7 (9.4%)
3	1055	1317	1.33	39.5 (22.2%)
4	1797	2491	1.43	74.7 (41.9%)
5	2661	4002	1.52	120.1 (67.4%)
6	3689	5941	1.62	178.2 (100%)

[a]Values are the means of 240 broilers from 4 groups received different dietary treatments; accumulatively on weekly basis.
[b]Body weight.
[c]Feed intake.
[d]Feed conversion ratio (feed intake (g) to body weight gain (g)).
[e]Based on 3% (w/w).

Fatty acid composition of meat tissues

The crude fat percentage and fatty acid analysis of broiler breast and thigh meat are presented in Table 4. Crude fat determination of lean meat tissues showed no significant difference between dietary groups, with thigh meat containing 2.0% and breast meat 1.9% fat on average, across all treatments. The broilers in the control (commercial diet) group had the same low level (2.5%) of total n-3 fatty acids in both types of meat. This percentage increased to 10.3%, 13.0% and 13.4% ($P < 0.001$) in breast meat, and 9.5%, 12.1% and 12.8% in thigh meat for the ALA2, ALA4 and ALA6 groups, respectively. The percentage increase in total n-3 PUFA content between the control and ALA2, ALA4 and ALA6 groups was greater in breast meat than thigh meat ($P < 0.001$). There was no significant difference in tissue total n-3 PUFA content between the ALA4 and ALA6 diet treatments, but both were significantly higher than

Table 4. Fatty acid profiles[1] (as % of total fatty acids) of meat tissues from male broiler chickens fed with different dietary treatments and sampled at 42 days of age.

	Dietary treatment				
	Control[2]	ALA2[3]	ALA4[4]	ALA6[5]	*P*-value
Breast meat					
Crude fat%[6]	1.8 ± 0.10	1.9 ± 0.17	1.8 ± 0.11	2.0 ± 0.15	0.584
Fatty acids (%)[7]					
Total SFA	34.5^{a}±0.24	32.6^{b}±0.45	32.9^{ab}±0.52	33.3^{ab}±0.65	0.046
Total n-9	34.2^{a}±0.55	32.1^{ab}±0.83	30.6^{b}±0.59	30.4^{b}±1.06	0.005
Total n-7	8.9^{a}±0.15	8.2^{b}±0.14	7.3^{c}±0.17	7.1^{c}±0.22	0.000
Total n-6	19.1^{a}±0.38	16.0^{b}±0.43	15.3^{b}±0.23	15.0^{b}±0.44	0.000
Total n-3	2.5^{a}±0.07	10.3^{b}±0.23	13.0^{c}±0.23	13.4^{c}±0.27	0.000
ALA	1.2^{a}±0.03	7.0^{b}±0.32	8.0^{b}±0.49	7.9^{b}±0.54	0.000
n-3 LCPUFA	1.2^{a}±0.02	3.0^{b}±0.11	4.7^{c}±0.17	5.1^{c}±0.24	0.000
EPA	0.3^{a}±0.01	1.0^{b}±0.09	1.3^{bc}±0.11	1.6^{c}±0.22	0.000
DPA	0.6^{a}±0.03	1.5^{b}±0.16	2.3^{c}±0.24	2.4^{c}±0.32	0.000
DHA	0.4^{a}±0.02	0.6^{a}±0.08	1.0^{b}±0.17	1.1^{b}±0.18	0.000
n-6:n-3	7.60	1.54	1.17	1.12	0.000
Thigh meat					
Crude fat%[6]	1.84±0.19	2.19±0.17	1.79±0.18	2.23±0.11	0.124
Fatty acids (%)[7]					
Total SFA	35.3^{a}±0.40	32.9^{b}±0.47	32.9^{b}±0.33	33.3^{b}±0.44	0.000
Total n-9	31.6±0.47	31.2±0.66	30.5±0.87	29.5±0.55	0.101
Total n-7	8.4^{a}±0.15	8.0^{a}±0.26	7.1^{b}±0.20	6.9^{b}±0.18	0.000
Total n-6	21.4^{a}±0.30	17.6^{b}±0.70	17.0^{b}±0.60	16.8^{b}±0.35	0.000
Total n-3	2.5^{a}±0.16	9.5^{b}±1.20	12.1^{c}±0.32	12.8^{c}±0.20	0.000
ALA	1.1^{a}±0.40	6.6^{b}±0.89	8.1^{c}±0.33	8.2^{c}±0.31	0.000
n-3 LCPUFA	1.3^{a}±0.09	2.7^{b}±0.11	3.7^{c}±0.07	4.3^{c}±0.06	0.000
EPA	0.3^{a}±0.08	0.8^{b}±0.12	1.1^{c}±0.08	1.2^{c}±0.06	0.000
DPA	0.7^{a}±0.18	1.4^{b}±0.21	1.9^{c}±0.16	2.2^{c}±0.10	0.000
DHA	0.4^{a}±0.09	0.5^{a}±0.09	0.8^{b}±0.06	0.9^{b}±0.07	0.000
n-6:n-3	8.43	1.84	1.41	1.32	0.000

[1]Values are means of 12 birds per treatment and their pooled standard error of the mean (SEM). Values in the same row without a common superscript alphabet are significantly different.
[2]A standard commercial broiler diet (Ridley Agriproducts Pty Ltd, Murray Bridge, Australia) was fed for the entire 6-week rearing period.
[3]Broilers were fed a macadamia oil diet in the first 4 weeks and then a flaxseed oil diet in the last 2 weeks of the 6-week rearing period.
[4]Broilers were fed a macadamia oil diet in the first 2 weeks and then a flaxseed oil diet in the last 4 weeks of the 6-week rearing period.
[5]Broilers fed the flaxseed oil diet throughout the 6-week rearing period.
[6]Based on the wet weight.
[7]SFA = saturated fatty acid; n-9 = omega 9 monounsaturated fatty acid; n-7 = omega 7 monounsaturated fatty acid; n-6 = omega 6 polyunsaturated fatty acid; n-3 = omega 3 polyunsaturated fatty acid; ALA = α-linolenic acid; n-3 LCPUFA = omega 3 long chain polyunsaturated fatty acids; EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid.

ALA2 (Table 4). The increase in total n-3 PUFA content was at the expense of most other groups of fatty acids, particularly n-6 PUFA and n-9 and n-7 MUFA, whereas the percentage of SFA was not different between treatments.

A detailed breakdown of the individual n-3 PUFA in both meat tissues indicated that the majority of the increase in total n-3 PUFA content (approximately two thirds) was due to an increase in ALA, with the remainder being n-3 LCPUFA (docosapentaenoic acid (DPA), EPA and DHA). The pattern of increase of ALA, DPA, EPA and DHA was similar to that seen for total n-3, in that the levels of all individual n-3 fatty acids were not significantly different between the ALA4 and ALA6 groups, but were higher in both these groups than in the ALA2 group (except that of ALA in breast meat). In addition, all ALA groups had higher levels of all n-3 fatty acids compared to the control group ($P < 0.001$).

Another nutritional benefit of feeding flaxseed oil diet to birds was a reduction in the n-6:n-3 ratio in the thigh and breast meat from 8.4 and 7.6 in the control group, to 1.3 (thigh meat) and 1.1 (breast meat) in the ALA4 and ALA6 birds (Table 4).

Discussion

The present study shows that feeding broilers a high ALA diet for any interval during the growing period does not significantly affect their growth performance (BW, FI, FCR or mortality rate). Indeed, broilers of all treatments performed better than the published Cobb 500™ (2015) performance objectives at the same age (42 d), with a higher BW (by 645 g/bird), a lower daily FI (by 91 g/bird) and lower FCR by 0.06. The observation that growth performance of broilers fed on n-3 PUFA-enriched diets was not different from those fed on a control diet is consistent with the outcomes of several previous studies (Fota *et al.*, 2010; Lopes *et al.*, 2013; Mandal *et al.*, 2014; Mirshekar *et al.*, 2015; Kanakri *et al.*, 2016), while other studies have reported that a high ALA diet was associated with improved growth and/or FCR (Carragher *et al.*, 2015; Zelenka *et al.*, 2006; Duarte *et al.*, 2014). Thus, the vast majority of studies conducted to date suggest that dietary ALA is not deleterious to broiler production and may even have potential advantages.

In this study, the duration of feeding broilers on the flaxseed oil diet did not change crude fat percentages in either breast or thigh meat. This indicates that the influence of the high ALA dietary treatment did not change the total fat content of the meat, but only altered the fatty acid composition of the lipid fraction. This finding agrees with a similar study in this field (Gonzalez-Esquerra and Leeson, 2000).

In general, the effect of feeding the high ALA diet in the present study was consistent with the published literature and with our previous findings, such that the broilers converted some of the dietary ALA to n-3 LCPUFA and

deposited these beneficial fatty acids in the meat tissues (Carragher *et al*., 2015; Kartikasari *et al*., 2012). However, the main aim of the present study was to determine whether feeding broilers on the high ALA diet for reduced periods prior to slaughter adversely affected the tissue accumulation of n-3 LCPUFA. There was no difference in the accumulation of n-3 LCPUFA between broilers that were fed the ALA diet for 4 or 6 weeks prior to slaughter. However, the n-3 LCPUFA content of broilers fed the diet for only 2 weeks was about 30% lower than ALA4, but still 2.3-fold higher than the control. This indicates that feeding 3% w/w flaxseed oil diet for only 2 weeks prior to slaughter is not sufficient for the birds to accumulate similar amounts of n-3 LCPUFA as feeding the same diet for 4 or 6 weeks. However, it appears that a feeding duration of between 2 and 4 weeks may be sufficient for the broilers to accumulate the same amount of total n-3 PUFA/LCPUFA as feeding the diet for 6 weeks.

A recent study by Mirshekar *et al*. (2015) reported the effect of various durations of feeding flaxseed-oil-based diet on the n-3 LCPUFA levels in broiler meat. However, in their study, the percentage of dietary flaxseed oil was 2.5 (w/w) in the starter diet and 5% (w/w) in the finisher diet. In the experimental substitute diet, soybean oil (48.8% n-6 PUFA and 9.0% n-3 PUFA) was used as a replacement lipid source and sex of the birds was not taken into account. These experimental differences mean it is not possible to extrapolate their findings to our experimental model. Nevertheless, on the basis of the results of their study, they recommended that feeding 5% flaxseed oil diet to mixed sex birds for 2.5 weeks prior to slaughter was sufficient for optimising n-3 LCPUFA accumulation in breast meat, but thigh meat required a shorter time (1 week). Our results do not, however, indicate that there was a difference between these tissues in the time taken to accumulate n-3 LCPUFA, although there were differences in the magnitude of the effect between tissues (discussed further below).

Gonzalez-Esquerra and Leeson (2000) found that feeding broilers a high (10% w/w) flaxseed oil diet for only 1 week before slaughter increased ALA and total n-3 content of the chicken meat, but did not increase in n-3 LCPUFA. This is similar to the findings of the current study, since ALA levels in the breast tissue were not significantly different between birds fed on the ALA diet for only 2 weeks and those fed it for 6 weeks. This therefore suggests that short-term feeding with a flaxseed oil diet will allow a similar amount of ALA to be deposited in breast meat tissue in comparison to a longer feeding duration, but that longer feeding periods are required for this to increase n-3 LCPUFA synthesis and tissue deposition. Furthermore, greater than two thirds of the total accumulated n-3 in meat tissues in ALA2 birds was ALA. In breast meat, this contribution decreased from 68.0% (ALA2) to 61.5% and 59.0% in ALA4 and ALA6, respectively. This may suggest that it takes some time for the elongase and desaturase enzymes to respond to the change in dietary fatty acids in order to synthesise the n-3 LCPUFA or deposit it in the phospholipid fraction. Previous studies have reported that increases in ALA consumption do not alter the mRNA expression of elongases and desaturases in the breast meat (Haug *et al*., 2014), and longer durations of ALA feeding have even been associated with reduced expression of these enzymes (Mirshekar *et al*., 2015). However, this is unlikely to affect the capacity for n-3 LCPUFA synthesis, since this process is regulated to a greater extent by substrate availability, rather than gene expression of the regulatory enzymes (Tu *et al*., 2010).

Interestingly, although the same changes in ALA and n-3 LCPUFA content were observed in breast and thigh meat, the magnitude of ALA contribution of the total n-3 PUFA was less (by 2% and 5% in ALA4 and ALA6, respectively). This may be because thigh meat has more extracellular fat, which is mostly triglycerides, and this fraction contains most of ALA (Betti *et al*., 2009). These data also suggest that increasing feeding time on a high ALA diet could be a reason to improve the ALA conversion efficiency to n-3 LCPUFA, and the deposition of n-3 LCPUFA in meat tissues, particularly in breast meat. A similar recommendation was reported by Zuidhof *et al*. (2009) using a diet containing ground full-fat flaxseed.

The trends of changes in the individual n-3 LCPUFA in both meat tissues demonstrated a similar pattern to that of the total n-3 PUFA content, as discussed previously. In general, EPA, DPA and DHA percentages were slightly higher in breast meat than in thigh meat, with DPA being the most prevalent n-3 LCPUFA (50% of all n-3 LCPUFA) in all treatments. In relative terms, EPA increased to the greatest extent, by 5.3- and 4.0-fold in breast and thigh meat, while DHA accumulated least, with a 2.8- and 2.3-fold increase in breast and thigh meat, respectively, in the broilers fed the high ALA diet for 6 weeks.

In terms of the potential of n-3 LCPUFA-enriched broiler meat to improve daily n-3 LCPUFA intake of consumers who follow a western-style diet, based on the n-3 LCPUFA percentages, the total amount of fat in the chicken meat, and the average daily consumption of chicken meat in Australia, the ALA6 treatment group would provide an additional 28.6, 41.2 and 15.5 mg/person/d of EPA, DPA and DHA, respectively, and a total n-3 LCPUFA intake of 117 mg/d compared to 31.7 mg/d from the control (commercial) chicken meat. The n-3 PUFA-enriched chicken meat product could therefore contribute 14% and 18.5% of the recommended daily intakes for men and women, respectively (NATIONAL HEALTH AND MEDICAL RESEARCH COUNCIL (NHMRC), 2006). Moreover, since the increase in total n-3 PUFA content was coupled with a decrease in total n-6 PUFA content, the n-6:n-3 PUFA ratio was lower by 77.9% and 85.5% in the breast and thigh meat, respectively, in comparison to the control meat. This is likely to provide additional nutritional and health benefits for the consumer, given recent evidence implicating the high n-6 PUFA intakes in modern western diets in a range of chronic diseases (Wijendran and Hayes, 2004; Wall *et al*., 2010).

Our results confirm that it is possible to implement a time-reduction strategy to lower the production cost of enriching broiler meat with n-3 LCPUFA from a flaxseed-oil-based diet. The estimated benefit of excluding the first 2–3 weeks of feeding broilers the relatively expensive high ALA diet is a saving of 9.4%–22.2% (16.7–39.5 g, Table 3) of the required amount of flaxseed oil. Based on the difference in price between flaxseed oil and other cheaper dietary fats, the additional cost of the enrichment process can be reduced by a similar percentage. Being able to feed broilers the high ALA diet for a shorter period will support commercialisation of n-3 LCPUFA-enriched broiler meat at an affordable price for the industry and consumers.

Acknowledgements

The authors wish to thank Ridley AgriProducts Pty Ltd, Murray Bridge, Australia, for providing the broiler feed. We are grateful to Mr Derek Schultz at SARDI, Roseworthy Campus, for his help in conducting the broiler experiment and to Ms Ela Zielinski at FOOD*plus* Research Centre for her technical assistance and advice in the fatty acid analysis. We acknowledge the financial support of the University of Adelaide and the South Australian Department of Further Education, Employment, Science and Technology, Australia (DFEEST).

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The experimental work was financed by a grant from the South Australian Department of Further Education, Employment, Science and Technology (DFEEST), Australia. Mr Khaled Kanakri received Research Training Program (RTP) from The Australian Government Research Training Program Scholarship and full PhD scholarship from the Faculty of Sciences, University of Adelaide. Beverly Muhlhausler is supported by a Career Development Fellowship from the National Health and Medical Research Council of Australia (NHMRC). Robert Gibson is supported by a NHMRC Senior Research Fellowship..

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Appendix4

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4.4. Published manuscript 4 for Chapter 5

Statement of Authorship

Title of Paper	*In ovo* exposure to omega-3 fatty acids does not enhance omega-3 long chain polyunsaturated fatty acid metabolism in broiler chickens
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Accepted 14.03.2017, Published online, Journal of Developmental Origins of Health and Disease Doi:10.1017/S2040174417000216.

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Name of Principal Author (Candidate)	Khaled Kanakri
Contribution to the Paper	Performed the experimental work (field part and fatty acid analysis), statistical analysis, data interpretation, wrote the manuscript and acted as corresponding author.
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 26.07.2017

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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Journal of Developmental Origins of Health and Disease (2017), **8**(5), 520–528.

doi:10.1017/S2040174417000216
Themed Issue: Australian Perspectives: Outcomes from the 2016 ANZ

ORIGINAL ARTICLE

In ovo exposure to omega-3 fatty acids does not enhance omega-3 long-chain polyunsaturated fatty acid metabolism in broiler chickens

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The content of omega-3 long-chain polyunsaturated fatty acids ($n-3$ LCPUFA) in chicken meat can be boosted by feeding broilers a diet containing α-linolenic acid (ALA, from flaxseed oil), some of which is converted by hepatic enzymes to $n-3$ LCPUFA. However, most of the accumulated $n-3$ polyunsaturated fatty acid (PUFA) in meat tissues is still in the form of ALA. Despite this, the levels of chicken diets are being enhanced by the inclusion of vegetable and marine sources of omega-3 fats. This study investigated whether the capacity of chicken for $n-3$ LCPUFA accumulation could be enhanced or inhibited by exposure to an increased supply of ALA or $n-3$ LCPUFA *in ovo*. Breeder hens were fed either flaxseed oil (High-ALA), fish oil (high $n-3$ LCPUFA) or tallow- (low $n-3$ PUFA, Control) based diets. The newly hatched chicks in each group were fed either the High-ALA or the Control diets until harvest at 42 days' post-hatch. The $n-3$ PUFA content of egg yolk and day-old chick meat closely matched the $n-3$ PUFA composition of the maternal diet. In contrast, the $n-3$ PUFA composition of breast and leg meat tissues of the 42-day-old offspring closely matched the diet fed post-hatch, with no significant effect of maternal diet. Indeed, there was an inhibition of $n-3$ LCPUFA accumulation in meat of the broilers from the maternal Fish-Oil diet group when fed the post-hatch High-ALA diet. Therefore, this approach is not valid to elevate *n*-3 LCPUFA in chicken meat.

Received 14 January 2017; Revised 7 March 2017; Accepted 14 March 2017; First published online 12 April 2017

Key words: chicks, fish oil, flaxseed oil, maternal diet, meat, n – 3

Introduction

The omega-3 long-chain polyunsaturated fatty acids ($n-3$ LCPUFA) have a number of important health benefits in humans, in particular in relation to inflammatory conditions such as rheumatoid arthritis and protection against cardiovascular diseases.[1,2] These effects rely on the incorporation of $n-3$ LCPUFA into the phospholipid fraction of the cell membrane, and subsequent release in the free fatty acid pool to give rise to bio-active mediators.[3] This has led to recommendations from a number of health agencies for humans to increase their consumption of fish and seafood, the richest dietary sources of these fatty acids. Despite this, consumption of these sources remains low in western countries,[1,4] and fish and seafood are also not environmentally sustainable sources of these fats.[5] In contrast to seafood, the global consumption of poultry, especially chicken, is steadily increasing and this is now the most popular type of meat in many societies.[6] This has led to suggestion that one strategy to increase dietary $n-3$ LCPUFA intake in western countries, and one which would avoid placing additional pressure on global marine resources, is to increase the $n-3$ LCPUFA content of chicken meat.[7,8]

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Chicken meat is naturally low in fat (~2.0%), and a poor source of $n-3$ polyunsaturated fatty acids (PUFA) at only ~2.5% of total fatty acids including $n-3$ LCPUFA at ~1.3%.[9] However, we and other researchers have demonstrated that increasing the amount of $n-3$ PUFA in the diet, by feeding chickens diets supplemented with flaxseed (*Linum usitatissimum*) oil [high in the short-chain $n-3$ PUFA, α-linolenic acid (ALA)], results in substantial increases in the ALA content of the meat, without increasing the overall fat content.[10] Importantly, chickens also possess the hepatic enzymes required to synthesize the $n-3$ LCPUFA; eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), from ALA.[7,11] Consequently, replacing standard fat sources in formulated chicken diets such as corn oil, canola oil, soybean oil (all rich in $n-6$ PUFA), animal fat or their blends, with flaxseed oil reduces the dietary $n-6$: $n-3$ PUFA ratio and increases the $n-3$ LCPUFA content of chicken tissues[9,10,12–14], as $n-6$ and $n-3$ PUFA precursors compete for the same hepatic enzymes in their elongation and desaturation pathways.[10,15] Despite the success of High-ALA diets for increasing the level of $n-3$ LCPUFA in chicken meat, the actual levels of $n-3$ LCPUFA which are achieved (76.5–108 mg/100 g meat)[9] are still substantially lower than those in oily fish (e.g. herring, salmon and mackerel), which contains >1500 mg in the similar amount of meat.[16]

Improving the efficiency through which High-ALA diets improve chicken meat $n-3$ LCPUFA content relies on altering

the processes which regulate ALA conversion to $n-3$ LCPUFA and/or deposition of fatty acids into different lipid fractions.[17,18] The egg yolk is the main reserve of energy and the sole source of essential fatty acids during embryogenesis,[19] and previous studies have demonstrated that feeding layer hens a diet enriched in ALA increases the $n-3$ LCPUFA content of the eggs[20–23] and of the chicks at hatch.[22] In addition, feeding hens a diet enriched with fish oil results in higher $n-3$ PUFA levels in cardiac tissues in their chicks.[24] However, no previous studies have determined the effect of *in ovo* exposure to an increased supply of $n-3$ PUFA on the chicken's subsequent capacity for ALA-derived $n-3$ LCPUFA accumulation in meat tissues. Therefore, the aim of the present study was to determine the effect of maternal dietary treatments that would expose developing chicks *in ovo* to an elevated level of $n-3$ LCPUFA, or its precursor (ALA) on the capacity of chickens to accumulate $n-3$ LCPUFA when fed a High-ALA diet post-hatch.

Methods

Maternal dietary treatments

A total of 324 of broiler breeder hens of the Cobb 500 strain were housed in the HiChick Breeding Company facility (Bethyl, SA, Australia). Before the study all the hens received the same commercial breeder diet. Hens were allocated to one of three dietary groups ($n = 108$/group): Control (basal diet mixed with 4% w/w beef tallow), High-ALA (basal diet mixed with 4% w/w flaxseed oil) or Fish-Oil (basal diet mixed with 4% w/w fish-oil) (Fig. 1.). The breeder basal diet used for all feeds was purchased from Lauke Mills, Australia. The three diets contained the same proportions of fat, carbohydrate and protein, and differed only in their fatty acid composition (Table 1). All three experimental diets contained the same levels of vitamins and minerals and these either met or exceeded the recommended levels.[25] Each group of hens was housed separately with eight roosters from the same strain, and fed the diets for the duration of the experiment (Fig. 1).

Egg sampling

All eggs that were laid in the 5th week of the maternal diet regimen (Control, $n = 132$ eggs; High-ALA, $n = 148$ eggs; Fish-Oil, $n = 80$ eggs) were collected and stored at holding temperature to temporarily prevent initiation of embryonic development. The yolk from five eggs selected at random from each maternal diet group were collected and stored at −18°C for subsequent fatty acid analysis. The remaining eggs were transferred to the South Australian Research and Development Institute (Roseworthy, SA, Australia) and immediately placed in incubators under standardized conditions (38°C and 55% humidity, increasing to 60% in the last 4 days of incubation). After 1 week of incubation, the fertility of each egg was assessed, and non-fertile eggs and eggs with dead embryos were discarded.

Chick hatch and sampling

Chicks were hatched at ~21 days after the start of the incubation, and at 1-day old were feather-sexed into pullets and cockerels[26]

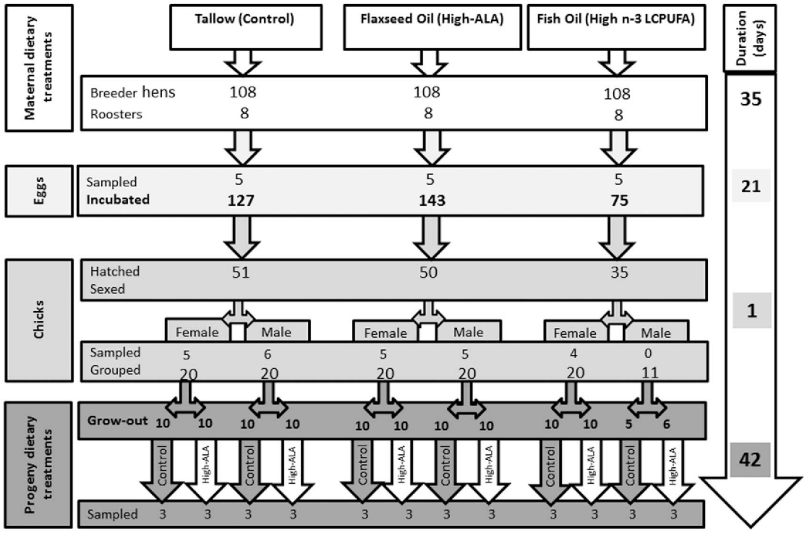

Fig. 1. The methodology and numbers of birds used during different experimental phases.

Table 1. *Fatty acid composition of the experimental diets*[a]

	Maternal diets[b]			Progeny diets[c]	
Fatty acid group	Control	Flaxseed oil	Fish oil	Control	Flaxseed oil
Crude fat %[d]	7.5	7.7	7.8	6.3	6.6
Total SFA[e]	33.0	15.3	25.4	36.7	19.3
Total *trans*	0.6	0.1	0.3	1.7	0.3
Total *n* – 9 MUFA[f]	37.1	25.9	27.1	30.5	18.2
Total *n* – 7 MUFA[g]	4.2	2.0	7.4	3.7	1.6
Total *n* – 6 PUFA[h]	19.8	25.2	18.9	24.4	30.5
Total *n* – 3 PUFA[i]	3.4	30.6	19.4	2.7	29.9
ALA[j]	3.2	30.6	3.3	2.4	29.8
Total *n* – 3 LCPUFA[k]	0.2	0.1	16.1	0.2	0.0
n – 6:*n* – 3	5.8	0.8	1.0	9.0	1.0

[a]Values are fatty acid group percentage of the total fatty acid.
[b]By mixing basal breeder hens diet with (4% w/w) beef tallow (Control), flaxseed oil-based diet or fish oil ($n = 3$).
[c]By mixing finisher basal diet with (4% w/w) beef tallow (Control), flaxseed oil ($n = 6$).
[d]Percentages are based on the wet weight.
[e]SFA, Saturated fatty acid.
[f]*n* -9 MUFA, Omega 9 monounsaturated fatty acid.
[g]*n* -7 MUFA, Omega 7 monounsaturated fatty acid.
[h]*n* -6 PUFA, Omega 6 polyunsaturated fatty acid.
[i]*n* -3 PUFA, Omega 3 polyunsaturated fatty acid.
[j]ALA, α-linolenic acid ALA.
[k]*n* -3 LCPUFA, Omega 3 long chain polyunsaturated fatty acid.

(Control: $n = 26$ females, $n = 25$ males; High-ALA: $n = 25$ females, $n = 25$ males; Fish-Oil: $n = 24$ females, $n = 11$ males). In total, 20 male and 20 female chicks in each maternal dietary group were then allocated to two separate raised floor pens (1.2×0.9 m each; $n = 10$ chicks/pen), except for the Fish-Oil group males where the pens held six and five chicks due to the smaller number of hatchlings (Fig. 1).

The unallocated 1-day old chicks (Control: six females and five males; High-ALA: five of each sex; and Fish-Oil: four females only) were euthanized by cervical dislocation and ~1–2 g of breast and leg meat were collected in plastic vials and immediately placed on dry ice. Samples then were transferred to the laboratory where they stored at −18°C until subsequent determination of crude fat content and fatty acid composition.

Housing of broilers

A complete factorial randomized block design ($3 \times 2 \times 2$) was implemented such that one pen of male and one pen of female chicks in each of the three maternal dietary treatment were fed either the control (4% w/w beef tallow) or the High-ALA (4% w/w flaxseed oil) progeny diets for the entire 6 weeks of grow-out. The two experimental progeny diets were nutritionally identical, met requirements for healthy growth and all vitamins and minerals met or exceeded the recommended levels.[25] Broilers were reared under controlled environmental conditions with free access to feed from hoppers and water from a nipple drinker line. The room temperature was 27°C for the first 4 days then gradually decreased to 20°C and maintained until harvest, with pens heated by infrared lamps (175 W) during the first 3 weeks. Feed intake and final body weights (BW) of broilers were recorded and feed conversion rate (FCR) during the final week before slaughter was calculated. The numbers of birds that were culled or died was recorded on daily basis.

Tissue sampling

On day 42 of grow-out, three birds from each pen ($n = 36$) were randomly selected and euthanized by cervical dislocation. Breast and leg meat tissues were sampled, frozen on dry ice and stored at −18°C for subsequent fatty acid profiling.

Fatty acid analysis

Crude lipid was extracted from a representative sample of homogenized feed, egg yolk and lean meat samples.[27] The gravimetric approach was utilized to estimate total crude lipid (% of wet weight). Fatty acid profiling was performed after transmethylation of the extracted crude lipids with 1% H_2SO_4 in methanol at 70°C for 3 hours. Briefly, after cooling to room temperature, the resulting fatty acid methyl esters (FAME) were extracted with *n*-heptane (2 ml) and transferred into gas chromatography (GC) vials containing about 30 mg of anhydrous sodium sulfate and stored at −18°C until GC

analysis. The FAMEs were separated using a Hewlett-Packard 6890 GC (Hewlett-Packard, CA, USA) equipped with a flame ionization detector, a split injector and a BPX-70 capillary column (50 m × 0.32 mm internal diameter) with a 0.25 µm film thickness (SGE, Victoria, Australia). The operating conditions of the GC, fatty acid identification using the GLC 463 external standard (Nu-Chek Prep Inc., MN, USA) and qualitative analysis were as described previously.[28]

Statistical analyses

The effects of dietary treatment on the fatty acid profile were tested by one-way, two-way and three-way analysis of variance (ANOVA) for egg, chick and broiler tissues, respectively, using SPSS version 21 for Windows (IBM Corp., NY, USA). Duncan's multiple comparison test was implemented where the ANOVA showed significant differences between groups ($P < 0.05$). Due to the uneven number of broilers in each pen, it was not possible to reliably assess the impact of the dietary treatments on growth performance.

Results

Fatty acid composition of the experimental diets

There was no difference in the crude fat percentages between the three breeder diets or the two progeny diets. The crude fat percentage of the progeny finisher diet (fed in the last 3 weeks of broilers grow-out) was lower than the breeder hens' diets by ~1.2%, due to the different nutritional requirements of birds, however, the fatty acid profiles of both the breeder and progeny diets similarly reflected the type of lipid added to the basal feed. Thus, the Control (beef tallow) diet comprised predominately of saturated fatty acid (SFA) and $n-9$ monounsaturated fatty acid (MUFA), the High-ALA diet comprised predominately of $n-3$ PUFA as ALA, whereas the Fish-Oil diet was the only one which contained $n-3$ LCPUFA (Table 1). The ratio of $n-6$:$n-3$ PUFA in the diets decreased from 5.8 in the maternal control diet to 0.8–1.0 in the flaxseed and fish oil diets (Table 1).

Productivity of breeder hens

The laying rate of the breeder hens (number of eggs/breeder) appeared to be lower in hens fed the Fish-Oil diet compared with those fed either the Control or High-ALA diets (Control, 1.22; High-ALA, 1.37; Fish-Oil, 0.74). The ratio of female:male chicks hatched, on the other hand, appeared to be higher in the Fish-Oil group compared with the other dietary treatments (Control, 0.96; High-ALA, 1.00; and Fish-Oil, 2.18). The hatchability of the eggs (number of chicks hatched/egg laid) was similar between groups (Control, 0.39; High-ALA, 0.33; Fish-Oil, 0.44).

Fatty acid composition of egg yolks

The crude fat content of egg yolks ranged from 32–38% of the yolk weight (data not shown), and did not differ between dietary treatments. The fatty acid composition of the yolk

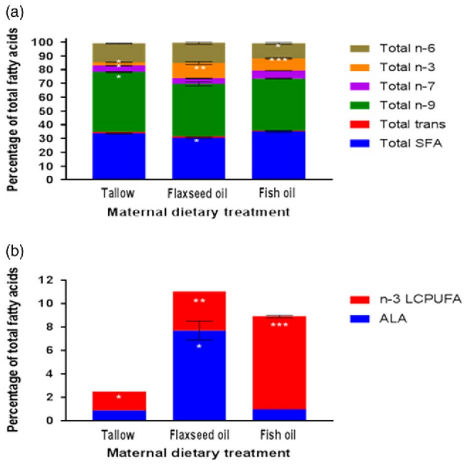

Fig. 2. *Fatty* acid profile (*a*) and omega-3 distribution (*b*) in egg yolk of breeder hens fed three different diets ($n = 5 \pm$ S.E.M.). Different stars within the same fatty acid indicate significant difference ($P < 0.01$ for $n-9$ and $n-6$) or ($P < 0.001$ for other fatty acids) between maternal treatments.

reflected that of the maternal diet (Fig. 2a). Thus, the ALA content of the yolk was higher in the High-ALA dietary group compared with the Control and Fish-Oil groups (Fig. 2b; $P < 0.0001$), whereas the $n-3$ LCPUFA level in the yolk was the highest in the Fish-Oil group compared with the other treatments (Fig. 2b, $P < 0.0001$). The yolk from hens in the Fish-Oil treatment group also contained less $n-6$ PUFA than both other groups ($P < 0.01$). The ratio of $n-6$:$n-3$ PUFA of the egg yolk was 4–5-fold lower in both the High-ALA (1.3) and Fish-Oil (1.2) groups compared with the Control (5.6) group ($P < 0.01$).

Fatty acid composition of day-old chicks

There were significant differences ($P < 0.0001$) in the levels of all major fatty acids in both the breast and leg tissues of the day-old chicks between dietary treatment groups, with the exception of the SFA content, which was similar between treatments. In the breast meat, the MUFA content was lower in the chicks in the High-ALA treatment group compared with both the Fish-Oil and Control treatments. The total $n-3$ PUFA content (ALA + $n-3$ LCPUFA) was highest (13.7%) in chicks in the Fish-Oil group, slightly lower (13.4%) in High-ALA and lowest (5.4%) in the Control group (Fig. 3a; $P < 0.0001$). A similar effect was observed for the $n-3$ LCPUFA content, which was 11.6, 9.0 and 4.9% in the Fish-Oil, High-ALA and Control groups, respectively. The $n-3$ LCPUFA made up a higher proportion of total $n-3$ PUFA in the Fish-Oil group compared with the High-ALA group (Fig. 3b; $P < 0.0001$). The ALA content of the chicks

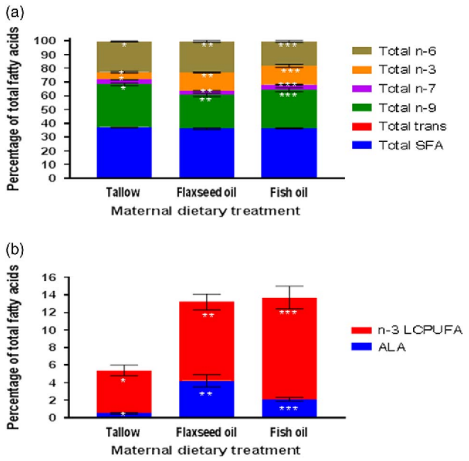

Fig. 3. Fatty acid profile (*a*) and omega-3 distribution (*b*) in breast meat of female day-old chicks from three different maternal dietary treatments ($n = 6.5$ and $4 \pm$ S.E.M. for control, flaxseed and fish oil treatments, respectively). Different stars within the same fatty acid indicate significant difference ($P < 0.0001$) between maternal treatments.

was also different between treatments, and was higher in the High-ALA (4.4%) and Fish-Oil (2.1%) groups compared with the Control group (0.5%) (Fig. 3b; $P < 0.0001$). The $n-6$ PUFA content was ~5% higher in the Control and High-ALA chicks compared with chicks in the Fish-Oil group (Fig. 3a; $P < 0.0001$). However, the $n-6$:$n-3$ PUFA ratio was reduced by 2.5-fold and 3.2-fold ($P < 0.0001$) in the High-ALA and Fish-Oil groups compared with the Control group. Similar effects were observed in the leg meat, and there were no differences between sexes (Control and High-ALA groups) or interactions between chick sex and maternal dietary treatment (males of Fish-Oil group excluded) in either tissue.

Growth of broilers

The final BW of the broilers at 42 days' post-hatch was 2.98 kg in females and 3.64 kg in males, and the FCR in the final week before tissue collection were 1.92 and 1.81 for females and males, respectively. Only two broilers were culled or died before tissue collection throughout the entire 6-week grow-out period.

Fatty acid composition of broilers

Overall, the fatty acid composition of the leg and breast meat tissues was broadly reflective of the diets that the offspring were fed post-hatch, independent of the diet to which they had been

Table 2. P values *at different levels of interaction of three experimental factors (maternal by progeny diets by gender) on 6-week-old broilers (n=3/group)*

		P-value of independent variable interaction (treatment)						
Fatty acid group	Tissue	G[a]	M[b]	P[c]	G × M	G × P	M × P	G × M × P
Total SFA[d]	Breast	<0.001	ns[k]	<0.001	ns	ns	ns	ns
	Leg	<0.001	ns	<0.001	ns	0.004	ns	ns
Total *trans*	Breast	0.001	ns	<0.001	ns	0.023	ns	ns
	Leg	0.004	ns	<0.001	ns	ns	ns	ns
Total $n-9$ MUFA[e]	Breast	0.008	ns	<0.001	0.025	ns	ns	ns
	Leg	0.047	ns	<0.001	ns	ns	ns	ns
Total $n-7$ MUFA[f]	Breast	0.033	ns	<0.001	ns	ns	ns	ns
	Leg	0.024	ns	<0.001	ns	ns	0.031	ns
Total $n-6$ PUFA[g]	Breast	ns	ns	ns	0.022	ns	ns	ns
	Leg	ns	ns	ns	ns	ns	ns	ns
Total $n-3$ PUFA[h]	Breast	ns	ns	<0.001	ns	0.004	0.015	ns
	Leg	0.002	ns	<0.001	ns	0.001	0.013	ns
ALA[i]	Breast	<0.001	0.028	<0.001	ns	0.000	0.036	ns
	Leg	<0.001	ns	<0.001	ns	0.001	ns	ns
Total $n-3$ LCPUFA[j]	Breast	<0.001	ns	<0.001	ns	0.004	ns	ns
	Leg	ns	ns	<0.001	ns	0.028	ns	ns

[a]G, gender.
[b]M, maternal diets (birds received 3 different diets).
[c]P, progeny (birds received two different progeny diets).
[d]SFA, saturated fatty acid.
[e]n – 9 MUFA, omega-9 monounsaturated fatty acid.
[f]n – 7 MUFA, omega-7 monounsaturated fatty acid.
[g]n – 6 PUFA, omega-6 polyunsaturated fatty acid.
[h]n – 3 PUFA, omega-3 polyunsaturated fatty acid.
[i]ALA, α-linolenic acid.
[j]n – 3 LCPUFA = omega-3 long-chain polyunsaturated fatty acid.
[k]ns, not significant ($P > 0.05$).

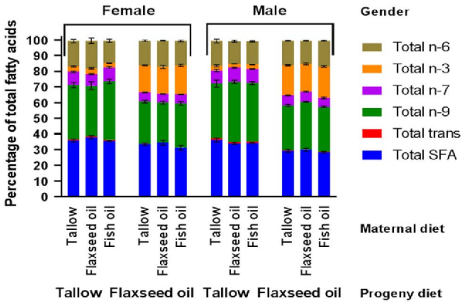

Fig. 4. Fatty acid profile in breast meat of 42-day-old broilers received three maternal by two progeny diets ($n = 3 \pm$ S.E.M.).

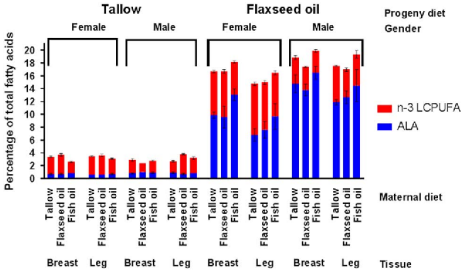

Fig. 5. Omega-3 distribution in meat tissues of 42–day-old broilers received three maternal by two progeny diets ($n = 3 \pm$ S.E.M.).

exposed *in ovo* (Table 2). Therefore, tissues from broilers fed on the High-ALA diet post-hatch had higher levels of total $n-3$ PUFA, ALA and $n-3$ LCPUFA, and lower levels of MUFA and SFA compared with those fed on the control diet, independent of the treatment the chicks had been exposed to *in ovo* (Figs 4 and 5). The percentage of SFA in the tissues ranged from ~28 to ~38% across the different treatments, and was higher in progeny fed the Control diet compared with those fed the High-ALA diet ($P < 0.0001$). Tissue levels of $n-9$ and $n-7$ MUFA were lower in broilers fed the High-ALA diet post-hatch compared with broilers fed a control diet, independent of *in ovo* diet exposure ($P < 0.0001$). The $n-6$ PUFA content of the meat was not different between broilers fed the Control and High-ALA diets. The $n-6$:$n-3$ ratio therefore reflected the variation in $n-3$ PUFA content, and was lower in broilers fed the High-ALA diet (0.8–1.4) compared with those fed the Control diet (4.7–6.4) (Table 2).

Broilers that were exposed to the Fish-Oil treatment *in ovo* had higher ALA levels in the breast meat compared with those exposed *in ovo* to the Control and High-ALA treatments, independent of the diet fed post-hatch, however, the magnitude of this difference was small (Table 2 and Fig. 5, $P < 0.05$). This was accompanied by a reduction in the EPA content of the meat from these broilers. There were no other differences in fatty acid composition of either the breast or leg meat between *in ovo* treatment groups (Table 2 and Fig. 5). There was, however, an interaction between *in ovo* dietary exposure and post-hatch diet on tissue $n-3$ PUFA concentrations (Table 2, $P < 0.05$), such progeny exposed *in ovo* to the Fish-Oil diet that were fed the High-ALA diet post-hatch had significantly higher levels of ALA in breast meat compared with other two maternal treatments fed this same diet (Fig. 5).

The fatty acid profiles of the leg meat tissue of progeny at 42 days of age were largely consistent with the breast meat with the exception that males had higher level of $n-3$ PUFA in their leg meat compared with females (Table 2, $P < 0.01$) and maternal High-ALA diet reduced ALA content only in breast meat (Table 2 and Fig. 5, $P < 0.05$).

Discussion

The reduced number of chicks in the Fish-Oil group appeared to be a result of reduced laying rate in the breeder hens fed the Fish-Oil diet, whereas the proportion of eggs that were laid that produced live chicks did not appear to be adversely affected. It is important to note, however, that the study was not powered to investigate differences in production characteristics between treatments and thus further studies are required to confirm if there are any adverse effects of the Fish-Oil diet on laying performance.

Although our study was not specifically designed to investigate differences in growth in the progeny, there are no suggestions of adverse effects of any of the diets on the productivity of the chicks. The broilers from all treatments grew at a normal rate, and BW, FCR and mortality in the progeny across the different treatments were within the normal standards for this strain[29] and in agreement with our previous findings.[9]

Our finding that feeding breeder hens a High-ALA diet increased not only the ALA content, but also the $n-3$ LCPUFA content of the eggs, supports the capacity of chickens for ALA conversion to $n-3$ LCPUFA, and confirms that ALA-supplementation of feed is an effective strategy for increasing the $n-3$ LCPUFA content of eggs.[20,22,30–32] This is likely to be due to the combined effects of the higher amount of ALA, and lower amount of $n-6$ PUFA in the feed, as the $n-3$ and $n-6$ PUFA compete with each other for both metabolic conversion and accumulation into tissues.[33]

Interestingly, although supplementing the breeder hens directly with $n-3$ LCPUFA, in the form of fish oil, led to the greatest increase in $n-3$ LCPUFA (five-fold of the control eggs), the $n-3$ LCPUFA content of the eggs (8%), was still only half of that of the feed (16.1%). This observation suggests there is a maximum level to which $n-3$ LCPUFA can be incorporated into egg yolk, which may be due to structural limitation of triglycerides and phospholipids. In contrast, the $n-3$ LCPUFA percentage was relatively increased in eggs of the other two treatments in comparison with the levels in the diets. This observation indicates the importance of optimizing the dietary content of $n-3$ LCPUFA in broiler feed, as the

incorporation of these fatty acids into eggs appears to follow a curvilinear, rather than linear, pattern and reach a plateau at high *n* – 3 LCPUFA intakes.

Previous studies have established that >80% of lipids deposited in the egg yolk are consumed by the developing embryo before hatch, and therefore represent a major source of nutrition for supporting chick growth and development.[34] In addition, ~50% of egg total fatty acids are incorporated into the newly hatched chick with embryonic preference to incorporate PUFA at the expense of MUFA.[35] Consistent with the findings of the current study, previous studies[22,32] have demonstrated that the relationship between fatty acid content of the egg yolk and post-hatch chick were closest for the essential fatty acids (*n* – 3 and *n* – 6 PUFA), compared with MUFA and SFA. Our finding that the *n* – 3 and *n* – 6 PUFA content in all groups were relatively higher in the meat of the newly hatched chicks than in the eggs provides evidence of continuous synthesis and accumulation of both of PUFA types in the muscle tissue *in ovo*. Hence, the ratio of *n* – 6:*n* – 3 PUFA in the meat tissues of chicks (especially in the high *n* – 3 PUFA treatments) did not shift as much as the levels of the individual PUFA. This was important for the second stage of the experiment, since it ensured that the capacity of the chicks for converting ALA to the *n* – 3 LCPUFA was unlikely to be limited by the presence of excessive amounts of *n* – 6 PUFA in the tissues.

Interestingly, the contribution of *n* – 3 LCPUFA to the total *n* – 3 PUFA pool in day-old chicks exposed to the High-ALA diet *in ovo* was ~37% greater than in eggs from High-ALA hens, suggesting that these chicks had the capacity for ALA conversion to LCPUFA during embryogenesis. As with the eggs, however, the ability of the chicks to accumulate *n* – 3 LCPUFA appeared to be limited at higher concentrations, as the *n* – 3 LCPUFA content of meat tissues in chicks exposed to the Fish-Oil diet *in ovo* was about half that in the yolk. Lin *et al.*[35] found a linear relationship in *n* – 3 and *n* – 6 PUFA levels between eggs and chicks. However, these authors also suggested that the synthesizing of *n* – 3 LCPUFA from ALA was suppressed at higher levels of dietary *n* – 3 LCPUFA.

The growth of the 42-day-old broilers (data not shown) in the current study agreed with our previous study[9] and was not affected by dietary fat exposure either *in ovo* or post-hatch.[24,36] At 6 weeks of age, the fatty acid profile of broilers was found to be mainly affected by the post-hatch diet and sex, with minimal influences of dietary exposure *in ovo*. As the post-hatch control diet contained more SFA, *trans*, *n* – 9 and *n* – 7 MUFA, these fatty acids were predominant in broilers fed this diet. On the other hand, *n* – 3 and *n* – 6 PUFA were the predominant fatty acid groups in broilers fed High-ALA diet, consistent with our previous findings.[9] Although the High-ALA diet was relatively higher than the Control diet in *n* – 6 PUFA content, this did not affect the *n* – 6 PUFA level in broiler meat. This is probably related to the preferential utilization of *n* – 3 PUFA substrates (ALA) by the enzymes involved in the metabolic conversion of shorter-chain fatty acids to their long-chain derivatives and preferential incorporation of *n* – 3 PUFA into tissues.[10,15]

The major finding of this study was that exposure to either a high *n* – 3 PUFA (ALA) or *n* – 3 LCPUFA (Fish-Oil) diet *in ovo* had very little impact on the capacity of the progeny for converting ALA to the *n* – 3 LCPUFA. Indeed, the only effect observed was an apparently inhibitory effect of *in ovo* exposure to maternal fish oil supplementation on ALA conversion to *n* – 3 LCPUFA. One possibility is that *in ovo* exposure to a high dietary *n* – 3 LCPUFA content may have acted to suppress the expression and/or activity of genes involved *n* – 3 PUFA metabolism, specifically the desaturase and elongase enzymes required for the conversion of ALA to *n* – 3 LCPUFA. This is supported by previous studies showing that DHA supplementation suppresses endogenous synthesis of *n* – 3 LCPUFA from ALA in human subjects[37] and that feeding chickens a diet enriched in fish oil-based diet resulted in an increase in the percentage of both of ALA and linolenic acid in the chicken meat, suggesting reduced conversion.[38] Similarly, in chicken, Ajuyah *et al.*[24] reported no effect of a reserve of yolk *n* – 3 LCPUFA from maternal Fish-Oil diet on the *n* – 3 PUFA in the offspring cardiac tissue, but showed an adverse effect on the EPA percentage. In contrast, *in ovo* exposure to the High-ALA diet had no effect on the subsequent capacity of the chickens for ALA conversion. Haug *et al.*[39] reported that the concentration of dietary ALA does not affect the gene expression of the elongation and desaturation enzymes in adult chickens, and the results of the current study suggest that this may also be the case during the embryonic stage. Thus, exposing embryos to either High-ALA or High-*n* – 3 LCPUFA environments do not enhance their subsequent capacity for depositing more *n* – 3 LCPUFA after hatch.

Although there were relatively few differences between the male and female chickens in their response to the diet, we did identify that male broilers fed the High-ALA diet post-hatch accumulated more total *n* – 3 PUFA in the leg meat, independent of their *in ovo* exposure. In addition, despite no differences in fatty acid profile of male and female chicks at 1-day post-hatch (Control and High-ALA groups), there were sex differences in the levels of all fatty acids (except *n* – 6 PUFA) in the 42-day-old broilers. Thus, tissues from male chickens contained relatively more *n* – 3 PUFA and lower *n* – 3 LCPUFA, which indicates their lower ALA conversion efficiency, a finding is consistent with our previous study.[14]

In summary, we have shown that exposing broiler chickens to elevated levels of ALA or *n* – 3 LCPUFA *in ovo* was effective in increasing *n* – 3 LCPUFA deposition into their meat tissues during embryonic development. However, neither of these strategies were effective at increasing the subsequent capacity of the chickens for accumulating *n* – 3 LCPUFA in their meat tissues when fed a High-ALA diet post-hatch. In fact, increased *in ovo* *n* – 3 LCPUFA exposure appeared to be associated with an impaired capability of the broilers to convert ALA to *n* – 3 LPCUFA.

Conclusion

In ovo *n* – 3 LCPUFA exposure appeared to be associated with an impaired capability of the broilers to convert ALA to *n* – 3

LPCUFA. Manipulation of dietary fatty acids can affect yolk composition, but on its own it is not an appropriate strategy for enhancing n-3 LCPUFA content in the offspring at market age.

Acknowledgments

The authors would like to thank Derek Schultz, Mandy Bowling, Kylee Swanson, Nicole Heberle, Natasha Edwards, Saad Gilani, Dr Carolyn Dekoning and Dr Reza Barekatain from Roseworthy campus for their assistance in sampling the animals and Dr Liu Ge from Waite campus, University of Adelaide for his assistance in the laboratory work.

Financial Support

K.K. received Research Training Program (RTP) from The Australian Government Research Training Program Scholarship and full PhD scholarship from the Faculty of Sciences, University of Adelaide. The experimental work was financed by a grant from the South Australian Department of Further Education, Employment, Science and Technology (DFEEST), Australia. B.M. is supported by a Career Development Fellowship from the National Health and Medical Research Council of Australia (NHMRC). R.G. is supported by a NHMRC Senior Research Fellowship.

Conflicts of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of animals. This study was approved by the Animal Ethics Committee of the University of Adelaide (approval S-2013-152) and the Department of Primary Industries and Regions South Australia, Australia (approval 15/13).

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