MODULATION OF AVIAN METABOLISM BY DIETARY FATTY ACIDS

BY

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DECLARATION

This thesis details the results of original investigations undertaken on the role of dietary polyunsaturated fatty acids and their effects on metabolic activity in the broiler chicken (*Gallus domesticus*). The studies described in this thesis are my own original work and have not been submitted previously for the purpose of obtaining any other degree or diploma in any other University.

R.E. Newman
SUMMARY

The role of dietary fatty acids and their subsequent effects on metabolism has received considerable attention in mammalian species. It is becoming increasingly clear that fatty acids have metabolic roles over and above their influence on energy density of the diet. Recent studies have linked changes in the fatty acyl composition of the plasma membrane, induced by the dietary fat profile, to alterations in both lipid and glucose metabolism. These dietary induced changes have profound effects on insulin action, glucose transport and enzyme activity that regulate triglyceride and fatty acid synthesis, factors that ultimately influence protein and lipid deposition of animals.

Because of their high growth rate, broiler chickens have a high requirement for energy and the use of triglycerides as a major energy source has resulted in a fat carcass. A change in the glucose-insulin balance has been suggested as being the main reason for differences in adiposity between broilers selected for fatness or leanness. The hypotheses of this thesis is based on the finding that dietary polyunsaturated fatty acids (PUFA’s) increase the sensitivity of muscle tissue to insulin and this would presumably augment insulin-stimulated glucose uptake into muscle cells. Therefore, increasing the capacity of broiler muscle tissue to utilise glucose as its principal energy substrate would reduce the bird’s reliance on triglycerides and this in turn would result in a leaner carcass.

The aims of this study are firstly to explore the role that dietary PUFA’s from the n-3 and n-6 series have on the growth and body composition of broiler chickens and secondly to determine the relationships between dietary fatty acid profile, tissue insulin sensitivity and lipid deposition. Because dietary fatty acids have been implicated in the modulation of hormones important for the growth and development of animals, a third aim of this thesis is to determine the effects of dietary n-3 and n-6 polyunsaturated fatty acids on pituitary and adrenal sensitivity. Since the modulation of metabolism by dietary fatty acids has been attributed to changes in the fatty acyl composition of the plasma membrane, the final aim of this study will be to investigate and characterise the molecular species of the breast muscle choline and ethanolamine phospholipids.

Feeding either fish oil a source of n-3 PUFA’s or sunflower oil a source of n-6 PUFA’s fatty acids to broiler chickens resulted in a significant (P<0.01) reduction in the abdominal fat pad mass and a modest increase in breast muscle mass when compared to
broilers fed edible tallow. Associated with the changes in carcass composition was an alteration in energy substrate utilisation. This was reflected by lower respiratory quotients and reduced triglyceride and insulin concentrations for the chickens fed the two PUFA diets. Coupled to the shift in energy metabolism was a significant (P<0.05) increase in the proportion of PUFA’s incorporated into the abdominal fat pad and breast muscle. The dietary fat supplements resulted in the incorporation of specific fatty acid subtypes. Feeding fish oil significantly increased the proportion of long-chain n-3 PUFA’s whereas feeding sunflower oil significantly increased the proportion of long-chain n-6 PUFA’s compared to tallow feeding whose tissues were dominated by a higher proportion of saturated fatty acids.

It was further shown that dietary n-3 and n-6 PUFA’s enhanced glucose /insulin action. Feeding either fish oil or sunflower oil to broiler chickens increased insulin action when examined by an intravenous glucose tolerance test. The maximal insulin release in response to the glucose infusion was higher in the tallow fed group compared to either the sunflower oil or fish oil groups. To estimate the disappearance rate of glucose from the plasma and its incorporation into tissues, 2-deoxy-D-\(^3\)H glucose was infused into each chicken. There were no significant differences in the clearance rate of 2-deoxy-D-\(^3\)H glucose from the plasma. However, when measured under steady state conditions, the labelled glucose incorporation into the breast muscle was greater in birds fed fish oil compared to either tallow or sunflower oil feeding.

The dietary fatty acid induced increase in insulin action suggests that the sensitivity of muscle cells to insulin was enhanced. This modulation of tissue sensitivity by dietary fatty acids was also shown to occur at the level of the pituitary. To provide an estimate of pituitary sensitivity, bolus GnRH and GHRH infusions were given on different days to chickens fed the three dietary treatments. Feeding sunflower oil (n-6 PUFA’s) increased the level of GH that was released in response to the GHRH infusion when compared to birds fed either tallow or fish oil (n-3 PUFA’s). This dietary fatty acid modulation appears to be specific to certain pituitary cell types as there was no effect on LH secretion following the GnRH infusion. Dietary fatty acid modulation of endocrine gland sensitivity is particular to the gland type. Although the dietary treatments mediated a distinct pattern in pituitary sensitivity to GHRH infusion, these same three diets did not
influence adrenal sensitivity, as there was no difference in the corticosterone profile following either ACTH or CRF infusion.

The previously observed physiological changes for the three dietary groups was expected to be positively correlated to an alteration of the plasma membrane phospholipids induced by the dietary fatty acids. Supplementation with fish oil (n-3 PUFA’s) significantly increased levels of both eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) into the choline (PC) and ethanolamine (PE) breast muscle phospholipids compared to either sunflower oil (n-6 PUFA’s) or tallow supplementation. The increase in n-3 PUFA incorporation was associated with a corresponding decrease in the proportion of arachidonic acid (AA; 20:4n-6) an event that would presumably alter substrate availability for the 1- and 2-series eicosanoids. However, feeding sunflower oil or tallow gave a molecular species profile that was remarkably similar in both fatty acid subtype and proportion. This suggests that the plasma membrane dynamics would be similar for these two dietary groups. Therefore, it is appears that factors other than a change in the fatty acyl- composition of the plasma membrane may be responsible for modifying the physiology of the broiler.
ACKNOWLEDGEMENTS

The original hypothesis for this thesis was the result of various ideas that were crystallised together with my friend and colleague Dr Jeff Downing whilst we were employed at CSIRO Division of Animal Production. Jeff’s invaluable contribution to this project after we moved to the University of Sydney, was not only manifested by his willingness to participate in almost every aspect of this research, but also for providing a friendly ear ‘when times got tuff’-for this I am eternally grateful.

I would like to thank my supervisor, Associate Professor Wayne Bryden from the University of Sydney, who was instrumental in initiating the PhD candidature. Wayne was initially sceptical by the capacity of dietary fatty acids to extensively modulate metabolism, but by projects end he had been persuaded by their potential for improving animal and human nutrition.

I would especially like to thank Eva Fleck from the CSIRO Division of Animal Production for her ‘brilliant’ technical expertise in lipid chemistry. Without Eva’s technical input particularly in respect to the molecular species analysis, this thesis would not be as incisive as I believe it to be. I would also like to thank Dr Trevor Scott, a former Chief of the CSIRO Division of Animal Production. Trevor’s wealth of knowledge of lipid biochemistry was invaluable and he provided a ready conduit for the many ideas and thoughts I had during the course of this thesis. His willingness to read and comment on the written material is also gratefully acknowledged. I would also like to thank Bob Weston, who now retired, was also once employed by the CSIRO Division of Animal Production. Bobs ability to critically evaluate what I had written was invaluable particularly when it concerned the area of nutrition, Bob’s forte.

I am especially grateful to my co-supervisor Professor Len Storlien from the Dept of Biomedical Sciences, University of Wollongong. Len’s enthusiasm for dietary fatty acids, particularly n-3’s, was infectious. He not only stimulated my own interest in understanding their significant nutritional role but this enthusiasm impacted on many others as well. I am also grateful to Associate Professor Tony Hulbert and Dr Bill Buttemer from the Dept of Biological Sciences, University of Wollongong. The discussions I had with Tony enabled me to appreciate the dynamics of cell membranes in a more holistic manner. The calorimetry studies conducted in Chapter 4 of this thesis
would not have been possible without the expertise provide by Bill Buttemer. I would also like to thank both Tony and Bill for the friendship they provided on my visits to Wollongong.

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I am greatly indebted to the RIRDC Chicken Meat Committee who provided the financial means to carry out this project. Without their commitment, this project would not have been possible. I hope that the results and their implications contained within this thesis will be of value to the industry.

Finally, I am forever grateful for the support and encouragement I received from my best friend and partner, Helen Campbell, who was the mainstay of my studies especially during the writing of the thesis.
DEDICATION

This thesis is dedicated to the memory of my mother, Dorothy Isobel Newman. Her love embraced not only her own family but was also there for those whose path crossed with hers.
LIST OF COMMONLY USED ABBREVIATIONS

%   Percent
AA (20:4n-6)  Arachidonic acid
ACTH   Adrenocorticotropic hormone
AME   Apparent metabolisable energy
ANOVA   Analysis of variance
cAMP   Cyclic adenosine monophosphate
CNS   Central nervous system
CPT   Carnitine palmitoyl-transferase
CRF   Corticotropin releasing hormone
DHA (22:6n-3)  Docosahexaenoic acid
EPA (20:5n-3)  Eicosapentaenoic acid
FA   Fatty acid
GH   Growth hormone
GHRH   Growth hormone releasing hormone
GLUT   Glucose transporter
GnRH   Luteinising hormone releasing hormone
LA (18:2n-6)  Linoleic acid
LH   Luteinising hormone
LNA (18:3n-3)  Linolenic acid
LT   Leukotriene
Mol   Amount of substance
NEFA   Non-esterified fatty acids
NIDDM  Non-insulin-dependent diabetes mellitus
PC   Phosphatidylcholine
PE   Phosphatidylethanolamine
PG   Prostaglandin
PI   Phosphatidylinositol
PKC   Protein kinase C
PS   Phosphatidylserine
P/S   Polyunsaturated to saturated fatty acid ratio
PUFA’s   Polyunsaturated fatty acids
RIA   Radioimmunoassay
RQ   Respiratory quotient
TLC   Thin-layer chromatography
TX   Thromboxane
VFI   Voluntary feed intake
VLDL   Very-low-density lipoprotein
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CHAPTER 1

INTRODUCTION

MANIPULATION OF GROWTH IN ANIMALS AND POULTRY

1.2. DEVELOPMENT OF THE HYPOTHESIS

The need to improve livestock productivity is a continual endeavour for most agricultural systems. Two major strategies have evolved to meet this purpose; firstly to increase the efficiency and the total amount of meat, eggs and milk produced and secondly to improve the nutrient composition of these products in line with consumer requirements (Beermann, 1989). The use conventional animal breeding practices equates to an ongoing annual increase of about 1-2%. This improvement has led to a substantial increase in the growth rate of the broiler chicken. The selection for increased growth has, however, produced a chicken with excessive fatness and this has become a problem in modern broilers strains; commercial broiler strains contain between 13-18% of their body weight as ether-extractable fat (Griffin, 1993).

Most strategies have been based on modulation of the hormonal control of energy and protein metabolism (McDowell and Annison, 1991). Various biological strategies have been used in an attempt to manipulate livestock productivity to improve growth rate and reduce body fat mass. Although these technologies have been successful in some animal production systems, their effectiveness in producing the desired biological outcomes has been inconsistent. This has certainly been the case for the broiler chicken.

1.1. MANIPULATION OF GROWTH IN ANIMALS AND POULTRY

Somatotropin (growth hormone; GH) has been used to alter nutrient use to improve growth rate and feed conversion and has been used successfully as an agent for enhancing productivity in various livestock industries. It results in leaner pork, in addition to increasing growth rates and feed efficiency in the pig (Beermann, 1989). However, the responses to GH administration to improve the growth rate and feed conversion of chickens have been variable. A daily intravenous injection of chicken GH
to cockerels resulted in only a transient improvement in body weight gain, with no differences in either feed efficiency or carcass composition when compared to control birds (Leung et al., 1984). However, it appears that it is the method of GH administration that determines the response, as the pulsatile infusion of GH into chickens improved growth, reduced body fat content and improved feed efficiency (Vasilatos-Younken et al., 1988). In contrast, single daily injections of GH are effective in stimulating growth in pigs.

The use of β-adrenergic agonists, such as clenbuterol, which are chemically and pharmacologically similar to natural catecholamines, have profound effects on skeletal muscle growth and fat deposition in livestock (Asato et al., 1984). These agents bind to the β-adrenergic receptor facilitating nutrient repartitioning (Beermann, 1989). They have been shown to stimulate metabolic rate, and alter carcass composition; reduce fat deposition and increase protein accretion (Lindsay et al., 1992). However, β-adrenergic agonists are species-specific as they are more effective in ruminants than in non-ruminants (Beermann, 1989). Reduced efficacy has been demonstrated in avian species as the administration of β-adrenergic agonists to broiler chickens resulted in only a small improvement in growth rate, feed efficiency and body fat content when compared to sheep and cattle (Dalrymple et al., 1984). The reduced avian response is consistent with the muted catecholaminergic effect of adrenaline which is weekly lipolytic in birds (Langslow and Lewis, 1974).

Vaccines that influence physiological activity by inducing antibodies to neutralise biological effects have also found a place in animal livestock practices (Hoskinson et al., 1990). However, few vaccines target improvements in growth and metabolism per se and may not result in the desired outcome for all species. Studies of Flint et al. (1994) of active immunisation against adipocyte plasma membranes to reduce fat deposition have resulted in a varied response in a range of animals. The immunisation procedure was most successful in the rat and the pig, resulting in a consistent reduction in adipocyte cell numbers and for the pig, a significant decrease in backfat thickness. However, in the sheep, the same immunisation procedure resulted in a decrease in liveweight gain with no appreciable effect on fat deposition. Similarly, when broiler chickens were passively immunised with sheep anti-chicken fat cell antibodies there was no effect on adipose
tissue deposition although the flesh turned a dark shade of green in response to immunisation (Butterwith et al., 1989).

The immunoneutralisation of somatostatin (an inhibitor of somatotropin release) has been used as an alternative approach to the injection of exogenous GH. Spencer and Garssen (1983) demonstrated substantial liveweight gain in sheep in response to somatostatin immunisation, although this was not accompanied by any changes in circulating GH concentrations. Moreover, subsequent immunisation studies in sheep have shown either a more modest increase in liveweight gain (Spencer and Garssen, 1983) or no increase (Varner et al., 1980). The active immunisation of broiler chickens to somatostatin has produced a more favourable response in modulating growth. Spencer et al. (1986) reported significant increases in growth rates and reduced fat deposition in birds that were actively immunised against somatostatin, although, like the sheep, the physiological effects were not correlated to circulating GH concentrations. Other studies in birds have shown very variable responses to immunisation (A.J. Husband and W.L. Bryden, unpublished). These different outcomes demonstrate one of the inherent problems of immunomodulation as a tool for manipulating growth in animals; namely a species-specific immune response to a given antigen.

Molecular biology was heralded as a new and exciting approach to improving animal production. It was hoped that GH transgenesis could be used to enhance productivity in both sheep and pigs after the initial report of a substantial improvement observed in transgenic mice (Palmiter et al., 1982). Transgenic pigs that expressed bovine GH grew 11-15% faster than their littermates when they were placed on a diet with elevated crude protein and additional lysine. The high concentration of plasma GH in these pigs did result in reduced fat deposition and increased feed conversion efficiency (Pursel et al., 1989). The growth promoting effects and the altered carcass composition produced by GH transgenesis have produced a number of severe and detrimental side effects in pigs and mice. These include gastric ulcers, synovitis, cardiac myocyte nuclear hypertrophy, dermatitis, nephritis, pneumonia and reduced life span (Doi et al., 1988; Brem et al., 1989; Pursel et al., 1989). However, these aberrant responses are most likely due to the lack of control over the promoter and associated transcriptional regulatory mechanisms in these studies.
The commercial failure of these technologies could be attributed to the scant regard given to an animal’s ability to maintain its interior milieu by physiological and behavioural homeostatic adaptations. The mechanisms that modulate homeostasis have long been recognised, having been described initially in 1878 by the physiologist Claude Bernard. These technologies appear to have failed because only a single biological pathway, receptor type or subtype was targeted and as a consequence, the animal’s homeostatic mechanisms have been able to circumvent the single point modulation of physiological function. This has certainly been the case for some of the vaccine technologies that have been developed to manipulate production traits in the sheep (Newman, 1995).

1.2 DEVELOPMENT OF THE HYPOTHESIS

In mammals, most of the energy required for muscle protein synthesis is supplied by glucose and fatty acids, the uptake of which is facilitated by insulin. However, when compared to mammals, birds are relatively insensitive to the actions of insulin (see review Simon, 1988). As a consequence of this insulin insensitivity and the need for high energy levels to sustain rapid growth, birds metabolise triglycerides in preference to glucose to supply the energy required for muscle protein synthesis (Griffin et al., 1991). Therefore, the selection for rapid growth has resulted in a major change in the way the broiler uses and partitions energy.

Nutritional studies have shown that the energy and fat balance of animals can be manipulated by altering the polyunsaturated to saturated fatty acid (P/S) ratio, specifically by the inclusion of the long chain polyunsaturated fatty acids (PUFA’s) (Field et al., 1990; Luo et al., 1996; Couet et al., 1997). The alteration in the energy balance may be the result of an increase in the fluidity of plasma membranes induced by the dietary fat profile. Studies conducted in vivo have shown that dietary fats can alter the nature of plasma membrane structural lipids (Clandinin et al., 1985;1991). In addition, the dietary fat profile has also been shown to regulate the interface between circulating hormones and hormone–activated receptor-effector systems (Clandinin et al., 1991). Studies in mammals show a positive correlation between diets with a high P/S fatty acid ratio,
membrane composition and the sensitivity of cells to insulin (Field et al., 1990; Shimomura et al., 1990; Storlien et al., 1991; 1995; Borkman et al., 1993).

A change in the glucose-insulin balance has been suggested as being the underlying reason for differences in adiposity between broilers selected for leanness or fatness (Leclercq et al., 1988). Increasing the sensitivity of muscle tissue to insulin increases utilisation of glucose by muscle, therefore decreasing the dependence on triglycerides as an energy source. If this could be achieved in birds by changing the P/S ratio of the diet the reduced reliance on triglycerides should induce a change in energy partitioning and reduce carcass fat deposition.
# CHAPTER 2

## LITERATURE REVIEW

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2.1. SCOPE OF THE LITERATURE REVIEW

The role of dietary polyunsaturated fatty acids (PUFA’s) from the n-3 and n-6 series and their associated affects on metabolic activity, particularly in respect to both carbohydrate and lipid metabolism has received much attention. Many studies have focused on the essential fatty acids and their role in orchestrating positive changes in insulin action by altering the fatty acyl composition of the plasma cell membrane and hence its fluidity (Clandinin et al., 1985; 1991; Field et al., 1990; Storlien et al., 1991; 1994; Borkman et al., 1993). Indeed, with the prevalence of obesity, insulin resistance and non-insulin-dependent diabetes mellitus (NIDDM) rapidly increasing worldwide, it is
not surprising that scientific journals have devoted entire issues to this subject (see Br. J. Nutr Vol 83 2000). Birds are similar to other meat-producing animals in that changes in nutrient partitioning are associated with an alteration in metabolism, particularly in respect to lipid and glucose metabolism (McDowell and Annison, 1991). The present review details studies that describe changes in cell membrane dynamics induced by dietary n-3 and n-6 PUFA’s and the associated affects on metabolism. Although these studies have chiefly been carried out using mammals, references to avian species are discussed where reported. To acquaint the reader with avian digestion, this review also describes the physical and chemical characteristics of the digestive tract with specific reference to carbohydrate, protein and lipid metabolism.

2.2. DIGESTION

The avian digestive system represents many adaptations that have evolved to meet the energy requirements of homiothermy and flight (Farner, 1960). The characteristics that distinguish the avian from mammalian digestive systems are many. Some of the more obvious differences are the specialised mouth area that includes the beak with lighter jawbones and muscle and the lack of true teeth. As a result birds do not chew their food and swallowing is assisted by the presence of a comparatively larger diameter oesophagus that can accommodate larger food items and a muscular stomach, the gizzard, for the mechanical breakdown of food. The lengths of various parts of the digestive tract vary with the size of the bird and the type of food eaten. Coarse fibrous food results in birds having large digestive tracts (Mc Lelland, 1979). The domestic chicken has been subjected to an intensive change through genetic, dietary and environmental manipulation resulting in the productivity of the modern broiler and layer. It is probable that such manipulations have caused modifications in the digestive activity of the chicken.

2.2.1. STRUCTURE OF THE ALIMENTARY TRACT.

**Mouth**

The tongue and beak are important in food manipulation (McLelland, 1979). Co-ordinated movement of the structures within the mouth and pharynx during swallowing are based on their innervation by the lingual and larngo-lingual branches of the
glossopharyngeal nerve. The cavity of the mouth and surfaces of the tongue are covered with thick stratified squamous epithelium. The taste buds in the chicken are few in number, for example Lindenmaier and Kare, (1959) observed about twelve in the young chick, which doubled, by 3 months of age. These receptor cells respond to stimulation in a similar way to mammalian cells when salt, bitter or acid solutions are applied (Kitchell et al., 1959; Halpern, 1962). Salivary glands are widely scattered throughout the mouth and pharynx and simple tubules open into a common cavity from which one or more ducts lead to the mouth. Each gland contains mucous cells that alternate in a cycle between accumulation and discharge of mucous, which is stimulated by both feeding and by parasympathetic stimulation (Chodnik, 1948). This secretory activity is well suited to the intermittent eating habits of the chicken.

**Oesophagus and crop**

The oesophagus of the chicken is comparatively long and consists of outer longitudinal and inner circular muscles that are covered with a loose adventitia of elastic and fibrous tissue containing blood vessels and the vagus and sympathetic nerves (Hill, 1971). Mucous glands discharge into the oesophageal opening and help to lubricate the passage of food. The crop is a thin walled storage pouch and lies on the ventral surface of the oesophagus at the entry point into the thoracic cavity that divides the upper and lower oesophagus (Hill, 1971). The microstructure of the crop is similar to the oesophagus, possessing a similar type of lining which is deeply folded to facilitate distension during food storage (Duke, 1986). The size and shape of the crop varies between breeds and between the sexes within a breed (Wehner and Harrold, 1982).

**Proventriculus**

The proventriculus lies between the lower oesophagus and the gizzard and is lined with a glandular mucous membrane containing the gastric secretory glands (Hill, 1971). The primary function of this organ is production and release of the gastric pepsin, hydrochloric acid and mucous (Duke, 1986). In chickens, there are no well-characterised oxyntic (acid secreting) or peptic (enzyme secreting) cells. The proventriculus has one cell type (oxyntico-peptic) possessing most of the features of the two separate cell types
found in mammals (Hill, 1971). The oxyntico-peptic cells contain zymogen or pepsinogen granules, the amount dependent on the degree of functional activity, increasing during fasting and decreasing immediately after feeding (Duke, 1985). Only a certain number of cells are involved in the secretory process at one time and each cell appears to act as an independent unit (Hill, 1971).

**Gizzard**

The gizzard or ventriculus serves two main purposes, as a food-grinding chamber and as a site for peptic proteolysis. The organ is composed of two lateral pairs of opposing muscles termed the thin and thick muscle pairs (Dziuk and Duke, 1972). When the gizzard contracts the mucosal surface is protected from damage by a lining that also protects it against the corrosive effect of the acid-enzyme mixture, which flows from the proventriculus. The gizzard receives extrinsic fibres, both stimulatory and inhibitory, from the vagus and the sympathetic nervous system (Burnstock, 1969). There is extensive communication between the adjacent smooth muscle cells and the nervous system suggesting an anatomical basis for the rapid propagation of impulses through the muscle mass and hence the rapid contractibility of the gizzard (Bennet and Cobb, 1969).

**Small intestine**

The small intestine consists of the duodenal loop, jejunum and ileum and is the principal site of chemical digestion, involving enzymes of both intestinal and pancreatic origin. The cellular structure of the small intestine is similar to mammals (Toner, 1965). Their being absorptive cells, large and cylindrical with basal nuclei and an apical membrane covered with long microvilli. The mucosa of the small intestine is characterised by crypts of Lieberkühn in varying degrees of development, which secrete the intestinal digestive enzymes. Unlike mammals, the chicken intestine does contain large duodenal digestive glands, the Brunners glands (Calhoun, 1954); their mucus-secreting role is carried out by goblet-type cells present between the columnar cells of the surface epithelium (Aitken, 1958). The layers of the epithelium are similar to mammals, having distinct stratum that include the mucosa, submucosa layers, inner circular and outer longitudinal muscles and the serosa. In addition, there are two major nerve plexi
that serve the small intestine. The submucosa nerve plexus innervates blood vessels and glands and the myenteric nerve plexus innervates the two muscle layers. This latter nerve plexus is associated with the gizzard and the proventriculus (Duke et al., 1975). Within mammalian villi of the absorptive region are central lacteals that contain arterioles, venules and lymph vessels, however, in the chicken the villous core is occupied by a capillary bed. The extrinsic innervation of the chicken intestine is extensive and includes pre- and post-ganglionic fibres that may be either vagal or sympathetic in origin (Everett 1968).

**Large intestine**

The large intestine consists of three distinct regions, the ceca and colon which function primarily as absorptive areas and the cloaca where the digestive and uro-genital tracts converge. Both the colon and the cloaca are involved in excretion and the control of mineral and water balance (Hill, 1971). The ceca are paired blind-ended tubes, which arise at the junction of the small and large intestine and are separated by ileocecal valves. Histologically, ceca are similar to the small intestine and in some bird species, exhibit a high degree of secretory activity (Duke, 1986). The cecum is the major site of microbial fermentation in the gut. There are three distinct regions in each cecum, each consisting of a well-defined muscle coat lined with columnar epithelium. Two of these regions have villus-like projections that correspond to the major absorptive area (Hill, 1971). The colon is a short narrow tubular structure that extends from the ileoceal junction to the cloaca and although histologically similar to the small intestine, the mucosa are sort broad villus-like projections lined with columnar cells (Hill, 1971). The cloaca is divided into three chambers the coprodeum, urodeum and the proctodeum each being defined by incomplete folds or flaps of mucosa. The coprodeum is the largest of the three chambers and serves as a reservoir for faces and urine. The oviduct opens into the urodeum that connects through to the proctodeum.

**Pancreas**

The chicken pancreas has three lobes and lies within the duodenal loop. There are three secretory glands that pass to the distal end of the duodenal loop and open into the
duodenum on a common papilla with the bile duct. Although the histology of the pancreas is similar to that of mammals the chicken pancreas has a less well-defined islet tissue (Hill, 1971).

**Gall bladder and bile ducts**

The biliary system is composed of two ducts. The cystic duct bears the gall bladder and drains bile mainly from the right lobe of the liver and the hepatic duct, which drains the left lobe of the liver (Hill, 1971). These two ducts are joined by a connecting branch in the liver on a common papilla with the pancreatic ducts.

**2.2.2. DEVELOPMENT OF THE GASTRO-INTESTINAL TRACT**

The weight of the gastro-intestinal tract and the activity of the digestive glands increase in response to the adaptation to exogenous food (Tarvid, 1995). Adaptive changes of the alimentary canal to the digestion of exogenous food are essential for the maximisation of growth and involve differential growth rates of internal organs. The relative maximum growth rate occurs after a post-hatch lag (Bjornhag, 1979) and is greater in faster growing strains than slower growing strains (Knizetova et al., 1985).

The development of the avian gastro-intestinal tract is a vital aspect of growth. The gastro-intestinal tract increases in both size and weight more rapidly in relation to body weight than any other organ or tissue during the post-hatching period (Dror et al., 1977; Lilja, 1983; Katanbaf et al., 1988). The major changes of the digestive tract include an increase in the weight of the small intestine and a decrease in the weight of the large intestine and this occurs during the first ten days after hatching (Crompton and Walters, 1979). This led Tarvid (1995) to suggest that the morphological and physiological development of the intestine may be completed by 10 days post-hatching.

The development of the alimentary canal is dependent on both body weight and growth rate (Nitzan et al., 1991). Relative weights of the storage segments of the gastro-intestinal tract (crop, proventriculus and gizzard) are reduced in heavier chickens. In broilers, these same segments are the largest at day 1 and smallest on day 15 when compared to chickens with a slower growth rate. Whereas, the absolute and relative weights of the small intestine and the duodenum are greater in broilers compared to layer-
type chickens, with the fastest period of growth for these two segments occurring at day 6 (Nitzan et al., 1991). Differences in the development of the gastro-intestinal tract between the broiler and layer-type chickens, may account to some extent, for the differences in food regulation between these two breeds. Studies have suggested that the capacity of the gastro-intestinal tract may be the limiting factor for food intake in broilers, whereas in layers, the hypothalamic regulation of food intake is considered to be more important (Nir et al., 1978; McCarthy and Siegel, 1983; Leenstra, 1986).

The intestinal weight, length and surface area are greater in broiler chickens compared to White Leghorn chickens at 6 and 20 weeks of age (Isshiki et al., 1992), indicating that the development of the intestine occurs earlier in the broiler and may contribute to their greater growth rate and higher food intake compared to layer-type birds (Tarvid, 1995). Therefore, the characteristics of intestinal development in birds are more determined by growth rate than by bird breeds (Tarvid, 1995). The accelerated growth of the gastro-intestinal tract of chicks in the early postnatal period demonstrates the importance of organs and tissues that fulfil a “supply” function for achieving early body development (Katanbaf et al., 1988). The relative growth rate of broilers is greatest during the first 2 weeks post-hatch and decreases thereafter (Marks, 1979; Nitzan et al., 1991). Growth of the digestive tract of broilers follows a pattern similar to that of relative growth suggesting that plasticity of the digestive tract of broilers is age dependent (Nitzan et al., 1991).

2.2.3. Regulation of Food Intake

Intensive genetic selection has resulted in large increases in the body weight of broilers and a bird type far removed from both layers and earlier generations of broilers. Broilers expend less energy on activities such as feeding and selectively partition resources to growth at the expense of other physiological processes including immunity (Siegel et al., 1989). The increased body weight is due largely to increases in feed intake (Denbow, 1994). Denbow (1994) suggests that alteration in food intake may be the result of reduced sympathetic nervous activity because injections or brain lesions that increase food intake generally decrease sympathetic nervous activity (Bray, 1991).
Regulation of food intake occurs at various levels. Both the crop and gizzard have distension-sensitive receptors and it is suggested that they respond to neural stimuli and act to restrict gut fill (Duke et al., 1977; Hodgkiss, 1981). Both the infusion of water during feeding (Lepkovsky and Furuta, 1960) and inflation of balloons located within the crop (Richardson 1970) decrease food intake in chickens. These observations suggest a correlation between crop distension and food intake.

Following a fast, food initially bypasses the crop and enters the gizzard (Henry et al., 1933; Vonk and Postma, 1949; Hill and Strachan, 1975) suggesting that crop filling and emptying are controlled by distension of the gizzard (Ihnen, 1928; Hill and Strachan, 1975). The crop is necessary for normal growth only when feed availability is restricted. Growth rate of Leghorn chickens following cropectomy is compromised only when feed is restricted but not when fed ad libitum (Richardson, 1970). Denbow (1994) suggests that the primary role of the crop is storage and that appetite regulation is controlled at sites other than the crop. In turkeys, satiety regulation is related to the inhibition of oesophageal peristalsis caused by the filling of the stomach (gizzard), duodenum and the proximal one-third end of the ileum (Chaplin et al., 1992).

While mechanical forces acting on the avian gastro-intestinal tract have been shown to effect regulation of food intake, chemosensitive and osmotic receptors within the tract also influence satiety. Duodenal osmotic receptors function in the regulation of food intake. The intra-duodenal infusion of hyperosmotic solutions of potassium chloride reduces food intake in free-fed chickens (Shurlock and Forbes, 1981a) and Leghorn cockerels (Lacy et al., 1986a). How osmotic receptors respond to hyperosmotic infusions in regulating food intake has not been elucidated. However, Denbow (1994) suggests that the motility of the gastro-intestinal tract may be an important satiety regulator as the injection of hypertonic solutions depresses gizzard motility. This finding is pertinent because of the temporal organisation between the gizzard, proventriculus and duodenum (Dziuk and Duke, 1972).

The role of both glucose and gluco-receptors within the avian gastro-intestinal tract in food intake regulation remains unclear. Some studies demonstrate a positive correlation between the administration of exogenous glucose and reduced food intake (Sonoda and Makino, 1980; Shurlock and Forbes, 1981b). While these studies suggest
that glucose may play a specific role in regulation of food intake, they could be compromised owing to the lack of osmotic controls. The responses could be attributable to either gluco- or osmo-receptors (Denbow, 1994). The effects of intra gastro-intestinal tract glucose infusions and the associated reduction in food intake may also be the result of hepatic glucose uptake (Denbow, 1994). Studies in layer cockerels indicate the presence of hepatic receptors, which respond directly or indirectly to glucose (Shurlock and Forbes, 1981b). The regulatory nature of these receptors appears to be modulated by genetic variability as demonstrated by Lacy and co-workers (1985) who showed reduced food intake in Leghorns but not in broilers following intrahepatic infusions of glucose.

Difference between layers and broilers also appear to be instrumental in modulating the response of hepatic lipid receptors. Intrahepatic infusion of lipid reduces food intake in Leghorns but has no effect in broilers (Lacy et al., 1986b). The chain length of fatty acid subtypes could play an important role in regulating food intake in chickens. Intrahepatic infusion of specific fat subtypes with varying chain lengths reduced food intake in fasted Leghorns. When these same fatty acids were administered intragastrically the short-chained fatty acids were more effective in suppressing food intake than the long-chained fatty acids (Denbow et al., 1992).

The role of protein or amino acids in the regulation of food intake in the chicken has been the subject of many studies. Feeding chickens diets with amino acids in either deficit (Almquist, 1954) or excess (D’Mello and Lewis, 1971) reduces food intake. The ability of chickens to select between adequate and low protein diets (Steinruck and Kirchgessner, 1993) or amino acid deficient and balanced diets (Picard et al., 1993) suggests a sensitive sensory mechanism controlling appetite (Denbow, 1994). However, the ability of the liver to detect the presence of amino acids and respond accordingly remains questionable. There is evidence that intrahepatic amino acid infusions decrease food intake in chickens (Shurlock and Forbes, 1984) while no effect was indicated by Lacy et al., (1986b).

In mammals, there is a definitive neural role for the vagus nerve in regulating food intake through interpreting signals that arise from the gastro-intestinal tract (Gieselman et al., 1980; Sawchenko and Gold, 1981). Although studies in chickens suggest that the vagus nerve transmits sensory information from the liver (Howes and
Delineation of a specific role for the vagus nerve in food intake regulation of birds is made more difficult as the chicken gastrointestinal tract is also innervated by Remarks nerve, an intestinal nerve unique to birds (Akester, 1979). This nerve may also play a role in the neural control of food intake in chickens (Denbow 1994).

Studies with rats by Hetherington and Ranson (1940) implicated the central nervous system, acting at the level of the hypothalamus, in the modulation of food intake. Early studies suggested a dual role for the hypothalamus; hypothalamic theory of food intake regulation where the ventromedial hypothalamus acts as the satiety centre and the lateral hypothalamus the feeding centre (Stellar, 1954). It is likely that the control of food intake involves neural circuits within the central nervous system rather than circumscribed sites (Sclafani and Kirchgessner, 1986). These hypothalamic regions have also been shown to mediate food intake in birds (Sonoda, 1978; Snapir and Robinson, 1985) but genetic variation may override these regulatory areas. Studies using lines of chickens selected for either high or low body weight indicate that lesions to the ventromedial hypothalamus in low-weight birds increased body weight and obesity with no increase in food intake while similar lesions in the heavier line had no effect (Burkhart et al., 1983).

The relationship between the catecholamines, adrenaline, noradrenaline and dopamine, and food intake has been assessed in both layers and broilers. Interestingly, a genetic component has also been observed in the food intake response following administration of these neurochemicals. Intracerebroventricular (ICV) adrenaline injection into broilers significantly increased food intake while dopamine and noradrenaline injection had no effect (Denbow et al., 1981). These same ICV injections had no effect on food intake in Leghorns (Denbow et al., 1983). Differences in response to the catecholamines have also been observed in chickens genetically selected for either low or high body weight. When injected ICV with an \( \alpha_1 \)-adrenergic agonist, food intake increased in the high body weight group but had no effect in the low body weight group (Denbow et al., 1986). There are other neurochemicals that not only alter the food intake response but also show varied sensitivity depending on genotype and these include 5-
hydroxytryptamine (Denbow et al., 1982; Denbow et al., 1983) and calcium (Denbow and Van Krey 1987).

Several peptides have been implicated in the regulation of food intake in birds. The secretion of cholecystokinin (Savory and Gentle, 1980; Savory and Gentle, 1983) and bombesin (Denbow, 1989) from the gastro-intestinal tract acts to reduce food intake in response to the presence of lipids and selective amino acids within the lumen of the duodenum (Dockray, 1979; Yang et al., 1989). The ICV injection of Neuropeptide Y or avian pancreatic polypeptide stimulates food intake in broilers (Kuenzel et al., 1987) and Leghorn chickens (Denbow et al., 1988) whereas, melatonin administration causes a decrease in food intake (Injidi and Forbes, 1983). Opioids have also been shown to reduce food intake by acting at both peripheral sites (Denbow 1994) and within the central nervous system (Denbow and McCormack 1990).

2.2.4. METABOLISM OF ENERGY SUBSTRATES

Lipid and carbohydrates act as the main energy sources in typical avian diets, and although protein can be used as an energy source, this only occurs when present in excess or where a shortage of fat and/or carbohydrate occurs (Stevens, 1996).

2.2.5. CARBOHYDRATE METABOLISM

Absorption and transport

Glucose functions as precursor for the synthesis of glycoproteins, triglycerides and glycogen and provides an important energy source by generating ATP through glycolysis. Because glucose is a polar molecule, it does not readily diffuse across the hydrophobic plasma membrane, therefore, specific carrier molecules exist to mediate cellular uptake (Olson and Pessin, 1996).

Avian intestinal glucose is absorbed both actively by Na⁺-dependent glucose transporters located within the luminal membrane of intestinal epithelial cells and passively via the paracellular route (Croom et al., 1998). In chickens, glucose transport activity increases for 2-3 weeks post hatch, a response that has been attributed to an increase in carrier availability rather than an increase in substrate affinity (Ziswiler and Farner, 1972). There exist several different Na⁺ dependent glucose transporter subtypes.
along the intestinal axis from the duodenum to the ileum (Hopfer 1987). These transporters differ in their relative affinities for glucose and galactose and have different Michaelis-Menten constants (Turner and Moran, 1982). There is preferential sugar absorption in the avian intestine and follows the pattern of D-galactose>D-glucose>D-xylose>D-fructose (Hazelwood, 1986).

Sodium dependent glucose transporters are found in the apical membrane of enterocytes in the upper villus of the jejunum (Silverman, 1991) and it is the middle segment of the avian small intestine that is most active in the transportation of carbohydrates (Ziswiler and Farner, 1972). Interaction of glucose and Na⁺ with the transporter initiates a conformational change and the intracellular transport of 1 mole of glucose is accompanied by the translocation of 2 moles of Na⁺ (Wright et al., 1991; Hediger and Rhoads, 1994). After the Na⁺ ions and glucose are internalised, the transporter returns to its original conformation exposing the substrate-binding site to the intestinal lumen (Croom et al., 1998). The energy required for this process is generated by a Na⁺-dependent electrochemical gradient and the Na⁺ ions driven by a Na⁺/K⁺ ATPase located on the basolateral membrane of the enterocyte (Horisberger et al., 1991).

Facilitative glucose transporter proteins (GLUT), GLUT1, GLUT2, and GLUT3, as well as the fructose transporter GLUT5 are found in the small intestines and kidneys of mammals (Fukumoto, et al., 1988; Thorens et al., 1988; Kern et al., 1990; Rand et al., 1993). The GLUT2 transporter is the primary isoform responsible for glucose transport across the basolateral membrane of intestinal epithelial cells to the extracellular space of the serosa (Wright et al., 1980; Maenz and Cheeseman, 1987; Thorens et al., 1990), whereas, the Na⁺-dependent glucose transporter is localised to the brush-border membrane (Olson and Pessin1996). The GLUT5 isoform is abundant in the apical and the basolateral membranes of the intestine and facilitates the uptake of fructose from the intestinal lumen and its exodus from the intestinal epithelia (Blakemore et al., 1995). Regulation of GLUT2 and GLUT5 is controlled by the concentrations of dietary sugars. GLUT2 mRNA is increased by D-glucose, D-galactose and D-fructose (Miyamoto et al., 1993), whereas GLUT5 mRNA is up regulated only by D-fructose (Miyamoto et al., 1993; Burant and Saxena, 1994). Once absorbed, carbohydrates enter the metabolic pool and are either polymerised to glycogen in the liver and kidney, or metabolised to release...
CO₂, H₂O and energy via the classical oxidative citric acid pathway or shunted into lipogenic paths (Hazelwood, 1986).

**Intracellular transport of glucose**

The principal form of stored carbohydrate in birds is glycogen, which is mobilised as glucose and transported between tissues. Avian plasma glucose concentrations and body weights are not affected by glucose uptake with the intestinal wall converting approximately 30% of the ingested carbohydrate to lactate, thereby regulating glucose homeostasis (Riesenfield *et al.*, 1982). This regulation of plasma glucose concentrations is also maintained during fasting (Hazelwood and Lorenz, 1959). By comparison, plasma glucose concentrations are higher and liver glycogen levels lower in birds than mammals (Sarker, 1971) and this may be indicative of the importance of the gluconeogenic pathway in birds especially during fasting (Langslow 1978).

The modulation of plasma glucose concentrations is characterised by hexokinase and glucose 6-phosphatase. These two enzymes are important in the control of glucose transport by a substrate cycle as they catalyse the phosphorylation and dephosphorylation of glucose, thereby exerting an effect on tissue uptake (Newsholme and Leech, 1983). Diffusion of glucose across the plasma membrane by facilitated GLUTs also mediates blood glucose concentrations (Stevens, 1996). In addition to the glucose transporters identified within the small intestines and kidneys of mammals, GLUTs are also expressed in other tissues.

Seven genes grouped into a supergene family are responsible for the glucose transporter (GLUT) proteins. Only four have been documented as authentic glucose transporters (Olson and Pessin, 1996). The GLUT1 transporter is expressed in all foetal tissue, whereas in adult tissues, GLUT 1 although widely expressed, is most abundant in fibroblasts, erythrocytes and endothelial cells with low levels of expression in muscle, liver and adipose tissue (Birnbaum *et al.*, 1986; Fukumoto *et al.*, 1988). The GLUT2 transporter is found in the small intestine and kidney and also on the basolateral membrane surfaces of liver cells and pancreatic β-cells (Thorens *et al.*, 1988). The GLUT3 transporter is found in highest levels in neonatal tissue and is considered the major GLUT responsible for transporting glucose into the brain and peripheral tissue.
(Kayano et al., 1988; Nagamatsu et al., 1992). The GLUT4 transporter is predominately expressed in adult skeletal and cardiac muscle, as well as in brown and white adipose tissue and positively correlates with the cell types that display insulin-sensitive glucose transport (Birnbaum, 1989; Charron et al., 1989). The GLUT6 is a pseudo gene containing multiple insertions and translation termination signals, and does not encode for an expressed protein (Kayano et al., 1990). Whereas, GLUT7 is located within the endoplasmic reticulum and has been identified as a component of the glucose-6-phosphatase complex in liver (Waddell et al., 1992). Although GLUTs have not been studied in detail in the bird, studies with avian tissues suggest a similar family of GLUTs probably exist. For example, Duclos and co-workers (1993) identified a protein in skeletal muscle, adipose tissue and brain that had a similar molecular weight that corresponded to that of the GLUT1 from mammals.

**Metabolic pathways**

The metabolic pathways that have been identified in mammals are also, on the whole, operative in birds. However, there are differences in the relative contribution of a given pathway to the overall energy requirements of a given tissue or to the organism itself. One example of this difference between mammals and birds is in the type of substrate in gluconeogenesis. Fasting chickens do not utilise alanine as a precursor to the same extent as mammals, but preferentially utilise both lactate and glycerol (Davison and Langslow, 1975). In addition, tricarboxylic acid cycle intermediates and amino acids are also sparsely used as precursors when compared to mammals (Brady et al., 1979). This may be due to the localisation of rate-limiting gluconeogenic enzymes within the mitochondria (Brady et al., 1979). Interestingly, carnivorous birds such as the vulture whose normal diet is carrion, have higher gluconeogenic enzyme levels when compared to more granivorous birds such as chickens, which have a “constant-nibbling” regimen of food intake. Hazelwood, (1987) has suggested that these higher enzyme levels reflect the difference in the meal eating nature between these birds.

Glucose metabolism occurs in all tissues, although the metabolic pathway resulting in glucose break down varies between tissues. In skeletal muscle, glycolysis is of prime importance in the generation of ATP for muscle contraction, whereas, for liver
the rate of glycolysis is much lower, its role being primarily to provide intermediary metabolites for biosynthesis (Stevens, 1996). This role is particularly important for triglyceride production as adipose tissue has very little capacity for de novo triglyceride biosynthesis (Saadoun and Leclercq, 1983). Avian muscle has the same fast-twitch glycolytic and slow-twitch oxidative fibre types as mammals with anatomic muscles being made up with varying proportions of each fibre type (Stevens, 1996). As in mammals, there is considerable difference in the importance that aerobic and anaerobic metabolism of glucose plays in different types of skeletal muscle. Studies by Blomstrand and co-workers (1983) compared the enzymes involved in both aerobic (oxoglutarate dehydrogenase) and anaerobic (6-phosphofructokinase) metabolism in pectoral and cardiac muscles from the pigeon and the chicken. They found that the cardiac muscle from both bird’s generated equal amounts of ATP from aerobic and anaerobic metabolism. However, the pectoral muscle from the chicken had a significantly reduced capacity for aerobic metabolism when compared to the pectoral muscle of the pigeon. These differences are related to specific muscle energy requirements as pectoral muscles from birds that fly have a greater capacity to carry out aerobic metabolism and this is correlated to the ability to perform flight (Marsh, 1981).

As in mammals, homeostatic mechanisms have evolved in birds to control glycolysis and gluconeogenesis. These mechanisms include feedback inhibition of specific metabolites of both pathways and regulation by hormones interacting with receptors on the cell surface that initiate the release of second messengers within the cell. Feedback inhibition involves either regulation of enzyme activity by effectors binding to regulatory sites and then either decreasing or increasing the activity, or reversible covalent modification, usually phosphorylation of regulatory enzymes. Whereas hormonal control involves a change in the amount of a particular enzyme either by changing its rate of synthesis or its degradation (Stevens, 1996).

Changes in intermediary metabolism during development

The chick embryo uses the egg-yolk and -white (albumin) as sources of nutrients during development. The egg-yolk contains approximately 55% protein, 44% lipid and 1% carbohydrate on a dry weight basis. The lipid acts as the main source of energy for
development (Bate and Dickson, 1986). During late embryo development, much of the lipid is used for gluconeogenesis although significant amounts are converted to ketone bodies to be used by the embryonic heart, brain and kidney (Bate and Dickson, 1986). Upon hatching, the source of energy changes from one derived almost exclusively from lipid to one that is predominately derived from carbohydrate, resulting in changes to intermediary metabolism. (Stevens, 1996). The liver is the principal organ that converts metabolisable fuels and accepts lipid derived from either dietary origin or triglycerides synthesised de novo. The liver has a high gluconeogenic capacity during embryonic development but following hatching glycolysis is favoured above gluconeogenesis (Langslow, 1978; Garcia et al., 1986).

2.2.6. PROTEIN METABOLISM

Absorption and transport

In chickens, metabolism of digested protein is initially characterised by the action of gastric enzymes secreted by the proventriculus followed by the secretion of proteinases and pancreatic juice from the pancreatic gland into the intestinal lumen. These enzymes hydrolyse protein molecules to smaller olygopeptides and dipeptides (Mc Donald and Barrett, 1986). The hydrolysis of these peptides is the final stage in protein degradation and is catalysed by the action of pancreatic and intestinal exopeptidases that are transported to the brush border zone of the digestive tract (Tarvid, 1995). This stage of protein digestion is classified as membrane hydrolysis and is followed by amino acid and/or peptide transport (Tarvid, 1995).

Amino acid transport in the chicken is assisted by Na\(^+\)-dependent and energy-dependent mechanisms (Lerner, 1984). A number of amino acid transport mechanisms have been observed for the small intestine of the chick that are specific for individual amino acids (Miller et al., 1973). The site of transport is also specific for individual amino acids and is dependent on their molecular weight. Studies in chickens and turkeys have shown that peptides with a molecular weight of between 8000 and 15000 accumulate in the proximal portion of the small intestine, whereas, low molecular weight peptides accumulate in its distal part (Sklan and Hurwitz, 1980a). In addition, Lerner and co-workers (1976) have shown that a genetically programmed transport distribution
pattern exists along the entire length of the small intestine with the maximal transport occurring in the mid region (ileum).

There is a general decline in the intestinal uptake of essential amino acids during the postnatal period of the chicken (Buddington and Diamond, 1989) and this is correlated to the intensity of the transport system which decreases after hatching (Tarvid, 1995). At hatching, transport rates are high to accommodate the absorption of high quantities of food necessary to meet the chick’s high metabolic requirements (Tarvid, 1995). However, the reduced uptake of amino acids during the postnatal period could be specific for different amino acids, as for example, the transport capacity for proline increases with the age of the bird (Buddington and Diamond, 1989). Mechanisms that may contribute to the developmental changes in amino acid transport capacity include changes in transporter number, the switch from one transport mechanism to another, and changes in membrane characteristics, especially membrane fluidity, which decreases with age (Hayashi and Kawasaki, 1982).

Protein turnover

The mechanisms involved in the synthesis of protein and nucleic acids for birds are essentially the same as those described for mammals (see Mathews and van Holde, 1990; Zubay, 1993) with the relative importance of anabolic and catabolic pathways varying for tissues and with age (Stevens, 1996). Intracellular proteins are continually being turned over throughout the life of a cell (Price and Stevens, 1989). These proteins are utilised as structural components of the body such as bone, muscle, skin and feathers and also function as transport proteins (Griminger and Scanes, 1986). Proteins also play a functional role by supplying energy in the course of their degradation (Griminger and Scanes, 1986).

Protein turnover studies in skeletal muscle indicate that birds selected for high growth rates accumulate body protein at a greater rate than do laying hens (Fisher, 1980). The principal sites of protein accumulation are also different between bird types. In laying hens, protein accumulates mainly in the oviduct and liver, whereas for broilers, protein accumulates in nearly all tissues (Fisher, 1980). Protein synthesis rate can vary between tissues. Isotope studies using $^{14}$C-labelled amino acids demonstrated that for
chickens incorporation rates are greater in the intestine, intermediate in the liver and kidney, and lowest in skeletal muscle (Saunderson and Whitehead, 1987). Studies in quail by Park and co-workers, (1991) showed that the rates of protein turnover measured in different tissues decreased in the order: liver>heart≈ brain>pectoral muscle. 

Protein degradation gives rise to amino acids which can act as precursors for the synthesis of new proteins or sources of energy. Studies by Swick (1982) showed that approximately 80% of the amino acids arising from degradation are reutilised. Proteins are characterised as short-lived or long-lived (Hershko and Ciechanover, 1982). Long-lived proteins are not normally tissue specific but are found in a wide range of tissues where they are taken up into lysosomes and degraded by cathepsins. Energy-dependent pathways degrade short-lived proteins (Stevens, 1996).

Most tissues maintain low concentrations of intracellular free amino acids, as there are no specific protein storage molecules. When dietary protein is in excess, it is degraded and deaminated resulting in carbon skeletons available for the synthesis of fats and carbohydrates (Stevens, 1996). In birds and reptiles, surplus nitrogen is excreted as uric acid, whereas in mammals, the principal end product is urea (Griminger and Scanes, 1986). In addition to uric acid, nitrogen is also excreted as ammonia and to a lesser extent urea in birds. However, nitrogen from certain amino acids and glutathione, a tripeptide found in many tissues especially red blood cells, are conjugated to xenobiotics prior to their excretion (Stevens, 1996). The percentage of uric acid in excreta is normally higher in carnivorous birds than granivorous birds. In turkey vultures the concentration of uric acid ranged from 75-85% of the total nitrogen (McNabb et al., 1980). In contrast, the excreta concentration of uric acid in laying hens ranged from 55-59% for a low protein diet to 72-79% for a high protein diet (Skadhauge, 1983).

2.2.7. LIPID METABOLISM

Absorption and transport

Lipid particles leaving the gizzard are initially subdivided by emulsification with bile salts and reach the duodenum as triglycerides and phospholipids. Biliary salts allow the adsorption of colipase at the lipid-water interface and act as an anchor for the binding of pancreatic lipase (Freeman, 1984) and also stabilises the enzyme against inactivation
Carboxylic ester hydrolase (cholesterol esterase) also secreted by the pancreas acts to hydrolyse cholesterol esters (Shiratori and Goodman, 1965) and catalyses the re-synthesis of cholesterol and free fatty acids (Freeman, 1984). In addition, the secretion of phospholipase A₁ and phospholipase A₂ from the pancreas acts to hydrolyse the ester bonds at the sn-1 and sn-2 position of the glycerol moiety respectively. The resultant lipolysis products are 2-monoglycerides and free fatty acids with cholesterol, lysophospholipids, 1,2-diglycerides and glycerol as minor components (Freeman, 1984). These compounds form micelles with biliary salts and are passively absorbed by an energy-independent mechanism by which the lipolytic products pass from the micelle into the mucosa. However, a bile-independent mechanism of fatty acid uptake has been demonstrated in the chick that is mediated through a fatty-acid/carrier-protein complex (Sklan and Hurwitz, 1980b).

The absorption of lipids into the enterocytes depends upon inward diffusion gradient (Brindley, 1984). The work of Ockner and co-workers (1972) and Brindley (1984) suggest that fatty acids upon entering the mucosa cells bind to intracellular proteins which have specific affinity depending on the degree of saturation and chain length. Long-chain unsaturated fatty acids are bound in preference to saturated short- and medium-chain fatty acids. The inward diffusion gradient is partly maintained by the re-esterification of absorbed lipids. For the chicken, resynthesis of triglycerides from long-chain fatty acids occurs in the small intestinal epithelium using both the monoglyceride and glycerol-3-phosphate pathways (Bickerstaffe and Annison, 1969). In monogastrics, Brindley, (1974) estimated that between 75-85% of dietary triglycerides can be metabolised via the monoglyceride pathway during absorption. The major intestinal site of lipid absorption in the chicken is the jejunum (Annison, 1983) although uptake also occurs from the ileum (Hurwitz, et al., 1973) and duodenum (Noyan et al., 1964; Hurwitz, et al., 1973).

While digestibility of intestinal fat is high in poultry, often more than 80% (Doreau and Chilliard, 1997) there may be limits to fatty acid digestibility (Wiseman, 1984). Digestibility improves with age and is greater in hens than cockerels (Griminger, 1976). Digestibility can differ between fatty acid subtypes; it decreases as chain length increases for saturated fatty acids from 14 to 18 C atoms in length (Lessire et al., 1996).
and increases with degree of unsaturation (Lessire and Leclercq, 1982). An interaction between saturated and unsaturated fats results in improved digestibility of saturated fats (Doreau and Chilliard, 1997). Sklan (1976) recorded higher absorption rates for the polyunsaturated fatty acids linolenic (C18:2) and linoleic acids (C18:3) compared to stearic acid (C18:0). In growing chickens, soybean oil and lard are well absorbed when compared to tallow with absorption values of 96%, 92% and 67% respectively (Sklan, 1976). For a particular fatty acid digestibility varies according to its position on the glycerol molecule and is higher for saturated fatty acids esterified at position 2 than at positions 1 or 3 (Doreau and Chilliard, 1997).

After re-esterification in the intestinal cells, transport of lipids in mammals occurs mainly in the lymph as chylomicrons. Short-chain fatty acids are transported directly through the cell into the portal system as albumin-bound free fatty acids, whereas, medium-chain (C8-C12) fatty acids are transported in lymph or in portal blood depending on their chain length (Freeman, 1984). In birds because of a poorly developed lymphatic system (Kiyasu, 1955), absorbed lipid is transported as triglycerides of the very-low-density lipoprotein (VLDL) fraction (Freeman, 1984). Compared to mammalian chylomicrons, chick VLDL are characterised by a relatively low content of triglycerides, but a significantly higher protein content than the serum VLDL fractions of pigs and man (Husbands, 1971).

The chylomicrons and VLDL produced in the small intestine enter the circulation and interact with other blood born lipoproteins resulting in phospholipid being lost from the surface coat with a net gain of cholesterol esters (Brindley, 1984). The surface of the chylomicrons also acquires the apoproteins, apo-C and apo-E (Zilversmit, 1978; Havel, 1982). In the circulation triglyceride in the chylomicron and VLDL particles is hydrolysed by lipoprotein lipase, a key enzyme that regulates the disposal of lipid fuels in the body and is present in the capillary beds of several tissues (Fielding and Frayn, 1998).

Lipoprotein lipase is synthesised within the parenchymal cells of tissues. A tissue-specific regulatory element in the lipoprotein lipase gene promotor region allows for differential regulation in different tissues (Fielding and Frayn, 1998). It has been reported that around forty lipoprotein lipase molecules may act on chylomicron and VLDL particles (Scow and Olivecrona, 1977). These particles are anchored by the interaction of
its apolipoproteins with the heparin sulfate proteoglycan chains and lipoprotein lipase (Golberg, 1996). Lipoprotein lipase is expressed to different levels in different tissues and is positively correlated with the animal’s nutritional state and therefore the tissues requirement for fatty acids (Fielding and Frayn, 1998). In the fed state, lipoprotein lipase is activated in white adipose tissue and down regulated in skeletal muscle and heart and therefore fatty acids are directed to adipose tissue for esterification and storage in a time of energy surplus (Cryer et al., 1976). During fasting, lipoprotein lipase will be up regulated in muscle and suppressed in adipose tissue, with fatty acids being directed to the tissue in which they are needed as an oxidative fuel (Cryer et al., 1976; Borensztajn, 1987).

As the triglyceride core is depleted, surface material from chylomicrons is lost resulting in the transfer of apo-C and apo-A to high-density lipoproteins (Havel, 1982). As a consequence of these changes there is a reduction in the affinity for lipoprotein lipase and chylomicrons are transferred from the capillary bed to the liver where lipid and protein components are degraded by lysosomal catabolism (Havel, 1982). The metabolism of VLDL’s is similar with remnant triglyceride particles being converted to low-density lipoproteins before being removed from the circulation by the liver or extrahepatic tissue (Brindley, 1984).

**Lipid biosynthesis**

Birds have a high capacity for lipid biosynthesis that is evident from early in life. Total body lipid of growing chickens doubles every 5.5 days from week 1 post-hatch (Scanes, 1987) and the rate of liver fatty acid synthesis can reach maximum capacity as early as 7 weeks of age (Furuse et al., 1991). In birds as in man the liver is the principal site of synthesis and is reported to have a capacity 20 times greater than adipose tissue when compared to an equal weight (Griminger, 1986). In contrast, in pigs, ruminants and laboratory rodents the principal site of lipid synthesis is adipose tissue (Pearce, 1980). Goodridge (1968) reports that little lipogenesis of any physiological significance takes place in chicken adipose tissue. Studies in chicks have demonstrated a high capacity for lipid synthesis in bone marrow (Nir and Lin, 1982). The synthesis of fatty acids also
occurs in the uropygial gland, which is the main source of fatty oils used to preen the feathers (Stevens, 1996).

The pathways involved in fatty acid synthesis in birds are the same as those that have been described for mammals (see Stryer, 1996). These pathways involve acetyl-CoA carboxylase catalysing the conversion of acetyl-CoA to malonyl-CoA and the formation of palmitate from acetyl-CoA and malonyl-CoA by fatty acid synthetase (Annison, 1983). Fatty acid synthetase is a multi-enzyme polypeptide, and in vertebrates contains the seven enzyme activities required for the synthesis of long-chain fatty acids from malonyl-CoA (Stevens, 1996). A sequence of six steps increases the fatty acid chain by 2 carbon atoms with the release of the free fatty acid as the final step (Stevens, 1996). Once synthesised, fatty acids are transported to adipose tissue as triglyceride in the VLDL’s where extracellular lipoprotein lipase catalyses the hydrolysis of triglyceride so that fatty acids can pass into the adipose tissue (Griffin et al., 1982).

The main controls that govern the amount of fatty acids that are synthesised are nutritional and hormonal (Wakil et al., 1983). For chickens, most of the accumulated fat is of dietary origin (Griffin and Hermier, 1988) with the level of intake influencing liver enzymes, ATP citrate lyase and malate enzyme and therefore de novo fatty acid synthesis (Griminger, 1986). Fatty acid synthesis increases in response to low dietary lipid intake and decreases when intake is high. Principal hormones influencing hepatic lipid biosynthesis are insulin, glucagon, triiodothyronine (T₃) and glucocorticoids (Fischer and Goodridge, 1978; Vives et al., 1981). Insulin and T₃ promote fatty acid synthesis by effects on gene transcription (Wilson et al., 1986). Glucocorticoids and glucagon have antilipogenic effects, with glucagon inhibiting the accumulation of fatty acid synthetase mRNA (Wilson et al., 1986).

**Lipid mobilisation**

Triglycerides are the major source of energy in birds, and the largest stores are found in adipose tissue and the liver in adult birds. Mobilisation of lipid from adipose tissue occurs when hormones bind to and then activate receptors on the adipocyte surface causing a cascade of biochemical reactions which terminate with the activation of hormone-sensitive lipase (Oscar, 1995). Activation of hormone-sensitive lipase requires
phosphorylation at its regulatory site and involves protein kinase A (Corbin et al., 1970; Huttunen and Steinberg, 1971), which in turn is activated by increasing levels of cAMP (Oscar, 1995). Cyclic AMP binds to the regulatory subunits of protein kinase A resulting in release of activated catalytic subunits (Oscar, 1995). Cyclic AMP level is regulated by the enzyme adenyl cyclase whose activity is dependent on the balance between activation by Gs, (the stimulatory guanine nucleotide binding protein, G protein) and inhibition by Gi, (the inhibitory G protein) (Spiegel et al., 1992). Hormones regulate the activity of Gs and Gi proteins by binding to receptors coupled to these G proteins (Oscar, 1995).

Glucagon is thought to be the major lipolytic hormone in poultry (Scanes, 1995). Its physiological action is to increase the release of glycerol and free fatty acids from chicken adipose tissue (Goodridge, 1968; Langslow and Hales, 1969). While T3 stimulates lipogenesis in chickens (Goodridge et al., 1989) it may also influence lipolysis. The pre-incubation of chicken adipocytes with T3 increases their sensitivity to the lipolytic influence of both glucagon (Harden and Oscar, 1993) and adrenaline (Hargis et al., 1991). The lipolytic actions of adrenaline and noradrenaline in chickens are less dramatic than in many mammals (Scanes, 1995). As in mammals, growth hormone (GH) administered to chickens promotes lipolysis and increases circulating concentrations of free fatty acids (Hall et al., 1987; Scanes, 1992) whereas somatostatin is anti-lipolytic in poultry (Kurima et al., 1994).

Fatty acids released from adipose tissue are transported in plasma bound to albumin and are taken up by the peripheral tissue across the plasma membrane. In the cytosol these fatty acids are converted to acyl-CoA and then acylcarnitine before entering the mitochondrial matrix where it is oxidised via β-oxidation to generate ATP (Stryer, 1996). The mitochondrial enzymes that transport acyl groups across the inner mitochondrial membrane act as a major regulatory point in fatty acid oxidation. The shuttle enzyme, carnitine palmitoyl-transferase and its inhibitor, malonyl-CoA, have been identified in both mammals and birds (Griffin et al., 1990). In mammals when fatty acid synthesis is active, β-oxidation is inhibited by an increase in malonyl-CoA that prevents acyl-CoA from entering the mitochondria. However, in the chicken increased malonyl-CoA concentrations alone are insufficient to inhibit carnitine palmitoyl-transferase and both lipogenesis and fatty acid oxidation can occur simultaneously (Griffin et al., 1990).
In the chicken, β-hydroxybutyrate and acetoacetate, are principal ketone bodies responsible for transport of endogenous lipid to peripheral tissues where they are converted into acetoacetyl-CoA and oxidatively metabolised (Stevens, 1996). During periods of fasting the concentration of β-hydroxybutyrate rises substantially compared to acetoacetate levels (Bailey and Horne, 1972). There is preferential utilisation by tissues of either ketone bodies or fatty acids as a source of metabolic fuel. Concentrations of β-hydroxybutyrate are low in all tissues of the chicken except the brain and kidney (Nehlig et al., 1980) whereas skeletal and cardiac muscle may selectively utilise fatty acids (Stevens, 1996).

2.3. DIETARY FATTY ACIDS

Fat is included in poultry diets as a concentrated energy source. While dietary fat can be reduced to a low level it cannot be entirely eliminated. In rodents a diet deficient in fat leads to retarded growth, dermatitis and eventual death (Burr and Burr, 1929). These conditions are eliminated by the incorporation of the “essential fatty acids”, linoleic (LA), linolenic (LNA) and arachidonic acids (AA). These fatty acids cannot be synthesised by either mammalian or avian cells, and therefore must be supplied by the diet (Stevens, 1996). Chickens have a similar requirement for LA and/or AA which are important for chick growth, egg production and egg size (Balnave, 1970; Watkins, 1991). However, the specific requirement of LNA for mammals (Spector, 1999) or poultry (Watkins, 1991) has yet to be established.

2.3.1. CHEMISTRY AND NOMENCLATURE OF FATTY ACIDS

Fatty acids consist of a chain of carbon atoms, the length of which varies according to the particular fatty acid, with a methyl (CH₃) group at one end and a carboxyl (COOH) group at the other. The number of known natural fatty acids exceeds 1000 although only a relatively small number (20-50) are of common significance (Gunstone, 1996). There are a number of alternative fatty acids descriptions. The common or trivial name was given prior to the elucidation of the chemical structure and was often based on the source of the discovery, such as, palmitic from palm oil and oleic from olive oil. While trivial names are easy to use they are not indicative of the fatty
acid’s structure and therefore, a systematic name has been applied. For example, oleic acid is \textit{cis}-9-octadecenoic acid, a carboxylic acid (oic) with 18 carbon atoms (octadec) and one olefinic centre (en) which lies between carbon 9 and 10 and has a \textit{cis} configuration.

\text{i.e. CH}_3(CH_2)_7CH=CH(CH_2)_7COOH

A simpler way of describing fatty acids involves the use of numbers such as 18:2n-6 linoleic acid. The chain length is given followed by a colon and the total number of double bonds in the molecule. The position of the first double bond in the carbon chain is designated by its relation to the methyl end of the molecule, which is counted as carbon 1 or, n-1. There are three major families of fatty acids, the n-3, n-6 and the n-9 series.

\textbf{2.3.2. BIOSYNTHESIS OF FATTY ACIDS}

\textbf{Elongation and desaturation}

Biosynthesis of long-chain polyunsaturated fatty acids (PUFA) in mammalian cells occurs through a sequence of alternating desaturation and chain-elongation reactions acting on the dietary fatty acid precursors, linoleic acid 18:2n-6 (LA) and linolenic acid 18:3n-3 (LNA) see Figure 2.1 (Cook, 1991). The same elongation and desaturation pathway involving 24-carbon intermediates and peroxisomal retroconversion is utilised by n-3 and n-6 PUFA (Sprecher \textit{et al.}, 1995). Arachidonic acid 20:4n-6 (AA), the major product of the n-6 series, generates from 18:2n-6 by the sequential action of $\Delta^6$-desaturase, an elongase and $\Delta^5$-desaturase. The same pathway acting on 18:3n-3 yields eicosapentaenoic acid 20:5n-3 (EPA) and docosahexaenoic acid 22:6n-3 (DHA), the most abundant PUFA of the n-3 series (Cook, 1991). The rate-limiting step in the enzymatic pathways of PUFA biosynthesis is thought to be $\Delta^6$-desaturase (see Yamazaki \textit{et al.}, 1992). The commonly accepted pathway for the synthesis of DHA (22:6n-3) consists of the elongation of 20:5n-3 to 22:5n-3 followed by a $\Delta^4$-desaturation (Cook, 1991). Whereas, saturated fatty acids (pamitic 16:0 and stearic 18:0 acids) are converted to monounsaturates (oleic 18:1 acid) by $\Delta^9$-desaturase (Jeffcoat, 1979).
Oleic Acid Series (n-9) Linoleic Acid Series (n-6) α-Linolenic Acid Series (n-3)

Figure 2.1 The biosynthesis of polyunsaturated fatty acids, showing pathways of elongation and desaturation. Fatty acid classes compete for the same enzymes at each level of the biosynthetic pathway. Abbreviations used: E, elongase; Δx, fatty acyl-CoA desaturase. (From Pan et al., 1994).

A recent study by Marzo and co-workers (1996) proposes that the synthesis of DHA occurs via a route independent of Δ⁴-desaturation (Figure 2.2). This pathway involves two successive elongations, followed by Δ⁶-desaturation of 24:5n-3 to 24:6n-3 and retroconversion to 22:6n-3. Whereas, the synthesis of 22:5n-6 from AA (20:4n-6) occurs via the combined effect of elongation to 24:4n-6, Δ⁶-desaturation to 24:5 and the retroconversion to 22:5n-6 (Spector, 1999).

The genes that encode for Δ⁹-desaturase can be regulated by diet (Douaire, et al., 1992; Elliott et al., 1993). Activity of this enzyme is reduced by the incorporation of dietary LA (18:2n-6) (Whale and Radcliffe, 1977) while its intrinsic activity is positively correlated with obesity (Whale and Radcliffe, 1977; Legrand et al., 1987). Liver Δ⁹-desaturase activity has been shown to be significantly greater in genetically fat chickens
(Legrand et al., 1987) and obese Zucker rats (Whale and Radcliffe, 1977) compared to leaner animals. While in humans, there is a significant positive relationship between $\Delta^9$-desaturase activity in vastus lateralis muscle and the percentage of body fat (Pan et al., 1994). Increased $\Delta^9$-desaturase activity may result in lowering the melting point of the fatty acids thereby facilitating their incorporation via triglycerides into VLDL (Legrand et al., 1987).

\[
\begin{align*}
\Delta^6 & \quad \Delta^5 & \quad \Delta^6 \\
18:3n-3 & \rightarrow 18:4n-3 & \rightarrow 20:4n-3 & \rightarrow 20:5n-3 & \rightarrow 22:5n-3 & \rightarrow 24:5n-3 & \rightarrow 24:6n-3 \\
\text{LNA} & \quad \text{EPA} & \quad \text{DHA} \\
\end{align*}
\]

\[
\begin{align*}
\Delta^6 & \quad \Delta^5 & \quad \Delta^6 \\
18:2n-6 & \rightarrow 18:3n-6 & \rightarrow 20:3n-6 & \rightarrow 20:4n-6 & \rightarrow 22:4n-6 & \rightarrow 24:4n-6 & \rightarrow 24:5n-6 \\
\text{LA} & \quad \text{AA} & \quad \text{22:5n-6} \\
\end{align*}
\]

Figure 2.2. Operation of the polyunsaturated fatty acid metabolic pathway, (A) with n-3 fatty acids and (B) with n-6 fatty acids. (From Spector, 1999)

In mammals, $\Delta^6$- and $\Delta^5$-desaturases and elongase enzymes produced in the liver are subject to hormonal and dietary regulation (Brenner, 1981). In rodents the production of $\Delta^6$-desaturase is influenced by temperature, nutritional state of the animal and essential fatty acid availability specifically, LA (18:2n-6) and AA (20:4n-6) (Brenner, 1989). Decreased temperature, a fed state and a deficiency in essential fatty acids stimulate the enzyme, whereas, fasting, low dietary protein, LA (18:2n-6) and AA (20:4n-6) decrease activity. Insulin, glucagon, adrenaline, glucocorticoids and adrenocorticotropin (ACTH) also play a regulatory role in $\Delta^6$- desaturase activity (Mandon et al., 1987; Brenner, 1989). Chronic alcohol consumption reduces $\Delta^6$- and $\Delta^5$-desaturase activities (Nakamura
et al., 1994) and the activity of Δ⁶-desaturase declines with age (Ayala et al., 1973; Bourre et al., 1990). The consequences of reduced enzyme activity can be protected against by dietary supplementation with γ-linolenic acid (18:3n-6) (Hrelia et al., 1991). In addition to their role of converting essential fatty acids to PUFA, Δ⁶- and Δ⁵-desaturases also facilitate transport of dietary and de-novo saturated fatty acids, and generate PUFA’s as components of phospholipids and precursors of eicosanoids (Watkins, 1995).

2.3.3. FUNCTION AND ACTION OF FATTY ACIDS

Membranes and membrane lipids

Membranes are assemblies of proteins and lipid molecules that are held together by non-covalent bonds and form a dynamic phospholipid bilayer that separates the internal components of the cell from the extracellular milieu (Singer and Nicholson, 1972). The lipid molecules are arranged as a continuous double layer (lipid bilayer) and it is this that forms the basic structure of the membrane. There are three major kinds of membrane lipids, the phospholipids, being the most abundant, glycolipids and cholesterol.

Phospholipids are important amphipathic molecules essential for cellular membrane formation and function (MacDonald and Sprecher, 1991) and are derived from either glycerol or sphingosine and called phosphoglycerides and sphingomyelin respectively (Stryer, 1996) A phosphoglyceride consists of a glycerol backbone, two fatty acid chains and a phosphorylated alcohol. The hydroxyl groups at C-1 and C-2 of glycerol are esterified to the carboxyl groups of two fatty acid chains. The C-3 hydroxyl group of the glycerol backbone is esterified to phosphoric acid and termed phosphatidate, a key intermediate in the biosynthesis of other phosphoglycerides (Stryer, 1996). Sphingomyelin consists of a sphingosine backbone, an amino alcohol that contains a long, unsaturated hydrocarbon chain that is linked to a fatty acid by an amide bond.

The main phospholipids are derived from phosphatidate where the phosphate group of phosphatidate becomes esterified to one of several alcohols. The common alcohol moieties of phospholipids are serine, ethanolamine, choline, glycerol and inositol. These form the major phospholipid classes found in the membrane lipids of mammals (Ashes et al., 1995) and birds (Hermier et al., 1999) and are named, phosphatidylcholine
(PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin. Of these, PC and PE are the predominant phospholipids found in birds (Hermier et al., 1999) and mammals (Schmid et al., 1995).

Glycolipids are sugar-containing lipids and in animal cells are derived from sphingosine. The amino group of the sphingosine backbone is acylated by a fatty acid, as in sphingomyelin. Glycolipids differ from sphingomyelin in the nature of the unit that is linked to the primary hydroxyl group of the sphingosine backbone. In glycolipids, one or more sugars are attached to this group. Cholesterol, the major steroid in animal tissues, is also an amphipathic molecule and contains four fused cyclohexane rings with a polar head group and a nonpolar hydrocarbon body. Compared to the fatty acids tails cholesterol is a bulky rigid structure and as such fits awkwardly into membrane lipids and tends to disrupt regularity in membrane structure (Evans and Hardison, 1985).

The fatty acyl chains in phospholipids and glycolipids usually contain an even number of carbon atoms, with 16- and 18-carbon fatty acids being the most common and form the hydrophobic section of the phospholipid and glycolipid molecule (Stryer, 1996). The degree of saturation, the type and number of double bonds contained within the fatty acyl chains determines the characteristics of the lipid bilayer (Hulbert and Else 1999). The fatty acyl chains in the membrane bilayer can exist in an ordered rigid state or a disordered state. When saturated acyl chains are packed closely together they form ordered, rigid arrays that allows free rotation around each of the C-C bonds and gives a greater flexibility to the fatty acyl chain. Whereas, unsaturated fatty acyl chains that contain bonds in the cis configuration do not allow rotation around the C=C linkage. The cis configuration produces a bend or “kink” in the hydrocarbon chain that interferes with the highly ordered packing structure of the chains. Therefore, unsaturated fatty acids cannot pack together as tightly as fully saturated fatty acids. Interactions of unsaturated fatty acids with each other are weaker than with saturated fatty acids and less thermal energy is required to disrupt these poorly ordered arrays and this, in turn, has major consequences for the lipid membrane environment (Hulbert and Else 1999).

Cell membranes of vertebrates contain $\Delta^6$, $\Delta^5$, and $\Delta^9$-desaurase enzyme complexes that are capable of converting the dietary and de novo fatty acids to unsaturated fatty acids by the insertion of double bonds at specific places in the in the
chain. Plants, microorganisms and some vertebrates have $\Delta^{12}$- and $\Delta^{15}$-desaurases in addition to the desaturase enzymes complexes found in vertebrates (Tocher et al., 1998). The products of $\Delta^{12}$-desaturase are called the linoleic (n-6) series and further processing by $\Delta^{15}$-desaurase produces the $\alpha$-linolenic (n-3) series (Tocher et al., 1998). A compelling observation by Hulbert and Else (1999) suggests that prior to the evolution of vertebrates, the $\Delta^{12}$- and $\Delta^{15}$-desaurase enzymes were lost and consequently the ability of vertebrates to directly place C=C units deep into the middle half of the membrane bilayer was lost. Therefore, the physical dynamics of biological membranes are subject not only to the degree of saturation of the fatty acyl chains but also to the type of PUFA series that comprises the lipid bilayer. For example, membranes without PUFA’s will have no C=C units in the middle half of the bilayer whilst membranes with only n-6PUFA’s will have no C=C units in the middle third of the bilayer whereas, only n-3 PUFA’s contribute C=C units to the middle portion of the bilayer (Hulbert and Else, 1999).

2.3.4. MEMBRANE FLUIDITY

The fluidity (or viscosity) of biological membranes results from the combination of lipid dynamics and fatty acyl chain order (Hulbert and Else, 1999). The lipid dynamic processes include lateral and rotational diffusion of the whole molecule as well as rotation around single C-C bonds (Hulbert and Else, 1999). Whereas, fatty acyl chain order refers to the average orientation of each C atom along the hydrocarbon chain (Seelig and Seelig, 1980; McDonald et al., 1985). The fatty acyl chain order decreases and mobility increases as the depth of the membrane bilayer increases, although the outside 50% of each leaflet of the bilayer is relatively constant and coincides with a hydrocarbon chain length of C1-C9 (McDonald et al., 1985). The fluidity of the lipid domain core correlates with the partial specific volume of the hydrocarbons present (Shinitzky and Henkart, 1979). Saturated fatty acids occupy a relatively small volume and confer rigidity whereas unsaturated fatty acids with cis double bonds occupy larger volumes and confer fluidity (Hulbert and Else, 1999). Therefore, the fluidity of biological membranes is dependent on the fatty acyl composition of the phospholipids both with respect to the degree of unsaturation and chain length. In some membranes, fluidity is also dependent on the ratio of these fatty acyls to cholesterol and other sterols.
Cholesterol is found predominately in plasma membranes and increases rigidity of the membrane compared to intracellular membranes (Schroeder et al., 1998). Cholesterol fits tightly between the phospholipids and prevents crystallization of the fatty acyl chains and its presence makes the bilayer less permeable to small molecules (Bretscher and Munro, 1993).

### 2.3.5. Regulation of Membrane Fatty Acids

Although biological membranes often contain hundreds of phospholipid molecular species (Dowhan, 1997), only four are synthesised de novo with all others remodelled from these four species by changes at either the sn-1 or sn-2 positions of glycerol (see Figure 2.3) (Schmid et al., 1995). The four molecular species are 16:0-18:2 (n-6), 16:0-18:1, 16:0-22:6 (n-3) and 18:1-18:2 (n-6) (Schmid et al., 1995).

The remodelling of molecular species is achieved by deacylation-reacylation from the combined actions of phospholipases, acyltransferases and transacylases (Balsinde et al., 1995; Yamashita et al., 1997). Saturated fatty acyl chains are largely incorporated via de novo synthesis whereas; polyunsaturated acyl chains are introduced into phospholipids via deacylation-reacylation (Valtersson et al., 1986). Vertebrates are only able to synthesise de novo saturated and monounsaturated acyl chains and obtain PUFA’s from their diet or intestinal biota (Hulbert and Else, 2000).
Figure 2.3 Schematic representation of specificity of fatty acid acylation to sn-1 and sn-2 positions of phospholipids and further remodelling via the deacylation-reacylation pathway. From Ashes et al., 1995.

2.3.6. PERIPHERAL MEMBRANE PROTEINS

Membrane lipids form a permeability barrier and thereby establish compartments, whereas specific proteins mediate nearly all other membrane functions. The biphasic environment of the plasma cell membrane influences both the structure and function of proteins. Membrane proteins are classified as either integral or peripheral, depending upon the nature of the protein-membrane association (Seaton and Roberts, 1996). Integral
proteins are anchored to lipid bilayers by one or more hydrophobic segments that traverse
the membrane. These proteins generally function as receptors, enzymes and ion channels
(Mourtisen and Kinnunen, 1996). Peripheral proteins associated at the lipid surface are
major components of biological membranes and function \textit{in situ} as electron carriers,
enzymes, signal transduction proteins or primarily as structural elements (Heimberg and

Many of the key proteins involved in signal transduction including phospholipase
A\textsubscript{2}, phospholipase C, G-proteins and protein kinase C (PKC) are extrinsic membrane
proteins. Some of these translocate from the cytoplasm to the cell membrane as part of
early signalling processes that initiate a cascade of events that lead to many biological
actions such as cell proliferation and differentiation (Slater \textit{et al}., 1996). The interaction
of these proteins with the membrane is not only lipid dependent but their activity is
dependent on the membrane lipid composition. Phosphatidylcholine (PC) generally
occurs on the outer leaflet of the bilayer membrane structure, whereas,
phosphatidylethanolamine (PE) occurs primarily on the inner leaflet of membranes
(Zachowski, 1993; Clore \textit{et al}., 1998). The hydrolysis of PC by phospholipase A\textsubscript{2}, C and
D produces second messengers, (free fatty acids, diacylglycerols) and phosphatidic acid
and this provides a mechanism for prolonged activation of PKC which regulates a diverse
range of cellular activities (Nishizuka, 1988). The hydrolysis of PC may therefore
produce significant changes in the structure of membrane domains and the transport of
proteins and electrolytes (Ashes \textit{et al}., 1995). Whereas, PE has been implicated in
hormone binding (Clandinin \textit{et al}., 1991).

Studies by Slater and co-workers (1993) have demonstrated that PKC action is
modulated via phosphatidylserine (PS). This is mediated by the structure of the
membrane bilayer specifically in response to other cell membrane lipids including PE and
PC. The degree of unsaturation of phospholipid comprising the bilayer is suggested to be
of prime importance in determining PKC activity (Slater \textit{et al}., 1993). If PS and PE
subclasses are saturated there is no PKC activity however, as the level of unsaturation
increases there is a corresponding increase in PKC activity. More recent studies indicate
that PKC activity is influenced by the phospholipid polar head group spacings and
associated energetics of the head group interactions in forming non-bilayer phases (Slater et al., 1996).

Influence of the phospholipid saturation on membrane protein interactions has also been observed in studies where the functions of ligand receptors and membrane lipid interactions have been investigated (Litman and Mitchell, 1996). These studies investigated rhodopsin receptor activity in response to the composition of the phospholipid fatty acyl chain of the bilayer membrane. The results indicate that the primary regulatory control was the degree of saturation of the phospholipid acyl chain. Bilayers containing lower levels of acyl chain unsaturation reduced the presence of the receptor and bilayers containing highly unsaturated levels of phospholipids promoted its formation (Litman and Mitchell, 1996). This was reported to be the result of the membrane acquiring unique structural properties provided by the phospholipids in the form of lateral packing domains and an increase in the fatty acyl chain packing free volume (Litman and Mitchell, 1996). Interestingly, in this study cholesterol content was considered to be a secondary regulatory point, its control dependent on the presence of a saturated fatty acyl chain (Litman and Mitchell, 1996). In the study by Slater and co-workers (1996) cholesterol was without affect on PKC activity.

The composition of cell membranes and hence their functional properties are regulated by both genetic and environmental components indeed, with diet probably the most important environmental factor (Pan et al., 1994).

2.3.7. Manipulation of Metabolism by Dietary Fatty Acids

In mammals, the dietary fat profile has been shown to affect several metabolic processes, including insulin action (Borkman et al., 1993, Storlien et al., 1987), lipid secretion (Berry et al., 1986) substrate oxidation (Couet et al., 1997) and weight gain (Cunnane et al., 1986). These metabolic processes are interrelated and result in the metabolic condition known as “Syndrome X” or “ Syndromes of Insulin Resistance” (Reaven, 1988; 1993). For this condition, hyperinsulinemia and impaired insulin action are common factors. Insulin resistance is an essential and early component of non-insulin-dependent diabetes mellitus (NIDDM) and is closely related to obesity (Campbell, 1996). In some instances, altering the dietary fat profile by including PUFA’s
successfully ameliorates this condition in animals and humans (Campbell, 1996; Storlien et al., 1996).

**Glucose metabolism and insulin action**

A change in the fatty acid composition of skeletal muscle and adipocyte cell membranes has a major influence on insulin action (Storlien et al., 1996, 1997). A high proportion of saturated fatty acids in the cell membrane can impair insulin action by:

(a) Altering insulin receptor binding/affinity  
(b) Altering ability to translocate/insert glucose transporters  
(c) Changing interactions with second messengers  
(d) Reducing ion permeability (Vessby, 2000).

The insulin receptor is embedded in the plasma membrane bilayer and appears to be sensitive to the surrounding lipid environment (Gould et al., 1982; Sandra et al., 1984). Studies in mammals show a relationship between dietary fat type, membrane composition and the sensitivity of the cell to insulin (Field et al., 1990; Shimomura et al., 1990; Borkman et al., 1993). The presence of PUFA’s and specifically the longer-chain 20 and 22 carbon fats in place of saturated fatty acids in the membrane bilayer improves insulin action (Storlien et al., 1991). This improved insulin sensitivity appears to result from an increased number of insulin receptors as incorporation of PUFA’s into the phospholipid bilayer increases the number of insulin binding sites (Ginsberg et al., 1982; Cheema et al., 1992). Rats fed a diet with a high polyunsaturated/saturated fatty acid (P/S) ratio increases insulin binding to muscle cells by 2-3-fold (Liu et al., 1994). Although there are few data relating dietary fat profile and insulin action in chickens there are reports of such a relationship in turkeys. Gould and co-workers (1982) have shown that the activity of the insulin receptor in the membrane of the turkey erythrocyte is affected by the fatty acid composition of the membrane surrounding the receptor. The more saturated the phospholipid environment the greater was the affinity for insulin. However, the insulin binding capacity was significantly reduced in these studies. Changes in the membrane lipid composition also affects receptor activity by altering the composition of the fatty acids that are released and then incorporated into second messenger molecules (i.e.}

41
diacylglycerol, and PKC), interactions that mediate insulin receptor function (Malanos and Stacpoole, 1991).

Increased dietary PUFA’s ameliorates insulin sensitivity and increases glucose transport rates in both skeletal muscle and adipose tissue (Field et al., 1990; Storlien et al., 1987). A dietary regimen having a low P/S ratio and therefore reduced membrane fluidity impairs insulin receptor signalling and reduces glucose transporter activity (Field et al., 1990; Podolin et al., 1998). Although the basis for improved insulin sensitivity and increased glucose transport has yet to be elucidated, a possible mechanism could involve an increase in the translocation of the insulin-sensitive transporter GLUT 4. Feeding a high fat diet to rats impairs both insulin-stimulated glucose transport and reduces GLUT 4 translocation to the membrane (Zierath et al., 1997; Hansen et al., 1998). Because GLUT 4 resides in the plasma membrane for a prolonged period of time (Gasperi kova et al., 1997), the relationship between membranes enriched with PUFA’s and improved glucose transport may result from increased GLUT 4 translocation induced by exposure to that membrane lipid environment (Clarke, 2000).

It appears that the long-chain PUFA’s from the n-3 series specifically EPA (20:5n-3) and DHA (22:6n-3) are more potent regulators of glucose metabolism and insulin action than PUFA’s from the n-6 series. Glucose uptake increases in rats when their skeletal muscle membranes were enriched with EPA (20:5n-3) and DHA (22:6n-3) (Storlien et al., 1987). Recent studies by Baur and co-workers (1998) demonstrate that the level of insulin sensitivity in humans is negatively correlated to the amount of DHA (22:6n-3) within skeletal muscle phospholipid. In addition, the ratio of dietary n-3:n-6 PUFA’s may be integral in the amelioration of insulin action. Studies in rats demonstrate that diets with a high level of PUFA’s but where they are primarily of the n-6 series leads to a profound insulin resistance in many tissues (Storlien et al., 1991).

Body composition: obesity

Insulin resistance and obesity have been closely linked (Dreon et al., 1988; Parker et al., 1993). Indeed, obesity has been shown to be one of the most significant causes of insulin resistance in humans (DeFronzo et al., 1985) and is accompanied by abnormalities in systemic carbohydrate and lipid metabolism (Clandinin et al., 1996).
The problem of obesity is essentially a problem of energy balance. Excess caloric intake relative to caloric expenditure results in storage of fat at rates depending on the degree of imbalance (Bray, 1976).

Dietary fat profile has been implicated in modulating body fat accumulation in mice (Cheema et al., 1992) and humans (Couet et al., 1997). Feeding specific fat types influences body weight gain. Feeding n-3 fatty acids reduce body weight gain in genetically ob/ob obese mice (Cunnane et al., 1986) and dietary n-6 PUFA’s reduce body fat in rats (Shimomura et al., 1990). The response is attributed to the dietary fatty acid profile as the rate of weight gain is positively correlated to tissue phospholipid fatty acid composition (Pan and Storlien, 1993). Diets high in saturated fatty acids result in increased body fatness when compared to highly enriched PUFA diets (Shimomura et al., 1990; Pan et al., 1994).

Obesity is also associated with altered metabolic state resulting in defective brown adipose tissue (BAT) thermogenesis (Coleman, 1982), lipid oxidation rates (Couet et al., 1997) and altered activities of enzymes involved in regulation of de novo fatty acid biosynthesis, acetyl-CoA carboxylase, fatty acid synthase and malic enzyme (Hasting and Hill, 1990; Yamini et al., 1991; Shillabeer et al., 1992). These metabolic processes are also responsive to changes in the dietary fatty acid profile. Diets having a high P/S ratio increase both resting metabolic rate, lipid oxidation (Couet et al., 1997) and enhance BAT thermogenesis (Clandinin et al., 1996).

**Fatty acid oxidation and enzyme activity**

Partitioning of dietary fatty acids for storage or oxidation depends on the fatty acid subtype with respect to both their chain length and degree of saturation. Saturated fatty acids are predominately stored, n-3 PUFA’s are preferentially oxidised and n-6 PUFA’s are intermediate with respect to these groups (Leyton, et al., 1987). Facilitation of lipid oxidation by dietary PUFA’s may result from an alteration in the activity of enzymes involved in the β-oxidative pathway. In Syrian hamsters (Surette et al., 1992) and rats (Kabir and Ide, 1996) there is an increase in both mitochondrial and peroxisomal oxidation following dietary n-3 PUFA consumption. For both studies, increased fatty acid oxidation was positively correlated with an increased in hepatic carnitine palmitoyl
transferase I activity. The enzyme facilitates the transfer of long-chain fatty acids across the outer mitochondrial membrane. Kabir and Ide (1996) report increased activities for other enzymes associated with β-oxidation following dietary supplementation with n-3 PUFA’s. These include carnitine palmitoyl transferase II, located on the inner mitochondrial membrane, acyl-CoA oxidase, acyl-CoA dehydrogenase and 2,4-dienoyl-CoA reductase.

The interaction of dietary PUFA’s and the activity of enzymes involved in β-oxidation and associated effects on body weight have not been delineated for poultry. However, it is plausible that a similar interaction between the activity of the β-oxidative enzymes and body weight gain also occurs in birds. When broiler chickens received diets supplemented with L-carnitine for four weeks, the abdominal fat pad weight was reduced (Rabie and Szilágyi, 1998). In this same experiment there was increased breast and thigh muscle mass and improved feed conversion in response to dietary L-carnitine.

Peroxisomal fatty acid oxidation is a major site for oxidation of the very long-chain fatty acids (22- and 24 carbon) as well as 16- and 18 carbon fatty acids (Reddy and Mannaerts, 1994) and takes place in both liver (Kondrup and Lazarow, 1985) and skeletal muscle (Baillie et al., 1999). The oxidative capacity of skeletal muscle, combined with the large size of the skeletal muscle pool has led to the suggestion that the peroxisome is a major site of fatty acid oxidation and diet-induced thermogenesis in mammals (Clarke, 2000). Enzymes of peroxisomal fatty acid oxidation are induced 2-3 fold by dietary n-3 PUFA’s and by peroxisome proliferator activated receptor-α specific ligands (Baillie et al., 1999).

**Lipid metabolism and enzyme activity**

Dietary PUFA’s are also potent inhibitors of hepatic fatty acid and triglyceride synthesis (Jeffcoat, 1979; Clarke et al., 1990). When Syrian hamsters were fed n-3 PUFA’s derived from fish oil, plasma triglyceride concentrations were reduced (Surette et al., 1992). A similar correlation between plasma triglyceride concentrations and dietary n-3 PUFA’s has also been shown for rats (Chait et al., 1974; Okuno et al., 1997) humans (see review of Harris, 1989) and chickens (Akiba et al., 1995). Fish oils have also been reported to affect the activity of enzymes involved in hepatic triglyceride synthesis. In
cultured hepatocytes, EPA (20:5n-3) reduced the activity of acyl-CoA;1,2-diacylglycerol O-acyltransferase which catalyses the final step of triglyceride synthesis (Rustan et al., 1988). The n-6 PUFA subtypes also inhibit the activity of enzymes associated with hepatic lipogenesis. Arachidonic acid (AA 20:4n-6) is more effective in reducing insulin-stimulated fatty acid synthesis in rat hepatocytes than linoleic acid (LA 18:2n-6) (Mikkelsen et al., 1993). This same study showed the greater the degree of unstauration of the fatty acid the more fatty acid synthesis was inhibited; DHA (22:6n-3) being more potent than EPA (20:5:n-3) or AA (20:4n-6).

Gene expression

Dietary fat is a significant modulator of hepatic gene regulation (Sessler and Ntambi, 1998). Clarke and co-workers (1990) suggest that dietary n-3 and n-6 PUFA’s modulate liver fatty acid synthesis and other lipogenic enzymes by regulating mRNA synthesis. Alterations in dietary fat influence enzyme activity by up or down regulating gene transcription (Clandinin et al., 1996). Clarke and Jump (1993) have elucidated a mechanism for PUFA regulation of gene expression for fatty acid synthetase. Dietary n-3 and n-6 fatty acids that inhibit fatty acid synthetase gene transcription are transported through the plasma membrane and subsequently bind to the cytosolic fatty acid binding protein (FABP). The FABP shuttles the 18-carbon fatty acids to the Δ^6-desaturase and subsequently carries fatty acid products to the nucleus, where the cytosolic FABP is transferred to a specific nuclear FABP. This nuclear FABP binds to a specific cis-acting element, which operates to govern gene transcription (Clarke and Jump, 1993).

The regulation of gene expression by n-3 and n-6 PUFA’s also occurs in adipose tissue. For example, Amri and co-workers (1991) have demonstrated adipocyte-specific gene regulation by linolenic acid (LNA 18:3n-3). Stearoyl-CoA desaturase 1 (SCD1) mRNA activity in rodent adipose tissue is reduced when fed a high n-6 PUFA diet (Jones et al., 1996). Modulation of SCD1 activity by n-3 and n-6 PUFA’s has also been observed in tissue culture. The n-6 PUFA’s AA (20:4n-6) and LA (18:2n-6) as well as the n-3 PUFA’s LNA (18:3n-3) and EPA (20:5n-3) depress SCD1 mRNA stability in a dose dependent manner in 3T3-L1 adipocyte cell lines (Sessler et al., 1996).
Metabolic rate and thermogenesis

Dietary fatty acids regulate membrane proteins, including transporters (Dwight and Hendry, 1995), ion channels (Lundbaek and Andersen, 1994), and transduction pathway proteins (Hulbert and Else, 1990) and are implicated in the control of metabolic rate and thermogenesis. Dietary fatty acids modulate BAT thermogenesis by influencing proton conductance of the inner mitochondrial membrane and this is achieved by altering the lipid environment of this pathway (Clandinin et al., 1996). Studies by Shimomura and co-workers (1990) demonstrated increased thermogenesis and oxygen consumption in rats fed n-6 PUFA’s compared to rats fed a diet of edible tallow. Increased metabolic rates have also been achieved in rats consuming n-3 PUFA’s compared to those consuming diets high in saturated fat (Pan and Storlien, 1993). From this latter experiment the suggestion is that it is a consequence of increased incorporation of long-chain highly unsaturated (n-3) fats into the phospholipid of skeletal muscle membranes (Pan et al., 1994).

2.4. EICOSANOIDS

Eicosanoids are a group of oxygenated 20-carbon compounds that include the prostaglandins (PG’s), thromboxanes (TX’s), leukotrienes (LT’s) and a variety of hydroxy and hydroperoxy fatty acids (Smith and Marnett, 1991). The conversion of specific long-chain fatty acids to these bioactive compounds plays an integral role in the overall regulation of cellular activity and hence metabolic control (Schmidt and Dyerberg, 1994). Most eicosanoids are derived from AA (20:4n-6), which is maintained in membrane phospholipids and they are referred to as series 2 eicosanoids (Watkins, 1995). Eicosanoids can also be synthesised from dihomogammalinolenic acid (DGLA 20:3n-6) and EPA (20:5n-3) and these are designated as series 1 and series 3 eicosanoids respectively (see Figure 2.4) (Watkins, 1995).

For most conditions, eicosanoids derived from AA (20:4n-6) are considered to have more potent biological functions than those derived from DGLA (20:3n-6) or EPA (20:5n-3) (Calder, 1998). Eicosanoids synthesised from EPA (20:5n-3) are associated with less vigorous responses than n-6 eicosanoids when bound to specific receptors (Lee et al., 1988). The rate of eicosanoid synthesis also determines its biological affect (Lands,
If the rate of synthesis is low there will be insufficient active eicosanoid to occupy specific receptors and if too high excess active eicosanoid can cause pathophysiology (Lands, 1992).

The conversion of AA (20:4n-6) to the eicosanoids is via three major enzymic pathways. The cyclooxygenase pathway which yields PGs and TX’s, the 5-, 12- or 15-lipoxygenase pathway which yield the LT’s and related eicosanoids and the epoxygenase pathway which yield the cis-epoxy-eicosatrienoic acids (EPETREs) and HETEs (Wainwright, 1997). The types and quantities of eicosanoids synthesised are determined by the availability of AA (20:4n-6), by the activities of phospholipase A2 and phospholipase C, which act on membrane phospholipids to release AA (20:4n-6) and by the activities of the above enzymic pathways (Calder, 1998). The n-3 PUFA’s, EPA (20:5n-3) and DHA (22:6n-3) moderate the intensity of n-6 eicosanoid formation (Lands et al., 1973) and function (Lee et al., 1988) by competitively inhibiting the oxygenation of AA (20:4n-6) by cyclooxygenase (Calder, 1998). In addition, EPA (20:5n-3) but not DHA (22:6n-3) is capable of acting as a substrate for both cyclooxygenase and 5-lipoxygenase (Calder, 1998). Increased concentrations of n-3 PUFA’s will consequently decrease the membrane AA (20:4n-6) concentrations and result in a concomitant decrease in the capacity to synthesise eicosanoids from AA (20:4n-6) (Calder, 1998) thereby modifying the physiological response (Lands, 1992).
Eicosanoids produce a range of biological effects (Table 2.1) with EPA (20:5n-3) derived eicosanoids not always having the same biological properties as the analogues derived from AA (20:4n-6) (Calder, 1998). Eicosanoids derived from AA (20:4n-6), DGLA (20:3n-6) or EPA (20:5n-3), have major affects on the growth and development of animals by modulating the pulsatile release of hypothalamic and pituitary hormones (Ojeda et al., 1981), a function that undoubtly underscores the importance of dietary n-3 and n-6 PUFA’s. Eicosanoids derived from n-3 and n-6 PUFA’s stimulate GH release.
from the pituitary by stimulating GRF release from the hypothalamus, mediate the release of ACTH from the pituitary, enhance the response of thyroid tissue to TSH, promote prolactin release by decreasing prolactin inhibitory factor and increasing stimulatory factors, and stimulate gonadotrophin (LH and FSH) release by stimulating LHRH release from the hypothalamus (Ojeda et al., 1981). In addition eicosanoids have profound affects on immunity (Calder, 1999) and bone development (Watkins 1995).

Table 2.1 Biological effects of selected eicosanoids (From Schmidt and Dyerberg, 1994)

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Eicosanoid</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA 20:4n-6</td>
<td>Thromboxane A2</td>
<td>Platelet aggregation, vasoconstriction</td>
</tr>
<tr>
<td></td>
<td>Leukotriene B4</td>
<td>Adhesion, degranulation, aggregation, chemotaxis</td>
</tr>
<tr>
<td></td>
<td>Prostacyclin PG12</td>
<td>Anti-aggregation, vasodilation vessel wall</td>
</tr>
<tr>
<td></td>
<td>Protaglandin E2</td>
<td>Proinflammatory, platelet aggregation</td>
</tr>
<tr>
<td>EPA 20:5n-3</td>
<td>Thromboxane A3</td>
<td>Inactive</td>
</tr>
<tr>
<td></td>
<td>Leukotriene B5</td>
<td>10% of (LTB4) activity</td>
</tr>
<tr>
<td></td>
<td>Prostacyclin PG13</td>
<td>Same as PG12</td>
</tr>
<tr>
<td>(DGLA 20:3n-6)</td>
<td>Protaglandin E1 (PGE1)</td>
<td>Anti-aggregation, vasodilation, reduced inflammation, promotes red cell flexibility</td>
</tr>
</tbody>
</table>

2.5 RESEARCH HYPOTHESIS

2.5.1 INDUSTRY PROBLEM

The high rate of growth of the broiler chicken has resulted in a bird with a very high requirement for muscle protein synthesis. However, associated with the faster growth rate has been an increase in fatness, which is a problem for all modern broiler
strains. In contrast, fast growing meat animals are leaner and a major question to be addressed by the broiler industry is why are broiler chickens fat?

2.5.2 BROILER ENERGY METABOLISM

Energy yielding substrates absorbed following digestion are metabolised by the liver to supply glucose and fatty acids. Of the three major dietary requirements, protein is considered the most important as it is able to act as a source of body protein, carbohydrate and fat. Dietary protein is broken down in the gut where the products of proteolysis are absorbed in the small intestine in the form of amino acids or small peptides (Mathews, 1975). Natural foodstuffs contain a mixture of a large number of different proteins, however, diets made up of plant proteins have limiting amounts of methionine, lysine and tryptophan (Stevens, 1996).

Essential amino acids are those required to maintain a growing animal in positive nitrogen balance, or an adult in nitrogen equilibrium. The essential amino acid requirements for maintenance of nitrogen balance in adult chickens, are arginine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine and histidine, whereas, glycine is necessary for growing chicks to maintain maximum growth rate (Almquist, 1952). Because of the rapid growth rate of broiler chicks, any deficiencies in amino acid requirements are likely to be more pronounced resulting in reduced growth rates and producing carcasses with higher fat content (Stevens, 1996).

In most mammals, the energy for muscle protein synthesis is largely supplied by glucose. Insulin facilitates cellular uptake of glucose and promotes a number of other cellular events including regulation of ion and amino acid transport, protein synthesis and lipid metabolism (Cheatham and Kahn, 1995). As outlined in chapter 1, birds, when compared to mammals, are relatively insensitive to the actions of insulin (see review; Simon, 1988). Avian muscle and adipose tissue have a limited capacity for de novo lipogenesis and most of the lipid accumulating in adipose or muscle tissue is derived from the diet or synthesised in the liver and transported as triglyceride (Griffin, 1993). There is a strong negative relationship between muscle triglyceride content and insulin-stimulated glucose uptake (Storlien et al., 1991). So, while broilers are comparatively less sensitive to insulin, glucose uptake would be further compromised.
Hepatic secretion of triglyceride and lipoprotein in broilers is about twice that for layer chicks (Griffin et al., 1991). Circulating triglyceride levels are therefore about 50% higher in broilers than layer chicks and coupled with this is an increased rate of triglyceride incorporation into adipose tissue. There is little mobilisation of adipose tissue triglyceride in broilers and this suggests that triglyceride production in the liver is greater than is needed to meet energy demands. It also suggests that food intake is not well balanced with the energy needs of the bird. Insulin sensitivity of peripheral tissues, glucose uptake and insulin levels are all monitored by the central nervous system (CNS) and function as one of the many signals that regulate satiety in mammals (see review; Schwartz et al., 1992). Disruption of the CNS through reduced insulin sensitivity may also be another factor that is associated with the highly complex regulatory mechanisms that influence food intake in broilers.

2.5.3 ALTERATION OF BROILER METABOLISM: MECHANISTIC APPROACH

As outlined above in the literature review, modulation of insulin sensitivity can be achieved by manipulating the lipid composition of the plasma membrane. A change in the dietary fat alters the nature of the membrane structural lipid components, thereby modifying the regulatory interface between circulating hormones and hormone-activated receptor effector systems (Clandinin et al., 1991). Events such as microaggregation of receptors, internalisation of the insulin-receptor complex and movement of glucose transporters to and from the membrane surface can all be affected by changes in the membrane composition.

Triglycerides are an important energy source for broilers but any mechanism that alters energy substrate use could reduce fatty acid synthesis. Increasing the sensitivity of muscle tissue to insulin would increase the muscle use of glucose and decrease dependence on triglyceride. Decreased need for triglyceride production by the liver would lower circulating levels and consequently the triglyceride available for deposition in adipose tissue. A reduced demand for acetyl-CoA or lipogenesis would also decrease catabolism of precursor substrates including the amino acids (Saunderson, 1988). This would increase amino acid availability for protein synthesis. An increase in insulin
sensitivity would also stimulate amino acid uptake and protein synthesis resulting in greater energy utilisation and would also reduce fat deposition.

Increasing muscle insulin sensitivity in broilers would result in:

1) A decreased reliance on liver lipogenesis to meet the energy demand for protein synthesis and would in turn decrease triglyceride production resulting in less being available for deposition in adipose tissue.
2) An enhanced rate of amino acid uptake into muscle cells with increased availability of amino acids for protein synthesis.
3) Restoration of the functional integrity of the CNS in the regulation of food intake. Food intake would therefore be more closely aligned to the energy requirements of growth.

2.6. RESEARCH OBJECTIVES

1) To examine the relationship between dietary n-3 and n-6 fatty acid composition on growth of broiler chickens.

2) To examine the relationship between dietary n-3 and n-6 fatty acid composition on insulin action in broiler chickens.

3) To examine the effects of dietary n-3 and n-6 fatty acids on pituitary and adrenal sensitivity in broiler chickens.

4) To characterise the molecular species of the choline and ethanolamine phospholipids in breast muscle of broilers fed n-3 and n-6 fatty acids.
CHAPTER 3

MATERIALS AND METHODS

3.1. EXPERIMENTAL DIETS

3.2. SURGICAL PROCEDURES
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3.3. LIPID ANALYSIS

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   3.4.2. Normal rabbit serum
   3.4.3. Normal guinea pig serum
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   3.5.2. Buffer B
   3.5.3. Buffer C
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   3.5.5. Reagent preparation
   3.5.6. Reaction procedure

3.6. INSULIN RIA
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   3.6.2. Buffer 2 ‘Insulin Diluent’ 0.05M PO₄ + 0.05M EDTA + BSA
   3.6.3. Primary antibody
   3.6.4. Insulin standard
   3.6.5. Assay procedure
   3.6.6. Assay parameters

3.7. GROWTH HORMONE RIA
   3.7.1. Buffer 3 0.01M PO₄ + BSA
   3.7.2. Buffer 4
3.1 EXPERIMENTAL DIETS

The diets were prepared by adding either 8.0 g/100g of edible grade tallow containing less than 1% free fatty acids, sunflower oil, or fish oil to the standard diet (Table 3.1). The fatty acid composition of the lipid supplements and for the three experimental diets is described in Chapter 4 and shown in Table 4.1 and Table 4.2 respectively. The diets were prepared weekly. Feed ingredients and diets were kept at room temperature with the exception of fish oil, which was maintained at 4°C. The fish oil was stabilised with an antioxidant by the manufacturer and the vitamin/mineral premix contained ethoxyquin.
### TABLE 3.1. *Composition (g/kg) of the experimental diets*

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Fish oil</th>
<th>Sunflower oil</th>
<th>Tallow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>566</td>
<td>566</td>
<td>566</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>318</td>
<td>318</td>
<td>318</td>
</tr>
<tr>
<td>Fish oil</td>
<td>80</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>-</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td>Tallow</td>
<td>-</td>
<td>-</td>
<td>80</td>
</tr>
<tr>
<td>Limestone</td>
<td>11.6</td>
<td>11.6</td>
<td>11.6</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>14.3</td>
<td>14.3</td>
<td>14.3</td>
</tr>
<tr>
<td>Salt</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Vitamin-trace mineral premix*</td>
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<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
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</table>

**Determined composition (g/kg)**

<table>
<thead>
<tr>
<th></th>
<th>Fish oil</th>
<th>Sunflower oil</th>
<th>Tallow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>202</td>
<td>209</td>
<td>221</td>
</tr>
<tr>
<td>Metabolisable energy (MJ/kg)</td>
<td>14.0</td>
<td>14.2</td>
<td>13.7</td>
</tr>
<tr>
<td>Total fat (g/kg)</td>
<td>111</td>
<td>107</td>
<td>111</td>
</tr>
</tbody>
</table>

* Supplied per kilogram diet: *trans*-retinol, 3.3 mg; cholecalciferol, 87.5 µg; dl-α-tocopheryl acetate, 20 mg; menadione, 2 mg; thiamine, 1.5 mg; riboflavin, 8 mg; calcium pantothenate 15 mg; niacin, 30 mg; pyridoxine, 5 mg; folic acid, 2 mg; cyanocobalamin, 15 g; biotin, 100 µg; Mn, 75 mg; Zn, 50 mg; Cu, 5 mg; Mo, 1.6 mg; Co, 300 µg; I, 1 mg; Fe, 20 mg; Se, 100 µg; choline chloride, 300 mg; ethoxyquin, 125 mg.

### 3.2. SURGICAL PROCEDURES

#### 3.2.1. Jugular vein catheterisation

Chickens, fasted for 24 h prior to the surgical procedure, were anaesthetised with 2-4% isoflurane (Abbott Laboratories, Kurnell, NSW Australia) in oxygen. The gases were delivered using a Flurotec 3 vaporiser at 30 mL/min and the isoflurane concentration regulated according to the reflex responses of the individual chicken. The
chickens were maintained throughout the surgical procedure at stage 3 (depression of respiratory, circulatory, protective reflexes and muscle tone) and plane 2 (defined as the point where eyeballs remain eccentrically fixed) of anaesthesia (Hall and Clarke, 1983). Feathers were removed from the surgical area and the skin disinfected with 70% ethanol and then 30% iodine solution. An incision, (5-cm) was made parallel to the trachea and the connective tissues separated by blunt dissection to expose the vein, which was then separated from the adjacent tissues. The vein was then cut, the catheter (clear vinyl tubing; ID 1.0 mm, O.D. 1.5 mm, Critchely Electrical Products Ltd, Parramatta, NSW) inserted with the opening towards the head and exteriorised through the skin between where the wings attach to the thoracic cavity. The catheter was secured in a canvas pouch (90 mm x 90 mm) attached to the back of the chicken between the wings. The pouch material and the lacing were sufficiently flexible to allow the bird unrestricted movement.

After surgery the birds were placed in single pens in a recovery room maintained at 32-35°C. Post-operative care consisted of a daily intramuscular injection of a mixture of penicillin 250 mg/ml and streptomycin 250 mg/mL at a dose rate of 0.1 mL/kg body weight for 3 days. In addition, the birds were also injected intramuscularly with the analgesic carprofen (Zenecarp, Heriot Ag Vet Pty Ltd, Rowville, Vic) at a concentration of 2 mg/kg body weight for 7 days. Water and feed were offered within 3 h after surgery and the chickens carefully monitored until feed intake returned to pre-operative levels usually within 24 h.

3.3 LIPID ANALYSIS

The methodology for the extraction and analysis of lipids from muscle and adipose tissue is fully described in Chapter 4. The methodology for the determination of the molecular species profile in breast muscle is fully described in Chapter 7.

3.4. PROTEIN ASSAYS

REAGENTS

All chemicals used in buffer preparations were of analytical grade and were dissolved in reverse osmosis water unless specified otherwise.
3.4.1. Stripped broiler plasma

Hormone free plasma was used in the preparation of standards for all hormone assays. It was prepared by adding Norit A charcoal (AJAX Chemicals, Auburn Australia) to pooled broiler plasma at a concentration of 10 mg/mL. The mixture was incubated at room temperature and stirred constantly for 5 h. This procedure was repeated 3 times. The solution was then centrifuged (Beckman, USA, J6-MI) at 5,000 g, 4°C for 15 min, the plasma was then removed and stored frozen until required.

3.4.2. Normal rabbit serum (NRS)

Normal rabbit serum was obtained from the ear vein of mature New Zealand White rabbits by withdrawing 5 mL of whole blood using a 25g needle (0.50 x 19mm, Terumo Pty Ltd, Melbourne Australia). Blood was allowed to clot at room temperature then held at 4°C for 10 hrs. The serum was collected and then centrifuged (Beckman USA Model J6-MI) at 5,000 g, 4°C for 30 min. The supernatant was removed and stored frozen until required.

3.4.3. Normal guinea pig serum (NGPS)

Normal guinea pig serum was obtained from mature guinea pigs by withdrawing 5 mL whole blood from the heart using 21 g needle 0.8 x 38mm. Prior to heart puncture, animals were anaesthetised with Anaesthetic Ether (Hoechst Limited Victoria Australia). The whole blood was processed and stored in a similar manner to that for the NRS.

3.4.4. Donkey Anti-Rabbit Serum (DARS) or Donkey Anti-Guinea Pig Serum (DAGPS)

To obtain the DARS or DAGPS a primary immunisation of 1 mg of rabbit gamma globulin was dissolved in 2.5 mL saline and this was emulsified with 2.5 mL Freunds Complete Adjuvant (Sigma Chemical Co. USA). At 4 weeks post primary, the donkeys were given a booster immunisation using a similar regime, however, the Freunds Complete Adjuvant was replaced with Freunds Incomplete Adjuvant. One week after the boost, blood was withdrawn from the jugular vein of the treated donkeys using a 13G needle (Solila Pty Ltd, Melbourne, Australia). Blood was allowed to clot at room
temperature and then kept at 4°C overnight before serum was harvested. Serum was centrifuged (Beckman, USA Model J2-21M) at 5000 g, 4°C for 30 min and the supernatant collected and stored at 4°C until required.

3.5. PROTEIN IODINATION

BUFFERS

Phosphate buffers for iodination

3.5.1. Buffer A
A 0.05M PO₄ buffer was prepared by dissolving 0.74 g NaH₂PO₄ 2H₂O and 2.68 g Na₂H PO₄ (anhydrous) into 500 mL of distilled water.

3.5.2. Buffer B
A 0.05M PO₄ + 0.25% BSA buffer was prepared by adding 1.25 g BSA, Fraction V (Sigma Chemical Co USA) to 500 mL 0.05M PO₄ buffer to give a concentration of 0.25% weight/volume.

3.5.3. Buffer C
A 0.25M PO₄ buffer was prepared by dissolving 1.48 g NaH₂PO₄ 2H₂O and 5.36 g Na₂H PO₄ (anhydrous) into 100 mL of distilled water.

3.5.4. Preparation of Sephadex column for protein and free label separation
Five g of Sephadex (Amrad Pharmacia, Melbourne, Aust.) was rehydrated in 100 mL of distilled water. The Sephadex solution was heated in a boiling water bath for 6 h and then allowed to cool to room temperature. The Sephadex solution was de-gassed before loading onto a glass column, (1.5 cm x 30cm) and then left to stand for 2 days to allow column to “bed down”. The column was equilibrated by gravity flow with 0.05M PO₄, (Buffer A) before use. A column containing Sephadex G 50 was used to separate
labelled growth hormone from free radioactive iodine and Sephadex G 100 was used to separate labelled insulin and luteinising hormone from free radioactive iodine.

3.5.5. Reagent preparation

Protein for iodination was dissolved in Buffer C to give a concentration of $1 \mu g/\mu L$. Chloramine T was used as an oxidising agent at a concentration of 1 mg/mL of Buffer A. Sodium Metabisulphide was used to stop the reaction and was used at a concentration of $0.2 \mu g/\mu L$ of Buffer A. Potassium Iodide was used as the eluent at a concentration of 1 mg/mL of Buffer B.

3.5.6. Reaction procedure

The iodination procedure was based on that reported by Greenwood and co-workers (1963). And was carried out in accordance with the University of Sydney’s health and safety guidelines.

To a glass reaction vial, containing 5 µg of protein was added 5 µL $^{125}\text{I}$ containing approximately 18 MBq. To this was added 5 µL of Chloramine T and this was allowed to react for 30-45 sec’s. To stop the reaction, 10 µL of Sodium Metabisulphide solution was added and this was followed by the addition of 0.2 ml Potassium Iodide solution. The mixture was then transferred onto the separation column and then eluted with Buffer B. The eluent was collected as fractions the sizes of which were determined by following the separation of labelled protein on a radiometric pen recorder. The desired fractions were then stoppered and kept frozen at -20°C until required.

3.6. INSULIN RIA

Plasma concentrations of insulin were determined in duplicate as described below using the double-antibody RIA method of Dr F. Thomas, CRC for Tissue Growth and Repair, Adelaide.
Assay reagents

Buffers

3.6.1. Buffer 1 ‘Insulin Buffer’ 0.05M PO₄ + 0.05M EDTA
This buffer was made by dissolving the following reagents in 1 litre distilled water.

- 9.0 g of Sodium Chloride (NaCl)
- 1.19 g of Sodium Dihydrogen Orthophosphate (NaH₂PO₄ 2H₂O)
- 4.63 g of Di-Sodium Hydrogen Orthophosphate (Na₂HPO₄2H₂O)
- 18.2 g Ethylenediaminetetra-acetic acid di-sodium salt (EDTA)

The pH was adjusted to 7.4 with 10N Sodium Hydroxidesolution.

3.6.2. Buffer 2 ‘Insulin Diluent’ 0.05M PO₄ + 0.05M EDTA + BSA
This buffer was made by dissolving 1.0 g Bovine Serum Albumin (BSA, Fraction V, Sigma Chemical Co., St. Louis, Missouri, USA) in Buffer 1.

3.6.3. Primary antibody
The antisera used in the assay was raised in a guinea pig and was the gift of Dr F. Thomas, CRC for Tissue Growth and Repair, Adelaide. The antiserum was found to have the following cross-reactivity’s:

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Percentage Cross-reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken insulin</td>
<td>100</td>
</tr>
<tr>
<td>Ovine insulin</td>
<td>23</td>
</tr>
<tr>
<td>Equine insulin</td>
<td>18</td>
</tr>
<tr>
<td>Pro-insulin</td>
<td>32</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>0</td>
</tr>
<tr>
<td>Chicken GH</td>
<td>0</td>
</tr>
<tr>
<td>Prolactin</td>
<td>0</td>
</tr>
</tbody>
</table>
3.6.4. Insulin standard

The insulin standards were prepared from purified chicken insulin (Litron Laboratories, USA). Initially the standards were made up to a concentration of 20 ng/mL in Buffer 1 and stored in aliquots at -20°C. When needed, 1 mL of the stock was serially diluted in Buffer 1 down to a concentration of 0.022 ng/mL.

3.6.5. Assay procedure

All samples were assayed in 10 mm x 75 mm P4 plastic tubes (John Morris Ltd., Sydney, Aust.). The samples and the quality controls were assayed in duplicate and the insulin standards were assayed in triplicate.

On day 1, 100 µL of sample was diluted with 200 µL of Buffer 1 using a mechanical diluter (Digiflex, ICN Biomedicals, Inc). The standard curve was prepared by adding 100 µL of the insulin standard, 100 µL of the hormone stripped plasma and then diluting this with 100 µL of Buffer 1. The insulin antiserum was made up to a working dilution of 1:10,000 in Buffer 1, which contained NGPS (1:100). A 50 µL volume of antiserum solution was added to each assay tube then vortexed and left to incubate at 4°C for 24 h. On the second day, insulin tracer was diluted in Buffer 2 and 50 µL containing 10,000 cpm was added to each tube before vortexing. The assay tubes were further incubated at 4°C for 48 h. On the fifth day DAGPS was diluted to a working dilution of 1:5 with Buffer 1. A volume of 100 µL of this mixture was added to each tube before being vortexed and then incubated 4°C for 18 h. On the following day, the assay tubes were centrifuged (Beckman USA) at 1,500 g for 45 min. The supernatant from each tube was removed and the pellet was then counted on a gamma counter (Gammamaster, LKB Wallac, Turku, Finland). The insulin concentrations of unknowns were determined from the level of bound label (cpm) in the unknown and comparing this with the level of bound label in the standard concentrations. This was carried out using a specific software (RiaCalc, Wallac Oy, Pharmacia, Finland) computer program. A standard curve was computed by plotting, log of the concentration versus B/Bo where B equals the amount of bound label in the standard and Bo equals the maximum amount of label bound by the antiserum at the dilution used in the assay. The software was programmed to calculate a spline smoothed standard curve. The spline function attempts to fit the ideal equation to
each part of the curve and then the different parts are joined in a smooth and continuous manner.

### 3.6.6. Assay parameters

For all assays the sensitivity is defined as the minimum amount of hormone that can be reliably detected within the assay. The accepted method of estimating the sensitivity is to take two standard deviations of the assay zero and compute this as concentration in the assay. The coefficient of variation was estimated for low, medium and high concentration recovery. The recoveries were always assayed in triplicate and therefore the variation between replicates could be estimated to give the intra-assay coefficient of variance. Inter-assay variation was determined by calculating the coefficient of variation for the triplicate mean of each recovery of each assay. The coefficient of variation was calculated as (Steel and Torrie, 1980);

\[
\frac{\text{sample standard deviation}}{\text{sample mean}} \times 100
\]

**Assay parameters**

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (n=4): 0.02 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coefficients of variation (%)</td>
<td>Intra</td>
</tr>
<tr>
<td>Low level (0.32 ng/mL)</td>
<td>8.9</td>
</tr>
<tr>
<td>Medium level (1.25 ng/mL)</td>
<td>7.4</td>
</tr>
<tr>
<td>High level (7.5 ng/mL)</td>
<td>10.6</td>
</tr>
</tbody>
</table>
3.7. GROWTH HORMONE RIA

Plasma concentrations of GH were determined by the double-antibody RIA assay method of Downing et al. (1995) as outlined below.

ASSAY REAGENTS

Buffers

3.7.1 Buffer 3 0.01M PO₄ + BSA

This buffer was made up by dissolving the following reagents in 1 litre distilled water

- 0.13 g Sodium Dihydrogen Orthophosphate (NaH₂PO₄ 2H₂O)
- 1.3 g Di-Sodium Hydrogen Orthophosphate (Na₂HPO₄2H₂O)
- 9.0 g Sodium Chloride (NaCl)
- 1.0 g Sodium Azide (NaN₃)
- 1.0 g Bovine Serum Albumin (BSA, Fraction V, Sigma Chemical Co., St. Louis, Missouri, USA).

The pH of the buffer was adjusted to 7.4 with 4N Hydrochloric acid solution.

3.7.2. Buffer 4

This was made by dissolving 18.62 g of Ethylenediaminetetra-acetic acid di-sodium salt in Buffer 3 and adjusting the pH to 7.4 with 10N sodium hydroxide.

3.7.3. Primary antibody

The antisera (AFP-551-11-86) used in the GH assay was raised in a rabbit and was supplied by the National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, California, USA. The levels of cross reactivity with chicken hormones are given below (details supplied with the antiserum);

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Percentage Cross-Reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken GH (AFP-9020C)</td>
<td>100</td>
</tr>
<tr>
<td>Chicken prolactin (AFP-10328)</td>
<td>0.75</td>
</tr>
</tbody>
</table>
The antisera was diluted in Buffer 3 to give a concentration of 1:5000 and stored in aliquots of 100-200 µL at -20°C. When required, sufficient antiserum was diluted in Buffer 3 to give an initial dilution of 1:80,000.

3.7.4. Growth hormone standard

The standard reference preparation used was AFP-9020C and obtained from the National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, California, USA.

3.7.5. Radioiodinated growth hormone

The chicken growth hormone used in the preparation of GH label was AFP-7678B supplied by National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, California, USA. For use in the assay the label was diluted in Buffer 4 to give 12,000 cpm-15,000 cpm in 50 µL

3.7.6. Assay procedure

Using an automatic diluter (Digiflex, ICN Biomedicals, Inc), 5-50 µL of the standard, internal standards or unknown plasma was added to 10 x 75 mm plastic tube and diluted with 300-345 µL of Buffer 3. All samples were assayed in triplicate while five replicates of the internal standards were assayed each time. On day one, 50 µL of the antiserum dilution containing NRS (1:600) was added to each tube then vortexed. The assay was incubated at 4°C overnight before adding 50 µL of 125I-chicken GH label and vortexing again. The assay was incubated for 2 days at 4°C and then 50 µL of DARS was added to each tube before vortexing. The assay was left overnight at 4°C and then the tubes were centrifuged (Beckman J6-Mi) at 1900-x g for 60 min. The supernatant from each tube was then aspirated and the level of bound label in the precipitate was determined on a gamma counter (LKB-Wallac 1277, Gammamaster automatic gamma counter; Wallac Oy, Turku, Finland). The hormone concentrations for the unknowns were determined as previously described for the insulin assay.
3.7.7. Assay parameters

Sensitivity (n=4): 0.34 ng/mL

Coefficients of variation (%)

<table>
<thead>
<tr>
<th>Level</th>
<th>Intra</th>
<th>Inter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low level (2.91 ng/mL)</td>
<td>8.7</td>
<td>9.8</td>
</tr>
<tr>
<td>Medium level (6.9 ng/mL)</td>
<td>5.4</td>
<td>8.9</td>
</tr>
<tr>
<td>High level (19.5 ng/mL)</td>
<td>9.6</td>
<td>11.1</td>
</tr>
</tbody>
</table>

3.8. LUTEINIZING HORMONE RIA

Plasma concentrations of LH were determined by the double-antibody RIA assay method of Downing et al. (1995).

ASSAY REAGENTS

3.8.1. Primary antibody

The antisera (USDA-AcLH-5) used in the GH assay was raised in a rabbit and was supplied by the National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, California, USA. The levels of cross reactivity with chicken hormones are given below (details supplied with the antiserum);

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Percentage Cross-Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken LH</td>
<td>100</td>
</tr>
<tr>
<td>Chicken FSH</td>
<td>&lt; 3</td>
</tr>
<tr>
<td>Turkey PRL</td>
<td>&lt; 9</td>
</tr>
<tr>
<td>Chicken GH</td>
<td>&lt; 0.9</td>
</tr>
<tr>
<td>Recombinant chicken GH</td>
<td>&lt; 0.9</td>
</tr>
</tbody>
</table>

3.8.2. Luteinizing hormone standard

The standard reference preparation used was USDA-cLH-K-3 and obtained from the National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, California, USA.
3.8.3. Radioiodinated luteinizing hormone

The chicken LH used in the preparation of LH label was USDA-cLH-I-3 supplied by National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, California, USA. For use in the assay the label was diluted in Buffer 4 to give 12,000 cpm-15,000 cpm in 50 µL

3.8.4. Assay procedure

The LH RIA protocol was identical as described for the GH assay.

3.8.5. Assay parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sensitivity (n=4): 0.2 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficients of variation (%)</td>
</tr>
<tr>
<td>Low level (0.625 ng/mL)</td>
<td>9.5</td>
</tr>
<tr>
<td>Medium level (1.25 ng/mL)</td>
<td>8.6</td>
</tr>
<tr>
<td>High level (2.5 ng/mL)</td>
<td>5.8</td>
</tr>
</tbody>
</table>

3.9 STEROID ASSAYS

ASSAY REAGENTS

Buffers

3.8.1. Buffer 5

This buffer was made up by dissolving the following reagents in 1 litre distilled water:

- 4.33 g Sodium Dihydrogen Orthophosphate (NaH2PO4 2H2O)
- 3.0 g Di-Sodium Hydrogen Orthophosphate (Na2HPO42H2O)
- 9.0 g Sodium Chloride (NaCl)
- 1.0 g Sodium Azide (NaN3)
- 1.0 g Gelatine

The solution was heated to dissolve the gelatine and the pH of the solution was then adjusted to 7.6 with 4M NaOH.
3.9.2. Dextran coated charcoal

The dextran-coated charcoal was prepared by dissolving 6.25 g of Norit A charcoal and 625 mg Dextran T 70 (Amrad Pharmacia, Melbourne, Aust.) in 250 mL of distilled water. The solution was stored at 4°C and was freshly prepared every three weeks.

3.10. CORTICOSTERONE RIA

Plasma concentrations of corticosterone were determined in duplicate using the method described ICN Biomedicals, Inc, page 1304, 1997 (California, USA).

3.10.1. Primary antibody

The corticosterone antibody used in this assay was raised in a rabbit and was obtained commercially from ICN Biomedicals, Inc (California, USA) Cat. No: 61-362, Lot No: 86370. The levels of cross reactivity with other steroids are given below (details supplied with the antiserum);

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Percentage Cross-Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androsterone</td>
<td>0.6</td>
</tr>
<tr>
<td>11-Desoxytocorticosterone</td>
<td>26.0</td>
</tr>
<tr>
<td>Progesterone</td>
<td>20.0</td>
</tr>
<tr>
<td>Cortisol</td>
<td>13.5</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>6.0</td>
</tr>
<tr>
<td>20α-Hydroxyprogesterone</td>
<td>11.3</td>
</tr>
<tr>
<td>Testosterone</td>
<td>4.0</td>
</tr>
<tr>
<td>11-Desoxycortisol</td>
<td>7.4</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>3.7</td>
</tr>
<tr>
<td>20β- Hydroxyprogesterone</td>
<td>7.7</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>0.6</td>
</tr>
<tr>
<td>Cortisone</td>
<td>0.86</td>
</tr>
<tr>
<td>5-α-Dihydrotestosterone</td>
<td>1.2</td>
</tr>
<tr>
<td>Estradiol-17B</td>
<td>0.25</td>
</tr>
</tbody>
</table>
### Steroid Percentage Cross-Reactivity

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Percentage Cross-Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrone</td>
<td>0.25</td>
</tr>
<tr>
<td>Estriol</td>
<td>0.18</td>
</tr>
<tr>
<td>17-Hydroxyprogesterone</td>
<td>6.4</td>
</tr>
</tbody>
</table>

3.10.2. Corticosterone standard  

The corticosterone standard was prepared by dissolving corticosterone (Cat. No: c-2505 Sigma, Australia) in ethanol to give a concentration of 1033.88 ng/mL. The prepared standard was further diluted in Buffer 5 to give a concentration of 103.388 ng/ml.

3.10.3. Assay procedure  

All assays were carried out in 12 mm x 75 mm ‘Borex’ glass tubes (Crown Scientific, NSW, Australia). The samples and the internal standards were assayed in duplicate and the standards for the standard curve were assayed in triplicate.

On day one, 100µL of sample was diluted with 300µL of Buffer 5. The standard curve was prepared by adding 100µL of the corticosterone standard with 100µL of the hormone stripped plasma and 200µL of Buffer 5. The samples and standards were extracted with 2 mL of diethyl/ether then shaken vigorously on a mechanical shaker for 10 min. The extracted standards and samples were then poured off into 12 mm x 75 mm ‘Borex’ glass tubes and placed into a heating block before being evaporated to dryness under N₂. After evaporation, 100µL of Buffer 5 was added to each tube and left to incubate for 30 min. The corticosterone antisera was diluted with Buffer 5 to give a dilution of 1:25 and 100µL of this solution was added to the assay tubes which were then vortexed. The corticosterone tracer, 1,26,7-³H corticosterone, (Code No: TRK 406, Amersham Pty Ltd., Sydney, Aust.) was diluted with Buffer 5 to give a count of 10,000cpm in 100µL. A 100µL aliquot of the tracer was added to all tubes before vortexing and then incubated overnight at 4°C. A 250µL volume of the dextran-coated charcoal was added to each tube before vortexing. The tubes were incubated for 15 min at 4°C before centrifuging at 1,500 g for 15 min. The supernatant was dispensed into miniature polyethylene vials (Packard Instrument Co, Meriden, USA) and 3 mL of the
scintillation cocktail added (Optiphase ‘Hisafe 3’, Wallac, Fisher Chemical Co, Leicestershire, England). The vials were then vortexed and counted (LKB-Wallac 1219-Rackbeta, Wallac Oy, Turku, Finland). A specific RiaCalc program was used to determine the corticosterone concentrations for the unknown samples.

3.10.4. Assay parameters

Sensitivity (n=2): 0.20 ng/mL

<table>
<thead>
<tr>
<th>Coefficients of variation (%)</th>
<th>Intra</th>
<th>Inter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low level (0.8 ng/mL)</td>
<td>9.7</td>
<td>11.8</td>
</tr>
<tr>
<td>Medium level (6.8 ng/mL)</td>
<td>7.3</td>
<td>9.9</td>
</tr>
<tr>
<td>High level (20.1 ng/mL)</td>
<td>9.6</td>
<td>10.1</td>
</tr>
</tbody>
</table>
CHAPTER 4

DIETARY POLYUNSATURATED FATTY ACIDS OF THE N-3 AND THE N-6 SERIES REDUCE ABDOMINAL FAT DEPOSITION AND INFLUENCE LIPID METABOLISM

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<th>Pages</th>
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<td></td>
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<td></td>
</tr>
<tr>
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<tr>
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<td>81</td>
</tr>
<tr>
<td>EXPERIMENT 2</td>
<td></td>
</tr>
<tr>
<td>4.3.6. Voluntary food intake, initial body weight, weight gain, feed/gain, abdominal fat pad mass, breast muscle mass</td>
<td>83</td>
</tr>
</tbody>
</table>
4.1. INTRODUCTION

Nutritional studies in humans and rats have shown that energy balance and body fat content can be manipulated by altering the dietary polyunsaturated to saturated fatty acid (P/S) ratio, specifically by inclusion of long chain polyunsaturated fatty acids (PUFA’s) (Field et al., 1990; Pan et al., 1994; Luo et al., 1996; Couet et al., 1997). An increase in the dietary P/S ratio by the inclusion of n-3 fatty acids has direct effects on glucose and lipid metabolism and decreases body fat mass. Storlien et al. (1991) and Luo and co-workers (1996) showed that a diet containing 30% fish oil reduced circulating insulin concentrations and improved insulin-stimulated glucose uptake when compared with diets containing equal amounts of vegetable or animal oils. Consumption of the fish oil diet also reduced the epididymal fat pad mass without any change in body weight. Similar metabolic and body compositional changes in response to the inclusion of n-3 PUFA’s have also been reported for man. When a diet enriched with fish oil was fed to young adult men and women, basal blood glucose concentrations increased and basal insulin concentrations decreased and there was a significant reduction in overall body fat mass (Couet et al., 1997). Associated with this reduced fat mass, these workers also reported an increase in the resting metabolic rate and a decrease in the basal respiratory quotient (RQ), thus indicating an increase in lipid oxidation.

Birds generally have a high capacity for lipid biosynthesis and the liver is the principal site of synthesis (Pearce, 1980; Klasing, 1998). The modern broiler or meat chicken has the propensity to become excessively fat and this is significant as carcass fat of these birds is an important source of dietary fat for humans. Much recent research has examined whether it is possible to select chicken genotypes that have a low tendency to accumulate triglyceride (Griffin, 1993) and to what extent the composition of carcass triglycerides can be modified by changes in dietary lipid intake (Leskanich and Noble, 1997). The aim of many of the latter studies has been to enhance the human dietary
intake of long chain polyunsaturates. Despite the many studies that have manipulated the n-3 and n-6 fatty acid content of carcass triglycerides, few have examined the effects of PUFA’s on avian metabolism. The present study examined a range of morphological and physiological responses of broiler chickens to different sources of dietary fatty acids; fish oil (n-3 PUFA’s), sunflower oil (n-6 PUFA’s) and edible tallow (mixture of saturated, monounsaturated and polyunsaturated fatty acids).

4.2. MATERIALS AND METHODS

EXPERIMENT 1

4.2.1. Animals and experimental diets

Twenty-four 4-week old broiler chickens of mixed sex (Inghams strain TM70) were divided into 3 groups (n=8) and placed into individual cages with free access to water and fed the experimental diets for 4 weeks. Fresh feed was offered at intervals of 2-3 days following the collection of feed residues from the previous period. Estimates of weekly feed intake were made by subtracting the total weekly residue weight from the total weight of feed offered for that week. At the end of week 4, a blood sample (5 ml) was collected by venipuncture from the jugular vein using heparinized syringes (Terumo, Japan). The chickens were then killed by cervical dislocation and the abdominal fat pad removed and weighed. To determine fatty acid incorporation into tissues a sample of the abdominal fat pad was taken from each bird and stored at −70°C until required. Blood samples were kept on ice until centrifugation within 1 h of collection and the harvested plasma then stored at −20°C until assayed.

4.2.2. Determination of apparent metabolisable energy

Apparent metabolisable energy was determined for the three experimental diets from the differences in feed energy intake and excreta energy output. Clean metal trays were placed beneath each cage during week three of the dietary treatment. Twenty-four hours later, feather and scurf contamination were removed by the use of forced air and the excreta collected into separate metal trays and placed in a forced draught oven (60°C) for 24 h. This procedure was repeated for three days. Oven-dried excreta was allowed to
equilibrate with atmospheric moisture for three days, weighed then ground. Representative samples of excreta and feed were stored in airtight containers prior to analysis. Gross energy determination was measured on excreta and diet samples using an automatic adiabatic bomb calorimeter (Gallenkamp and Co. Ltd., London) that had been standardised using benzoic acid (Mollah et al., 1983).

4.2.3. Determination of plasma insulin and metabolites

Plasma insulin concentrations were determined by radioimmunoassay procedures and have been described previously in Chapter 3. Plasma glucose, triglyceride, total cholesterol, and phospholipid measurements were determined with commercially available kits (Glucose Unimate 5 Gluc HK; Triglycerides Unimate 5 Trig:, Australia, Phospholipids bioMérieux, Marcy-l’Etoile, France) using a Roche Cobas Mira automatic analyser.

**EXPERIMENT 2**

4.2.4. Animals and experimental diets

Thirty-five mixed male and female broiler chicks (Inghams strain TM70) obtained from a commercial hatchery were placed in a temperature-controlled battery brooder with raised wire floors. The chicks were exposed to constant light illumination (24L:0D) that consisted of six fluorescent tubes (40-43 W) positioned 2.3 m from the floor. All chicks were given free access to a commercial starter diet and water for a period of three weeks. At the end of this period, 30 of these chicks were randomly divided into 3 groups (n=10) and placed in individual cages with free access to water and fed the experimental diets for 5 weeks. The dietary rations and the estimation of voluntary food intake (VFI) were identical to that described for Expt. 1. During weeks 4 and 5 the resting RQ and metabolic rate were determined for 5 birds from each group. At the end of week 5, a blood sample (5ml) was collected by venipuncture from the jugular vein of each bird using heparinized syringes. The chickens were euthanased by cervical dislocation and the abdominal fat pad and breast muscles removed and weighed. Muscle and fat samples were taken from each bird for lipid analysis and stored at −70°C until required. Blood
samples were kept on ice until centrifugation within 1 h of collection, and the harvested plasma then stored at −20 °C until assayed.

4.2.5. Determination of the resting respiratory quotient and metabolic rate

Oxygen consumption (\(V_{O2}\)) and carbon dioxide production (\(V_{CO2}\)) rates were evaluated using an open-system respirometer chamber, by measuring oxygen and CO\(_2\) content of inlet and outlet air using a Sable Systems FC-1 oxygen and CA-1 CO\(_2\) analysers. The chambers consisted of rectangular plastic box fitted over a standard broiler chicken holding cage (645 x 355 x 615 mm) with a transparent top and front panel, to permit illumination and opaque sides to minimise interaction between adjacent birds. Birds were provided with free access to food and water throughout the measurement period which took place between 0800 and 1700h in a lighted room maintained at 25ºC. Air was provided to each box at 3.5 litres/min using calibrated rotameters. After a 2-h adjustment period, chambers were selectively sampled for air composition using a Sable Systems Respirometer Multiplexer (V 2.0). Voltage output from the oxygen and CO\(_2\) analysers were recorded at 5-second intervals and each bird was sampled for 11 minutes per hour. Reported values for \(V_{O2}\) consumption and \(V_{CO2}\) have been corrected to standard temperature and pressure (STP) conditions after appropriate adjustment for volume effects associated with respiratory quotients different to unity (Withers 1977). Resting rates of oxygen consumption (RMR) for each bird represent the mean of the 2 lowest 10-minute averages of oxygen uptake recorded during two separate sampling periods during the 8-hour measurement period (Buttemer et al., 1991). Respiratory quotients (\(V_{CO2}/V_{O2}\)) are based on 8-h averages of \(V_{CO2}\) and \(V_{O2}\).

4.2.6. Determination of water and fat content of the breast muscle

Water and fat determinations were analysed on tissue samples taken from eight of the ten birds from each dietary treatment. Tissues were allowed to thaw to RT, weighed, and dissected into 1 mm pieces before placing into cellulose extraction thimbles (single thickness, 28 mm internal diameter x 80 mm length, Whatman, Cat No 2800288) that had been equilibrated to 60ºC. To determine the percentage of water, the muscle tissue was incubated at 60ºC for 24 h, weighed and further incubated at 60ºC until tissue weights
had stabilised. Muscle samples were extracted with petroleum spirit (Unilab Ajax Chemicals, Sydney Australia) for 24 h using Soxhlet extraction then re-weighed to determine the percentage of fat in each sample.

4.2.7. Extraction and analysis of lipids

Total lipids were extracted from 10 g of muscle tissue with chloroform: methanol (2:1 v/v) containing 0.01% butylated hydroxy toluene using procedures described previously by Ashes et al. (1992) and Folch et al (1957). Fatty acid methyl esters were prepared on an aliquot taken from the extracts using the toluene / sulphuric acid procedure as described by Christie (1989). This procedure was also used to prepare fatty acid methyl esters from 50-100 mg of abdominal fat tissue as well as 500 mg of diet samples and individual dietary fat additives. Individual fatty acids were separated and quantified by gas chromatography (Perkin Elmer Autosystem, FID and PE Nelson data system, model 10202), fitted with a BPX 70 Capillary column (50 mm x 0.25 mm) (SGE Australia Pty Ltd). Helium was used as the carrier gas with an injection split ratio of 100:1. The GLC was temperature programmed from 150° C to 210° C at a rate of 2°C per minute with an injection temperature of 210° C and a detector temperature of 250° C. Peaks separated were identified by comparison with standard samples of known composition.

4.2.8. Determination of plasma metabolites

Plasma cholesterol measurements was analysed with a commercially available kit (Cholesterol Unimate 5 Chol, Roche Products Pty. Ltd., NSW, Australia) and colorimetrically determined using a Roche Cobas Mira automatic analyser. Plasma glucose and triglycerides were colorimetrically determined using the enzymic assay kits as described for Expt 1. Plasma long-chain, non-esterified fatty acids (NEFA) were determined by enzymic analysis using reagents and methods as supplied by Boehringer Mannheim, Germany.
4.2.9. Statistical analysis

All data are presented as the mean ± the standard error of the mean. Statistical examination of treatment effects was determined by ANOVA and the Turkey-Kramer multi comparison test. For voluntary food intake, repeated measures analysis of variance was used to analyse for the effects of treatment, time and their interaction (CLR ANOVA program; Clear Lake Research Inc., Houston TX, USA).

4.3. RESULTS

EXPERIMENT 1

As there were no significant differences between male and female birds in the results for any of the parameters measured, the data for the two sexes was pooled.

4.3.1. Fatty acid composition for the dietary lipid supplements

The fatty acid profile for the dietary lipid additives is given in Table 4.1. As would be expected, the fish oil contained a higher proportion of n-3 PUFA’s compared with either the sunflower oil or tallow. The principal n-3 FA’s for this lipid were eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). These two FA’s were not present for either the sunflower oil or tallow additives. In contrast, the predominant FA’s for the sunflower oil were linoleic acid (LA; 18:2n-6) and oleic acid (18:1n-9). Whereas, tallow consisted of a mixture of saturated (palmitic acid; 16:0), monounsaturated (oleic acid; 18:1) and PUFA’s (LA 18:2n-6).

4.3.2. Fatty acid composition for the experimental diets

The fatty acid profile for the dietary treatments are given in Table 4.2. The fish oil (n-3 PUFA’s) and the sunflower oil (n-6 PUFA’s) diets contained significantly less saturated (palmitic C16:0; stearic C18:0) and monounsaturated (oleic C18:1c9) fatty acids than the tallow diet (P<0.01). In contrast, the sunflower oil diet contained a significantly higher (P<0.01) proportion of LA (18:2n-6) when compared with either the fish oil or the tallow diets. The fish oil diet contained the n-3 fatty acids, EPA (20:5n-3) and DHA (22:6n-3), and these fatty acids were not detected in either the sunflower or the
tallow diets. As expected, the sunflower oil diet and fish oil diets had higher P/S ratios than the tallow diet (4.95, 1.59 and 0.84 respectively).

**TABLE 4.1**

*Fatty acid composition of the dietary lipid additives*

<table>
<thead>
<tr>
<th>Fatty Acid (%w/w)</th>
<th>Fish Oil</th>
<th>Sunflower Oil</th>
<th>Tallow</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>7.85</td>
<td>_</td>
<td>1.46</td>
</tr>
<tr>
<td>C16:0</td>
<td>18.34</td>
<td>4.69</td>
<td>27.96</td>
</tr>
<tr>
<td>C16:1</td>
<td>9.66</td>
<td>_</td>
<td>1.38</td>
</tr>
<tr>
<td>C18:0</td>
<td>3.79</td>
<td>5.50</td>
<td>8.20</td>
</tr>
<tr>
<td>C18:1</td>
<td>14.03</td>
<td>42.02</td>
<td>39.96</td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>1.75</td>
<td>45.66</td>
<td>14.62</td>
</tr>
<tr>
<td>C18:3n-3</td>
<td>1.05</td>
<td>0.32</td>
<td>1.04</td>
</tr>
<tr>
<td>C20:0</td>
<td>3.64</td>
<td>0.40</td>
<td>0.42</td>
</tr>
<tr>
<td>C20:1</td>
<td>1.74</td>
<td>0.20</td>
<td>0.33</td>
</tr>
<tr>
<td>C20:5n-3</td>
<td>16.69</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>C22:5n-3</td>
<td>1.69</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>C22:6n-3</td>
<td>12.36</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>∑ Sat</td>
<td>33.6</td>
<td>10.6</td>
<td>38.0</td>
</tr>
<tr>
<td>∑ Monounsat</td>
<td>25.4</td>
<td>42.3</td>
<td>41.7</td>
</tr>
<tr>
<td>∑ Poly n-6</td>
<td>1.8</td>
<td>45.7</td>
<td>14.6</td>
</tr>
<tr>
<td>∑ Poly n-3</td>
<td>30.1</td>
<td>0.32</td>
<td>1.0</td>
</tr>
</tbody>
</table>

_not detectable_

Trace components of fatty acids not included
### TABLE 4.2

*Fatty acid composition of the experimental diets*

<table>
<thead>
<tr>
<th>Fatty Acid (%w/w)</th>
<th>Fish Oil</th>
<th>Sunflower oil</th>
<th>Tallow</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>5.17</td>
<td>_</td>
<td>1.04</td>
</tr>
<tr>
<td>C16:0</td>
<td>16.03</td>
<td>8.55</td>
<td>25.24</td>
</tr>
<tr>
<td>C16:1</td>
<td>6.03</td>
<td>_</td>
<td>1.12</td>
</tr>
<tr>
<td>C18:0</td>
<td>3.43</td>
<td>4.31</td>
<td>6.06</td>
</tr>
<tr>
<td>C18:1e9</td>
<td>14.07</td>
<td>23.27</td>
<td>38.03</td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>19.77</td>
<td>62.73</td>
<td>25.80</td>
</tr>
<tr>
<td>C18:3n-3</td>
<td>1.81</td>
<td>1.13</td>
<td>1.49</td>
</tr>
<tr>
<td>C20:0</td>
<td>2.94</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>C20:5n-3</td>
<td>13.41</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>C22:6n-3</td>
<td>8.77</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>∑ Sat</td>
<td>27.6</td>
<td>12.9</td>
<td>32.3</td>
</tr>
<tr>
<td>∑ Monounsat</td>
<td>20.1</td>
<td>23.3</td>
<td>39.2</td>
</tr>
<tr>
<td>∑ Poly n-6</td>
<td>19.8</td>
<td>62.7</td>
<td>25.8</td>
</tr>
<tr>
<td>∑ Poly n-3</td>
<td>23.9</td>
<td>1.1</td>
<td>1.5</td>
</tr>
<tr>
<td>*P/S</td>
<td>1.59</td>
<td>4.95</td>
<td>0.84</td>
</tr>
</tbody>
</table>

_not detectable_

*P/S  \( P = n-6 + n-3 / S = C14:0 + C16:0 + C18:0 + C20:0 \)

#### 4.3.3. Gross energy (GE), apparent metabolisable energy (AME), voluntary food intake (VFI), initial body weight, weight gain, feed/gain, fat pad mass

Although the GE for the three experimental diets was similar, the AME for the birds fed the tallow diet was somewhat lower when compared with the chickens fed the sunflower and fish oil diets (Table 4.3). The chickens fed the tallow diet tended to
consume more feed, although this was not significant, compared with the sunflower and fish oil dietary groups. The initial body weight for the three dietary groups did not differ significantly, however, there was a significant increase (p<0.05) in the weight gain for the chickens fed the tallow diet compared with either fish or sunflower oil feeding. The higher weight gain for the tallow fed chickens was reflected in a significantly lower (P<0.05) feed/gain ratio compared with the fish oil and sunflower oil dietary groups. This lower feed/gain ratio for the tallow group equated to an improvement of 4.9% when compared to fish oil feeding and 6.5% when compared to those fed sunflower oil. Regardless of this improvement, the chickens fed the fish and sunflower oil diets had significantly smaller (p<0.01) abdominal fat pad masses compared with the tallow fed chickens.

**TABLE 4.3**

*Gross energy (GE), apparent metabolisable energy (AME), intakes, initial body weight, feed/gain, and fat pad mass in chickens fed fish oil (n-3 PUFA’s), or sunflower oil (n-6 PUFA’s) or tallow supplements. Values are mean ± SEM.*

<table>
<thead>
<tr>
<th></th>
<th>Fish Oil</th>
<th>Sunflower Oil</th>
<th>Tallow</th>
</tr>
</thead>
<tbody>
<tr>
<td>GE, MJ/kg</td>
<td>17.4</td>
<td>17.6</td>
<td>17.6</td>
</tr>
<tr>
<td>AME, MJ/kg</td>
<td>14.0</td>
<td>14.2</td>
<td>13.7</td>
</tr>
<tr>
<td>Diet intake, grams</td>
<td>4509 ± 289</td>
<td>4731 ± 429</td>
<td>5042 ± 214</td>
</tr>
<tr>
<td>Initial body wt, grams</td>
<td>1333 ± 60</td>
<td>1284 ± 51</td>
<td>1325 ± 20</td>
</tr>
<tr>
<td>Weight gain, grams</td>
<td>2125 ± 76 a</td>
<td>2194 ± 168 a</td>
<td>2495 ± 57 b</td>
</tr>
<tr>
<td>Feed/Gain</td>
<td>2.12 ± 0.04 a</td>
<td>2.16 ± 0.03 a</td>
<td>2.02 ± 0.03 b</td>
</tr>
<tr>
<td>Fat Pad, grams</td>
<td>69.3 ± 11.4 a</td>
<td>63.9 ± 4.9 a</td>
<td>119.4 ± 11.6 b</td>
</tr>
</tbody>
</table>

Values with different superscripts are significantly different when compared by ANOVA.
4.3.4. Plasma insulin and metabolites

The plasma triglyceride concentrations were significantly lower (p<0.05) in the birds fed the sunflower and fish oil diets compared with the tallow fed group (Table 4.4). The plasma phospholipids reflected the triglyceride levels, being lower for the sunflower oil fed chickens and significantly reduced (p<0.05) for the fish oil group compared with the tallow fed chickens. There were no significant differences for the dietary treatments for either plasma glucose or insulin concentrations, although plasma insulin levels tended to be lower for the fish and sunflower oil fed chickens compared with those fed tallow.

**TABLE 4.4**

*Plasma concentrations of triglycerides, phospholipids, glucose and insulin in chickens fed fish oil (n-3 PUFA’s), or sunflower oil (n-6 PUFA’s) or tallow supplements. Values are means ± SEM*

<table>
<thead>
<tr>
<th></th>
<th>Fish Oil</th>
<th>Sunflower Oil</th>
<th>Tallow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides, mmol/L</td>
<td>0.51 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.48 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.10 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phospholipids, mmol/L</td>
<td>2.08 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.64 ± 0.27&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.19 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>14.08 ± 0.42</td>
<td>15.58 ± 0.48</td>
<td>14.88 ± 0.37</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.045 ± 0.011</td>
<td>0.046 ± 0.021</td>
<td>0.052 ± 0.020</td>
</tr>
</tbody>
</table>

Values with different superscripts are significantly different when compared by ANOVA.
4.3.5. Fatty acid content of the abdominal fat pad

The fatty acid types found in the abdominal fat pads reflected the dietary fatty acid profile (Table 4.5). The fatty acids EPA (20:5n-3) and DHA (22:6n-3) were only observed in the fat pads of the chickens fed the fish oil diet (n-3 PUFA’s). Feeding sunflower oil (n-6 PUFA’s) significantly reduced (p<0.01) the proportion of saturated (C16:0) and monounsaturated (C16:1, C18:1) fatty acids and significantly increased (p<0.01) the proportion of LA (18:2n-6) compared with the fish oil or tallow fed birds. The P/S ratio of the abdominal fat pad for the tallow (0.47) fed birds was lower when compared with the chickens fed the fish oil (0.64) diet, while consistent with the dietary fatty acid values, the P/S ratio was higher in the chickens fed sunflower oil (1.69).
<table>
<thead>
<tr>
<th>Fatty Acid (% w/w)</th>
<th>Fish Oil</th>
<th>Sunflower Oil</th>
<th>Tallow</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>3.26 ± 0.13</td>
<td>0.97 ± 0.0</td>
<td>1.01 ± 0.15</td>
</tr>
<tr>
<td>C16:0</td>
<td>24.10 ± 0.72</td>
<td>16.60 ± 0.26</td>
<td>25.55 ± 0.43</td>
</tr>
<tr>
<td>C16:1c</td>
<td>7.67 ± 0.46</td>
<td>2.87 ± 0.28</td>
<td>5.35 ± 0.48</td>
</tr>
<tr>
<td>C18:0</td>
<td>6.72 ± 0.35</td>
<td>5.28 ± 0.23</td>
<td>5.87 ± 0.11</td>
</tr>
<tr>
<td>C18:1c9</td>
<td>30.79 ± 1.02</td>
<td>33.69 ± 0.69</td>
<td>44.93 ± 0.86</td>
</tr>
<tr>
<td>C18:1c7</td>
<td>2.63 ± 0.06</td>
<td>1.24 ± 0.08</td>
<td>1.89 ± 0.07</td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>12.55 ± 0.51</td>
<td>38.00 ± 2.35</td>
<td>14.47 ± 0.80</td>
</tr>
<tr>
<td>C18:3n-3</td>
<td>1.08 ± 0.0</td>
<td>0.71 ± 0.0</td>
<td>0.76 ± 0.0</td>
</tr>
<tr>
<td>C20:0</td>
<td>1.36 ± 0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20:5n-3</td>
<td>5.39 ± 0.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C22:6n-3</td>
<td>3.51 ± 0.26</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

∑ Sat          35.4  22.9  32.4
∑ Monunsat     41.1  37.8  52.2
∑ Poly n-6     12.6  38.0  14.5
∑ Poly n-3     9.9   0.7   0.8
*P/S            0.64  1.69  0.47

_ not detectable amount of substance (mol%)  
*P/S    P = n-6 + n-3 / S = C14:0 + C16:0 + C18:0 + C20:0

Composition expressed as mean (mol %) ± SEM for analysis of abdominal fat samples from four fish oil, sunflower oil and tallow supplemented chickens. Values with different superscripts are significantly different when compared by ANOVA.
EXPERIMENT 2

As there were no significant differences between male and female birds in the results for any of the parameters measured, the data for the two sexes was pooled.

4.3.6. Voluntary food intake (VFI), initial body weight, weight gain, gain/feed, abdominal fat pad mass, breast muscle mass.

Although there was no significant differences in VFI for the three treatments, the chickens fed the tallow diet tended to consume more feed compared with either the sunflower oil or fish oil fed chickens (Table 4.6). The weight gain of the birds given the 3 dietary treatments was not different. However, the feed/gain ratio for the fish oil dietary group was lower and was significantly lower (P<0.05) for the sunflower oil dietary group compared with those fed tallow. Despite this, the birds fed sunflower and fish oil diets had significantly smaller abdominal fat pad masses when compared with the chickens consuming the tallow diet (P<0.01). Birds fed the fish and sunflower oils tended to have the largest breast muscle mass however, these values did not differ significantly between the dietary treatments. While fish and sunflower oil feeding resulted in a significant increase (p<0.05) in the water content of the breast muscle there was no significant difference in the fat content of this muscle type, although the fat content tended to be higher for the tallow fed chickens.

4.3.7. Fatty acid content of the abdominal fat pad

The fatty acid profile and their proportions observed in the abdominal fat pads were similar to those for Expt 1 and reflected the dietary fatty acid profile (Table 4.7). As was shown in Expt 1, the n-3 fatty acids EPA (20:5n-3) and DHA (22:6n-3) were only observed in the fat pads of the chickens fed the fish oil diet. The rates of incorporation for these two fatty acids were similar, as the ratio of EPA (20:5n-3) and DHA (22:6n-3) for the fat pad resembled the ratio of the diet, the percentage of incorporation being 4.7% and 4.4% respectively. Feeding fish oil or the sunflower oil diets significantly reduced (P<0.01) the proportion of palmitic (16:0) and oleic acids (18:1) compared with tallow feeding. In contrast, the fat pads from the chickens fed the sunflower oil diet had a significantly higher (P<0.01) proportion of LA (18:2n-6) compared with either fish oil or
tallow feeding. The P/S ratio of the abdominal fat pad for the three dietary groups was similar, although somewhat higher than observed for Expt 1. The P/S ratio for the chickens fed tallow (0.51) was lower than those fed fish oil (0.81), while this ratio was higher in the chickens fed the sunflower oil diet (2.17).

**TABLE 4.6**

*Intakes, initial body weight, weight gain, gain/feed, fat pad mass breast muscle mass and breast muscle water and fat concentrations in chickens fed fish oil (n-3 PUFA’s), or sunflower oil (n-6 PUFA’s) or tallow supplements. Values are means ± SEM*

<table>
<thead>
<tr>
<th></th>
<th>Fish oil</th>
<th>Sunflower oil</th>
<th>Tallow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet intake, grams</td>
<td>5212 ± 213</td>
<td>5225 ± 218</td>
<td>5531 ± 175</td>
</tr>
<tr>
<td>Initial body wt, grams</td>
<td>584 ± 26</td>
<td>582 ± 31</td>
<td>608 ± 20</td>
</tr>
<tr>
<td>Weight gain, grams</td>
<td>2714 ± 121</td>
<td>2808 ± 151</td>
<td>2712 ± 129</td>
</tr>
<tr>
<td>Feed/Gain</td>
<td>1.92 ± 0.04&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.87 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.05 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fat pad, grams</td>
<td>45.3 ± 7.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.2 ± 8.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>107.4 ± 10.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Breast muscle, grams</td>
<td>572 ± 20</td>
<td>585 ± 30</td>
<td>542 ± 28</td>
</tr>
<tr>
<td>Breast muscle water, %</td>
<td>24.3 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.1 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.1 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Breast muscle fat, %</td>
<td>0.63 ± 0.02</td>
<td>0.72 ± 0.13</td>
<td>0.88 ± 0.28</td>
</tr>
</tbody>
</table>

Values with different superscripts are significantly different when compared by ANOVA.

**4.3.8. Fatty acid composition of the breast muscle**

The fatty acid profiles in muscle for the three experimental dietary treatments (Table 4.8) showed many similarities to the abdominal fat pad profiles. However, unlike the abdominal fat pad, fatty acids were identified which were not present in the diet. The most notable change was the presence of arachidonic acid, (AA; 20:4n-6), presumably synthesised from LA (18:2n-6) within the muscle tissue. The proportion of AA (20:4n-6)
for the sunflower oil diet group was not significantly different compared with the tallow fed birds, however, the proportion of this fatty acid was significantly lower (P<0.01) for the fish oil group. The high concentration of LA (18:2n-6) in the sunflower oil diet was reflected by its significantly greater incorporation into muscle compared with the other dietary groups (P<0.01). The ratio of EPA (20:5n-3) and DHA (22:6n-3) in the muscle of the chickens fed the fish oil was the inverse of both the diet and the abdominal fat pad. The P/S ratio for the muscle was similar for that observed for the abdominal fat pad being lower for tallow fed chickens (0.65) compared with either the fish oil (0.94) or sunflower oil (1.51) fed birds.

**4.3.9. Plasma insulin, metabolites, metabolic rate and respiratory quotient (RQ)**

Values for plasma insulin, metabolites, metabolic rate and the RQ for the 3 dietary treatments are shown in Table 4.9. The plasma triglyceride concentrations tended to be lower in the birds fed the sunflower and fish oil diets than the tallow fed group with the value being significantly lower (P<0.05) for the fish oil group than for the tallow fed group. Plasma NEFA concentrations were also elevated, although not significantly, for the chickens fed the fish oil and sunflower oil diets. Fish oil fed chickens had significantly lower (P<0.05) total plasma cholesterol concentrations when compared with those fed either sunflower or tallow. Plasma glucose concentrations did not differ between the three dietary groups however plasma insulin concentrations tended to be lower for birds fed the fish oil and the sunflower oil diets. Birds fed the sunflower oil or the fish oil diets tended to have higher metabolic rates than those fed tallow. In contrast, the RQ for the chickens fed fish oil or sunflower oil tended to be lower than that of birds fed tallow.
### TABLE 4.7

*Fatty acid composition of the abdominal fat pad in chickens fed fish oil (n-3 PUFA’s) or sunflower oil (n-6 PUFA’s) or tallow supplements*

<table>
<thead>
<tr>
<th>Fatty Acid (%w/w)</th>
<th>Fish oil</th>
<th>Sunflower oil</th>
<th>Tallow</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>3.74 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.47 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.94 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C16:0</td>
<td>19.86 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.75 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.02 ± 0.26&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C16:1c</td>
<td>7.53 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.83 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.46 ± 0.18&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:0</td>
<td>5.63 ± 0.18</td>
<td>6.26 ± 0.36</td>
<td>5.57 ± 0.11</td>
</tr>
<tr>
<td>C18:1c9</td>
<td>21.75 ± 0.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.13 ± 0.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43.64 ± 0.26&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:1c7</td>
<td>2.94 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.59 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.33 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>12.38 ± 0.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.04 ± 0.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.80 ± 0.28&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:3n-3</td>
<td>1.15 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.58 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.77 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:0</td>
<td>1.87 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.15 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.22 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:1</td>
<td>0.94 ± 0.01</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C20:5n-3</td>
<td>7.31 ± 0.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C22:6n-3</td>
<td>4.42 ± 0.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>∑ Sat</td>
<td>31.1</td>
<td>20.6</td>
<td>30.8</td>
</tr>
<tr>
<td>∑ Monounsat</td>
<td>33.2</td>
<td>31.6</td>
<td>49.4</td>
</tr>
<tr>
<td>∑ Poly n-6</td>
<td>12.4</td>
<td>44.0</td>
<td>14.8</td>
</tr>
<tr>
<td>∑ Poly n-3</td>
<td>12.9</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>*P/S</td>
<td>0.81</td>
<td>2.17</td>
<td>0.51</td>
</tr>
</tbody>
</table>

_ not detectable amount of substance (mol%)_

*P/S = P = n-6 + n-3 / S = C14:0 + C16:0 + C18:0 + C20:0

Composition expressed as mean (mol %) ± SEM for analysis of abdominal fat samples from eight fish oil, sunflower oil and tallow supplemented chickens. Values with different superscripts are significantly different when compared by ANOVA.
### TABLE 4.8

**Fatty acid composition of the total fatty acids in breast muscle in chickens fed fish oil (n-3 PUFA’s), or sunflower oil (n-6 PUFA’s) or tallow supplements**

<table>
<thead>
<tr>
<th>Fatty Acid (%w/w)</th>
<th>Fish Oil</th>
<th>Sunflower Oil</th>
<th>Tallow</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>$2.78 \pm 0.30^a$</td>
<td>_</td>
<td>$0.82 \pm 0.1^b$</td>
</tr>
<tr>
<td>C16:0</td>
<td>$20.79 \pm 0.20^a$</td>
<td>$15.91 \pm 0.50^b$</td>
<td>$23.36 \pm 0.21^c$</td>
</tr>
<tr>
<td>C16:1c</td>
<td>$4.37 \pm 0.14^a$</td>
<td>$1.05 \pm 0.1^b$</td>
<td>$2.58 \pm 0.24^c$</td>
</tr>
<tr>
<td>C18:0</td>
<td>$9.92 \pm 0.5$</td>
<td>$10.41 \pm 0.5$</td>
<td>$9.25 \pm 0.53$</td>
</tr>
<tr>
<td>C18:1 c9</td>
<td>$16.65 \pm 0.39^a$</td>
<td>$20.47 \pm 0.94^a$</td>
<td>$31.89 \pm 2.01^b$</td>
</tr>
<tr>
<td>C18:1 w7</td>
<td>$3.13 \pm 0.13^a$</td>
<td>$1.73 \pm 0.12^b$</td>
<td>$2.59 \pm 0.17^a$</td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>$10.1 \pm 0.49^a$</td>
<td>$31.29 \pm 1.81^b$</td>
<td>$14.84 \pm 0.40^c$</td>
</tr>
<tr>
<td>C18:3n-3</td>
<td>$0.93 \pm 0.12$</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>C20:0</td>
<td>$1.35 \pm 0.10$</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>C20:4n-6</td>
<td>$2.14 \pm 0.20^a$</td>
<td>$8.51 \pm 0.81^b$</td>
<td>$6.05 \pm 0.89^b$</td>
</tr>
<tr>
<td>C20:5n-3</td>
<td>$7.21 \pm 0.32$</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>C24:0</td>
<td>_</td>
<td>$1.66 \pm 0.37$</td>
<td>$1.22 \pm 0.31$</td>
</tr>
<tr>
<td>C24:1</td>
<td>$3.22 \pm 0.18^a$</td>
<td>$1.24 \pm 0.24^b$</td>
<td>$0.91 \pm 0.14^b$</td>
</tr>
<tr>
<td>C22:6n-3</td>
<td>$12.29 \pm 0.9^a$</td>
<td>$2.30 \pm 0.4^b$</td>
<td>$1.60 \pm 0.26^b$</td>
</tr>
</tbody>
</table>

| $\sum$ Sat | 34.8 | 27.9 | 34.7 |
| $\sum$ Monounsat | 27.4 | 24.5 | 37.9 |
| $\sum$ Poly n-6 | 12.2 | 39.8 | 20.9 |
| $\sum$ Poly n-3 | 20.4 | 2.3 | 1.6 |
| P/S            | 0.94 | 1.51 | 0.65 |

*P/S \quad P = n-6 + n-3 / S = C14:0 + C16:0 + C18:0 + C20:0 + C24:0

Composition expressed as mean (mol %) ± SEM for analysis of breast muscle samples from eight fish oil, sunflower oil and tallow supplemented chickens. Values with different superscripts are significantly different when compared by ANOVA.
**TABLE 4.9**

*Plasma concentrations of triglycerides, NEFA’s, cholesterol, insulin, glucose, RQ and metabolic rate of chickens fed fish oil (n-3 PUFA’s), or sunflower oil (n-6 PUFA’s) or tallow supplements. Values are means ± SEM*

<table>
<thead>
<tr>
<th></th>
<th>Fish oil</th>
<th>Sunflower oil</th>
<th>Tallow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides, mmol/L</td>
<td>0.24 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.28 ± 0.03&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.42 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NEFA’s, mmol/L</td>
<td>0.20 ± 0.04</td>
<td>0.15 ± 0.02</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>2.40 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.77 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.81 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>13.7 ± 0.3</td>
<td>13.9 ± 0.4</td>
<td>13.4 ± 0.3</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.031 ± .002</td>
<td>0.033 ± .010</td>
<td>0.043 ± .020</td>
</tr>
<tr>
<td>RQ</td>
<td>0.89 ± 0.04</td>
<td>0.84 ± 0.02</td>
<td>0.94 ± 0.02</td>
</tr>
<tr>
<td>Metabolic Rate</td>
<td>1.019 ± 0.035</td>
<td>1.084 ± 0.087</td>
<td>0.938 ± 0.038</td>
</tr>
</tbody>
</table>

mlO₂/g body mass/h

Values with different superscripts are significantly different when compared by ANOVA.

**4.4. DISCUSSION**

The major finding of this study was that broilers fed diets containing 80g/kg of either fish oil (n-3 PUFA’s) or sunflower oil (n-6 PUFA’s) had significantly smaller abdominal fat pad mass than birds fed tallow, a more saturated fat. When calculated on a percentage basis, the fat pad mass observed in experiment 1 made up 1.8% of the final body weight for the two PUFA dietary groups compared with 2.9% for the chickens fed tallow. Similarly, the fat pad mass observed in experiment 2, although somewhat lower, made up 1.1% and 0.8% of the body weight for the fish oil and the sunflower oil dietary groups, respectively, compared with 2.7% for the tallow diet. In this experiment, the lower fat pad mass occurred without affecting the final body mass with an improved feed/gain ratio. These findings are consistent with broiler genotypes that have been selected for leanness which also show an improved feed efficiency (Cahaner, 1988;
and genotypes selected for improved feed efficiency that have lower body fat content (Leenstra, 1988; Jørgensen et al., 1990).

In Expt 1, the weight gain for the chickens fed the tallow diet was significantly higher than the two PUFA dietary groups. The discrepancy between these two experiments is difficult to reconcile. Although the birds used in both experiments were obtained from the same commercial source, the chickens used in Expt 1 were biologically older compared with their counterparts used in experiment 2. In addition, the birds in Expt 1 were fed the experimental diets for a shorter period of time, 4 instead of 5 weeks. Therefore, it is conceivable that this discrepancy between the two experiments may simply be the result of using biologically older birds resulting in less growth and more nutrients directed towards lipid deposition, or a difference in the time of exposure to these experimental diets or the interaction of both factors.

The abdominal fat pad mass has been shown to be highly correlated with the total fat content of both the carcass and the edible meats of chickens (Akiba et al., 1995). The significant reduction in the abdominal fat pad mass for the broilers fed the fish and sunflower oil diets presumably reflects a lower total body fat content. All three diets contained the same dietary fat content, demonstrating that the type of dietary fat and not just the quantity of fat ingested influences body fat deposition.

In Expt 2, the decrease in the fat pad mass for the two PUFA dietary groups was associated with an increase in lipid oxidation, suggesting an increase in the mobilisation and/or oxidation of lipids (Halminski et al., 1991; Raclot and Groscolas, 1993). This is supported by the lower resting RQ observed for the chickens fed fish oil or sunflower oil compared with those fed the tallow diet. The change in lipid partitioning observed when feeding dietary PUFA’s has also been reported for human subjects where the dietary inclusion of n-3 PUFA’s for three weeks, reduced body fat mass and increased basal lipid oxidation (Couet et al., 1997). Similar results have also been obtained with rodents (Hill et al., 1993; Su and Jones, 1993). These mammalian studies suggest that the dietary fatty acid composition can modulate the partitioning of fat between oxidation and storage.

The changes in metabolic rate and fat balance seen following fish oil and sunflower oil feeding may not be primarily due to a change in the P/S ratio. It may also be a function of the percentage of monounsaturated fatty acids fed or the feeding of
specific fatty acid sub-types or a combination of both. When the proportion of monounsaturated fatty acids are considered, the abdominal fat pad and breast muscle tissue from the birds fed the tallow diet contained a significantly higher percentage of monounsaturated fatty acids compared with the birds fed fish oil or the sunflower oil. The higher proportion of monounsaturated fatty acids in the tallow diet may explain these differences. This observation is consistent with the study of Doucet and co-workers (1998), who have shown that the waist circumference of humans increased in individuals with a higher total dietary saturated and monounsaturated fat but not PUFA content in their diets.

The reduced triglyceride concentrations and enhanced NEFA concentrations observed for the n-3 and n-6 PUFA dietary groups are concordant with animals that are metabolising lipid (Newman et al., 1998). The lower plasma triglyceride concentrations observed in broilers fed fish oil are consistent with other studies of chickens (Akiba et al., 1995; Phetteplace and Watkins 1990; Leskanich and Noble, 1997) and rats (Chait et al., 1974; Shimomura et al., 1990; Okuno et al., 1997). Current studies demonstrate that reduced circulating triglyceride concentrations are not specific to the n-3 PUFA’s alone as birds fed sunflower oil also had reduced plasma triglyceride concentrations, a result consistent with studies in humans (Weintraub et al., 1988). The lower plasma insulin concentrations observed for both PUFA groups could also facilitate lipid oxidation in view of the hormone’s lipogenic effects. Reduced plasma insulin concentrations also occur in humans (Couet et al., 1997) and rats (Fickova et al., 1998) fed fish oil. Although the mechanisms responsible for this are unknown, it is possible that there is a reduced insulin secretion (Fickova et al., 1998) or perhaps a higher rate of receptor-mediated hormone clearance. It may also be that the lower insulin concentrations for the two PUFA groups simply reflects a decreased requirement for glucose as the principal energy substrate in view of the increased oxidation of fat for these two dietary groups.

The increase in lipid oxidation observed in the chickens fed the fish oil and sunflower oil diets may also be a response to the action of specific fatty acids to stimulate enzymes of the β-oxidative pathway. Fish oil feeding enhances carnitine palmitoyltransferase-1 (CPT1) activity in rats (Berge et al., 1988), mice (Borgeson et al., 1989) and Syrian hamsters (Surette et al., 1992). Fish oil diets have also been shown to
decrease the sensitivity of CPT1 to malonyl-CoA inhibition in liver mitochondria (Wong et al., 1984). Therefore, an increase in CPT1 activity and/or reduction in its malonyl-CoA inhibition would render fatty acids more available for the β-oxidative pathway. Support for preferential oxidation of n-3 and n-6 PUFA’s over saturated fatty acids has been demonstrated by Leyton (1987). In Leyton’s (1987) study the n-3 were oxidised for energy in higher proportions than the n-6 PUFA, which in turn were used for energy more readily than saturated fats that were preferentially stored. The preference for fatty acids to be either oxidised or stored was found to be dependent on their chain length and the degree of saturation.

The reduction in the abdominal fat pad mass seen in the fish oil and sunflower oil dietary groups could be more than just a shift in energy substrate oxidation. Polyunsaturated fatty acids act as regulators of genes controlling the expression of enzymes involved in fatty acid synthesis and oxidation (Cheema and Clandinin, 1996; Clarke et al., 1997). Fatty acid synthetase, an important enzyme in the hepatic biosynthetic pathway that controls de novo fatty acid synthesis is inhibited by both n-3 and n-6 PUFA’s. In broiler chickens fed n-3 PUFA’s for 2 weeks, the activity of hepatic fatty acid synthetase was reduced (Akiba et al., 1995). The reduction in fat pad mass observed in chickens fed fish oil or sunflower oil may be due, in part, to inhibition of fatty acid synthetase activity by these dietary fats.

The higher resting metabolic rate of broilers fed the fish oil and sunflower oil diets may be a consequence of the greater proportion of metabolically active tissues in these treatment groups. In addition to the greater fat-free mass, these two groups also had a larger breast muscle mass compared with the chickens fed the tallow diet. Alternatively, the increased resting metabolic rate could be due to an increase in ion “leakage”, perhaps through the activation of Na⁺/K⁺ ATPase induced through an increase in the PUFA content of cell membranes (Else and Hulbert, 1987). These remain possibilities because fish oil and sunflower oil feeding increased levels of n-3 and n-6 PUFA’s in the breast muscle.

The fatty acid composition of both the breast muscle and the abdominal fat pad reflects the dietary fat profile and is consistent with other reports (Jen et al., 1971; Phetteplace and Watkins, 1989; O’Neil et al., 1998). In the present study, the feeding of
fish oil increased the proportion of the long chain n-3 PUFA’s, EPA (20:5n-3) and DHA (22:6n-3) and decreased the proportion of AA (20:4n-6). However, the ratio between EPA (20:5n-3) and DHA (22:6n-3) in the muscle was the inverse of that found in the diet and the fat pad suggesting a preferential incorporation of DHA (22:6n-3) into muscle tissue. Feeding diets enriched in sunflower oil increased the proportion of LA (18:2n-6) and AA (20:4n-6) whereas the feeding of diets enriched with saturated fatty acids increased the proportion of palmitic and oleic acids. The greatest percentage of AA (20:4n-6) was observed in the breast muscle of animals fed the sunflower oil. This result was not surprising as the biosynthesis of arachidonic acid in mammalian cells occurs through a sequence of alternating desaturation and chain-elongation reactions using linoleic acid as the initial substrate (Watkins, 1995).

Arachidonic acid, and EPA (20:5n-3) are two of the most important fatty acids associated with the membrane phospholipids and are the major precursors for the biologically active molecules the eicosanoids (Lands, 1992). The physiological actions of eicosanoids need to be considered in light of our results as the eicosanoids have major influences on the pulsatile release of the hypothalamic and pituitary hormones, including growth hormone (Ojeda et al., 1981). In view of the lipolytic nature of growth hormone, it is conceivable that an alteration in basal growth hormone secretion may have also contributed to the reduced abdominal fat pad mass observed for the fish oil and sunflower oil dietary treatments.

The use of fats and oils as a supplementary energy source in poultry diets is a common practise. The present investigation shows clearly the effects that changes in dietary fatty acid profile can exert on tissue fatty acid composition. Feeding n-3 and n-6 fatty acids resulted in a leaner bird with an accompanying improvement in feed conversion efficiency, both important criteria for economically sustainable animal production systems. These effects reflect changes in avian metabolism through the modulation of lipid deposition and oxidation by n-3 and n-6 fatty acids and concur with mammalian studies in rats and man. In addition, the incorporation of these fatty acids into the tissues would impact favourably on consumers.

In view of the positive relationship between obesity and insulin sensitivity that has been demonstrated in mammals (Dreon et al., 1988; Parker et al., 1993) a second
study was undertaken in broilers using these same three dietary treatments to determine their effects on avian glucose metabolism.
CHAPTER 5

DIETARY (N-3) POLYUNSATURATED FATTY ACIDS ALTER
GLUCOSE METABOLISM

5.1. INTRODUCTION

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GLUCOSE METABOLISM STUDIES

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5.2.3. 2-deoxy-D-3H glucose infusions

5.2.4. Determination of plasma hormones and metabolites

5.2.5. Determination of glucose concentration in tissues

5.2.6. Statistical analysis

5.3. RESULTS

INTRAVENOUS GLUCOSE TOLERANCE TEST

5.3.1. Plasma glucose response

5.3.2. Plasma insulin response

2-DEOXY-D-3H GLUCOSE INFUSIONS

5.3.3. 2-deoxy-d-3H glucose clearance rate from the plasma

5.3.4. Plasma insulin response

5.3.5. Tissue uptake of 2-deoxy-d-3H glucose

5.4. DISCUSSION

5.1. INTRODUCTION

Dietary fatty acid profile has been shown to be a potent modulator of insulin action. Diets high in saturated fats (a low P/S ratio) or with a high n-6/n-3 ratio of PUFA’s lead to insulin resistance in both skeletal muscle, the most important tissue for insulin-stimulated glucose uptake, and adipose tissue (Storlien et al., 1996, 1997). In skeletal muscle, alterations in dietary lipid profile lead to changes in the fatty acid composition of phospholipids, the main membrane structural lipid (Pan et al., 1994). In turn, changes in skeletal muscle phospholipid fatty acid composition are closely
associated with changes in insulin action (Field et al., 1990; Pan et al., 1995). The more saturated the fatty acid within the membrane phospholipid the greater the insulin resistance (Grunfeld et al., 1981; Field et al., 1988). Field and colleagues (1990) have shown that the polyunsaturated to saturated fatty acid (P/S) ratio of diets influences the rate of insulin stimulated glucose transport, glucose oxidation and lipogenesis. For rats consuming a low P/S diet, insulin action is muted whereas when fed a high P/S diet, the addition of insulin stimulates glucose transport and its oxidation.

While the P/S ratio of a diet may be instrumental in modulating whole body insulin action and glucose metabolism, it is also plausible that the individual fatty acid subtype is important. Ingestion of fatty acids from the n-3 series, particularly eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) have been shown in man to have beneficial health effects. For example, these fatty acids have been reported to decrease coronary artery disease (Kromhout et al., 1985). It has been suggested that this effect results from the ability of the n-3 fatty acids to lower plasma cholesterol and triglyceride concentrations (Dyerberg et al., 1975; Fasching et al., 1991). The primary sources of the long-chain n-3 fatty acid metabolites are marine products and include EPA (20:5n-3) and DHA (22:6n-3). Studies in animals (Storlien et al., 1987) and patients with non-insulin-dependent diabetes mellitus (NIDDM) (Popp-Snijders et al., 1987) used fish oil as a dietary supplement to improve insulin resistance, insulin sensitivity and intravenous glucose tolerance. Dietary fatty acids from the n-6 series have also shown to be successful in rodents improving both insulin binding and insulin action (Cheema et al., 1992; Field et al., 1990). However, the use of n-6 fatty acids for these purposes remains controversial. Studies carried out in man show clearly that the higher the n-6/n-3 ratio the poorer is insulin action (Storlien et al., 1995).

The role of dietary polyunsaturated fatty acids (PUFA’s) have in influencing glucose metabolism in mammals has received considerable attention but there is little information for avian species. A study in turkeys suggests that dietary fatty acids may indeed have similar effects as those observed in mammals. It was demonstrated by Gould and co-workers, (1982) that the activity of the insulin receptor in the erythrocyte membrane is influenced by the fatty acid composition of the membrane surrounding the
receptor. The greater the degree of unsaturated fatty acids contained within the membrane the greater the number of insulin binding sites.

In view of the scant information relating dietary fat profile to insulin action and glucose metabolism in birds, the present study was designed to examine if the inclusion of n-3 and n-6 PUFA’s in the diet could induce similar changes in glucose/insulin action to those reported for rodents and man. Accordingly, the effects of dietary fish oil (n-3 PUFA’s), sunflower oil (n-6 PUFA’s) or edible tallow on insulin action and glucose uptake in broiler chickens have been investigated.

5.2. MATERIALS AND METHODS

5.2.1. Animals and diets

Eighteen male broiler chicks (Inghams strain TM70), obtained from a commercial hatchery were placed in a temperature-controlled battery brooder with raised wire floors. The chicks had free access to a commercial starter diet and water for three weeks and were exposed to the same photoperiod regime as that described in experiment 2, Chapter 4. At the end of this period, the chicks were randomly divided into three groups (n=6) and housed as groups with free access to water and the experimental diets for six weeks. The three experimental diets were prepared as described in Chapter 3. Jugular catheterisation was performed under general anaesthesia as previously described in Chapter 3 during week 4 of the dietary treatments and the birds allowed 7 days post-surgery to recover. Tests to assess whole body insulin action and glucose metabolism were done during week 6 of the dietary treatments.

GLUCOSE METABOLISM STUDIES

5.2.2. Intravenous glucose tolerance test

After six weeks of feeding with the experimental diets and following an overnight fast, D-glucose (1 g/kg body weight) was administered via the jugular catheter to each bird. Sequential blood samples (1.0 mL) were obtained via the catheter before and at –10, 0, 5, 10, 20, 30, 40, 50 and 60 minutes after the glucose dosing.

5.2.3. 2-deoxy-D-3H glucose infusions
To estimate the disappearance rate of glucose and its incorporation into body tissues, a single dose of 2DG-\textsuperscript{3}H glucose (50 µCi) (Amersham Pharmacia Biotech, UK) made up in 0.5 ml sterile physiological saline was infused into each chicken two days after the intravenous glucose tolerance test. The line was then flushed with 0.5 ml sterile saline immediately following the 2DG-\textsuperscript{3}H glucose infusion to remove any trace of the isotope. Blood samples were obtained via the catheter at 3, 5, 10, 15, 20, 25, 30, 40 and 60 minutes relative to the infusion. At the end of the sampling period, the chickens were euthanased with an overdose of sodium pentothal and samples of abdominal fat pad, breast and thigh muscle and the liver were taken. The tissues were immediately placed on ice and then transferred to \(-70^\circ\text{C}\) until assayed. Blood samples were kept on ice until centrifugation within 1 h of collection and the harvested plasma then stored at \(-20^\circ\text{C}\) until assayed.

5.2.4. Determination of plasma hormones and metabolites

Plasma insulin concentrations were determined by radioimmunoassay procedures as was previously described in Chapter 3. Plasma glucose measurements were determined with a commercially available kit (Glucose Unimate 5 Gluc HK, Roche products Pty Ltd, Frenchs Forest, Australia) using a Roche Cobas Mira automatic analyser.

5.2.5. Determination of glucose concentration in tissues

Tissue samples were dissolved in Soluene 350 (Packard Instrument Co, Meriden C.T. USA) at a ratio of 100 mg/mL and digested at 50 °C for 4 h in a shaking water bath. The digested samples were then allowed to cool before the addition of Hionic-Flour (Packard Instrument Co, Meriden C.T. USA). The samples were equilibrated then counted on a beta counter (LKB Wallac, Turku, Finland).

5.2.6. Statistical analysis

All data are presented as the mean ± the standard error of the mean. The statistical analysis of the data on 2DG-\textsuperscript{3}H glucose tissue uptake was performed by the analysis of variance (ANOVA). The plasma insulin and glucose data were log transformed because
of the heterogeneity of variance. The statistical analysis was then determined by repeated measures analysis of variance using SAS.

5.3. RESULTS

INTRAVENOUS GLUCOSE TOLERANCE TEST

5.3.1. Plasma glucose response

There were no significant differences in glucose clearance rate or the maximal plasma glucose concentration attained in response to the glucose tolerance test (Figure 5.1). Pre-infusion plasma glucose concentrations (mmol/L) for the three dietary groups were similar at, 14.8 ± 1.6, 13.9 ± 0.6 and 12.7 ± 0.6 for the tallow, fish and sunflower oil groups respectively. The corresponding mean concentrations 5 min after the infusion were also similar at, 33.5 ± 0.7, 33.5 ± 2.6 and 33.1 ± 3.7.

5.3.2. Plasma insulin response

Although the plasma glucose responses for the three dietary groups were similar, plasma insulin concentrations were elevated in response to the IVGTT for the tallow fed chickens when compared to either the sunflower or fish oil fed birds Figure 5.2. Pre-infusion plasma insulin concentrations (ng/mL) were similar at 0.028 ± 0.004, 0.024 ± 0.005 and 0.026 ± 0.001 for the tallow, sunflower and fish oil dietary groups respectively. Although, the maximal mean plasma insulin concentration for each dietary group coincided with the maximal glucose concentration, there was a graded insulin release for the three dietary treatments in response to the glucose tolerance test. Circulating plasma insulin concentrations were elevated in the tallow group (0.128 ± 0.062) when compared to either the sunflower oil fed group (0.063 ± 0.021) or to the fish oil fed group (0.041 ± 0.014).
Intravenous Glucose Tolerance Test
7 Week Old Broiler Chickens Fed 3 Different Fat Sources

Figure 5.1

Intravenous Glucose Tolerance Test
7 Week Old Broiler Chickens Fed 3 Different Fat Sources

Figure 5.2
2-DEOXY-D-\textsuperscript{3}H GLUCOSE INFUSIONS

5.3.3 2-deoxy-D-\textsuperscript{3}H glucose clearance rate from the plasma

The disappearance rate of 2DG-\textsuperscript{3}H glucose from the plasma is given in Figure 5.3. Experimental diets had no significant affect on the disappearance rate and the isotope was cleared from the plasma at a similar rate that resembled the glucose clearance following the glucose tolerance test.

5.3.4. Plasma insulin response

As shown in Figure 5.4, there were no significant differences in circulating insulin levels between the three dietary groups over the sampling period. However, the average plasma insulin values (ng/mL) for the tallow fed chickens tended to be higher (0.048 ±0.012) over this period when compared to either the sunflower (0.035 ± 0.003) or fish oil fed birds (0.031 ± 0.003). These average values were higher compared to those measured during the fasted state in the glucose tolerance test study (Figure 5.2).

5.3.5. Tissue uptake of 2-Deoxy-D-\textsuperscript{3}H glucose

Values for labelled glucose uptake into tissues as measured when birds had access to feed are shown in Figure 5.5; there is no abdominal fat value for the sunflower oil dietary group as there was little tissue present from the birds for this group. The concentration of 2DG-\textsuperscript{3}H glucose (cpm/gram) in the breast muscle was greater for the chickens consuming the fish oil diet (12572 ± 5360) compared to those fed tallow (3521 ± 447) and significantly higher (P<0.05) compared to chickens fed the sunflower oil (2147 ± 570). There were no significant differences for the dietary treatments in the labelled-glucose incorporation into the thigh muscle, liver or the abdominal fat tissues.
Plasma Disappearance of 2-Deoxy 3H Glucose
7 Week Old Broiler Chickens Fed 3 Different Fat Sources

Figure 5.3

2-Deoxy 3H Glucose Infusion
7 Week Old Broiler Chickens Fed 3 Different Fat Sources

Figure 5.4
5.4. DISCUSSION

These studies show that feeding of specific dietary fat types to chickens alters both insulin action and glucose metabolism. These findings demonstrate that the influence of dietary fatty acids have on glucose/insulin metabolism is not confined to just mammals, but also have similar effects in avian species. Using an intravenous glucose tolerance test, the results show that feeding either fish or sunflower oils to chickens increases tissue sensitivity to insulin. While there was no significant difference in plasma glucose response to the glucose tolerance test, circulating plasma insulin responses were substantially reduced in the fish and sunflower oil dietary groups compared to the tallow fed birds.
The insulinotropic effect of dietary fats has been well documented for mammalian species with long-chain fatty acids shown to acutely stimulate insulin secretion (Seyffert and Madison, 1967; Malaisse and Malaisse-Lagae, 1968; Balasse and Ooms, 1973 and Warnotte et al., 1999). More recently it has been shown that insulin release is modified by chain length and the degree of saturation (Stein et al., 1997). Warnotte and co-workers (1999) demonstrated that the saturated fatty acids, palmitate and stearate are more effective in initiating insulin release than are the monounsaturated fatty acid, oleate and the polyunsaturated fatty acid linoleate. Their rank order of potency was determined to be palmitate∼stearate>oleate≥linoleate. This finding has been corroborated by Stein et al., (1997) who showed a similar insulinotropic effect for these same fatty acids. In addition, Warnotte and co-workers (1999) observed that it was the fraction unbound to albumin rather than the total concentration of saturated and unsaturated fatty acids that determines their insulinotropic action. This same group also suggested that the fatty acids themselves may act as acute regulators of insulin secretion and this occurs if the concentration of unbound fatty acids close to the \(\beta\)-cells is sufficient to exert direct effects on insulin secretion. Differences in the potency of specific fatty acids may account for the graduation in insulin release observed in our study. Greatest insulin release was in birds fed the tallow diet and this diet had significantly more palmitate and stearate compared to either the fish or the sunflower oil diets.

Compared to mammals, birds are relatively insensitive to the actions of insulin (review: Simon, 1988). This insensitivity is manifested in the kinetics of both insulin action and the receptor, as exogenous insulin is rarely lethal in chickens (review: Simon, 1988). Although chickens have a higher affinity for the insulin receptor when compared to mammals, as well as a slower dissociation rate of the hormone-receptor complex, it has been suggested by Simon (1988) that the lack of insulin sensitivity is the result of lower insulin receptor numbers. Early studies revealed a reduced number of insulin receptors in both hepatocytes and thymocytes from growing chickens when compared to corresponding tissues taken from young rats (Simon et al., 1977; Kemmler et al., 1978; Simon, 1979 and Cramb et al., 1982). In addition, the coupling of the hormone-receptor complex between intracellular metabolism and exocytosis appears to be inefficient for the
chicken pancreatic β-cells. This feature has been suggested by Simon (1979) to resemble that observed in the β-cells from non-insulin dependent diabetic animals.

Although insulin receptor numbers were not measured in the present study, the lower concentration of insulin required to reduce the plasma glucose concentrations to pre-infusion levels in the two PUFA dietary groups is indicative of an increase in either receptor affinity or the number of receptor binding sites. Studies in rodents by Cheema and co-workers (1992) show that dietary fatty acids modulate the phospholipid fatty acid profile and this in turn alters insulin binding. The work demonstrated that feeding a high P/S diet increased insulin binding to liver cell nuclei from both lean and obese mice, a result assessed to be due to an increase in the number of binding sites. This result agrees with that of Ginsberg et al., (1982) who suggest that an increase in the polyunsaturated nature of the membrane environment is associated with an increase in the number of binding sites. Of some relevance to our own study is that of Gould and co-workers (1982) who solubilised and then reconstituted turkey erythrocyte membrane insulin receptors in lipid environments of differing degrees of unsaturation. They found that an increase in the unsaturated lipid membrane environment increases the number of available insulin binding sites. In our own study, feeding fish and sunflower oil to chickens could be having a similar effect. These two diets had a significantly higher P/S ratio when compared to the tallow diet and this may have resulted in an increase in the number of binding sites on the insulin receptors of these two groups. Further support for this is given by the findings that feeding either fish or sunflower oil at a concentration of 8g/100g to chickens increased the polyunsaturated environment in both adipose and muscle tissue (Chapter 4).

It is noteworthy that the insulin concentrations for the pre-and post-infusion periods for the three dietary treatments were all similar. This finding could be attributed to the nutritional state of the birds as they were all fasted for 24h prior to the intravenous glucose tolerance test. Chickens normally have lower plasma insulin concentrations in the fasted than in the fed state (Rideau, 1988). This nutritional regulation of plasma insulin is dramatically demonstrated in the non-fasted chickens that were infused with the 2DG-3H glucose. Plasma insulin concentrations for all three dietary groups were increased some 10 fold when compared to the plasma insulin concentrations obtained from the same
birds during the fasted state prior to the intravenous glucose tolerance test. It is plausible that this difference in plasma insulin could be attributed to the nutritional state of the chickens, as the plasma insulin concentrations for the three dietary groups remained relatively constant in response to the infused 2DG-\(^3\)H glucose. Results from the previous chapter (Chapter 4) showed that chickens fed these same experimental diets during the non-fasted state not only had higher circulating plasma insulin concentrations but also there was a graded response in circulating insulin levels similar to that observed for the intravenous glucose tolerance test. The birds fed the tallow diet had higher plasma insulin concentrations compared to those fed either the fish or sunflower oil diets.

While insulin action was enhanced for both PUFA dietary groups compared to tallow feeding, it was only for the chickens fed fish oil that there was an increase in the uptake of 2DG-\(^3\)H glucose into breast muscle tissue. In mammals, glucose uptake by skeletal muscle cells is mediated by two isoforms of the facilitated glucose transporter family. One is GLUT 1, which functions primarily as a regulator of basal glucose transport activity, and the other is GLUT 4, the insulin-regulable carrier, responsible for insulin-regulated glucose disposal (Olson and Pessin, 1996). The GLUT 4 transporter is thought to be translocated from intracellular membranes to the plasma membrane by insulin and this results in an increased muscle glucose uptake (Stephens and Pilch, 1995). It is plausible that the increase in 2DG-\(^3\)H glucose concentration into the breast muscle of the birds fed the fish oil diet could be a response to increased GLUT 4 transporter activity. However, as insulin concentrations for the three dietary treatments were similar, a mechanism to stimulate GLUT 4 transporter activity other than insulin may be responsible. It is conceivable that the fatty acid composition of the cell membrane may influence the activity of glucose transporters as studies have shown membrane bound enzymes are modulated by the surrounding lipid environment (Else and Hulbert, 1987).

The translocation of GLUT 4 is also influenced by the membrane lipid domain with unsaturated phospholipids stimulating and saturated phospholipids inhibiting glucose transport (Sandra et al., 1984). It is of interest, that glucose uptake into the breast muscle of the birds fed the sunflower oil was significantly reduced compared to the fish oil fed birds. This finding could be attributed to the activity of the GLUT 4 transporter being regulated in part by specific dietary fat subtypes (Mori et al., 1997). Feeding highly
purified EPA (20:5n-3) to rats significantly increases GLUT 4 mRNA in skeletal muscle when compared to rats fed a control diet. Whereas studies by Tebbey and co-workers (1994) have shown that arachidonic acid (AA; 20:4n-6) suppresses gene expression of GLUT 4 in mature adipocytes. *In vitro* studies by Van Epps-Fung et al., (1997) suggests that saturated fatty acids impair GLUT 4 transporter activity. Therefore, the increase in glucose uptake into the breast muscle of the fish oil fed birds may be the result of elevated concentrations of EPA (20:5n-3) that has been assimilated from the diet into the tissues of these birds. Conversely, the reduced glucose uptake into the breast muscle of the sunflower oil fed and the tallow birds may be due to the elevated levels of both AA (20:4n-6) and saturated fatty acids observed in the tissues of these two dietary groups (see Chapter 4).

It may not be just the GLUT 4 transporter that is influenced by the dietary fatty acid profile. Studies by Picinato and co-workers (1998), demonstrate increased GLUT 2 content in the pancreatic islets of rats fed a polyunsaturated fat diet and reduced GLUT 2 content in rats fed a saturated fat diet. There is some evidence to suggest that these same glucose transporter proteins may also be present for avian tissues. Duclos and co-workers (1993) used antibodies raised against rat GLUT 1 on chicken tissues and detected its presence in skeletal muscle, adipose tissue and the brain.

These data suggest that the type of fat added to broiler diets can influence glucose metabolism. The inclusion of fish oil in place of either tallow or sunflower oil increased insulin action and increased the rate of glucose uptake into the breast muscle. Consequently, this change in glucose utilisation may alter the energy metabolism of the broiler.

Dietary fatty acids have been implicated in the modulation of hormones important for the growth and development of mammals (Clandinin and Jumpsen, 1997). However, there is little information relating the dietary fat profile to hormone release in birds. In view of the increased insulin sensitivity induced by PUFA’s in the present experiment, a third study was undertaken in broilers to examine the effects of dietary n-3 and n-6 PUFA’s on pituitary and adrenal sensitivity.
CHAPTER 6

DIETARY INDUCED REGULATION OF PITUITARY SENSITIVITY
BY N-3 AND N-6 FATTY ACIDS

6.1. INTRODUCTION

The essential fatty acids, linoleic acid (LA; 18:2n-6) and linolenic acid (LNA; 18:3n-3) and their longer chain polyunsaturated derivatives have been well documented as forming integral structural components of all cell membranes. These fatty acids also contribute in the regulation of many physiological functions, through their contribution as precursors for the formation of the biologically active eicosanoids (Smith and Marnett, 1991). Eicosanoids are a group of oxygenated 20-carbon compounds that are derived from arachidonic acid (AA; 20:4n-6), dihomogammalinolenic acid (DGLA; 20:3n-6) eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3).
These long-chain polyunsaturated fatty acids (PUFA's) can be synthesized from their dietary precursors, LA (18:2n-6) and LNA (18:3n-3) or can be obtained directly from the diet (Gunstone and Norris, 1983). Eicosapentaenoic acid (20:5n-3) is structurally similar to AA (20:4n-6) and competes as a substrate for the major enzymic cyclo-oxygenase and lipooxygenase pathways (Simopoulos, 1991). In addition, they can also inhibit these enzymic pathways (Careaga and Sprecher, 1984). The type of PUFA involved in eicosanoid formation modulates the rate of eicosanoid biosynthesis. For example, the intensity of n-6 eicosanoid signaling is greater as AA (20:4n-6) forms a greater proportion of the PUFA's that are available for eicosanoid production. The majority of eicosanoids are biosynthesized from AA (20:4n-6), which is the precursor of the 2-series prostaglandins (Watkins, 1995). Its formation is often associated with an explosive, but transient burst of synthetic activity and provides active eicosanoids that can activate specific receptors before selective catabolic enzymes are able to convert the eicosanoids to inactive metabolites (Lands, 1992). Eicosanoids, which are formed from the n-3 series, have a slower rate of synthesis than those formed from the n-6 series do; therefore, the n-3 fatty acids act to competitively attenuate the rate of n-6 formation (Lands et al., 1973).

Eicosanoids formed from the n-6 and n-3 PUFA's play an integral role in the growth and development of an organism as they modulate the pulsatile release of the hypothalamic and pituitary hormones (Ojeda et al., 1981). Consequently, they have major effects on many physiological conditions. For example, early studies by Greenberg and Ershoff (1951) demonstrated a relationship between dietary fatty acids of the n-6 series and reproductive performance. They showed that the low weights of testes, prostate and seminal vesicles of animals could be restored to normal by either the injection of gonadotrophin or the ingestion of dietary LA (18:2n-6). Eicosanoids derived from dietary fatty acids modulate hypothalamic function in stimulating GH and ACTH release from the pituitary in promoting prolactin release by inhibiting prolactin inhibiting factor and increasing prolactin releasing factor, and in stimulating gonadotrophin (LH and FSH) release by stimulating LHRH release from the hypothalamus (Ojeda et al., 1981).

More recent studies have shown that specific fatty acids are also involved in both the signal and the release mechanism of peptides (Pacheco and Jope, 1996). For example,
the addition of AA (20:4n-6) (Ikawa et al., 1996) and oleic acids (Kennedy et al., 1994) to culture media enhances the release of the gonadotrophin-releasing hormones from cultured mammalian anterior pituitary cells. Earlier studies in vitro have demonstrated that eicosanoids derived from AA (20:4n-6) added to culture media modulated corticotrophin-releasing hormone secretion (CRF) from rat hypothalamic explants (Bernardini et al., 1989).

As dietary fatty acids have been implicated in the modulation of hormones important for the growth and development of mammals, the present study was designed to examine the effects of dietary n-3 and n-6 PUFA's on pituitary and adrenal sensitivity in birds. We examined the effects of dietary fish oil (n-3 PUFA's), sunflower oil (n-6 PUFA's) and edible tallow (mixture of saturated monounsaturated and polyunsaturated fatty acids) on plasma GH and LH concentrations in response to infusions of GHRH and GnRH and circulating corticosterone concentrations in response to CRF and ACTH infusions in a cohort of broiler chickens.

6.2. MATERIALS AND METHODS

6.2.1. Animals and experimental diets

Male broiler chicks (n=18) (Inghams strain TM70) were obtained from a commercial hatchery and placed in a temperature-controlled battery brooder with raised wire floors. All chicks had free access to a commercial starter diet and water for a period of three weeks and were exposed to the same photoperiod regime as that described for experiment 2, Chapter 4. At the end of this period, the chicks were randomly divided into three groups (n=6) and housed as groups with free access to water and the experimental diets until week seven of the experiment. The three experimental diets were prepared as described in Chapter 3. Jugular catheterisation was performed under general anaesthesia during week 4 of the dietary treatments and the birds allowed 7 days post-surgery to recover. To assess the tissue sensitivity of the pituitary and adrenal, LHRH, GHRH, CRF and ACTH challenges were undertaken during weeks 6 and 7 of the dietary treatments.
6.2.2. Jugular vein catheterization

The catheterization procedures for this experiment was identical to those performed in Chapter 5 and are fully described in Chapter 3.

6.2.3. Hypothalamic and adrenal releasing factor infusions

After six weeks of feeding the experimental diets the hypothalamic and adrenal releasing factor infusions were administered to non-fasting birds. Chicken GnRH (LHRH-1 Auspep Pty Ltd Parkville Vic, Australia) was injected at a concentration of 20 µg/bird. Sequential blood samples (1.0 mL) were obtained via the indwelling catheter at -20, -10, 0, 5, 10, 15, 20, 30, 40 and 60 minutes relative to the infusion. Two days later, chicken GHRH (Auspep Pty Ltd Parkville Vic, Australia) was administered at a concentration of 12.5 µg/kg and the same sampling regime as for the GnRH challenge carried out. The chickens were allowed to recover a further two days then infused with ovine CRF (Auspep Pty Ltd Parkville Vic, Australia) at a concentration of 0.2 µg/kg and then with ACTH 1-24 (0.01 IU/g) 3 days afterwards (Auspep Pty Ltd Parkville Vic, Australia). The blood sampling regimen for the CRF infusion was identical as described for the GnRH and GHRH infusions, whereas blood samples were collected at -20, -10, 0, 5, 10, 15, 20, 25, 30, 60, 90, 120, 150, and 180 minutes for the ACTH infusion. Blood samples were kept on ice until centrifugation within 60 minutes of collection and the harvested plasma then stored at -20°C until assayed.

6.2.4. Plasma growth hormone, luteinising hormone and corticosterone assays

Plasma GH, LH and corticosterone concentrations were determined by radioimmunoassay procedures as described previously in Chapter 3.

6.2.5. Statistical Analysis

All data are presented as the mean ± the standard error of the mean. The statistical analysis for the dietary fatty acid data was performed by the repeated measures analysis of variance (ANOVA) using SAS. The plasma LH, GH and corticosterone values were log-transformed prior to the statistical analysis to address heterogeneity of variance. The
pre-infusion plasma LH concentrations were used as the covariate for the analysis of plasma LH. Differences between the three dietary groups for the GnRH pre-infusion period were assessed by the least significant difference (5%). A polynomial decomposition of the time and time by treatment terms in the analysis of GH from 5 min post-infusion was obtained to establish the rate of disappearance of GH from the circulation.

6.3. RESULTS

HYPOthalamic AND ADRENal RELEASING FACTOR INFUSIONS

6.3.1. GHRH infusion

Circulating plasma GH concentrations are shown in Figure 6.1. The pre-infusion GH concentrations (ng/mL) were not significantly different for the tallow, sunflower and fish oil dietary groups, (5.1 ± 3.9, 6.1 ± 3.1 and 5.2 ± 1.0 respectively). Birds from all three dietary groups responded to the GHRH infusion. The maximal GH concentration for the experimental groups occurred 5 min after the onset of the challenge and the dietary treatments resulted in a distinct graded release of plasma GH. The plasma GH concentrations (ng/mL) were elevated in the sunflower oil group (44.7 ± 5.7) when compared to chickens fed the tallow and the fish oil (33.7 ± 9.7 and 21.3 ± 5.0 respectively). The rate of return to basal GH concentrations for the fish oil dietary group was significantly lower (P<0.05) than for the sunflower oil dietary group.

6.3.2. GnRH infusion

The plasma LH concentrations for the three dietary treatment groups are given in Figure 6.2. The pre-infusion plasma LH concentrations (ng/mL) for the chickens fed the tallow diet were significantly elevated (P<0.05) when compared to the birds fed either sunflower or fish oil diets (3.19 ± 0.67, 0.84 ± 0.25 and 0.93 ± 0.22 respectively). The plasma LH time-related response to the GnRH infusion was similar to the GHRH challenge. The maximal LH concentration for chickens fed the three experimental diets occurred 5 min after the onset of the infusion. However, there was no significant difference between the dietary groups in either maximal plasma LH concentration or the
rate of return of plasma LH to basal levels in response to the LHRH challenge.

6.3.3. CRF infusion

The corticosterone profiles for the three dietary groups are shown in Figure 6.3. The mean pre-infusion corticosterone values (ng/mL) were not significantly different for either the sunflower oil (10.95 ± 2.09), tallow (6.61 ± 1.94) or fish oil (7.68 ± 1.97) dietary groups. Unlike either the GHRH or GnRH infusions plasma corticosterone concentrations for the three dietary groups rose more slowly, within 10 min from the onset of the infusion and these levels were maintained over a longer period. The maximal corticosterone concentration (ng/mL) for the sunflower oil, tallow and fish oil fed birds were not significantly different (16.69 ± 3.97, 11.88 ± 3.22 and 13.94 ± 6.89 respectively). There was no significant difference for the rate of return of plasma corticosterone for the three dietary groups, with corticosterone concentrations returning to similar pre-infusion levels within 40 min from the onset of the infusion.

6.3.4. ACTH infusion

The plasma corticosterone concentrations are shown in Figure 6.4. The mean pre-infusion plasma corticosterone concentrations (ng/mL) for the three dietary groups were similar, being 1.8 ± 0.1 for the tallow fed chickens, 1.5 ± 0.2 for the sunflower oil group and 1.5 ± 0.2 for the fish oil group. However these values were significantly lower (P<0.05) than the CRF pre-infusion concentrations (Figure 3) and may reflect an adaptation to the handling regime imposed on these birds. Unlike the CRF infusion or the hypothalamic releasing factor infusions, the plasma corticosterone concentrations rose more slowly in response to the ACTH infusion, with the maximal plasma ACTH concentration for all three treatment groups occurring approximately 2 h after the onset of the infusion. There was no significant difference between the dietary groups for either the maximal plasma ACTH concentration or its rate of return to basal levels.
GH Response To GHRH Infusion
7 Week Old Broiler Chickens Fed 3 Different Fat Sources

LH Response To LHRH Infusion
7 Week Old Broiler Chickens Fed 3 Different Fat Sources
Corticosterone Response To CRF Infusion
7 Week Old Broiler Chickens Fed 3 Different Fat Sources

Figure 6.3
Time relative to CRF infusion (min)

Corticosterone Response To ACTH Infusion
7 Week Old Broiler Chickens Fed 3 Different Fat Sources

Figure 6.4
Time relative to ACTH infusion (min)
6.4. DISCUSSION

A major finding from this study was to show that the feeding of specific dietary fat types can modulate the pituitary sensitivity of the broiler chicken. In addition, this study also shows that dietary fats have a differential effect on pituitary cell activity and are specific to certain pituitary cell types. Polyunsaturated fatty acids from the n-6 series were more effective than PUFA's from the n-3 series in their ability to modulate the somatotrophic axis in stimulating the release of GH. However, these same dietary treatments had no effect on the modulation of either the gonadotrophic axis or hypothalamic-pituitary-adrenal (HPA) axis.

When infused into the portal circulation, GHRH has been shown to act directly on avian pituitary somatotropes to stimulate GH release (Harvey, 1993). In the present study, infusion of GHRH to chickens fed sunflower oil (n-6 PUFA's) resulted in a greater increase in circulating GH concentrations when compared to the feeding fish oil (n-3 PUFA's). Interestingly, when the tallow fed birds were infused with GHRH the rise in circulating plasma GH concentrations fell between the two PUFA groups. Previous studies have shown that fatty acids from the n-6 series, specifically LA (18:2n-6), enhance the release of GHRH (Seflaris et al., 1992; Barb et al., 1995; Ikawa et al., 1996). In addition, Seflaris and co-workers (1992) have also shown an inhibitory effect of LA (18:2n-6) on the growth hormone inhibiting factor, somatostatin, when cultured hypothalamic cells were exposed to oleic acid. These same authors have also demonstrated suppressed levels of pre-prosomatostatin mRNA to the same treatment. In the present study, the sunflower oil and tallow diets contained significantly higher concentrations of both LA (18:2n-6) and oleic acid when compared to the fish oil diet. Therefore, the known physiological actions of LA (18:2n-6) and oleic acid in stimulating GH release and inhibiting somatostatin may explain to some extent the increase in circulating plasma GH concentrations observed for the sunflower oil and tallow fed groups.

The finding that dietary fatty acids modulate GH release is in concert with studies that demonstrate a causal link between the action of eicosanoids and the modulation of the pulsatile hypothalamic and pituitary hormones (Ojeda et al., 1981; Bernardini et al., 1989). We have shown previously that the feeding of these same experimental diets to
broiler chickens significantly increased the concentration of EPA (20:5n-3), DHA (22:6n-3), and AA (20:4n-6) into the breast muscle (see Chapter 4). These results showed that chickens fed the sunflower oil and tallow diets exhibited significantly enhanced concentrations of AA (20:4n-6) compared to the fish oil fed birds. Whereas, chickens fed the fish oil diet had significantly enhanced concentrations of EPA (20:5n-3) and DHA (22:6n-3). Therefore, it is also plausible that the increase in plasma GH concentrations observed in the sunflower oil and tallow fed chickens may be a response to the increased concentration of AA (20:4n-6) and or its metabolites.

The dietary treatments used in the present study modulated GH secretion in response to the GHRH infusion, whereas they did not affect the response in the pattern of plasma LH secretion to GnRH stimulation. This suggests that in birds, not all pituitary cell types are sensitive to the modulating action of fatty acids. This difference in pituitary sensitivity may also be the result of intracellular mechanisms involved in the induction and release of GH and LH from the pituitary. Studies in mammals have shown that when GnRH binds to its specific gonadotrope receptor, multiple second messenger pathways are activated including calcium/calmodulin, phosphoinositide turnover, cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA), diacylglycerol/protein kinase C (PKC), and AA (20:4n-6) second messenger systems (Naor, 1990). Moreover, in vitro studies using chicken pituitary cells have also provided evidence for multiple second messenger systems being involved in GnRH induced LH secretion (Johnson and Tilly, 1991). Whereas, increased levels of cAMP are considered to induce the release of both prolactin (PRL) and GH in mammals (Tixer-Vidal and Gourdji, 1981; Clayton et al., 1986; Bole-Feysot et al., 1998). A similar pathway has also been suggested for birds. Studies by Kansaku and co-workers (1995) have indicated that the release of PRL and GH may be induced via the activation of adenyl cyclase, accumulation of cAMP and activation of PKA in the chicken anterior pituitary gland.

It is thought that AA (20:4n-6) and its metabolites, 5-HPETE and 12-HEPTE, in addition to some leukotrienes may act as intracellular messengers in a manner analogous to cAMP, Ca^{2+} and inositol 1,4,5-triphosphate (Barritt, 1996). It is conceivable therefore, that AA (20:4n-6) and or its metabolites may also act as intracellular second messengers in the chicken. In the present study, GH concentrations in response to GHRH were higher.
in both the sunflower oil and tallow-fed birds and this was positively correlated with elevated AA (20:4n-6) concentrations observed in the breast muscles of these two dietary groups (Chapter 4). In contrast, GH concentrations for the fish oil fed birds were lower when compared to either the sunflower oil and tallow groups and this was associated with lower AA (20:4n-6) breast muscle levels. Therefore, it is conceivable that in this study, GH release is mediated via AA (20:4n-6) and its metabolites that act as a key intracellular second messenger. This explains why GH synthesis and release is dependent on dietary fatty acids. Whereas, the secretion of LH may not be as sensitive to the dietary fat profile because of the multiple intracellular second messenger systems involved in the secretion of this hormone.

Although the plasma GH response was reduced in the birds fed the fish oil diet it is plausible that the reduction in pituitary sensitivity may reflect an increase in whole body tissue sensitivity to GH and that this may in turn influence carcass composition as GH is well known for its lipolytic action in mammals (Duquette et al., 1984) and chickens (Campbell and Scanes 1985). An alteration in GH sensitivity in the fish oil dietary group may in part explain the significant reduction in the abdominal fat pad mass reported for this dietary group in Chapter 4. To our knowledge, this is the first reported finding that the dietary fat profile can influence the sensitivity of the avian pituitary. This result is in keeping with studies that show a dietary fat-induced modulation of tissue sensitivity to the metabolic hormones. For example, tissue sensitivity to insulin is positively correlated to the dietary fat type through the alteration of the phospholipid composition of the skeletal muscle membrane (Grunfield et al., 1981; Field et al., 1988 & 1990; Cheema et al., 1992; Pan et al., 1995). Therefore, it is conceivable that the altered pituitary sensitivity observed in the present study might be the result of a modification of the phospholipid composition of the pituitary induced by the dietary fat profile. This notion is supported by a recent study that shows specific dietary fat types are assimilated into the phosphatidylethanolamines (PE) and phosphatidylcholines (PC) of rat brains (Weber et al., 1999). Further, this same study also shows and that the feeding of specific fat types can modulate the proportion of DHA (22:6n-3) and AA (20:4n-6) that are assimilated into the PE and PC phospholipids.
The results from the above experiments including the current data show a positive relationship between specific fatty acid subtypes and the stimulation of GH release. This finding needs to be considered in light of the clear in vivo and in vitro evidence that demonstrate a negative correlation between GH release and elevated non-esterified fatty acids (NEFA's) (Widmaier, 1997). This may simply be the result of a negative feedback effect of excess energy substrate. However, it is also plausible that the difference between these studies could be the fat-type or types and their concentrations that have been used. In the present study, GH release was substantially reduced in birds which were fed the fish oil, a diet rich in the n-3 PUFA's, compared to birds fed the sunflower oil, a diet rich in n-6 PUFA's. Many reported in vivo studies have used an emulsion of soybean oil (Intralipid) to measure the GH response to fatty acids (review see Widmaier, 1997). Although soybean oil contains both LA (18:2n-6) and oleic acid there concentrations are less than what is found in sunflower oil (Gunstone, 1996). In addition, soybean oil also contains a high percentage of LNA 18:3n-3). This n-3 fatty acid or its longer-chained derivatives may also reduce the GH response to GHRH infusion.

The modulation and release of GnRH from the hypothalamus and LH from the pituitary have been shown to be responsive to specific fatty acid subtypes and eicosanoids (Yehuda et al., 1997). In addition to modulating GH secretion, AA (20:4n-6) and oleic acid also enhance the release of GnRH (Ikawa et al., 1996; Kennedy et al., 1994). In vitro studies have demonstrated the efficacy of the eicosanoids derived from AA (20:4n-6), prostaglandin E2 (Kennedy et al., 1994) and leukotriene C4 (Hulting et al., 1985) to increase LH secretion when isolated rat anterior pituitary cells are cultured in their presence.

However, these above studies were conducted in an in vitro environment and may not necessarily reflect the true biological nature of these fatty acids and their effects on gonadotrophin secretion in vivo. For example, when sheep were infused acutely with NEFA, GH concentrations were inhibited but this same infusion had no effect on plasma LH levels (Estienne et al., 1989). These results may simply reflect the fact that LH, unlike GH, does not play a role in regulating energy substrate mobilization.

Although there were no significant differences in the plasma LH response to the LHRH infusion the pre- and post-infusion plasma LH levels for the chickens fed the two
PUFA diets were consistently lower when compared to tallow feeding. This result was surprising as the fatty acids that have been shown to stimulate LH release in mammals (Ikawa et al., 1996; Kennedy et al., 1994) were present in both the diet and tissues of the sunflower oil fed birds. In addition, the fatty acids contained in this diet were similar in both concentration and subtype for the tallow fed chickens. This suggests that in chickens, other factors may override the stimulatory nature of fatty acids and eicosanoids and therefore impinge on reproductive status. A possible candidate could be the hormone leptin.

The secretion and regulatory levels of leptin are positively correlated with obesity (Maffei et al., 1995; Considine et al., 1996; Hardie et al., 1996). Leptin is synthesized in adipose tissue in response to nutritional and metabolic changes (Houseknecht et al., 1998) with concentrations rising after feeding and with increasing body fat content (Hamann and Matthaei, 1996). The role of leptin in the regulation of reproduction has been extensively studied with leptin receptor mRNA detected in the ovary, testis, uterus, hypothalamus and pituitary gland of rats (Schwartz et al., 1996; Zamorano et al., 1997). Both in vivo studies (Brash et al., 1996) and in vitro studies (Barb et al., 1997; Liou et al., 1997; Yu et al., 1997) have shown that leptin enhances gonadotrophin secretion at the level of the pituitary and or hypothalamus (Yu et al., 1997). We have shown previously a significant decrease in the abdominal fat pad mass when broiler chickens were fed the two PUFA diets when compared with the addition of tallow to the diet (Chapter 4). Based on this, the higher body fat status of the tallow supplemented birds would dictate that basal gonadotrophin concentrations may be higher in this treatment group, which is consistent with our results (see Figure 2). Despite this, gonadotrophin sensitivity to stimulation was not altered by the fatty acid composition of the diet. Thus, plasma leptin concentrations could reflect the body fat content of these birds; being higher in the tallow fed chickens and lower in the chickens fed the PUFA diets. Therefore, it is conceivable that lower leptin concentrations could result in a reduced secretion of LH thereby explaining the lower basal levels in the present study.

The interrelationship between the sympathomedullary and HPA endocrine axes involved in modulating stress responses and dietary fatty acids has been well documented (Munk et al., 1984; Reed et al., 1987; Odio and Brodish, 1988). Both in vivo (Widmaier
et al., 1992) and in vitro (Sarel and Widmaier, 1995) studies have demonstrated a positive relationship between fatty acid subtypes and the modulation of cortisol and ACTH secretion. The infusion of intralipid, a source of saturated fatty acids, into unrestrained rats significantly increased plasma cortisol concentrations (Widmaier et al., 1992), a finding that is contrary to the need to increase insulin sensitivity to promote lipogenesis. In contrast, cortisol secretion was increased when dispersed rat adrenocortical cells were incubated in the presence of long-chain unsaturated fatty acids but not saturated or short-chained fatty acids. Earlier studies conducted by Bernardini and co-workers (1989) have demonstrated that eicosanoids derived from AA (20:4n-6) stimulated CRF from cultured rat hypothalami. These results may be attributed to the role of second messenger systems involved in ACTH stimulated steroidogenesis and the influence of specific fatty acid subtypes on their activity.

Studies using cAMP analogs have been shown to activate rat, mouse and bovine adrenal steroidogenesis thereby implicating its role as a second messenger in these species (Schimmer, 1980). More recent studies by Yamazaki and co-workers (1998) have reported that both Ca$^{2+}$ and lipoxygenase metabolites of AA (20:4n-6) can also act as dual second messengers in bovine adrenal fasciculate and reticularis cells. More importantly, they have also shown that both Ca$^{2+}$ and 15-lipoxygenase metabolites function at the same time and modulate steroidogenesis independently. In view of the nature of the above second messenger systems it is not surprising that long-chain fatty acids, specifically AA (20:4n-6) and its metabolites, can influence steroidogenesis in mammals.

Although chicken second messenger systems that regulate steroidogenesis have not, to our knowledge, been identified, it is plausible that second messenger systems similar to those reported for mammals are involved. However, in the present study, there was no discernible difference in corticosterone concentrations in response to either CRF or ACTH infusions between the three dietary groups. This result is difficult to explain in the light of studies conducted in mammalian species showing a positive relationship between fatty acid subtypes and HPA modulation. Indeed, when the breast muscle fatty acid profile for the three dietary groups is considered (Chapter 4) one would expect a difference in the corticosterone response between the fish oil group provided with low dietary AA (20:4n-6) concentrations compared to the sunflower oil group provided with
higher concentrations of this fatty acid. This result may simply reflect a difference between mammalian and avian species in adrenal sensitivity and their sensitivity to dietary fatty acids.

Studies indicate that adrenocortical responsiveness in some avian species may be affected by circannual events. This has been shown in north-temperate migratory species, whose pattern of glucocorticoid secretion is strongly affected by the time of year (Wingfield et al., 1982; Silvern, 1986). Peczely, (1976) observed seasonally related changes in corticosterone synthesis being higher in spring and autumn in migratory than sedentary songbirds. A more recent study has shown that the corticosterone concentrations of breeding male and female white-crowned sparrows are significantly greater than those of non-breeding females and males (Astheimer et. al., 1994). The timing of seasonal breeding animals is controlled by photoperiod (Marshall, 1937). Indeed, the circadian rhythm for glucocorticoid secretion is entrained to the prevailing photoperiod (Fischman et al., 1988), with peak levels corresponding to the period of darkness. Photic information received by the eye modulates a circadian pattern of release of melatonin, which is translated into a neural signal to the hypothalamus. This release pattern of melatonin has been demonstrated to mediate the circadian rhythm for other endocrine profiles including those of the pituitary-adrenal axis, in sheep (Jones, 1990) and deer (Newman et al., 1991).

In the present study, the day-old chickens used were entrained to a constant light photoperiod (24L:0D) and this was maintained throughout the experimental period. Therefore, it is conceivable that this photoperiodic regime may have altered the sensitivity of the HPA axis and this may explain the lack of adrenal sensitivity to dietary fatty acids. This photoperiod-induced alteration in adrenal sensitivity may also explain the delayed corticosterone response to the ACTH infusion (Figure 4). Studies by Astheimer and co-workers (1994) have shown that corticosterone concentrations increase within 10 minutes of an ACTH infusion in white-crowned sparrows. This result was achieved in birds that were entrained to the prevailing normal photoperiod, the rise in corticosterone being greater in birds exposed to longer day lengths than those exposed to shorter day lengths. Although photoperiod does have a significant effect in the modulation of steroidogenesis, our results also suggest that each of the components of the
HPA axis must be activated to achieve a normal physiological response in corticosterone release (Figure 3). This is supported by the observation that corticosterone levels increased after nearly 2 hours in response to ACTH infusion but only after 10 minutes in response to CRF infusion. The result also implies a possible synergy in the actions of CRF and ACTH at the level of the fasculata cell, with CRF having a modulatory influence on steroidogenesis.

In summary, this study has shown that dietary fatty acids modulate pituitary sensitivity in the chicken. However, there is a differential effect on pituitary cell activity that is dependent on the type of fatty acid as n-6 PUFA’s were more effective than n-3 PUFA’s in modulating GH secretion in response to GHRH infusion. In addition, this study has also demonstrated that dietary fatty acids are specific to certain pituitary cell types as these same dietary treatments had no effect on LH secretion following LHRH infusion.

The results from the present study and those from the previous experiments have adequately shown that dietary PUFA’s albeit from the n-3 or n-6 series significantly alter broiler metabolism. This dietary fatty acid induced response may be the result of a change in the phospholipid molecular species profile of the cell membrane as the fatty acyl chains of phospholipids of plasma membranes play an active role in determining cellular function (Hulbert and Else, 1999). Therefore, a final study was undertaken to determine the molecular species profile of choline and ethanolamine phospholipids using breast muscle samples taken from broilers fed the three dietary treatments described for Chapter 4, experiment 2.
CHAPTER 7

EFFECT OF DIETARY N-3 AND N-6 FATTY ACIDS ON THE COMPOSITION OF MOLECULAR SPECIES OF CHOLINE AND ETHANOLAMINE PHOSPHOLIPIDS IN BREAST MUSCLE

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7.1. INTRODUCTION

The plasma cell membrane is increasingly an important consideration in many biological studies. It is involved in a variety of cellular functions, including receptor mediated signal transduction, the processing of receptor ligand complexes, the passage of hydrophobic effectors such as steroid hormones that interact with their cytoplasmic membrane receptors and the passage and transport of ions and nutrients (Birnbaumer et al., 1977; Steinman et al., 1983; Robertson, 1983), factors critical to normal cellular function. Although membrane composition and functional properties are regulated by both genetic and environmental components, it has become increasingly clear that membrane lipids, particularly the fatty acyl chains play an active role in determining cellular function (see review Hulbert and Else 1999).

Alteration of the dietary fatty acid profile has been shown to affect several physiological processes, including the lowering of plasma lipid concentrations, modulation of eicosanoid formation and alteration of cell membrane properties (Harris, 1989; Kinsella et al., 1990). Consumption of n-3 polyunsaturated fatty acids (PUFA’s) has numerous beneficial effects, including improved cardiovascular health (Bang and Dyerberg, 1972; Leaf and Weber, 1988; Leaf, 1990), reduction of carcinogenesis (Schut, 1993; Caygill and Hill, 1993), as well as the amelioration of certain autoimmune diseases such as rheumatoid arthritis (Kremer et al., 1987; Robinson et al., 1993) and improved insulin sensitivity (Storlien et al., 1996; Storlien et al., 1997). Although n-3 PUFA’s have received greater attention because of their perceived beneficial health effects, consumption of n-6 fatty acids, and more specifically, linoleic acid (LA; 18:2n-6) also have been shown to modulate metabolism in rats by reducing lipid secretion (Strum-Odin et al., 1987) and improving both insulin binding and insulin action (Field et al., 1990; Cheema et al., 1992).

Several studies have found that fatty acids such as eicosapenaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3) and arachidonic acid (AA; 20:4n-6) are absorbed and incorporated into muscle phospholipids of ruminants (Scott et al., 1993) and monogastrics (dogs: Gross, 1985). The same fatty acids have a high affinity for acylation to the sn-2 position of plasmalogen phospholipids (Blank et al., 1989; Robinson et al., 1993; Blank et al., 1994). These plasmalogens may act as a reservoir for PUFA’s
(Ford and Gross, 1994; Thomas et al., 1990) which when released by a calcium-independent, plasmalogen-specific, phospholipase A\(_2\) can form bioactive molecules such as prostaglandins and leukotrienes (Hazen et al., 1991) and these have effects on many physiological systems (Samuelsson et al., 1987; Feuerstein and Hallenbeck, 1987).

Although there is a growing literature for mammals relating dietary fat profile to fatty acid incorporation into cell membrane phospholipids, there are few studies examining the same relationship in birds. The study of Kelso and co-workers (1997) showed that chickens fed a diet supplemented with fish oil for 30 and 48 weeks, there was a significant increase in the proportion of DHA (22:6n-3) incorporated into the spermatozoan phospholipid. In this same study, this increase in DHA (22:6n-3) was associated with a decrease in the proportion of AA (20:4n-6).

In view of the limited information available on the influence of n-3 and n-6 fatty acids in the diet on membrane phospholipids in birds, we investigated and characterised the molecular species of the choline and ethanolamine phospholipids in breast muscle of the domestic chicken when fed diets containing 8% fish oil (n-3PUFA’s), 8% sunflower oil (n-6 PUFA’s) and 8% tallow (mixture of saturated, monounsaturated and polyunsaturated fatty acids).

7.2. MATERIALS AND METHODS

7.2.1. Animals

The breast muscle tissue for the molecular species analysis was obtained from four broiler chickens fed each of the experimental diets collected previously from experiment No 2 (Chapter 4).

7.2.2. Materials:

Phospholipase C (Bacillus cerus Type XIII), benzoic anhydride, and 4-dimethylaminopyridine were purchased from Sigma Chemical Co. (St Louis, MO). Phospholipids and diacylglycerol standards were obtained from Serdary, London, Ontario Canada. Thin-layer plates, Silica Gel 60F 254 (20cm x 20cm), were from E. Merck (Darmstadt, Germany). The solvents used for the TLC solvent system were AR grade and
for HPLC separation were HPLC grade and supplied by Mallinckrodt, Selby Biolab (Sydney, Australia).

LIPID EXTRACTION, PHOSPHOLIPID SEPARATION AND FATTY ACID ANALYSIS

7.2.3. Lipid extraction

Total lipids were extracted from 10g of muscle tissue with chloroform: methanol (2:1 v/v) containing 0.01% butylated hydroxy toluene. The tissue was macerated for 2 min using a Waring blender, equilibrated for 4 h. at R.T. then filtered (Watman No 1 filter paper). The filtrate was washed with H₂O (Folch et al., 1957) and the phases allowed to separate before aspirating and discarding the top phase. The bottom layer was retained, evaporated to dryness and redissolved in chloroform: methanol (2:1 v/v).

7.2.4. Separation of phospholipids and neutral lipids

Twenty ml glass columns were prepared by loading with 3-5 g silicic acid that was suspended in 10-15 mL of chloroform. The lipid samples were dissolved in chloroform (0.5 mL) and applied to the top of the column and then eluted with 60 mL of chloroform to separate the neutral lipids. The phospholipids were then eluted with 60 mL of methanol and their concentration determined by phosphorous (P) estimation using a factor of P x 25 (Bartlett, 1958).

7.2.5. Separation of total phospholipids into individual phospholipid classes

All of the remaining phospholipid extracts were evaporated to dryness and then redissolved in 100-200 µL chloroform: methanol (2:1 v/v). Samples and phospholipid standards were separated by TLC using silica gel G plates that had previously been activated by incubating at 100°C for 1 h. The plates were then run in solvent system No 1 (chloroform 60 mL/methanol 25 mL/acetone 50 mL/ acetic acid 2 mL/ H₂O 5 mL), air-dried for 20-30 min then dried under vacuum for 30 min. The silica plates were then run in the same direction in solvent system No 2 (chloroform 60 mL/methanol 50 mL/ acetic acid 10 mL/ H₂O 3.3 mL). Individual phospholipids and standards were initially visualised by spraying the plate which encompassed the standards and a proportion of the
samples with ninhydrin, incubating at 100°C and then re-spraying to detect phosphorous (Vaskovsky, et al., 1975). Final visualisation of the lipids was achieved by spraying the plate with 2,7-dichlorofluorescein (0.02% w/v in ethanol) and exposed to ultraviolet light. Areas of gel corresponding to the phospholipids were scraped off the plate and the lipid was extracted with chloroform-methanol- H2O 5:5:1 (by volume). The phospholipid extracts were washed 3x in H2O and the volumes were adjusted to 10 mL with chloroform-methanol 2:1 v/v. A portion of this extract was used to prepare fatty acid methyl esters according to Christie (1989) and separated and quantified by gas chromatography (Perkin Elmer Autosystem, FID and PE Nelson data processing system, model 10202), fitted with a BPX 70 Capillary column (50 mm x 0.25 mm) SGE Australia Pty Ltd). Helium was used as the carrier gas with an injection split ratio of 100:1. The GLC was temperature programmed from 150 °C to 210 °C at a rate' of 2 °C per minute with an injection temperature of 210 °C and a detector temperature of 250 °C. Peaks separated were identified by comparison with standard samples of known composition. An aliquot of the phospholipid extract was also used to determine the amount of phospholipid phosphorous and the remainder was used for analysis of molecular species.

7.2.6. Separation of molecular species

The purified phospholipids were evaporated to dryness, redissolved in 1.25 mL 10 mM Tris-HCL (pH 7.5) containing 30 mM H3BO3 and 10 mM CaCl2 then sonicated in a cold water bath (2°C) for 5 min. Individual phospholipids (1-2 mg) were hydrolysed with 50 units of phospholipase C, then extracted with 2 mL diethyl-ether, mixed by vortexing and incubated overnight at R.T. while being constantly agitated. The ether layer was removed and the released 1, 2-diacylglycerols (DG) were extracted in diethyl-ether and dried under N2 to prepare benzoate derivatives (Blank, et al., 1984).

7.2.7. Benzoate derivatives

The extracted DGs were dissolved in 0.3 mL of benzene containing benzoic anhydride (10 mg) and 4-dimethylaminopyridine (4 mg) and allowed to react for 2 h. The benzoate derivatives were extracted three times with 2 mL of hexane and separated into three subclasses viz. diacyl, alkylacyl, and alkenylacyl by TLC on silica gel G in a
solvent system of toluene-hexane-diethyl ether 50:45:5 (by volume). The diradylglycerol-benzoates were visualised under ultraviolet light and the areas of gel corresponding to these derivatives were scraped from the plates and extracted with hexane. The DG extract was evaporated to dryness, and the DGs redissolved in acetonitrile-2-propanol 80:20 (v/v), and the distribution of the three subclasses in the ethanolamine and choline phospholipids was determined by measuring the absorbance at 230 nm.

7.2.8. Separation by HPLC

The molecular species of the diacyl and alkenylacyl derivatives were separated using a Waters HPLC system comprised of 501 pump, a 484 UV detector (operating at 230 nm), and a 740 data module. An SGE ODS 2-8/5 column (5 µm, 25cm x 4 mm id) was used at an operating temperature of 24 °C. Samples of the benzoate derivatives were positioned using two solvent systems, viz acetonitrile-2-propanol 80:20 (v/v) and methanol-2-propanol 95:5 (v/v). The molecular species were separated by isocratic elution at a flow rate of 1.0 mL/min and 0.8 mL/min, respectively a C12:0/C12:0 diacylglycerol-benzoate derivative was used as an internal standard.

7.2.9. Identification of molecular species

Identification of the individual molecular species was achieved by a combination of HPLC analysis of standards, graphing their relative retention times using the methods outlined by Takamura, et al., (1986); Bell, (1989) and Takamura and Kito (1991) and GLC analysis of the fatty acid methyl esters of the peaks corresponding to the eluted species.

7.3. RESULTS

7.3.1. Distribution of choline phospholipid (PC) subclasses in muscle

The percent distributions of the PC subclasses, diacyl, alkyl-acyl and alkyl-enyl for the three dietary treatments are shown in Figure 7.1. The diacyl subclass accounted for the highest percentage distribution of lipid in all three dietary groups. The feeding of the different dietary fats had no significant effect on the distribution of the PC subclasses.
For the birds fed the fish oil diet, the diacyl subclass distribution was 81.8 ± 2.9% and for the sunflower oil and tallow fed chickens the distribution was 78.9 ± 1.8% and 76.8 ± 2.5% respectively. The alkylacyl and alkenyl subclasses for the three dietary groups were, 6.7 ± 0.9% and 11.5 ± 3.1% for the fish oil fed birds, 7.71 ± 0.6% and 13.5 ± 1.9% for the sunflower oil fed chickens and 6.9 ± 0.7% and 16.3 ± 1.9% for the tallow group.

7.3.2. Phospholipid distribution of ethanolamine phospholipid (PE) subclasses in muscle

The phospholipid distribution for the PE subclasses, diacyl, alkyl-acyl and alkyl-enyl for the three dietary treatments are shown in Figure 7.2. Similar to the phospholipid distribution for PC subclasses, the feeding of the dietary fats had no significant effect on the distribution of the PE subclasses. However, unlike the PC subclasses the percentage phospholipid distribution of the diacyl and the alkyl-enyl subclasses were similar. The feeding of fish oil to chickens resulted in a phospholipid distribution of 49.2 ± 14.9% and 46.1 ± 15.9% for the diacyl and alkyl-enyl subclasses respectively. Similarly, the phospholipid distribution for the diacyl and alkyl-enyl subclasses were 55.2 ± 8.0%, 41.5 ± 7.3% for the sunflower oil and 45.7 ± 6.8%, 38.4 ± 14.1% for the tallow fed birds. Whereas, the alkyl-acyl subclass was present at a significantly lower (P<0.01) level in all dietary treatments when compared to both the diacyl and alkyl-enyl subclasses.

7.3.3. Molecular species: effect of fish oil and sunflower oil supplementation on composition of choline phospholipid diacyl subclass

The composition of the molecular species of the breast muscle for the PC diacyl subclass is shown in Table 7.1. The predominant molecular species observed for this phospholipid was found to be 16:0/18:1(n-9) and 16:0/18:2(n-6). These species accounted for 37.2 ± 3.9% and 13.7 ± 1.2% respectively for the fish oil group, 30.45 ± 6.7% and 27.3 ± 2.2% respectively for the sunflower oil fed chickens and 40.6 ± 5.1% and 28.8 ± 5.1% respectively for tallow fed chickens. The proportion of 16:0/18:1 was similar for all three dietary treatments. However, 16:0/18:2 was significantly reduced (P<0.01) in the muscle of the birds fed the fish oil when compared to birds fed either the sunflower oil or tallow. Fish oil feeding resulted in a sixteen fold increase in the
proportion of 16:0/20:5(n-3) (7.9 ± 2.7%) and a five fold increase in 16:0/22:6(n-3) (11.1 ± 2.0%) when compared to chickens fed the sunflower oil diet and this increase was slightly greater when compared to chickens fed the tallow diet. In addition, this increased concentration of 20:5(n-3) and 22:6(n-3) for the fish oil fed chickens was reflected in a corresponding decrease in the concentration of 20:4(n-6) (Figure 7.3). Although the proportion and concentration of the molecular species for the sunflower oil and tallow fed chickens were similar the molecular species 16:0/20:4 and 18:0/20:4 were significantly (P<0.05) elevated in the sunflower oil fed chickens when compared to those fed tallow. On the whole, sunflower oil resulted in an increase in the molecular species containing two or more double bonds and a proportional decrease in molecular species containing one double bond when compared to the tallow fed chickens.
Figure 7.1 Distribution (mean ± SEM; n = 4) of choline phospholipid (PC) subclasses, diacyl [■], alkyl-acyl [□] and alkyl-enyl [▩] in breast muscle of chickens fed fish oil, sunflower oil or tallow. Figure 7.2 Distribution (mean ± SEM; n = 4) of ethanolamine phospholipid (PE) subclasses, diacyl [■], alkyl-acyl [□] and alkyl-enyl [▩] in breast muscle of chickens fed fish oil sunflower oil or tallow.
7.3.4. Composition of the choline phospholipid alkyl-enyl subclass

The composition of the molecular species for the PC alkyl-enyl subclass of the three dietary treatments is given in Table 7.2. The predominant molecular species that was observed in the muscle of birds fed the sunflower oil and tallow diets were 16:0/18:1(n-9) and 16:0/20:4(n-6). However, for the chickens fed the fish oil the predominant species was 16:0/22:6(n-3). In addition, there was a significant decrease (P<0.01) in the concentration of 16:0/18:1 in the muscle phospholipids of the fish oil fed chickens when compared to the sunflower oil and tallow fed groups (18.6 ± 3.6%, 26.9 ± 5.1% and 34.6 ± 7.8% respectively). Conversely, the proportion of 16:0/22:6 was increased 6-fold in the fish oil group when compared to the sunflower oil group and 9-fold when compared to the tallow group (39.1 ± 4.3%, 6.4 ± 0.1% and 4.1 ± 1.1% respectively). As was detected for the PC diacyl subclass, fish oil feeding also increased the proportion of 16:0/20:5(n-3), resulting in a 10-fold increase when compared to the sunflower oil group and a 9-fold increase when compared to the tallow fed chickens. As was shown for the diacyl subclass the increased concentration of 20:5(n-3) and 22:6(n-3) was associated with a decrease in 20:4(n-6) (Figure 7.4). The feeding of 8% sunflower oil, a diet high in n-6 PUFA's, resulted in a molecular species profile that contained individual FA’s that were similar in proportion to that found in the profile of the chickens fed the 8% tallow diet. However, as was observed for the molecular species of the diacyl subclass, the sunflower oil resulted in increased FA’s that contained two or more double bonds and a decrease in species containing one double bond when compared to the tallow fed chickens.

7.3.5. Composition of ethanolamine phospholipid diacyl subclass

The molecular species profile for the PE diacyl subclass is shown in Table 7.3. As observed for the subclasses of the choline phospholipids, the molecular species profile and their proportions were similar for both the tallow and sunflower oil fed chickens. However, unlike the species profile for the choline phospholipids the predominant species of the ethanolamine phospholipid subclass was 18:0/20:4 for the sunflower oil and tallow fed chickens which accounted for 51.1% and 46.3% respectively of the total lipid. However, as for the choline phospholipids, feeding fish oil changed the
predominant molecular species with 18:0/20:5 and 18:0/22:6 accounting for 21.3% and 29.1% of total lipid respectively. These concentrations were significantly higher (P<0.01) than those found in muscle from birds offered the sunflower oil or the tallow diets. As for the choline phospholipids, fish oil feeding increased the concentration of the long-chained PUFA’s 20:5(n-3) and 22:6(n-3) and significantly reduced (P<0.01) the concentration of 20:4(n-6) when compared to the sunflower oil and tallow fed chickens (Figure 7.5).

7.3.6. Composition of ethanolamine phospholipid alkyl-enyl subclass

The distribution of molecular species in the alkyl-enyl PE subclass for the three dietary groups is shown in Table 7.4. As was observed for the other three phospholipids, the feeding of sunflower oil and tallow resulted in profiles that were similar in species type and concentration. The dominant species observed for these dietary groups were 16:0/18:1, 16:0/20:4 and 18:0/20:4, accounting for 17.9%, 10.7% and 14.1% of total lipid respectively for the sunflower oil fed chickens and 24.1%, 12.7% and 10.9% respectively for the tallow fed group. The dominant FA species for the fish oil fed chickens were 16:0/22:6 and 18:0/22:6 and these accounted for 40.1% and 18.9% respectively of the total lipid. Consistent with the observations for the other phospholipids, the feeding of fish oil significantly increased (P<0.01) the proportion of the n-3 species 16:0/22:6 and 18:0/22:6 and significantly decreased (P<0.01) the proportion of 16:0/20:4 and 18:0/20:4 (Figure 7.6) compared to both the sunflower oil and tallow fed chickens.
Table 7.1 Comparison of the molecular species of choline phospholipid diacyl subclass in chicken breast muscle (mol %)

<table>
<thead>
<tr>
<th>Molecular Species</th>
<th>Fish Oil</th>
<th>Sunflower Oil</th>
<th>Tallow</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:1-20:5</td>
<td>0.47 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.09 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.06 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0-20:5</td>
<td>7.91 ± 2.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.48 ± 0.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.27 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0-22:6</td>
<td>11.05 ± 2.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.98 ± 0.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.98 ± 0.19&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:1-22:5</td>
<td>0.58 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.22 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.38 ± 0.17&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:1-20:4</td>
<td>_</td>
<td>0.33 ± 0.15</td>
<td>0.34 ± 0.06</td>
</tr>
<tr>
<td>16:0-22:5</td>
<td>3.40 ± 0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.92 ± 0.86&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.16 ± 0.30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0-20:4</td>
<td>3.11 ± 0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.43 ± 2.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.34 ± 1.17&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0-20:5</td>
<td>2.39 ± 0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.75 ± 0.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.35 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:1-18:2</td>
<td>0.62 ± 0.43</td>
<td>1.56 ± 2.25</td>
<td>1.72 ± 0.47</td>
</tr>
<tr>
<td>18:0-22:6</td>
<td>_</td>
<td>_</td>
<td>2.23 ± 0.26</td>
</tr>
<tr>
<td>16:0-18:2</td>
<td>13.73 ± 1.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.28 ± 2.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.81 ± 5.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0-22:5</td>
<td>1.45 ± 0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.72 ± 0.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.39 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0-20:4</td>
<td>1.25 ± 0.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.92 ± 0.72&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.65 ± 0.39&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0-16:1</td>
<td>0.55 ± 0.08</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>18:1-18:1</td>
<td>1.20 ± 0.48</td>
<td>1.50 ± 0.44</td>
<td>2.13 ± 0.49</td>
</tr>
<tr>
<td>18:0-18:2</td>
<td>3.89 ± 1.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.53 ± 2.94&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.94 ± 1.11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0-18:1</td>
<td>37.18 ± 3.90</td>
<td>30.45 ± 6.66</td>
<td>40.45 ± 5.14</td>
</tr>
<tr>
<td>18:0-22:4</td>
<td>2.83 ± 0.75</td>
<td>3.07 ± 1.23</td>
<td>2.33 ± 0.84</td>
</tr>
<tr>
<td>18:0-18:1</td>
<td>3.35 ± 0.75</td>
<td>3.39 ± 0.63</td>
<td>3.27 ± 0.39</td>
</tr>
<tr>
<td>18:0-16:0</td>
<td>0.43 ± 0.13</td>
<td>0.94 ± 0.76</td>
<td>0.37 ± 0.14</td>
</tr>
<tr>
<td>95.39</td>
<td>96.55</td>
<td>100.16</td>
<td></td>
</tr>
</tbody>
</table>

Composition expressed as mean (mol %) ± SEM for duplicate analysis of muscle samples from four fish oil, sunflower oil and tallow supplemented chickens. Values with different superscripts are significantly different when compared by ANOVA. Total defined as the sum of the molecular species listed.
### Table 7.2 Comparison of the molecular species of choline phospholipid alkyl-enyl subclass in chicken breast muscle (mol %)

<table>
<thead>
<tr>
<th>Molecular Species</th>
<th>Fish Oil</th>
<th>Sunflower Oil</th>
<th>Tallow</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:1-20:4</td>
<td>0.24 ± 0.10</td>
<td>0.40 ± 0.29</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td>18:1-20:5</td>
<td>1.28 ± 0.45</td>
<td>0.64 ± 0.14</td>
<td>0.44 ± 0.07</td>
</tr>
<tr>
<td>16:0-20:5</td>
<td>9.50 ± 4.86</td>
<td>0.92 ± 0.71</td>
<td>1.02 ± 0.41</td>
</tr>
<tr>
<td>18:1-22:6</td>
<td>3.80 ± 0.19</td>
<td>1.05 ± 0.65</td>
<td>0.36 ± 0.14</td>
</tr>
<tr>
<td>18:1-22:5</td>
<td>0.93 ± 0.07</td>
<td>0.61 ± 0.05</td>
<td>0.42 ± 0.18</td>
</tr>
<tr>
<td>18:1-20:4</td>
<td>_</td>
<td>8.05 ± 1.92</td>
<td>6.46 ± 1.85</td>
</tr>
<tr>
<td>16:0-22:5</td>
<td>4.99 ± 0.36</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>16:0-20:4</td>
<td>6.47 ± 1.48</td>
<td>24.39 ± 3.25</td>
<td>22.69 ± 3.61</td>
</tr>
<tr>
<td>18:0-20:5</td>
<td>1.25 ± 0.40</td>
<td>2.21 ± 0.51</td>
<td>1.94 ± 0.41ab</td>
</tr>
<tr>
<td>18:1-18:2</td>
<td>_</td>
<td>3.48 ± 0.83</td>
<td>1.89 ± 0.33ab</td>
</tr>
<tr>
<td>18:0-22:6</td>
<td>4.22 ± 1.08</td>
<td>1.05 ± 0.12</td>
<td>0.78 ± 0.15ab</td>
</tr>
<tr>
<td>16:0-18:2</td>
<td>1.87 ± 0.12</td>
<td>4.12 ± 0.56</td>
<td>6.77 ± 1.99ab</td>
</tr>
<tr>
<td>16:0-22:4</td>
<td>_</td>
<td>5.06 ± 0.90</td>
<td>5.85 ± 1.00</td>
</tr>
<tr>
<td>18:0-22:5</td>
<td>0.33 ± 0.05</td>
<td>0.76 ± 0.39</td>
<td>_</td>
</tr>
<tr>
<td>18:0-20:4</td>
<td>1.83 ± 0.60</td>
<td>5.86 ± 0.87</td>
<td>4.04 ± 0.41ab</td>
</tr>
<tr>
<td>18:1-18:1</td>
<td>1.44 ± 0.37</td>
<td>3.28 ± 0.70 ab</td>
<td>3.15 ± 0.55b</td>
</tr>
<tr>
<td>16:0-18:1</td>
<td>18.55 ± 3.57</td>
<td>26.95 ± 5.07</td>
<td>34.58 ± 7.76</td>
</tr>
<tr>
<td>18:0-18:2</td>
<td>1.10 ± 0.40</td>
<td>1.75 ± 0.41</td>
<td>1.59 ± 0.40</td>
</tr>
<tr>
<td>18:0-22:4</td>
<td>0.71 ± 0.33</td>
<td>0.93 ± 0.47ab</td>
<td>1.35 ± 0.29b</td>
</tr>
<tr>
<td>18:0-18:1</td>
<td>0.62 ± 0.37</td>
<td>1.21 ± 0.26  ab</td>
<td>0.79 ± 0.23ab</td>
</tr>
<tr>
<td>18:0-16:0</td>
<td>0.09 ± 0.05</td>
<td>0.11 ± 0.07</td>
<td>_</td>
</tr>
</tbody>
</table>

Composition expressed as mean (mol %) ± SEM for duplicate analysis of muscle samples from four fish oil, sunflower oil and tallow supplemented chickens. Values with different superscripts are significantly different when compared by ANOVA. Total defined as the sum of the molecular species listed.
Distribution (mean ± SEM; n = 4) of EPA (20:5n-3), DHA (22:6n-3) and AA (20:4n-6) in choline phospholipid (PC) diacyl subclass (Figure 7.3) and choline phospholipid (PC) alkyl-enyl subclass (Figure 7.4) in breast muscle of chickens fed fish oil [■], sunflower oil [□] or tallow [▩].
Table 7.3 Comparison of the molecular species of ethanolamine phospholipid diacyl subclass in chicken breast muscle (mol %)

<table>
<thead>
<tr>
<th>Molecular Species</th>
<th>Fish Oil</th>
<th>Sunflower Oil</th>
<th>Tallow</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:1-20:5</td>
<td>1.45 ± 0.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>_</td>
<td>0.25 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0-20:5</td>
<td>3.91 ± 0.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.50 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.45 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0-22:6</td>
<td>7.01 ± 1.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.66 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.53 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:1-20:4</td>
<td>0.73 ± 0.32</td>
<td>0.51 ± 0.14</td>
<td>0.55 ± 0.19</td>
</tr>
<tr>
<td>16:0-22:5</td>
<td>1.23 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.36 ± 0.49&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.09 ± 0.67&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0-20:4</td>
<td>1.71 ± 0.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.65 ± 0.93&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.30 ± 0.46&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0-20:5</td>
<td>21.31 ± 5.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.73 ± 0.87&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.79 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:1-18:2</td>
<td>1.90 ± 2.47</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>18:0-22:6</td>
<td>29.09 ± 7.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.48 ± 1.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.05 ± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0-18:2</td>
<td>1.70 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.37 ± 0.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.92 ± 1.46&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0-22:5</td>
<td>3.04 ± 0.77&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.11 ± 0.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.67 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0-20:4</td>
<td>10.25 ± 0.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.09 ± 2.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46.25 ± 5.21&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0-16:1</td>
<td>0.62 ± 0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.20 ± 0.73&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.93 ± 0.37&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:1-18:1</td>
<td>0.71 ± 0.74</td>
<td>0.65 ± 0.49</td>
<td>1.02 ± 0.27</td>
</tr>
<tr>
<td>18:0-18:2</td>
<td>5.16 ± 0.90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.01 ± 0.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.23 ± 0.55&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0-18:1</td>
<td>4.80 ± 0.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.89 ± 1.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.20 ± 3.47&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0-22:4</td>
<td>0.20 ± 0.11</td>
<td>0.18 ± 0.04</td>
<td>_</td>
</tr>
<tr>
<td>18:0-18:1</td>
<td>4.09 ± 0.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.39 ± 0.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.86 ± 0.61&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0-16:0</td>
<td>0.13 ± 0.08</td>
<td>0.20 ± 0.08</td>
<td>0.21 ± 0.06</td>
</tr>
<tr>
<td>99.81</td>
<td>97.98</td>
<td>98.30</td>
<td></td>
</tr>
</tbody>
</table>

Composition expressed as mean (mol %) ± SEM for duplicate analysis of muscle samples from four fish oil, sunflower oil and tallow supplemented chickens. Values with different superscripts are significantly different when compared by ANOVA. Total defined as the sum of the molecular species listed.
Table 7.4 Comparison of the molecular species of ethanolamine phospholipid alkyl-enyl subclass in chicken breast muscle (mol %)

<table>
<thead>
<tr>
<th>Molecular Species</th>
<th>Fish Oil</th>
<th>Sunflower Oil</th>
<th>Tallow</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:1-20:4</td>
<td>0.12 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.10 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.06 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:1-20:5</td>
<td>0.78 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.95 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.44 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0-20:5</td>
<td>3.04 ± 1.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.30 ± 0.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.92 ± 0.24&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:1-22:6</td>
<td>6.44 ± 2.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.43 ± 0.67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.79 ± 0.46&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0-22:6</td>
<td>40.99 ± 1.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.35 ± 1.92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.88 ± 0.99&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:1-20:4</td>
<td>1.47 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.03 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.83 ± 0.14&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0-22:5</td>
<td>3.63 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.87 ± 0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.39 ± 1.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0-20:4</td>
<td>2.30 ± 0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.71 ± 0.99&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.73 ± 0.74&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0-20:5</td>
<td>2.80 ± 0.66</td>
<td>2.87 ± 0.69</td>
<td>3.40 ± 0.72</td>
</tr>
<tr>
<td>18:1-18:2</td>
<td>_</td>
<td>3.53 ± 0.54</td>
<td>3.14 ± 0.99</td>
</tr>
<tr>
<td>18:0-22:6</td>
<td>18.94 ± 2.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.40 ± 2.65&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.44 ± 2.58&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0-18:2</td>
<td>0.68 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.78 ± 0.51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.24 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0-22:4</td>
<td>6.22 ± 1.20</td>
<td>7.22 ± 1.20</td>
<td>7.22 ± 1.20</td>
</tr>
<tr>
<td>18:0-22:5</td>
<td>1.51 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.26 ± 0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.94 ± 0.31&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0-20:4</td>
<td>1.68 ± 0.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.08 ± 2.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.92 ± 1.66&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:1-18:1</td>
<td>1.10 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.12 ± 0.89&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.88 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0-18:1</td>
<td>10.22 ± 3.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.88 ± 3.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.11 ± 3.77&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0-18:2</td>
<td>0.78 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.63 ± 0.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.71 ± 0.59&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0-22:4</td>
<td>0.37 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.10 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.90 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0-18:1</td>
<td>1.11 ± 0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.67 ± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.13 ± 0.40&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0-16:0</td>
<td>0.12 ± 0.11</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td></td>
<td>98.08</td>
<td>97.25</td>
<td>99.05</td>
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</table>

Composition expressed as mean (mol %) ± SEM for duplicate analysis of muscle samples from four fish oil, sunflower oil and tallow supplemented chickens. Values with different superscripts are significantly different when compared by ANOVA. Total defined as the sum of the molecular species listed.
Distribution (mean ± SEM; n = 4) of EPA (20:5n-3), DHA (22:6n-3) and AA (20:4n-6) in ethanolamine phospholipid (PE) diacyl subclass Figure 7.5 and (D) ethanolamine phospholipid (PE) alkyl-enyl subclass Figure 7.6 in breast muscle of chickens fed fish oil [■], sunflower oil [□] or tallow [▩].
7.4. DISCUSSION

The present study demonstrates that the feeding of specific dietary fats to broiler chickens alters the FA molecular species profile of the breast muscle phospholipids. Feeding 8% fish oil to chickens increased the total content of long chained PUFA’s from the n-3 series, those being EPA (20:5n-3) and DHA (22:6n-3) into all subclasses from the PC and PE glycerophospholipids. Whereas, the feeding of 8% sunflower oil, an oil high in LA (18:2n-6), increased the total content of the n-6 PUFA’s, LA (18:2n-6) and AA (20:4n-6).

A major finding of this study was that feeding commercial grade edible tallow at the same dietary concentration gave a similar molecular species concentration and profile in both the PC and PE phospholipids and their subclasses as observed for the chickens fed the sunflower oil diet. This finding is of interest, as the dietary fatty acid concentration of LA (18:2n-6) for the tallow diet was approximately 60% less than that was found in the sunflower oil diet. This suggests that a primary role for the essential dietary fatty acids in chickens is to provide the substrate that can then be selectively assimilated into cell membranes to ensure a consistency of the physical nature of the lipid bilayer. Studies by Hazel and Williams (1990) and Schmid et al. (1995) led them to propose a similar notion. They suggest that the membrane bilayer is regulated to maintain a relatively constant physical state, referred to as ‘homeoviscous’ adaptation. This could simply provide the cell membrane with a uniform fatty acyl environment. Individual phospholipids have the potential to ‘gel out’ at different temperatures and this can cause phase separation, resulting in a membrane environment consisting of solid gel patches whilst others remain in the liquid crystalline phase (Schroeder et al., 1998). Our results support the classical physiological structure with the sn-1 positions occupied by a saturated fatty acid and the sn-2 positions occupied by an unsaturated fatty acid. This form of ‘compulsory mixing’ has been suggested by Hulbert and Else (1999) as a means of limiting phase separation in natural membranes.

In the present study, LA (18:2n-6), EPA (20:5n-3) and DHA (22:6n-3) were the principal dietary fatty acids that were predominantly paired with a 16:0 phosphatidylcholine (PC) moiety, whereas, AA (20:4n-6), EPA (20:5n-3) and DHA
(22:6n-3) were the principal fatty acids that were predominantly paired with 18:0 phosphatidylethanolamine (PE) moieties (see Figure 7.7).

Fig. 7.7

Schematic Diagram of the Incorporation of Dietary Fatty Acids into Phospholipids

**Acylation**

18:2 → 20:5 → 22:6

- **PC**
  - C → 16:0
  - C → 18:1
  - C → 18:2
  - C → P-Choline

**Elongation and Desaturation**

- **PE**
  - C → 18:0
  - C → 20:4
  - C → P-Ethanolamine

- **PE**
  - C → 18:0
  - C → 20:5
  - C → 22:6
  - C → P-Ethanolamine
Studies in rats have shown that EPA (20:5n-3) and DHA (22:6n-3) have a high affinity for acylation to the sn-2 position of plasmalogen phospholipids (Blank et al., 1989; Blank et al., 1994). In addition, AA (20:4n-6) has also been shown to occur in high proportions at the sn-2 position of the molecular species of diacyl PE from ruminant (Scott et al., 1993) and chicken (Blank et al., 1992) muscle. The concentration of the fatty acids from the n-3 and n-6 series incorporated into the phospholipids reflected their dietary fatty acid concentration. For example, chickens fed fish oil displayed a greater proportion of EPA (20:5n-3) and DHA (22:6n-3) than AA (20:4n-6) in both PC and PE phospholipids of the breast muscle. The chickens fed sunflower oil and tallow diets exhibited a greater proportion of LA (18:2n-6) and AA (20:4n-6). This finding is consistent with studies in chickens that show increased levels of EPA (20:5n-3) and DHA (22:6n-3) in the spermatozoan phospholipids after 30 weeks of fish oil supplementation (Kelso et al., 1997) and a corresponding decrease in n-6 fatty acids, specifically LA (18:2n-6) and AA (20:4n-6). A similar result has also been demonstrated in mammals: when rats were fed fish oil there was a significant increase in both the concentration of EPA (20:5n-3) and DHA (22:6n-3) into the ethanolamine plasmalogens while impairing the movement of AA (20:4n-6) between the lipid classes (Blank et al., 1994).

The n-3 fatty acids, EPA (20:5n-3) and DHA (22:6n-3) seem to preferentially displace LA (18:2n-6) rather than oleic acid (18:1n-9) from the sn-2 position in the diacyl subclass of the PC phospholipids. Whereas, in the alkenyl subclass of PC phospholipids, oleic acid (18:1n-9) and LA (18:2n-6) were displaced by EPA (20:5n-3) and DHA (22:6n-3). Scott and co-workers (1993) have also shown a similar substitution for oleic acid (18:1n-9) in ruminants fed fish oil, however, this occurred only in the PC diacyl subclass. This difference in substitution of oleic acid (18:1n-9) by EPA (20:5n-3) and DHA (22:6n-3) may simply reflect a difference in metabolism between avian and ruminant species. This present study has also shown specificity for the n-3 and n-6 fatty acids for different PE and PC phospholipid subclasses. For example, the concentration of LA (18:2n-6) and AA (20:4n-6) was found to be greater for the diacyl subclasses for both PE and PC phospholipids, whereas, the concentration of EPA (20:5n-3) and DHA (22:6n-3) was found to be greater for the alkenyl subclasses of both these phospholipids.

The major tissue phospholipids are PC and PE. Hermier et al., (1999) have shown
in both Landes and Poland geese that PC accounted for approximately 70% and PE accounted for approximately 25% of the total phospholipid. The dominance of PE and PC as the major phospholipids also occurs in mammals (Schmid et al., 1995). In addition, membrane studies using cultured murine neuroblastoma cells have shown that fatty acids are rapidly incorporated and esterified into phospholipids and that the incorporation into PC is more rapid when compared to other phospholipids (Chakravarthy et al., 1986). The current study also suggests that both EPA (20:5n-3) and DHA (22:6n-3) are remoulded between the PE and PC phospholipids (see Figure 7.8).

Fig. 7.8

Remodelling of EPA (20:5n-3) and DHA (22:6n-3)

Remodelling of phospholipid molecular species has been shown to involve the deacylation-reacylation pathways from the combined actions of phospholipases and acyltransferases, and transacylases (Balsinde et al., 1995; Tamashita et al., 1997). Both EPA (20:5n-3) and DHA (22:6n-3) are present in a high proportion for the PE and PC phospholipids. The high concentration of these two species may also be due to the dietary intake as vertebrates are only able to synthesise de novo saturated and monounsaturated
chains (Hulbert and Else 2000). Schmid and co-workers (1995) have reported that only a limited number of molecular species of PC and PE are remodelled by deacylation-reacylation at either the $sn$-1 or $sn$-2 position of glycerol. Included among this limited number is DHA (22:6n-3). Other studies indicate that PC DHA (22:6n-3) can be formed from the specific methylation of PE DHA (22:6n-3) (Vance 1988; Samborski et al., 1993).

The PC phospholipid generally occurs on the outer leaflet of the cell membrane structure, whereas PE occurs primarily on the inner leaflet of cell membranes (Zachowski, 1993; Clore et al., 1998). The importance of these two phospholipids and their associated molecular species may have direct affects on physiological function. In addition to their influence on membrane fluidity, cell fusion, protein activity and VLDL synthesis and secretion (Yao and Vance, 1988; Yeagle, 1989) these phospholipids have also been shown to modulate tissue sensitivity to the metabolic hormones. For example, a recent study by Clore and co-workers (1998) demonstrated altered insulin sensitivity in man that was negatively correlated to both the degree of membrane saturation and fatty acid elongation. Interestingly, this effect was only observed for the PC phospholipids and not in the PE phospholipids. Indeed, the ability of the n-3 fatty acids to interchange between the PE and PC phospholipids may explain to some extent some of the reported findings that show a positive relationship between the consumption of fish and other marine oils containing high concentrations of EPA (22:5n-3) and DHA (22:6n-3) and insulin sensitivity (Storlien et al., 1987; Popp-Snijders et al., 1987).

The major essential fatty acid for poultry has been shown to be LA (18:2n-6) and is important for chick growth, egg production and egg size (Balnave, 1970; Watkins 1991). However, our results also suggest that the n-3 series fatty acids may also be important, as both EPA (20:5n-3) and DHA (22:6n-3) were significantly incorporated into the breast muscle phospholipids from the chickens fed the three dietary treatments. Interestingly, EPA (20:5n-3) and DHA (22:6n-3) were not present in the dietary fatty acid profile for either the sunflower oil or tallow diets. While these diets did contain a small concentration of $\alpha$-linolenic acid (LNA 18:3n-3), it was only 1.5% of the total dietary lipid. Therefore, EPA (20:5n-3) and DHA (22:6n-3) of the phospholipids from these birds presumably were synthesised from LNA (18:3n-3) through the sequence of alternating
desaturation and chain-elongation reactions (Cook, 1991). The importance of n-3 fatty acids to animals is indicated by studies showing significant concentrations of DHA (22:6n-3) in the lipids of chicken retina (Rezanka, 1989) and nervous tissue (Budowski and Crawford, 1986; Anderson et al., 1989). Fatty acids from the n-3 and n-6 series are important in the development of the neuronal membranes of the retina and brain of mammals (Clandinin et al., 1980; Fleisler and Anderson, 1982; Martinez, 1992). Recent studies in guinea pigs by Sinclair and co-workers (1997) shows a positive correlation between retinal function and DHA (22:6n-3) concentrations as retinal function was reduced following reductions in retinal DHA (22:6n-3) concentrations. In addition to these effects, n-3 fatty acids have been implicated in the mediation of immunity (Klassing and Johnson 1991; Calder, 1999) and bone development (Watkins, 1995).

The occurrence of n-3 fatty acids in major phospholipids also provides a mechanism for cells to modulate the availability of different precursors for eicosanoid production (prostaglandins, leukotrienes and lipoxins) (Watkins, 1995). The fatty acids, dihomogammalinolenic acid (DGLA; 20:3n-6), EPA (20:5n-3) and AA (20:4n-6) are the precursors for the series 1- 3- and 2- eicosanoids respectively (Watkins, 1995), whereas DHA (22:6n-3) also produces prostaglandins termed docosanoids (Clandinin and Jumpsen, 1997). Eicosanoids are highly potent and can function locally as autocrine/paracrine cell-to-cell regulators (Watkins, 1995). Their biological effects are often antagonistic and are closely associated with their rate of synthesis (Lands et al., 1992). For example, AA (20:4n-6) is synthesised via the cyclo-oxygenase, lipoxygenase and epoxygenase enzymic pathways (Wainwright, 1997); its rate of synthesis can be modulated by n-3 PUFA competition for the desaturase/elongase enzymes in PUFA formation or by n-3 PUFA inhibition of cyclo-oxygenase (Simopoulos, 1991). Thus, the n-3 fatty acids can act to competitively attenuate the rate of n-6 formation (Lands et al., 1973). It is conceivable therefore, that the presence of both EPA (20:5n-3) and DHA (22:6n-3) observed in the PE and PC phospholipids of the breast muscle in the present study may be to modulate the synthesis of the series 2 eicosanoid formation derived from AA (20:4n-6) by simply supplying the substrate for the series 3 eicosanoids.

In summary, we have shown that the n-3 and n-6 long-chain PUFA’s are preferentially assimilated into the phospholipids of the plasma membrane of chicken
breast muscle. The finding that both these fatty acid series were present in the membranes of all chickens regardless of their dietary regime underscores their importance as an essential requirement for poultry diets. In addition, the finding that the molecular species concentration and profile was similar for both tallow and sunflower oil fed chickens demonstrates the necessity for nutritionists to supply these essential fatty acids at an adequate concentration for growth and development.
CHAPTER 8

GENERAL DISCUSSION

8.1. SYNTHESIS

The studies conducted in this thesis were based largely on the hypothesis that an increase in muscle insulin sensitivity would result in:

1) A decreased reliance on liver lipogenesis to meet the energy demand for protein synthesis and would in turn decrease triglyceride production resulting in less being available for deposition in adipose tissue.

2) An enhanced rate of amino acid uptake into muscle cells with an increased availability of amino acid for protein synthesis.

3) Restoration of the functional integrity of the CNS in regulating food intake. Food intake would therefore be more closely aligned to energy requirements of growth.

To test this hypothesis, the approach taken was to alter the fatty acyl-composition of plasma membranes of muscle and adipose cells by changing the relative dietary intake of polyunsaturated and saturated fatty acids. The altered phospholipid environment would, in turn, modulate the physical and chemical dynamics of the cell membrane and affect its fluidity (or viscosity). Altering cell membrane fatty acyl chains was expected to affect nutrient partitioning by modulating metabolic hormones that influence lipid and protein deposition. This could result from an increase in the sensitivity of both endocrine tissues to stimulation and target tissues to the metabolic hormones so released.

The results of the studies demonstrate that both lipid and protein deposition can be altered by the intake of saturated and polyunsaturated fatty acids from either n-3 or n-6 series. Feeding broilers n-3 or n-6 PUFA’s achieved a similar outcome, a significant
reduction in the abdominal fat pad mass that was accompanied by a modest increase in breast muscle mass. These changes were associated with an alteration in energy substrate utilisation (Chapter 4) and an increase in insulin sensitivity (Chapter 5). When the molecular species profile for the breast muscle for these two dietary groups were examined in Chapter 7 there were significant differences in the incorporation of specific fatty acids into their respective plasma membranes. Broilers fed fish oil had increased levels of the n-3 PUFA’s, EPA (20:5n-3) and DHA (22:6n-3), whereas, birds fed sunflower oil had increased levels of the n-6 PUFA’s, LA (18:2n-6) and AA (20:4n-6).

However, the results from Chapter 7 suggest that the alterations in metabolism and the consequent carcass composition changes may have been achieved through the direct action of excess specific fatty acid subtypes rather than the manipulation of the plasma membrane fatty acid composition. The most significant finding from this thesis was the demonstration that feeding chickens a diet high n-6 PUFA’s resulted in a molecular species profile that was almost identical in both fatty acid subtype and proportion to birds fed tallow. As the physical and chemical dynamics of cell membranes is dependent on the fatty acyl-composition of the phospholipids both with respect to the degree of unsaturation and their chain length, it would suggest that the membrane dynamics from the sunflower oil and tallow dietary groups would be comparable. This raises the obvious question: why are there significant physiological differences between these two dietary groups when their membrane characteristics are similar?

The phospholipid composition of the membrane is important, particularly in respect to changes in hormone signalling and receptor activity (Spector and York 1985; Clandinin et al., 1991). However, this membrane adaptation may be considered to be long-term rather than of a shorter duration where excess specific fatty acid subtypes may be responsible for rapid physiological responses. Support for this notion comes from enzyme studies in rodents that show the activities of fatty acid synthetase, malic enzyme and glucose-6-phosphate dehydrogenase are altered within 2 days of adding 2% linoleic acid (18:2n-6) to a high-carbohydrate, fat-free diet (Allmann and Gibson, 1965). The in vitro modulation of gene transcription rates for fatty acid synthetase mRNA have shown a rapid response, within 3 hours, to dietary n-3 PUFA’s (Clarke et al., 1990) following addition to cell culture media. Indeed, Clarke and Jump (1996) have suggested that
PUFA’s may act directly to modulate gene transcription rather than modifying membrane fatty acid composition and hence altering hormone release and signalling. It is plausible therefore that the physiological effects that are attributed to changes in the plasma membrane lipid composition may result from two discreet biochemical events. Firstly, dietary fatty acids induce a change in the phospholipid composition of the plasma cell membrane that modifies hormone signalling and receptor activity. These changes occur gradually over time, weeks, rather than days and reflect cell turnover rates. While these changes are occurring, rapid physiological effects mediated by specific PUFA’s, are taking place. The data from this thesis suggest that the most fundamental role for the essential dietary fatty acids, from either the n-3 or n-6 series, is to provide a phospholipid environment that maintains the functional integrity of the plasma cell membrane bilayer, the “primary response element”. Cell membrane integrity is fundamental to the maintenance of homeostasis and when this primary requirement is met, additional fatty acids could then be re-directed to other physiological processes, including the modulation of metabolism, the “secondary response element” (Figure 8.1).

The molecular species profile of breast muscle was similar for both the sunflower oil and tallow-fed birds but the dietary supply of linoleic acid (18:2n-6) was 60% greater for the sunflower oil group. The physiological differences observed between these two dietary groups, therefore, may be the result of the increased availability of n-6 PUFA’s. The concentration of PUFA’s from the n-6 series that were fed to the tallow group may have only been sufficient to meet the phospholipid requirements for cell membranes with little left to influence metabolism. The notion that a specific threshold concentration of either n-3 or n-6 fatty acids is required to influence metabolic responses is supported by studies that show dietary n-3 and n-6 fatty acids suppress hepatic lipogenesis. This occurs only if the level of fatty acid intake is four- to five-fold greater than that needed to fulfil the essential fatty acid requirement for optimal growth (Clarke and Jump, 1993).

The modulation of lipid metabolism by increasing the availability of PUFA’s may account for the significant reduction in the abdominal fat pad mass seen in broilers fed the 8% fish or sunflower oil diets compared to the birds fed 8% tallow (Chapter 4). The results demonstrate that the feeding of either fish or sunflower oil changes the pattern of energy utilisation compared to feeding tallow. The lower respiratory quotients (RQ)
observed in birds offered the fish and sunflower oil is consistent with the preferential oxidation of excess n-3 and n-6 PUFA’s: the reduction in circulating triglyceride and insulin concentrations support this observation. Increased availability of n-3 and n-6 PUFA’s may alter carcass composition simply by stimulating fatty acid oxidation, thereby decreasing the availability of fatty acids for triglyceride synthesis; thus less triglyceride is available for storage in adipose tissue resulting in a leaner bird. This proposed mechanism is consistent with a recent study in man, where it was shown, that as glucose oxidation rates increase there is a corresponding inhibition of fatty acid oxidation, resulting in accelerated triglyceride production (Sidossis et al., 1999).

8.2. FUTURE RESEARCH AND INDUSTRY APPLICATION

Carcass composition can be favourably modulated from a broiler industry perspective using n-3 and n-6 fatty acids. However, these studies suggest that the concentration of dietary fatty acids used may be in excess of the threshold required to achieve these changes. The need to establish the concentration of dietary fatty acid that gives the optimal result is self-evident. The results from this thesis also show that the n-3 and n-6 fatty acids mediate well-defined physiological differences. For example, the findings from Chapter 5 demonstrate that there is a positive relationship between n-3 and n-6 fatty acids and insulin sensitivity, but it is only the n-3 fatty acids that significantly stimulate glucose uptake into breast muscle. Distinct differences between these two fatty acid series were also observed in pituitary sensitivity to growth hormone releasing hormone infusions (Chapter 6). Significantly, both n-3 and n-6 fatty acids modulate energy partitioning and have similar consequences for carcass composition, but appear to do so by different mechanisms. It appears that not only is the n-3:n-6 ratio important in affecting changes in carcass composition of the broiler but these studies suggest that n-3 fatty acids could also be regarded as an essential requirement of broiler diets. Moreover, this notion is particularly relevant in respect to eicosanoid synthesis and their established effects on stress hormone and cytokine production both of which are so important to maintaining normal metabolic function in birds.
**Figure 8.1** PROPOSED MECHANISM OF FATTY ACID MODULATION OF METABOLISM

Dietary Essential Fatty Acids

![Diagram of proposed mechanism]

**RAPID RESPONSE**

- Fatty Acid Synthetase
- (De Novo Fatty Acid Synthesis)

**Primary Response Element**

- Available n-3 & n-6 PUFA’s Re-directed

**Secondary Response Element**

- SUBSTRATE OXIDATION

**LONG TERM EFFECTS**

- n-3 + n-6 PUFA’s Assimilated Into Phospholipids
  - Structure of Membranes
  - Eicosanoid Synthesis
  - Hormone Signalling and Receptor Activity

- FA Oxidation
- Glucose Oxidation
- (Insulin and Triglyceride Levels Decrease)
LITERATURE CITED


Sinclair, A. (1990).**************


Thomas, S.E., Byers, D.M., Palmer, F.B.St.C., Spence M.W. & Cook H.W. (1990). Incorporation of polyunsaturated fatty acids into plasmalogens, compared to other phospholipids of cultured glioma cells, is more dependent on chain length than on selectivity between (n-3) and (n-6) families. Biochem. Biophys. Acta. 1044: 349-356.


