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# Genetic factors affecting the omega-3 and omega-6 fatty acid variation in egg yolk

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*Dedicated to my beloved husband, Le Hoang Tam, for his supports throughout my Ph.D program. This research is also dedicated to my parents, brothers, sisters and all members of my family for their concerns and their encouragements*

Genetic factors affecting the omega-3 and omega-6 fatty acid variation in egg yolk

This study aimed to elucidate the genetic divergence of quail lines selected for high and low ω-6:ω-3 PUFAs ratio in the egg yolk by estimation of the genetic parameters, to clone and characterize of the direct candidate genes, FADS1 and FADS2, and to elucidate the effects of polymorphisms of these genes on the ω-6 and ω-3 fatty acid contents in egg yolk. Furthermore, the expression of the FADS1 and FADS2 genes as well as their polymorphisms in different European (LSL) and Vietnamese chicken breeds (Ac, Noi, H'mong, Ri and Te) was investigated.

The AA and DHA content were significantly lower in the high line than in the low line ($P<0.01$). The ω-6 and ω-3 PUFA ratio was significantly reduced between the low and high lines ($P<0.01$). Moderate heritabilties were found in the C22:6 (ω-3) and ω-6:ω-3 PUFA ratio and the low line is more efficient than the high line.

The quail and chicken cDNA sequences of FADS1 and FADS2 genes were obtained. No significant difference in expression of the two genes was found in both quail and chicken. However, the expression of both genes in the Te and LSL chicken breeds were significantly higher than Ac, Noi, Ri and H'mong chicken breeds.

In quail FADS2 five synonymous SNPs were found, while in FADS1 two of five SNPs resulted in an amino acid substitution. FADS2 was significantly associated with C20:4 (ω-6), C22:6 (ω-3) and the ω-6:ω-3 PUFA ratio ($P<0.05$), whereas FADS1 was significantly associated not only with C14:0, C16:0 and C16:1 (ω-7), but also with C18:2 (ω-6) ($P<0.05$).

Analysis of the quail SNPs in the different European and Vietnamese local chicken breeds revealed that only SNP4 of FADS2 segregated in five out of the six chicken breeds. The two SNPs within the FADS1 gene at position 391 and 468 segregated in Te, Noi, Ri and LSL chicken breeds.

Genetische Einflussfaktoren auf die Omega-3 und Omega-6 Fettsäurenvariation in Eidotter

Ziel der Vorliegenden Arbeit war es, die genetische Divergenz in Wachtellinien, die auf hohes („high line“) bzw. niedriges („low line“) ω-6:ω-3 PUFA Verhältnis selektiert waren, zu eruieren. Die direkten Kandidatengene FADS1 und FADS2 wurden geklont und charakterisiert, sowie der Einfluss von Polymorphismen in diesen Genen auf den ω-6 und ω-3 Fettsäuregehalt in Eidotter untersucht. Weiterhin wurden Expression und Polymorphismen von FADS1 und FADS2 in verschieden europäischen (LSL) und vietnamesischen Hühnerrassen (Ac, Noi, H'mong, Ri und Te) verglichen.

Der AA und DHA Gehalt waren in der „high“ Linie signifikant niedriger als in der „low“ Linie ($P<0.01$). Das ω-6 und ω-3 PUFA Verhältnis zwischen der "low" und der "high" Linie war signifikant reduziert ($P<0.01$). Moderate Heritabilitäten konnten für das C22:6 (ω-3) und für das ω-6:ω-3 PUFA Verhältnis geschätzt werden, wobei die "low" Linie höhere $h^2$ als die "high" Linie erbrachte.

Die cDNA Sequenz von FADS1 und FADS2 wurden von Wachtel und Huhn gewonnen. Allerdings konnte kein signifikanter Unterschied in der Expression zwischen den beiden Arten festgestellt werden. Die Expression der beiden Gene in den Hühnerrassen Te und LSL war jedoch signifikant höher als in den Rassen Ac, Noi, Ri und H'mong.

Im FADS2 der Wachtel konnten fünf synonyme SNPs gefunden werden, während in FADS1 zwei von fünf SNPs zu Aminosäuresubstitutionen führten. FADS2 war signifikant assoziiert mit den C20:4 (ω-6), C22:6 (ω-3) und dem ω-6:ω-3 PUFA Verhältnis ($P<0.05$), wohingegen FADS1 signifikant mit C14:0, C16:0, C16:1 (ω-7) sowie C18:2 (ω-6) assoziiert war ($P<0.05$).

Die Analyse von Wachtel SNPs in den europäischen und vietnamesischen Hühnerrassen ergab, dass nur ein SNP, in FADS2, in fünf von sechs Rassen segregierte. Zwei SNPs im FADS1 Gen an der Position 391 und 468 segregierten in den Rassen Te, Noi, Ri und LSL.

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List of abbreviations

| | |
|---|---|
| AA | : Arachidonic acid, C20:4 ω-6 |
| ACP | : Acyl carrier protein |
| ALA | : Alpha-Linolenic acid, C18:3 ω-3 |
| BLUP | : Best linear unbiased prediction |
| BHT | : Butylhydroxitoluol |
| cDNA | : Complementary deoxy ribonucleic acid |
| CDS | : Coding sequence |
| CHD | : Coronary heart disease |
| COX | : Cyclooxygenase |
| CVD | : Cardiovascular disease |
| DHA | : Docoxahexaenoic acid, C22:6 ω-3 |
| DMSO | : Dimethyl sulfoxide |
| DNA | : Deoxy ribonucleic acid |
| dNTP | : Deoxy nucleotide triphosphate |
| DPA | : Docoxapentaenoic acid, C22:5 ω-3 |
| DTT | : 1, 4, Dithio theritol |
| EDTA | : Ethylenediaminetetraacetic acid |
| EPA | : Eicosapentanoic acid, C20:5 ω-3 |
| ER | : Endoplasmic reticulum |
| EST | : Expressed sequenced tag |
| ExoSAP | : Exonuclease I and Shrimp Alkaline Phosphatase |
| FA gel | : Formaldehyde agarose gel |
| FADS1 | : Fatty acid desaturase 1 |
| FADS2 | : Fatty acid desaturase 2 |
| FAME | : Fatty acid methyl ester |
| FAO | : Food and Agriculture Organization |
| FBAT | : Family based association tests |
| FFA | : Free fatty acid |
| FID | : Flame ionization detector |
| GLA | : Gamma-linolenic acid, C18:3 ω-6 |

GLM : General Linear Models

HDL : High density lipoprotein

ISSFAL : International society for the study of fatty acids and lipids

LA : Linoleic acid, C18:2 ω-6

LDL : Low density lipoprotein

LSL : Lohman selected light

LSM : Least square means

mRNA : Messenger RNA

MUFA : Monounsaturated fatty acid

MW : Molecular weight

NCEP : National Cholesterol Education Program

NRC : National Research Council

OD260 : Optical density at 260 nm wavelength (UV light)

PAGE : Polyacrylamide gel electrophoresis

PC : Phosphatidylcholine

PCR : Polymerase chain reaction

PE : Phosphatidylethanolamine

PI : Phosphatidylinositol

pmol : Picomolar

PS : Phosphatidyl serine

PUFA : Polyunsaturated fatty acid

RACE : Rapid amplification of cDNA end

REML : Restricted maximum likelihood

RNA : Ribonucleic acid

SAP : Shrimp alkaline phosphatase

SFA : Saturated fatty acid

SLS : Sample loading solution

SMART : Switching mechanism at 5' end of RNA transcript

SNP : Single nucleotide polymorphism

SSCP : Single strand conformation polymorphism

TAE : Tris-acetate buffer

TBE : Tris-borate buffer

TE : Tris-EDTA buffer

TEMED : N,N,N',N'-tetramethylethelenediamine

TMSH : Trimethylsulfonium hydroxide

URT : Untranslated region

VLDL : Very low density lipoprotein

WHO : World Health Organization

X-gal : 5-Bromo 4-chloro-3-indolyl-β-D-galactoside

μg : Microgram

μl : Microliter

μM : Micromolar

ω-3 : Omega-3

ω-6 : Omega-6

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# 1 Introduction

Polyunsaturated fatty acids (PUFAs), especially docosahexaenoic acid (DHA, C22:6 ω-3) and eicosapentanoic acid (EPA, C22:5 ω-3) of the ω-3 type are essential for normal development and play an important role in the improvement of human health with respect to e.g. cardiovascular disease, inflammatory response and brain development (Simopoulos 2000). Therefore, decreasing the ω-6:ω-3 PUFA ratio to 5:1 in human diets is considered to improve human health. Research on alternative food sources enriched with ω-3 fatty acids indicated that birds are able to synthesize DHA and EPA from ω-3 PUFAs from the diet by carbon chain elongation and desaturation and deposit these substances into the yolk (Klassing 1998, Bavelaar et al. 2004). There are clear differences between poultry species such as chicken, turkey, duck and goose, given the same basic feed, regarding the deposition of DHA and EPA. In the chicken yolk there clearly was a higher enrichment of DHA than in other poultry species (Surai et al. 1999). The modification of fatty acid composition in the egg yolk by feeding is possible and well-examined (Simopoulos 1988, Caston et al. 1990, Watkins 1991, Cherian et al. 1992). Increasing the ω-3 fatty acid and at the same time reducing the ω-6 fatty acid content of eggs is considered to be a feasible way to improve their nutritional value and make them a beneficial source of DHA and EPA in terms of functional food for improving people's health (Van Elswyk 1997, Leskanich et al. 1997, Simopoulos 2000). Recently, a selection experiment demonstrated that selection for high and low ω-6:ω-3 PUFA ratio is possible in Japanese quail (Mennicken et al. 2005). Until now, however, little is known about the genetic basis of the variation of the ω-3 and ω-6 fatty acid content of the egg yolk and to what extent the ω-3 fatty acid absorption - mainly linoleic acid (LA, C18:2 ω-6) and α–linolenic acid (ALA, C18:3 ω-3) - and endogenous biosynthesis rate or deposition in the egg yolk - mainly arachidonic acid (AA, C20:4 ω-6) and DHA - contribute to the variation.

Advances in molecular techniques elucidate that the biosynthesis of ω-3 and ω-6 fatty acids from the dietary essential fatty acid LA and ALA is catalyzed by the activity of the fatty acid desaturase (FADS) 1 and 2 enzymes. Many studies have focused on the activity of these enzymes, for example, cDNA encoding FADS1 has been isolated in human (Cho et al. 1999b, Leonard et al. 2000), *C. elegans* (Michaelson et al. 1998a, Watts et al. 1999) and fungi (Knutzon et al. 1998, Michaelson et al. 1998b). In addition to FADS1, the activity of FADS2 has been found in vertebrate species such as human (Cho et al. 1999a) and rat (Aki et al. 1999), but also in plant (Sayanova et al. 1997), moss (Girke et al. 1998) and fungi (Zhang et al. 2004). Both genes are members of the

FADS gene cluster which is located on chromosome 5 in chicken and on chromosome 11 in human. The FADS cluster is thought to arise evolutionarily from gene duplication based on its similar exon/intron organization. FADS family members are considered fusion products composed of an N-terminal cytochrome $b_5$-like domain and a C-terminal multiple membrane-spanning desaturase portion, both of which are characterized by conserved histidine motifs.

On the basis of molecular techniques that can be used to elucidate the genetics underlying this variation through the identification of functional candidate genes, the objectives of this study were:

To estimate genetic parameters and direct selection response for the ω-6 and ω-3 PUFA ratio in egg yolk of lines of the differently selected Japanese quail

To clone and characterize the direct candidate genes, FADS2, FADS1 and to elucidate the effects of polymorphisms of these genes on the ω-6 and ω-3 fatty acid contents in egg yolk in these divergently selected Japanese quail lines

To detect the expression of the FADS2 and FADS1 as well as their polymorphisms in different European and Vietnamese chicken breeds.

For this study, the divergently selected Japanese quails for the high and low ω-6:ω-3 PUFA ratio as well as different European and Vietnamese chicken breeds were used.

# 2 Literature review

## 2.1 Roles of omega-3 and omega-6 polyunsaturated fatty acids for human health

The quantity and quality of fat in the diet play an important role in maintaining human health. Many studies have directly concerned the amount and type of fat intake to specific diseases such as cardiovascular disease, hypercholesterolemia, cancer, high blood pressure and obesity. To appreciate the functional roles of fatty acids on human health, the chemical structure of fatty acids, their metabolism and relationship to various diseases are reviewed in this part.

### 2.1.1 Chemical structure and nomenclature of fatty acids

Fatty acids contain a straight chain of carbon atoms with a carboxyl (COOH) group at one end and a methyl group ($CH_3$) at the other end. There are many names for the fatty acids (Table 1). The common name was based on the source of the discovery, for example, palmitic from palm oil or oleic from olive oil. The systematic name is using IUPAC nomenclature to indicate the chemical structure of fatty acids. The carboxyl-reference system indicates the number of carbons, the number of double bonds and the positions of the double bonds counting from the carboxyl carbon. The last name is based on the first double bond counting from the methyl end or omega end of the molecule.

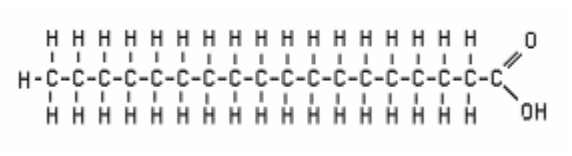
Table 1: Nomenclature of fatty acids

| Names | | Abbreviations | |
|---|---|---|---|
| Trivial | IUPAC | Carboxyl-reference | ω-reference |
| Saturated fatty acids | | | |
| Myristic acid | Tetradecanoic | 14:0 | 14:0 |
| Palmitic acid | Hexadecanoic | 16:0 | 16:0 |
| Stearic acid | Octadecanoic | 18:0 | 18:0 |
| Monounsaturated fatty acids | | | |
| Palmitoleic acid | 7-Hexadecenoic | 16:1 $\Delta^7$ | 16:1 ω-7 |
| Palmitoleic acid | 9-Hexadecenoic | 16:1 $\Delta^9$ | 16:1 ω-9 |
| Oleic acid | 9-Octadecenoic | 18:1 $\Delta^9$ | 18:1ω-9 |
| Polyunsaturated fatty acids* | | | |
| Linoleic acid | 9,12-Octadecenoic | 18:2 $\Delta^{9,12}$ | 18:2 ω-6 |
| | 5,9-Octadecadienoic | 18:2 $\Delta^{5,9}$ | |
| Linolenic acid | 9,12,15-Octadecenoic | 18:3 $\Delta^{9,12,15}$ | 18:3 ω-3 |
| γ-Linolenic acid | 6,9,12-Octadecatroenoic | 18:3 $\Delta^{6,9,12}$ | 18:3 ω-6 |
| Dihomo-γ-linolenic | 8,11,14-Eicosatrienoic | 20:3 $\Delta^{8,11,14}$ | 20:3 ω-6 |
| Arachidonic acid | 5,8,11,14-Eicosatetraenoic | 20:4 $\Delta^{5,8,11,14}$ | 20:4 ω-6 |
| | 5,8,11,14,17-Eicosapentaenoic | 20:5 $\Delta^{5,8,11,14,17}$ | 20:5 ω-3 |
| Docoxahexaenoic acid | 4,7,10,12,16,19-Docosahexaenoic | 22:6 $\Delta^{4,7,10,12,16,19}$ | 22:6 ω-3 |

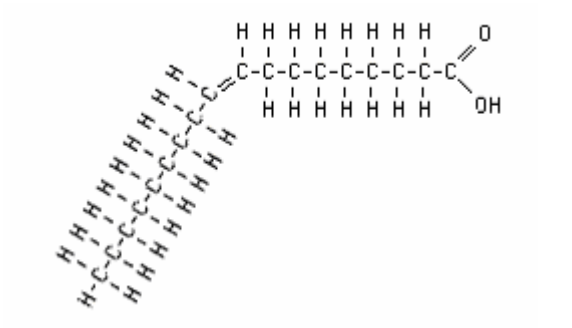
* all double bonds are of the *cis* configuration

Fatty acids can be divided broadly into saturated, monounsaturated and polyunsaturated fatty acids and the properties of these fatty acids depend on the fatty acids composing them. Fatty acids with no double bond are called saturated. Monounsaturated fatty acids have one double bond while polyunsaturated fatty acids have two or more double bonds. Saturated and monounsaturated fatty acids are synthesized independently, while polyunsaturated fatty acids (PUFAs), an important constituent of the diet, cannot be synthesized in the body and they contribute to a multitude of cellular pathways and functions. PUFAs are divided into two main types: the ω-6 and the ω-3 families, which are characterized by the position of first double bond. The ω-3 PUFAs have a terminal double bond at the third carbon from the methyl end of the acyl chain, while the ω-6 PUFAs have a double bond at the sixth carbon

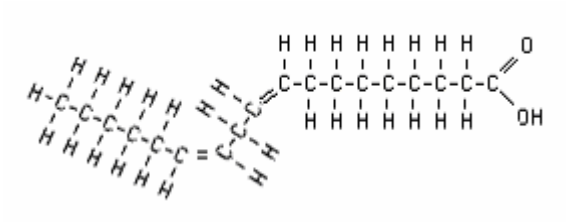
from the methyl end of the chain. The chemical structure of fatty acids is illustrated in Figure 1.



Saturated fatty acid



Monounsaturated fatty acid



Polyunsaturated fatty acid

Figure 1: The chemical structure of fatty acids

### 2.1.2 Digestion, absorption and transportation of fatty acids

Dietary lipids consist mainly of triglycerides and small amounts of phospholipids, cholesterol and its ester. Digestion of fat food is initiated mixing with lingual lipase, followed by hydrolysis of triglycerides in the stomach. Gastric lipase is also important in the initial hydrolysis of fat, especially for the short and medium triglycerides or those with mixed chain lengths (Nelson 2000). In birds, after leaving the gizzard lipid particles are initially sub-solubilized by bile salts and reach the duodenum as triglycerides and phospholipids (Freeman 1984). In addition, pancreatic lipase catalyses the hydrolysis of triglycerides at the first and third position of natural glycerides, leaving 1,2-diacylglycerol and 2-monoacylglycerides. By the action of pancreatic lipase, phospholipids are also hydrolyzed at the second position to free fatty acid with cholesterol, lysophospholipids and glycerol (Freeman 1984, Nelson 2000). These compounds combine with biliary salt to form micelles which are passively absorbed by

an energy-independent mechanism by which the lipolytic products pass from the micelle into the mucosa.

The absorption process from the intestinal lumen to the enterocyte is by passive diffusion across the plasma membrane (Carey et al. 1983). The fatty acids, upon entering the mucosa cells, bind to intracellular proteins depending on the degree of saturation and chain length. Long chain unsaturated fatty acids are bound in preferentially before short and medium chain fatty acids (Ockner et al. 1972, Brindley 1984). In chicken, the jejunum is the major intestinal site of lipid absorption compared to the ileum and duodenum (Newman 2000).

After re-esterification in the intestinal cells, the absorbed lipids are mainly transported in the lymph as chylomicrons, short chain fatty acids enter directly into the portal vein system, whereas medium chain fatty acids are transported in lymph or portal blood depending on their chain length.

Triglycerides, phospholipids, cholesterol and cholesterol esters are present in the plasma lipids as lipoproteins. In addition, there is also a much smaller fraction of unesterified long chain fatty acids (free fatty acids, FFAs) in the plasma.

The FFA is actively metabolized in the plasma membrane during the uptake of plasma triglycerides into the tissues. The rate of FFA production by adipose tissue controls the FFA in plasma because of the directly close relationship between the FFA turnover and the FFA concentration. Therefore, the nutritional diet has not only a strong effect on the fractional uptake of FFA but also alters the proportion of the oxidized and esterified uptake. These FFA are attached to a membrane fatty acid binding protein in the cell, the short chain fatty acids are more hydrophilic and are thus absorbed directly through the cell membrane.

In addition to FFA, lipoproteins are divided into four major groups based on their density that play an important role in physiological and in clinical diagnosis. Chylomicrons are derived from intestinal absorption of triglycerides; very low density lipoproteins (VLDL) are derived from liver for the export of triglycerides; low-density lipoproteins (LDL) are representative final stages in the catabolism of VLDL; and high density lipoproteins (HDL) are involved in VLDL and chylomicrons metabolism and in cholesterol transport. Chylomicrons and VLDL are predominantly represented in triglyceride whereas LDL and HDL are mostly found in cholesterol and phospholipids (Mayes 1996). In the circulating triglycerides, both chylomicrons and VLDL contain apolipoprotein (apo) C and E and are hydrolysed by lipoprotein lipase which is found in the endothelial cells of the liver and is related to chylomicron remnant and HDL metabolism. Moreover, lipoprotein lipase activity involves both phospholipids and apo C-II as cofactors. Thus, chylomicrons and VLDL provide the enzyme for their

metabolism with both substrates and cofactors. Lipoprotein lipase is expressed at different expression levels in different tissues such as heart, adipose tissue, spleen, lung, kidney and it is linked with the nutritional condition. The heart lipoprotein lipase has 10 times lower Km for triglycerides than in adipose tissue enzyme. In the starving condition the decreasing concentration of plasma triglycerides leads to diminish the saturation enzyme in adipose tissue while the heart enzyme remains saturated with substrate, therefore redirecting circulating substrates from adipose tissue toward the heart, resulting in an increase the uptake of lipoprotein triglycerides (Mayes 1996).

### 2.1.3 Membranes and membrane lipids

Membrane lipids serve as biological boundaries for the various cell compartments, thus are important for the life of organisms. The membranes consist of two major components, proteins and a bi-layer lipid membrane. Hulbert and Else (1999) showed that the degree of saturation, the type and the number of double bonds of the lipid are related to the characteristics of the lipid bilayer and the fluidity of membrane lipids. The bi-layer lipid is composed predominantly of phospholipids, cholesterol and a small amount of glycolipids. The glycolipids have a role in the cell surface associated antigens, whereas the cholesterol serves to regulate fluidity and can be found in the lipid portion of the plasma membranes. Phospholipids are important amphipathic molecules essential for cellular membrane formation and function, and are derivatives from either glycerol back bone called glycerophospholipid (phosphoglycerides) or sphingomyelin back bone. The phospholipids have fatty acids which are esterified to the hydroxyl on the first and second carbon, while the third carbon hydroxyl is esterified to phosphate. In membrane lipids, phosphate is in turn esterified to an alcohol of one of the following polar head groups of ethanolamine, choline, serine, glycerol and inositol, they are named phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol, phosphatidylinositol (PI) and diphosphatidylglycerol. PE and PC are the most predominant phospholipids in human (Schmid et al. 1995) and birds (Hermier et al. 1999). The polar position of the phospholipids requires for the electrostatic charge that is needed for the surface associations of specific cell surface proteins.

Furthermore, the activity of cell membranes as well as the equilibrium and dynamical properties of the bi-layer lipids depend on the physical state of the lipids. Cell membranes work well when their lipids are in the liquid crystal state which relies on the chain length and saturation of fatty acids attached at the first and second carbon of the

phospholipid. Membranes whose phospholipid fatty acids are saturated are less fluid than those membranes containing polyunsaturated fatty acids in their phospholipids.

### 2.1.4 Functional fatty acids

As mentioned above, dietary fats including quality and quantity of fats have effects not only on their absorption, transportation and metabolism but also on the membrane physiological properties.

Saturated fatty acids (SFA) are nonessential fatty acids because they can be synthesized by human. It is commonly accepted that diets high in saturated fat raise plasma total cholesterol and LDL-cholesterol leading to a high risk of coronary artery disease, diabetes and obesity (Grundy 1997), while monounsaturated fatty acids, oleic and stearic acid, may decrease plasma total cholesterol and LDL-cholesterol (Kris-Etherton et al. 1999). A similar result is found when SFAs are replaced with polyunsaturated fatty acids (Goodnight et al. 1982). Linoleic acid (LA, ω-6) and α-linolenic acid (ALA, ω-3) are called essential fatty acids that cannot be synthesized by humans and thus must be obtained from diets. They serve as precursor for eicosanoids. The principal eicosanoids of biological significance to humans are a group of molecules derived from C20 fatty acid, arachidonic acid (AA, ω-6) and eicosopentaenoic acid (EPA, ω-3), especially. These eicosanoids reside in the membrane phospholipid bilayer of cells (Nakamura et al. 1996, Zhou et al. 2001) and have a variety of physiological functions including eicosanoid signalling (Funk 2001), pinocytosis (Schmidt et al. 1999), ion channel modulation (Kang et al. 1996) and regulation of gene expression (Clarke et al. 1994).

In addition, studies have focused on prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs) derived from those of AA and EPA which are produced via cyclooxygenase (COX) and lipoxygenase (LOX). Supplement dietary of DHA and EPA conduce to reduced production of 2-series prostaglandins (PG2), thromboxanes (TX2) and 4-series leukotrienes (LT4) which can promote inflammation and vasoconstriction, stimulate platelet aggregation. Moreover, the 3-series prostaglandins (PG3) and thromboxanes (TX3) and 5-series leukotrienes (LT5), are also potent pro-inflammatory mediator that acts as chemotaxis for neutrophils and increases their adhesion to vessel endothelium, respect to active vasodilators and inhibitors of platelet aggregation (Lewis et al. 1986, Weber et al. 1986, Hutchins 2005). Furthermore, DHA is a prominent fatty acid in cell membranes and especially abundant in neural and retinal tissue. For that reason, DHA is essential in visual and neurological development, particularly in premature infants. Whereas, increasing the ω-6 fatty acids, particularly AA in the diet,

contributes to the eicosanoid formation and the competition with ω-3 fatty acids for the COX or LOX enzymes, thus leading to antagonistic action in physiological functions.
When interpreting the physiological significance of ω-3 fatty acids in human health, more attention should be paid to the correct balance of ω-6 and ω-3 fatty acids in diets with respect to not only the various diseases but also the neonatal growth and development.

### 2.1.5 Dietary recommendations for the polyunsaturated fatty acids

Understanding the important role of dietary fatty acids associated with human diseases, the developed countries especially in North America and Northern European countries have implemented new policies to improve the current consumption patterns that provide health benefits. The typical Western diets are currently related to high intake of SFA that are linked to health problems such as cardiovascular disease, diabetes and obesity. For optimal human health, total fat and cholesterol should be reduced in the diet, particularly, the intake of saturated fatty acids should be decreased, the intake of polyunsaturated fatty acids increased. According to the recommendation of the National Cholesterol Education Program (NCEP) and the American Heart Association, the total fat intake should be less than 30% of energy content in the diet and saturated fat intake limits less than 10% of energy from fat (Krauss et al. 1996, Lichtenstein 2003). In association with the recommendation to reduce intake of saturated fatty acids, trans-fatty acids should be less than 0.7% of total energy intake (Okamoto et al. 1999) and daily intake of cholesterol should be no more than 300 mg (Dixon et al. 2001). Moreover, the National Research Council (NRC) recommendation for the total PUFA intake should be at 7% of energy (NRC 1989). In addition to optimal PUFA daily intake, the recommendation of linoleic acid is 1% of energy (Sanders 2000), α-linolenic acid is 0.5 to 2.5 % of energy (Voskuil et al. 1996) and ω-3 PUFA including EPA and DHA is 0.5% of energy (British Nutrition Foundation, 1992). Based on these recommendations, a number of dietary supplements containing EPA and DHA have been proposed for different lifestyle choices for the overall health, for example daily intake of EPA and DHA for adults is 0.65 g, during pregnancy and lactation a DHA intake of 300 mg/d must be ensured (Simopoulos et al. 1999), and the daily intake for the mental health is 2 to 4 g (Haag 2003). The recommendations of EPA+DHA intake for the general population are shown in Table 2.

Table 2: Dietary recommendations for intake of polyunsaturated fatty acids

| | EPA + DHA (g/d) | Source |
|---|---|---|
| Canada | 1.2-1.6 | Scientific review committee (1990) |
| NATO | 0.8 | Leskanish et al. (1997) |
| ISSFAL | 0.65 | Simpoulos et al (1999) |
| US | 0.65 | Kris-Etherton et al. (2000) |
| WHO-NATO | 0.3-0.5 | Kris-Etherton et al. (2002) |
| WHO FAO | 0.4-1.0 | 2003 |
| Amerian Heart Association | 1.0 | Kris-Etherton et al. (2002) |
| UK SACN | 0.45 | Gebauer et al. (2006) |
| US Food and Drug Administration | <3.0 | Smith (2005) |

The current diets in Western countries contain low levels of ω-3 PUFAs with concomitant high levels of ω-6 PUFAs, i.e. the ω-6: ω-3 PUFAs ratio is about 25:1 (Simopoulos 1991). Therefore, the desirable dietary ω-6:ω-3 PUFA ratio recommendations in some countries are shown in Table 3.

Increased ω-3 PUFA intakes vitamin E levels which is an antioxidizable substrate should be considered. Daily intake of vitamin E is 0.4 mg/g LA, with 3.2 to 10.4 mg for men and 2.5 to 8 mg for women (Leskanich et al. 1997).

Despite guidelines for an adequate quantity of these essential fatty acids have been well established, scientists are still searching for better ratio of ω-6:ω-3 PUFA as well as finding suitable food sources of long-chain polyunsaturated fatty acids to satify the discussed health guidelines.

Table 3: Recommendations for intake of the ω-6:ω-3 PUFA ratio

| Country | ω-6:ω-3 PUFA ratio | Reference |
|---|---|---|
| Australia | 8:1 | Ollis et al (1999) |
| Canada | 4:1 | Holub (2002) |
| UK | 6:1 | Widdowson (2005) |
| USA | 9.8:1 | Kris-Etherton et al. (2002) |
| Japan | 4-4.5:1 | Okita et al. (1995) |
| Japan | 4:1 | Sugano et al. (2000) |
| FAO/WHO | 5-10:1 | Trautwein (2001) |
| Germany | 5:1 | Trautwein (2001) |

## 2.2 Sources of long-chain polyunsaturated fatty acids

Seafoods, e.g. salmon, hering, mackerel, trout and sardin are considered to be a good source for the ω-3 fatty acids, especially EPA and DHA. The dietary recommendations for optimal health are at least two meals per week with a high amount of sea-fish. However, the modification of food enriched with fish does not correspond to the consumer preferences because of a number of factors including the preference for fresh or frozen steaks, price perception and income. Besides, the world population increased in recent years while marine resources continued decreasing because of the expansion of the deep-sea fishing and also the environmental impacts on fish farms (Pauly et al. 2002, Hites et al. 2004). Furthermore, the high levels of mercury accumulates with in some kinds of fish are also of concern. Therefore, seafoods are unlikely to be a sustainable solution to efficiently supply these valuable fatty acids in a long term approach.

An alternative approach for increasing ω-3 fatty acid supplements may be by pharmacologic intake. However, the danger of an uncontrolled overdose by the user exists and it is also problematic because of palatability when consuming these "enriched" foods.

Poultry products, e.g. poultry meat and in particular eggs, may provide an exciting alternative food source due to its large acceptance as well as a source of ω-3 fatty acids (Van Elswyk et al. 1992), despite a consistently high fat content in the egg yolk which contains appoximately 33g fat/ 100g yolk or 6g fat/ egg (Noble et al. 1990). The enrichment of ω-3 fatty acids in eggs plays an important role in increasing the nourishing food for a part of the population. In fact, the egg yolk has a high digestibility, a balanced amount of amino acids, bioavailable sources of carotenoids, lutein and

zeaxanthin (Handelman et al. 1999). Besides, egg yolk contains high vitamins (A, E, K, B1, B2, B6), folic acid and particular mineral sources (Na, K, Ca, P, Mg, F, Fe) (Burrington 2000).

The egg is regularly consumed in large quantities in many Western countries and it seems to have increased when measured by the total egg consumption or per capita egg consumption. According to Speedy (2003), per capita egg consumption is 384 eggs in Japan, 320 eggs in France, 322 eggs in the Netherlands, 294 eggs in Denmark, 290 eggs in America, 288 eggs in Mexico and 274 eggs in Malaysia. In Germany, the average egg consumption is 244 eggs per-capita (Speedy 2003). From these data, the average daily consumption is about 3,7 g yolk fat which comprises about 34% saturated fatty acids, 45% monounsaturated fatty acids and 18% polyunsaturated fatty acids, thus yolk fat contains less saturated than unsaturated fatty acids. With regard to the beneficial effects of unsaturated fatty acids, yolk fatty acid composition corresponds to the recommendations of NCEP and DGE (1991), i.e. the dietary intake of fat should not exceed 10% saturated fatty acids as well as at least 30% monounsaturated fatty acids and polyunsaturated fatty acids. Furthermore, an enriched egg can provide approximately 400 mg ω-3 PUFA containing about 290 mg of EPA and DHA, thus the hen egg can supply a half amount of the recommended daily intake of the ω-3 PUFA (Farrell 1998). However, the ω-6:ω-3 PUFA ratio in the egg yolk is still too high.

## 2.3 Changing the fatty acid profile in the egg yolk

### 2.3.1 Changing by feeding

Poultry species are able to convert from the diet to long chain polyunsaturated fatty acids, DHA and EPA, by desaturation and elongation from their preliminary fats such C18:2 (ω-6) and C18:3 (ω-3) (Farrell 1994). The ω-3 PUFA in the egg yolk can be increased by changing the dietary fat of the laying hens and it has been well examined (Leskanich et al. 1997).

The distribution of various dietary fats contribute to increasing the ω-3 PUFA of yolk fat. Fish oil and seed oil products are successfully used as an alternative source of DHA and EPA in the laying hen's diet. For example, hens fed with a diet containing 10% of flax seed oil could transfer ω-3 fatty acid to the egg with approximately 264 mg of ALA and 92 mg of DHA and EPA (Ferrier et al. 1995). Farrell (1995) showed that hens fed with a diet of alga product or fish oil increased the ω-3 PUFA deposition in yolk to 470

mg/egg, containing 250 mg EPA and DHA. Furthermore, the ω-6:ω-3 PUFA ratio in the yolk fatty acid was reduced approximately from 11-14:1 to less than 2:1 (Caston et al. 1990, Cherian et al. 1992, Farrell 1995, Eder et al. 1998). Thereby, an enriched ω-3 egg can meet the daily consumption of ω-3 PUFA as the current recommendations (Table 2 and 3).

However, Van Elswyk et al. (1992) have shown that a hen's diet containing 3% menhaden oil was able to differently taste and flavour between ω-3 enriched and control scrambled eggs. It is suggested that to enhance the yolk ω-3 fatty acid, fish oil should be not over 3% in the hen diet. Besides, the levels of dietary antioxidants should be increased in order to prevent unwanted smell and flavour derives in the product (Leskanich et al. 1997).

### 2.3.2 Breeding changes the fatty acids

The nutritional manipulation in the laying hen's diets including the sources of ω-3 fatty acids promotes the deposition of these nutrients into egg yolk (Leskanich et al. 1997). There are clear differences between poultry species such as chicken, turkey, duck and goose, given the same basic feed, regarding the deposition of DHA and EPA. In the chicken yolk there was clearly a higher enrichment of DHA (Leskanich et al. 1997, Surai et al. 1999) (Table 4). Differences in yolk fat content have been found on different strains and breeds (Washburn 1979) or different chicken breeds and hybrid origins (Ahn et al. 1995, Scheideler et al. 1998). In addition, the fatty acid profiles of the yolk can be greatly affected by age or between breed and age of the hens (Noble et al. 1986, Washburn 1990, Scheideler et al. 1998). The body fat content of the hen also affects to the yolk fat. The study of Hargis (1988) supported that the egg yolk cholesterol are not correlated to cholesterol levels of laying hens, however an increase of the antioxidable substrates as vitamin E, carotenoide with the increase of the PUFA content in the yolk is desirable (Cherian et al. 1997, Hartfiel et al. 1997, Surai et al. 1997). The absorption rate of linoleic acid (LA C18:2 ω-6) and particularly α-linolenic acid (ALA C18:3 ω-3) increases with the age of the hens (Scheideler et al. 1998) and the ω-6:ω-3 PUFA ratio in the egg yolk changes in the process of the laying period.

Table 4: Fatty acid composition of the egg yolk of different poultry species

| Fatty acid | Chicken | Turkey | Goose | Duck |
|---|---|---|---|---|
| 14:0 | 0.4 | 0.5 | 0.7 | 0.5 |
| 16:0 | 25.8 | 28.9 | 31.2 | 26.4 |
| 16:1 ω-7 | 2.1 | 8.8 | 3.8 | 2.7 |
| 18:0 | 8.6 | 7.9 | 7.0 | 6.4 |
| 18:1 ω-9 | 40.5 | 39.5 | 41.9 | 47.0 |
| 18:1 ω-7 | 1.6 | 3.3 | 2.0 | 1.9 |
| 18:2 ω-6 | 14.7 | 8.6 | 9.3 | 5.6 |
| 18:3 ω-3 | 0.4 | 0.3 | 0.4 | 0.3 |
| 20:1 ω-9 | 0.3 | 0.2 | 0.4 | 0.5 |
| 20:4 ω-6 | 1.7 | 1.2 | 2.3 | 4.0 |
| 22:6 ω-3 | 1.6 | 0.4 | 0.3 | 0.6 |

Adapted from Surai et al. (1999)

Furthermore, results of Zaky et al. (1996) pointed out that a selection on "egg mass feeding utilization" over eight to nine generations, reduced the content of saturated fatty acids in chickens eggs and increased the ratio of polyunsaturated fatty acids to saturated fatty acids (P/S ratio).

Recently, our research group showed that genetic variance is present in the poultry which can be used for the enrichment of ω-3 fatty acids and lowering of the ω-6:ω-3 PUFA ratio (Mennicken et al. 1997, Mennicken et al. 2000). However, up to now little is known about the genetic basis of the variation of the ω-3 and ω-6 fatty acid content of the egg yolk and to what extent the ω-3 fatty acid absorption, mainly on LA and ALA, and endogenous biosynthesis rate or deposition in the egg yolk, mainly on AA and DHA, contribute to variation.

## 2.4 Egg formation and fat deposition

### 2.4.1 Composition of eggs

The egg contains three components including shell, yolk and egg white. The corresponding proportions are given in Table 5. The major components of yolk are lipids and proteins (2:1). The yolk contains 15.7 to 16.6% protein, 31.8 to 35.5% lipid, 0.2 to 1.0% carbohydrate and 1.1% ash in water (Powrie and Nakai 1986). All lipids are deposited into the yolk during maturation. The yolk lipids consist of triacylglyceride

(63%) which is considered a neutral lipid, phospholipid (30%) which consists of phosphatidyl choline or lecithin and cholesterol (5–6%) (Table 6). These lipids are associated with at least two proteins, vitellin and vitellenin (Shenstone 1968).

Table 5: The components of the egg

| | Estimated mean | Reported values |
|---|---|---|
| | % of whole egg | |
| Shell | 10.5 | 7.8 – 13.6 |
| Yolk | 31.0 | 24.0 – 35.5 |
| White | 58.5 | 53.1 – 68.9 |
| Total edible contents | 89.5 | 86.4 – 92.2 |

(Shenstone 1968)

Within yolk triacylglyceride, oleic acid is the most predominant and it is twice as much as the phospholipids (Table 7). A high amount of AA and DHA in comparison with other components are present in phospholipids (Leskanich et al. 1997). Many studies have shown that the fatty acid composition of the diet directly affects the fatty acid composition of the total phospholipid of the yolk (Balnave 1970, Halle 1997). However, the dietary fatty acid composition will not alter the total amount of saturated fatty acids in total yolk lipid. The alteration in the yolk fatty acids is effected mainly by an increase in the linoleic acids with concurrent decrease in the oleic acid content when there is an increase in dietary fatty acids containing two or more double bonds (Coppock et al. 1962, Chen et al. 1965, Summers et al. 1966).

Table 6: Major lipids (% of total weight) in the yolk

| | Chicken[1] | Pharaoh[2] | Golden[2] | White[2] |
|---|---|---|---|---|
| Cholesterol esters | 1.3 | 1.13 | 1.16 | 1.00 |
| Triglycerides | 63.1 | 58.67 | 64.12 | 52.13 |
| Free fatty acids | 0.9 | - | - | - |
| Free cholesterol | 4.9 | 3.51 | 3.89 | 2.98 |
| Total cholesterol | 6.2 | 4.64 | 4.85 | 3.98 |
| Phospholipids | 29.7 | - | - | - |
| Phosphatidylethanolamine | 23.9 | - | - | - |
| Phosphatidylcholine | 69.1 | - | - | - |
| Phosphatidylserine | 2.7 | - | - | - |

[1] chicken yolk (Noble 1987)

[2] quail yolk of different origin (Tarasewicz et al. 2004)

Table 7: Fatty acid compositions of the yolk (% total weight)

| Fatty acid | Triacylglyceride | Phospholipid |
|---|---|---|
| 16:0 | 24.5 | 28.4 |
| 16:1 ω-7 | 6.6 | 1.9 |
| 18:0 | 6.4 | 14.9 |
| 18:1 ω-9 | 46.2 | 29.5 |
| 18:2 ω-6 | 14.7 | 13.8 |
| 18:3 ω-3 | 1.1 | 0.3 |
| 20:4 ω-6 | 0.3 | 6.2 |
| 22:6 ω-3 | <0.2 | 4.1 |

Leskanich et al. (1997)

### 2.4.2 Yolk formation and fat deposition

The yolk is formed not only in the growing follicle and the ovary but also in the liver of the laying hens and it is prompted by estrogenic stimulation (Gilbert 1972). The yolk comprises two kinds of yolk; white yolk is formed in the second stage and contains more protein and yellow yolk is accumulated in the third stage of the yolk formation and contains more fat (Figure 2).

Yolk is formed in one of many follicular sacs of the ovary; the cell membrane of ovum is surrounded by a non-cellular vitellin membrane and then hen's liver takes lipid nutrients from the bloodstream to the ovary and turns them into yolk.

During the yolk development the liver produces two major yolks, vitellogenin and VLDL which are taken up from the blood by developing oocytes in the ovary via receptor-mediated endocytosis. This oocyte vitellogenesis receptor (OV receptor) belongs to the LDL receptor superfamily that shows high sequence identity with the mammal VLDL receptors, which is an essential receptor in avian species. Receptor-deficient mutant hens are sterile and exhibit severe hyperlipaemia with aortic artherosclerosis (Schneider 1996, Bujo et al. 1996). Vitellogenin is synthesized by estrogenic hormones from the ovary and is taken up into the ovum.

When yolk matures, the follicle ruptures along a line relatively free from blood vessels known as the stigma, and the yolk is released. After a few hours the yolk is coated with an albumen layer and the shell membranes are deposited during the egg passing into the isthmus. Continuously the calcification takes place in the uterine region and also pigment is formed. Finally, the egg passes into the vagina and cloaca for laying.

These physiological bases of the egg formation show that the nutrient composition of the yolk depends substantially on the synthesis of the components and their preliminary stages in the liver. The liver supplies the lipid stored in the yolk and this organ is considered as "candidate tissue" for the identification of fatty acids of the yolk.

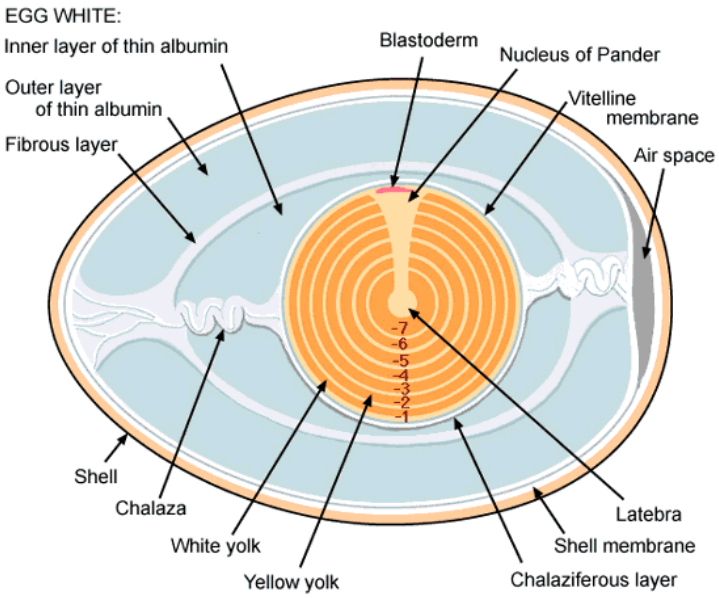



Figure 2: Egg yolk formation
(adapted from Mountney 1989)

## 2.5 Biochemical metabolism of unsaturated fatty acids - candidate genes for fatty acid profiles in the egg yolk

### 2.5.1 Classification and characteristics of desaturase enzymes

Fatty acid desaturases can be classified in two general classes:
(i) The acyl carrier protein (ACP) desaturases are soluble desaturases which are localized in plant plastid (Shanklin et al. 1998);
(ii) The membrane-bound desaturases are divided into two subgroups, acyl-CoA desaturases which are located in endoplasmic reticulum (ER); and acyl lipid desaturases which are localized in the membranes of cyanobacterial thylakoid, plant endoplasmic reticulum and plastid (Shanklin et al. 1994, Murata et al. 1995, Shanklin 1998).
In animals, fatty acid desaturases are nonheme iron-containing enzymes that introduce a double bond at a defined carbon at the $\Delta$4, $\Delta$5, $\Delta$6, $\Delta$8 and $\Delta$9 position but not at the

Δ12 or Δ15. These reactions occur in the endoplasmic reticulum and utilise acyl-CoA as substrates and require $O_2$ and NAD(P)H which comprises NAD(P)H-cytochrome $b_5$ reductase, cytochrome $b_5$ and a terminal desaturase. In the process of double bond formation, the membrane bound cytochrome b5 transfers electron by lateral diffusion from NADH cytochrome b5 reductase to the terminal desturase. Alignment of the amino acid sequences of membrane-bound desaturases reveal three conserved his-boxes that contain eight histidine residues $HX_{(3-4)}H$, $HX_{(2-3)}HH$ and $H/QX_{(2-3)}HH$. These histidine residues have been implicated in the binding of di-iron, necessary for catalytic activity (Napier et al. 1997, Michaelson et al. 2002).

### 2.5.2 Biosynthesis pathways of unsaturated fatty acids

Birds are able to synthesize DHA and EPA from ω-3 PUFAs from the discriminate foods by carbon chain elongation and desaturation and deposit these substances into the yolk. In birds, liver is an active site for the synthesis of fatty acids compared to adipose tissue (Volpe and Vagelos 1973, Bloch and Vance 1977, McGarry and Foster 1980). The biosynthesis pathways of fatty acids in birds are similar to those that have been described in mammals (Sprecher 1981). The biosynthesis of unsaturated fatty acids are supported by desaturase enzymes, which catalyze the introduction of double bonds into preformed acyl chains by removal of a pair of hydrogens, concomitant oxidation of an electron donor and reduction of $O_2$ (Shanklin et al. 1998, Girke et al. 1998). The essential fatty acids have different metabolic pathways which are influenced by dietary fat, type and the amount of essential fatty acids. The main pathway for *de novo* synthesis of fatty acids occurs in the cytoplasm.

*Δ9-desaturase or stearoyl-CoA-desaturase (SCD)*

Stearoyl–CoA desaturase (SCD) is the rate-limiting enzyme in the biosynthesis of monounsaturated fatty acids by introducing the double bond at the 9 to 10 position of the carboxyl end of fatty acids (Nakamura et al. 2004). The preferred desaturation substrates are mainly palmitoleic and oleic acids which are converted to palmitoleoyl–CoA (C16:1) and oleoyl–CoA (C18:1), respectively (Volpe et al. 1973, Enoch et al. 1976, Sprecher 1981, Kasturi et al. 1982, Wakil et al. 1983, Ntambi 1995, Ntambi et al. 1999). These reactions require oxygen ($O_2$), NADH and an electron transport sequence comprising NADH-cytochrome $b_5$ reductase, cytochrome $b_5$ and SCD (Nakamura et al. 2004). The deduced amino acid sequences indicate that this enzyme contains three

conserved histidine motifs which are essential for enzyme activity (Shanklin et al. 1994).

SCD has been cloned in rat (Thiede et al. 1985, 1986, Strittmatter et al. 1988), mouse (Madsen et al. 1997, Ntambi et al. 2003), chicken (Prasad et al. 1979), human (Li et al. 1994, Cadena et al. 1997) and carp (Tiku et al. 1996, Macartney et al. 1996).

*Δ6-desaturase or FADS2*

LA (C18:2 ω-6) and ALA (C18:3 ω-3) are essential fatty acids (EFAs) and considered as precursors of long chain ω-6 and ω-3 fatty acids. Δ6-desaturase, one of these rate-limiting enzymes, catalyzes the bioconversion of the C18:2 into C18:3 and C24:4 into C25:5 in the ω-6 series and of the C18:3 into C18:4 and C24:5 into C24:6 in the ω-3 series (Voss et al. 1991, Sprecher et al. 1995) (Figure 3). LA is rapidly incorporated into tissue and complex lipids and elongated and desaturated to AA (Lands et al. 1990), whereas ALA is even more strikingly eliminated from the tissues. ALA is slowly converted to EPA and DHA. In the contrary, AA may be metabolized at a much faster rate than DHA (Adam et al. 1986).

When in diet essential fatty acids are deficient or absent, Δ6-desaturase enzyme will introduce double bonds into the n-9, n-12, and n-15 positions of the carbon chain of n-9 series (Figure 4) by desaturation of eicosaenoic acid to eicosatrienoic acid or “mead acid” which is characteristic for essential fatty acid deficiency (Fulco et al. 1959, Mead 1968, Retterstol et al. 1995, Mayes 1996, Fokkema et al. 2002). This long chain PUFA is neither an “essential fatty acid” nor replaces AA or compensate for the deficiency of EFAs symptoms, however, it could be combined into the same tissues and complex lipids as AA (Nelson 2000).

So, the Δ6-desaturase participates in at least three reactions for the conversion of LA, ALA and C24:5 (ω-3) into their respective products of fatty acids (Inagaki et al. 2003).

The activity of Δ6-desaturase enzyme has been studied in vertebrate species such as human (Cho et al. 1999a) and rat (Aki et al. 1999), but also in plant (Sayanova et al. 1997), moss (Girke et al. 1998) and fungi (Zhang et al. 2004).

Analysis of amino acid sequences has shown that Δ6-desaturase contains an N-terminal cytochrome $b_5$-like domain together with heme binding motifs. HDxGH, HFQHH and QIEHH are the three histidine motifs that characterize the membrane-bound desaturase (Shanklin et al. 1994, Los and Murata 1998, Marquardt et al. 2000, Nakamura et al. 2004).

*Δ5- desaturase or FADS1*

Δ5-desaturase is involved in the last step of biosynthesis of long chain PUFAs AA (C20:4 ω-6) from the dihomo-γ-linoleic acid (C20:3 ω-6); and EPA (C20:5 ω-3) from C20:4 (ω-3) (Leikin et al. 1992, Horrobin 1992, Leonard et al. 2000) (Figure 3). Because of competition between ω–6 and ω–3 fatty acids for desaturase and elongase enzymes, the quantity of linoleic acid in the diet can affect the extent of ALA conversion to EPA and DHA (Simopoulos 1988, Ackerman 1995). cDNAs encoding Δ5-desaturase have been isolated in human (Cho et al. 1999b, Leonard et al. 2000), *Caenorhabditis elegans* (Michaelson et al. 1998a, Watts et al. 1999) and *Mortierella alpine* (Michaelson et al. 1998b).

### 2.5.3 Function of Δ9-, Δ6- and Δ5-desaturases

*Function of Δ9-desaturase*

SCD catalyses the synthesis of oleic acid (18:1 ω-9), the main product of this enzyme, present in most tissues as an energy reserve. This fatty acid is a requisite component of membrane phospholipids, triglycerides, cholesterol esters and wax esters that can affect lipoprotein metabolism and adiposity (Miyazaki et al. 2001a,b,c). High SCD activity has been implicated in a wide range of disorders including diabetes, artherosclerosis, cancer, obesity and viral infection (Enser 1975, Khoo et al. 1991, Li et al. 1994, Pan et al. 1994, Jones et al. 1996, Lee et al. 2001, Miyazaki et al. 2001b).

*Function of Δ6- and Δ5-desaturases*

Δ6- desaturase and Δ5- desaturase are the key enzymes for the synthesis of long chain polyunsaturated fatty acids such as AA (C20:4 ω-6) and DHA (C22:6 ω-3) (Emken et al. 1992, Pawlosky et al. 1992, Sprecher et al. 1995, Sprecher 1996) that are incorporated in phospholipids (PLs) and perform essential physiological functions. The Δ5- and Δ6-desaturases are considered as the rate limiting steps in the biosynthesis of long chain PUFAs. Both prefer as substrate fatty acids with double bonds in the ω-6 and, secondarily, the ω-3 position of the carbon chain. DHA (C22:6 ω-3) is the most predominant product whilst DPA (C22:5 ω-3) does not accumulate appreciably when adequate ω-3 fatty acids are in the diets (Sprecher et al. 1995,

Sprecher 1996). Sprecher and co-workers (Sprecher and Lee 1975, Bernert and Specher 1975, Sprecher 1991) have shown that dietary PUFAs are not elongated and then desaturated but rather are desaturated and then elongated. The vital functions of AA and DHA in human health are discussed in the section 2.2.1

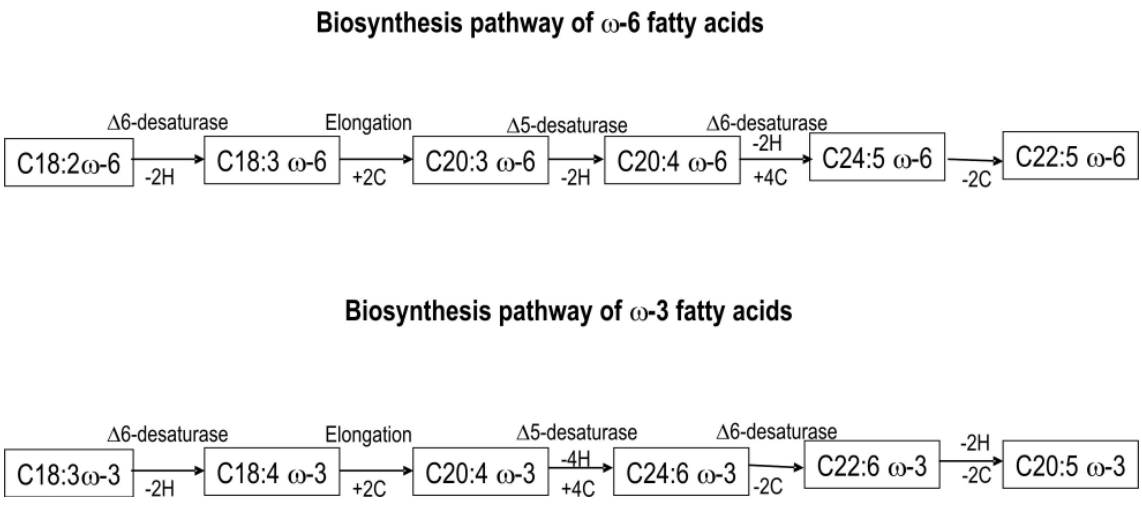



Figure 3: Metabolic pathways for the conversion of dietary UFAs to their LC-PUFA (adapted from Sprecher 1981)

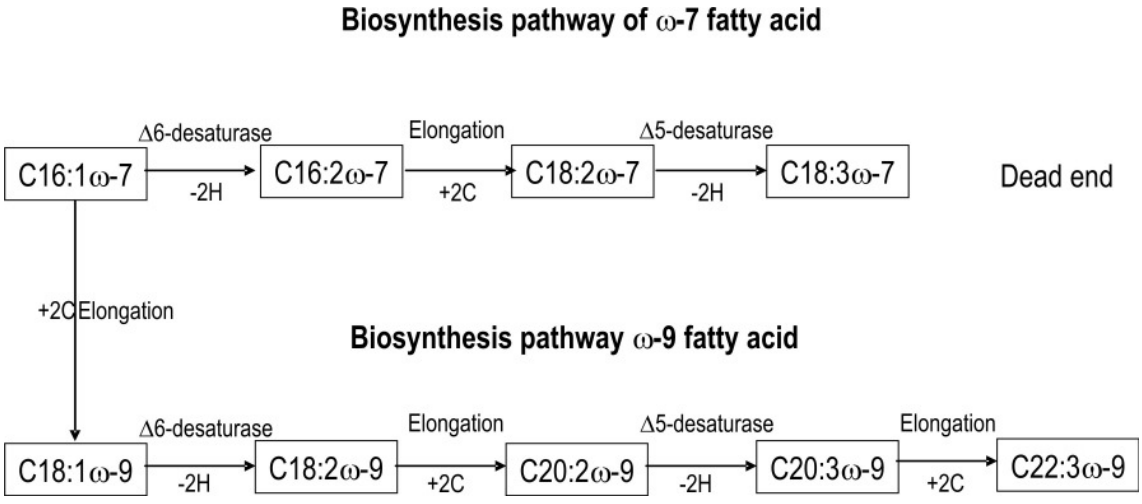



Figure 4: Metabolic pathway for the conversion of dietary C16:1 (ω-7) and C18:1 (ω-9) by the Δ5-, Δ6- desaturases
(adapted from Sprecher 1981)

### 2.5.4 Expression and factors regulating Δ6- and Δ5- desaturases

The availability of long chain C20 and C22 PUFAs greatly depends on the activity of the enzymes involved in the biosynthesis pathways by Δ6- and Δ5- desaturases and elongases (Specher 1981). Both, Δ6- and Δ5- desaturases are expressed in different tissues, such as in adrenal gland, liver, brain, testis in both rat (Matsuzaka et al. 2002) and human (Cho et al. 1999b) with the highest level of expression in liver (Scott et al. 1989). In rat testis, the Δ6- and Δ5- desaturases have the same expression pattern (Matsuzaka et al. 2002). However, the expression and activity of the Δ6- and Δ5- desaturases depend on age which depletes the Δ6- desaturase level in the testes and liver in rat (Horrobin 1981).

The activity of the Δ6- and Δ5- desaturase enzymes is mainly controlled by nutritional and hormonal factors (Wakil et al. 1983, Brenner 1989). The synthesis of fatty acids increases in response to the low dietary fat intake and decreases when the intake is high (Newman 2000). Furthermore, dietary ω-3 and ω-6 use the same enzymes of Δ5- and Δ6- desaturases in the biosynthesis pathway leading the competition between ω-3 and ω-6 acids for these enzymes in order to produce their final products. Therefore, the activities of these enzymes can probably be altered by diet and by hormone status (Brenner 1989).

*Food*

Dietary saturated fatty acids are effective in suppressing *de novo* fatty acid synthesis. A higher content of saturated fats and trans fatty acids can inhibit the activity of Δ6-desaturase and thereby lead to a decrease of the ratio between ALA and LA (Wahl et al. 2002). Moreover, studies on rats showed that rats fed with a diet of fish oil displayed less desaturase activity than rats fed a beef tallow diet or a linseed oil diet. When these diets were enriched with cholesterol, the desaturase activity was reduced, especially the Δ5-desaturase (Garg et al. 1988). Another study has demonstrated that the higher the corn oil contents in the diet, the lower the expression of both desaturases (Rodriguez-Cruz et al. 2006).

Dietary PUFAs suppressed the genes involved in fatty acid synthesis including SCD, Δ5- and Δ6- desaturases (Holloway et al. 1975, Clarke et al. 1994, Ntambi et al. 1996). In diets high in ω-3, most of Δ5-desaturase is used for the ω-3 pathway and thus little is available to convert DGLA into AA while in a low ω-3 diet, most of Δ5-desaturase is

ready for conversion DGLA to AA (Emken et al. 1992). The enzymatic activity of both desaturases is reduced by diabetes (Holman et al. 1983, Igal et al. 1991, Poisson et al. 1991) or by fasting and it is induced by re-feeding carbohydrate (Brenner 1989, Poisson et al. 1991).

*Hormones*

Despite these different physiological roles of SCD, Δ5- and Δ6-desaturases, these desaturases share common regulatory features including dependence of expression on insulin, glucagons, adrenaline, glucocorticoids and andrenocorticotropin hormones (Mandon et al. 1987, Nakamura et al. 2002). Many studies have concerned the effect of insulin, which up regulates the desaturase activities, especially in Δ6-desaturase (Mandon et al. 1987, Brenner 1989, Saether et al. 2003). Besides, the activity of Δ5-desaturase is suppressed by the hormone glucagons (Brenner 2003) while Δ6-desaturase is inhibited by adrenaline (Joshi and Aranda 1979, Brenner 2003).

## 2.6 Molecular genetic background and strategies for candidate gene identification and influence on the fatty acid profiles.

Nowadays, the combination of genetic and molecular approaches has given more evidences how genes determine the physical traits to develop products and practices for use by society. For example, in plant it can be used to alter the amount of the acyl groups normally in a species or to introduce exotic acyl groups (Slack and Browse 1984, Somerville and Browse 1988, Hammond and Glatz 1989, Hills et al. 1991, Somerville 1993, Kinney 1997). In animals, the genetic selection as well as feeding practices resulted in a considerable reduction in muscular fat (Rhee 1992) or milk fatty acids (Karijord et al. 1982). Furthermore, linkage mapping or positional cloning has successfully characterized the affect of back fat on porcine chromosome 4 by a large QTL (Andersson et al. 1994, Knott et al. 1998, Walling et al. 1998), fatness traits on chicken (Jennen 2004). Therefore, based on the selection of a population, genetic regions associated with the fatty acid profiles can be identified. This approach could compliment classical genetic selection programs currently used to modify the fatty acid composition of egg yolk. ω-6:ω-3 PUFA ratios are desired in the first step of the selection process. Through selection, the genetic value of the animals in a population is estimated and it is affected by genetic variation in the population, accuracy of selection and generation (Mennicken et al. 2000, 2005).

Quails (*Coturnix coturnix*) are particularly used as avian model in many fields of biological and medical research, especially it is considered as a standard laboratory animal because it requires short generation intervals and also their genetic parameters are similar to the other poultry species (Wilson et al. 1961). Because of that, quail contribute an important role in agricultural production. The first genetic linkage map of the Japanese quail was reported by using microsatellites (Pang et al.1999, Kayang et al. 2000, 2004) or the amplified fragment length polymorphism (AFLP) markers (Roussot et al. 2003). Recently studies have found the association between the QTL and egg production traits involved in shaping the egg laying curve (Minvielle et al. 2006). However, the limited information that is available on the genetics of the Japanese quail.

Along with quantitative traits, candidate gene identification provides new opportunities for the exploitation by understanding the fundamental biological mechanism. Considerable attention has focused on the beneficial effects of polyunsaturated fatty acids that are converted into long chain polyunsaturated fatty acids, including AA and DHA by the enzymes involved in the conversion of essential fatty acids into longer-chain and highly unsaturated fatty acids e.g. Δ5-desaturase (FADS1) and Δ6-desaturase (FADS2).

Finally, selected candidate genes need to be characterized and examined for their ability to be used in the breeding schemes.

# 3 Material and Methods

## 3.1 Chemicals, reagents and media and commercial kits

### 3.1.1 Chemicals and kits

Biomol (Hamburg): Phenol, Lambda DNA *Eco*91I (BstE II) and Lambda DNA *Hind*III

Biozym Diagnostik (Hessisch-Oldendorf): Sequagel XR sequencing gel (National Diagnostics)

Roth (Karlsruhe): Acetic acid, Ampicillin, Ammonium peroxydisulphate (APS), Butylhydroxitoluol (BHT), Boric acid, Bromophenol blue, Calcium chloride, Chlorofrom, Dimethyl sulfoxide (DMSO), dNTP, Ethylenediaminetetraacetic acid (EDTA), Ethanol, Ethidium bromide, Formadehyde (37%), Formamide, Glycerin, Hydrochloric acid, Hydrogen peroxide (30 %), Isopropyl b-D-thiogalactoside (IPTG), Methanol, N,N´-dimethylform-amide, Nitric acid, Peptone, Proteinase K, Sodium dodecyl sulphate (SDS), Silver nitrate, Sodium carbonate, Sodium chloride, Sodium hydroxide, N,N,N´,N´-Tetramethylethylene-diamine (TEMED), Tris, 5-bromo-4-chloro-3-indolyl-b-D-galactopyra-noside (X-gal), Xylencyanol and Yeast extract.

Larodan Fine Chemical AB: Mixture Me 61, Mixture Me 63, Mixture Me 81

Serva Electrophoresis GmbH (Heidelberg): Acrylamide (molecular biology grade) and Bisacrylamide.

Sigma-Aldrich Chemie GmbH (Taufkirchen): Agarose, Blue dextran, Calcium chloride, Diethyl barbituric acid, Ethylene glycol-bis (2-amino- ethylether)-N,N,N´,N´-tetraacetic acid (EGTA), Isopropanol, Magnesium chloride, Penicillin, Sodium barbiturate, Tri reagent.

### 3.1.2 Reagents and media

All solutions used in this investigation were prepared with deionized and demineralized (Millipore) water and pH was adjusted with sodium hydroxide or hydrochloric acid.

| | | |
|---|---|---|
| APS solution: | Ammoniumpersulfat | 5 g |
| 10% (w/v) | water added to | 50 ml |
| Acrylamide 40% : | Acrylamide | 76g (78.4g) |
| 19:1 (49:1) | Bis-acrylamide | 4g (1.6g) |
| | water added to | 200 ml |
| Acrylamide gels 6% (12%): | 40 % Acrylamide | 6.75 ml(4.5 ml) |

| | | |
|---|---|---|
| | water | 46.25 ml (12.75 ml) |
| | 10% APS (100 mg/ml) | 400 µl (130 µl) |
| | TEMED | 40 µl (10 µl) |
| Acetic acid 10% | Acetic acid | 100 ml |
| | water added to | 1000 ml |
| Blue dextran buffer: | Blue dextran (50 mg/ml) | 1 ml |
| | EDTA 0.5M (186.1 mg/ml) | 50 µl |
| | Formamide | 5 ml |
| IPTG solution: | IPTG | 1.2 g |
| | water added to | 10 ml |
| LB-agar plate: | Sodium chloride | 8 g |
| | Peptone | 8 g |
| | Yeast extract | 4 g |
| | Agar-Agar | 12 g |
| | Sodium hydroxide (40 mg/ml) | 480 µl |
| | water added to | 800 ml |
| LB-broth: | Sodium chloride | 8 g |
| | Peptone | 8 g |
| | Yeast extract | 4 g |
| | Sodium hydroxide(40 mg/ml) | 480 µl |
| | water added to | 800 ml |
| Lysis buffer: | SDS (10%) | 200 µl |
| | Tris-HCl 1M (pH 8.0) | 4 ml |
| | EDTA 0.5M (pH 8.0) | 4 ml |
| | Proteinase K 2% (w/v) | 4.44 ml |
| | Mercaptoethanol | 4 ml |
| | water added to | 200 ml |
| Natrium acetate solution (3M): | Natrium acetate (pH 5.3) | 133.05 g |
| | water added to | 500 ml |
| Nitric acid (1%) | Nitric acid (66%) | |
| | water added to | |
| PAA loading buffer: | Formamide | 98 % (v/v) |
| | EDTA 0.5M (pH 8.0) | 10 mM |
| | Bromophenol blue | 0.5 mg/ml |
| | Xylenzyanol | 0.5 mg/ml |

| | | |
|---|---|---|
| Proteinase K solution: | Proteinase K in 1× TE-buffer | 2% (w/v) |
| Saline | $Na_2HPO_32H_2O$ | 6.19 g |
| | $KH_2PO_4$ | 2.54 g |
| | NaCl | 4.14 g |
| | Formaldehyde (37%) | 125 ml |
| | Distilled water | 100 ml |
| SDS solution: | Sodium dodecylsulfat in water | 10% (w/v) |
| Sequence loading buffer: | Formamide | 83% (v/v) |
| | EDTA 0.5M ( pH 8.0) | 4 mM |
| | Blue dextran | 10m g/ml |
| Silane solution: | Silane | 3 µl |
| | Ethanol 95% (added) | 1 ml |
| Silver staining solution: | Sodium carbonate | 30 g |
| (Development solution) | water added to | 1000 ml |
| | Formaldehyde | 1500 µl |
| Silbernitrate solution: | Silbernitrate | 5 g |
| (0.2%) | water added | 2500 ml |
| SSCP loading buffer: | Formamide | 47.5 ml |
| | Sodium hydroxide | 200 mg |
| | Bromophenol blue | 125 mg |
| | Xylenecyanol | 125 mg |
| 50× TAE-buffer, pH=8 | Tris | 242 mg |
| | Acetic acid | 57.1 ml |
| | EDTA 0.5M (186.1 mg/ml) | 100 ml |
| | water added to | 1000 ml |
| 10× TBE-buffer: | Tris | 108 g |
| | Boric acid | 55 g |
| | EDTA 0.5M (186.1mg/ml) | 40 ml |
| | water added to | 1000 ml |
| 1× TE-buffer: | Tris 1M | 10.0 ml |
| | EDTA 0.5M (186.1 mg/ml) | 2.0 ml |
| | water added to | 1000 ml |
| X-gal: | X-gal | 50 mg |
| | N, N´-dimethylformamide | 1 ml |
| Glycogen | Glycogen | 20 mg |
| | water added to | 1 ml |

### 3.1.3 Commercial kits

| | |
|---|---|
| CEQ™ DNA Size Standard 80 Kit | BECKMAN COULTER™ |
| CEQ™ SNP-Primer Extension Kit | BECKMAN COULTER™ |
| Dye Terminator Cycle Sequencing | BECKMAN COULTER® |
| Exo-SAP-IT® | usb® |
| Gen Elute™ Plasmid Miniprep Kit | Sigma, Eppendorf |
| Oligonucleotide primers | MWG Biotech, Ebersberg |
| PGEM®-T and PGEM®-T Easy Vector Systems | Promega |
| QIA quick PCR purification Kit | Qiagen, Hilden |
| Recombinant RNasin® Ribonuclease Inhibitor | Promega |
| Rneasy Mini Kit | Qiagen |
| RQ1 Rnase-free DNAse | Promega |
| SequiTherm EXCEL™II | Biozym Dianostic |
| Shrimp Alkaline Phosphatase | usb® |
| SMART™ RACE cDNA Amplification Kit | BD Biosciences Clontech USA |
| SuperScript™ II Reverse Transcriptase | Invitrogen |
| Taq DNA polymerase | GENCRAFT |

## 3.2 Equipments

| | | | |
|---|---|---|---|
| Automated sequencer | LI-COR | 4200 | MWG (Ebersberg) |
| Automated sequencer | CEQ 8000 | | Beckman Coulter |
| Centrifuge | HERMLE | Z233MK | HERMLE (Wehingen) |
| Centrifuge | HERMLE | Z323K | HERMLE (Wehingen) |
| Electrophoresis(horizontal) | SUB-cell | GT | BIO RAD (München) |
| Electrophoresis(vertical) | UniEquip | S2S | Uniequip (Martinsried) |
| Electrophoresis(vertical) | Sequi-Gen | GT | BIO RAD (München) |
| Electrophoresis(vertical) | UniEquip | DAIICHI | Uniequip (Martinsried) |
| Gas-chromatograph | | 8500 | Perkin Elmer Autosystem |
| Gel dryer | BIO RAD | 583 | BIO RAD (München) |
| Incubator | Memmert | BB16 | Memmert (Schwabach) |
| Power supply | BIO RAD | Pac3000 | BIO RAD (München) |
| Power supply | BIO RAD | Pac300 | BIO RAD (München) |
| Spectrophotometer(UV) | DU®-62 | PM2K | Unterschleissheim-Lohhof |
| Thermocycler | MJ Research | PTC100 | Biozym, Hess Oldendorf |

| | | | |
|---|---|---|---|
| Thermocycler | Minicycler | PTC150 | Biozym Hess. Oldendorf |
| Thermocycler | BIO RAD | iCycler | BIO RAD (München) |
| Thermoshaker | Gerhardt | - | Gerhardt (Bonn) |
| UV Transilluminator | UniEquip | Uvi-tec | Uniequip (Martinsried) |
| UV/Visible Spectrophoto | Utrospec | 2100pro | Amersham Biosciences |
| Wasserreinigungsanlage | Millipore | Milli Q | Millipore (Eschborn) |
| Wasserreinigungsanlage | Millipore | Milli R | Millipore (Eschborn) |

## 3.3 Softwares

| | |
|---|---|
| BBSRC chickEST database | http://www.chick.umist.ac.uk/ |
| BCM search launcher | http://searchlauncher.bcm.tmc.edu/ |
| BLAST program | http://www.ncbi.nlm.nih.gov/blast/ |
| CEQ 8000 software | Beckman, Coulter, USA |
| ClustalW Multiple Sequence Alignment | http://searchlauncher.bcm.tmc.edu/multialign/Options/clustalw.html |
| Compute pI /Mw tool program | http://www.expasy.ch/tools/pi_tool.html |
| Genepop 3.4 | http://wbiomed.curtin.edu.au/genepop/index.html |
| Image Analysis program (Version 4.10) | LI-COR Biotechnology, USA |
| Multiple sequence alignment by Florence Corpet | http://prodes.toulouse.inra.fr/multalin/multalin.html |
| One-Dscan program | Scanalytics Inc., Billerica, MA |
| Primer design program | Primer express software version 2.0 |
| Primer3 Input | http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi |
| SAS Version 8.2 | SAS Institute Inc., Cary, NC, USA |
| UCSC genome bioinformatics | http://www.genome.ucsc.edu/index.html?org=Chicken&db=galGal2&hgsid=30444323 |
| Webcutter 2.0 | http://rna.lundberg.gu.se/cutter2/ |
| Weight to molar quantity for nucleic acid | http://www.molbiol.ru/eng/scripts/01_07.html |

## 3.4 Animals

### 3.4.1 Selection experiments

The basis breeding population of Japanese quails was kept since 1966. It consisted of four quail lines, with three lines selected for dust bathing activity (NN, HH and KK) and one representing an unselected control line (N24) (Gerken and Petersen 1992). The divergent selection within each of the four pure breed quail lines was performed. The ω-6:ω-3 PUFA ratio and C22:6 (ω-3) were desired in the first step of the selection process. The egg yolks were examined for the fatty acid contents in order to establish the pedigree for the next generation. The breeding value of individuals was estimated and quails were ranked from best to worst. The six hens with highest and six hens with lowest ω-6:ω-3 PUFA ratio in egg yolk of 30 hens were kept by mass selection. The hens were randomly mated with selected cocks of the same line. After four generations of divergent selection, eight sublines of the HIGH and LOW lines are produced (Mennicken et al. 2005). Animals of the 4$^{th}$ generation of these divergently selected lines were previously shown to differ in the ω-6:ω-3 ratio by 2.4 units, i.e. a difference of 1.6 phenotypic standard units ($s_p$=1.57) and four genetic standard units ($s_g$=0.64) (Mennicken et al. 2005). The selection was continuously carried out for three generations of S5, S6 and S7. The hens were kept in individual cages and fed a commercial layer diet.

Selection of generation S5

The production of the S5 generation was performed by selecting approximate six hens of each of the eight sublines from the S4 generation and the cocks were randomly selected from S4 generation due to the estimated breeding value (EBV) for the ω-6:ω-3 PUFA ratio. Therefore, generation five consisted of 78 hens from low line and 74 hens from high lines and 24 cocks per line.

Selection of generation S6

The S6 generation consisted of totally 170 Japanese laying quails (67 hens in the high line and 103 hens in the low line) and 24 cocks from generation S5 based on the breeding value obtained information from 6 hens with highest and six hens with lowest ω-6:ω-3 PUFA ratio of each sub line.

Selection of generation S7

Generation S7 consisted of the hens and cock from generation 6 and produced based on the estimated breeding value for the ω-6:ω-3 PUFA ratio. Therefore, six hens with highest and six hens with lowest ω-6:ω-3 PUFA ratio were selected. Finally, the S7 generation consisted of 58 hens from high line and 81 hens from low line and 24 cocks per line.

The selection process of the high and low lines of Japanese quail is described in Figure 5.

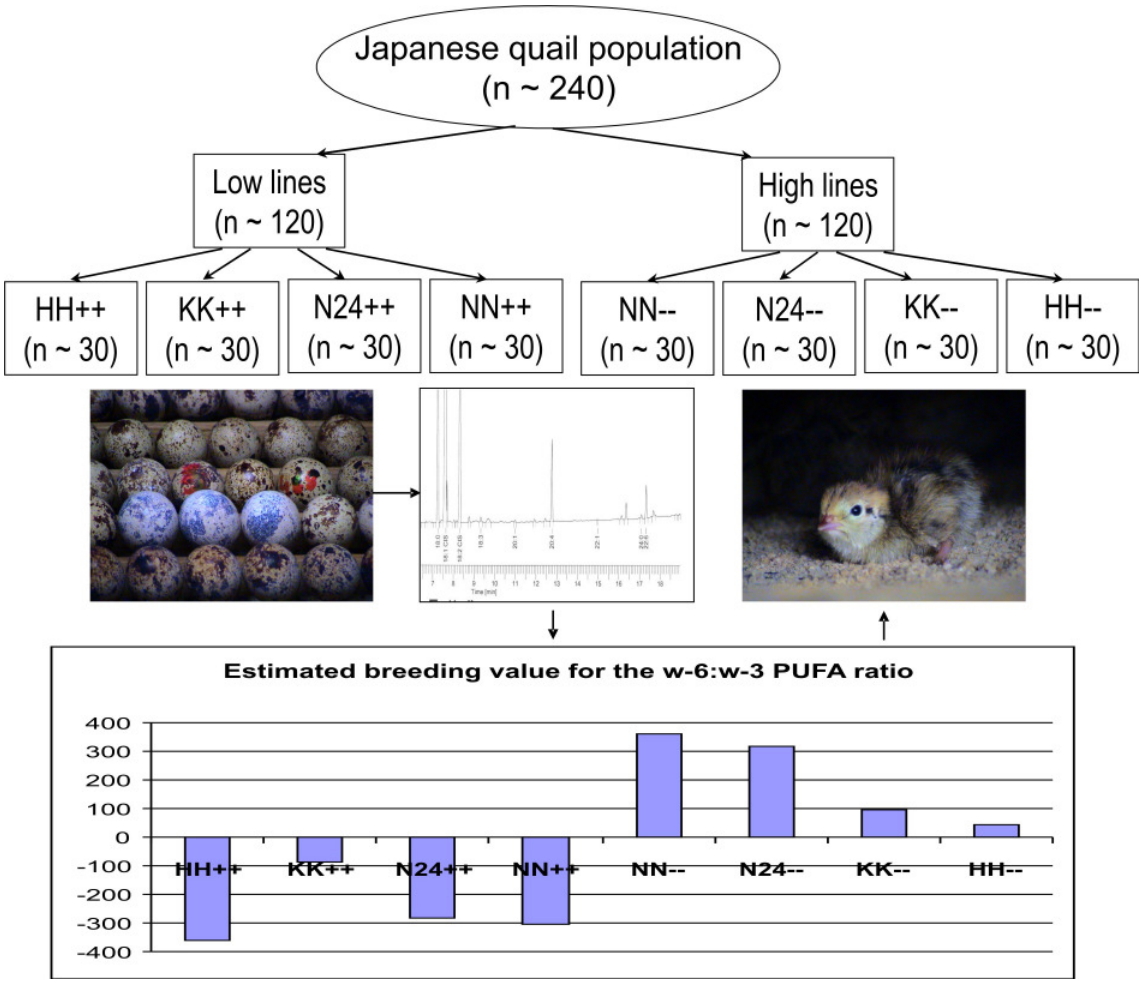



Figure 5: Selection scheme of the low and high lines of Japanese quail based on EBV

### 3.4.2 Feed composition

Quail hens were given a commercial diet containing 19% crude protein, 6% fat and 10.4 MJ/kg ME. Content of linoleic acid was 5 g/kg and vitamin E was 100 mg/kg. Fatty acid composition of feed is presented in Table 13.

### 3.4.3 Phenotypic trait records

#### Egg collection

Three eggss per hens were collected in two times, from day 15 after the first egg was layed onwards and repeated one week later after the first collection.
All hens of the 5$^{th}$, 6$^{th}$ and 7$^{th}$ generation were used for analysing the fatty acid profiles to evaluate the heritability of the ω-6:ω-3 PUFA ratio and C22:6 ω-3 of the eight divergent selected lines of Japanese quails.
In addition, Ri chicken eggs (n=11) were collected from Thuyphuong farm, National Institute of Animal Husbandry, Hanoi, Vietnam.

#### Fatty acid analyses

Yolk lipid was extracted according to the method of Folch et al. (1957). Three egg yolks per hen were magnetically homogenized and 1 g of yolk was dried with 6 g sodium sulphate. It was suspended in 10 ml chloroform-methanol mixture (1:1) containing 0.02% BHT and shaked for 20 min. The supernatant were transferred to a 2.0 ml tube and plunged in a stream of nitrogen. Analysis of the fatty acid composition was carried out by gas chromatography using trimethylsulphoniumhydroxide (TMSH) (Schulte and Weber 1989). A 30 µl of TMSH was added in a total volume of 100 µl sample in a vial for analysis. The fatty acid methyl esters (FAME) were analyzed by gas chromatography (Perkin Elmer Autosystem model 8500) equipped with a flame ionization detector (FID) and a split injector and a capillary column (30 m×0.32 mm inner diameter) with a 0.25 µm film thickness. Helium was used as gas carrier. The GC conditions were as follows injector temperature at 250 °C, the detector 260°C. The column temperature program started at 150°C for 2 min, follwed by an increase of 10°C/min to 180°C/min and held for 5 min, and then 5°C/min to 225°C. Identification of fatty acid methyl ester peaks was based upon retention times obtained for methyl esters prepared from the three standards of fatty acid (Mixture Me 61, 63 and 81) (Larodan, Sweden).

## 3.5 Molecular genetics methods

### 3.5.1 RNA isolation

Total RNA was isolated from quail liver by using the guanidium thiocyanate method (Chomczynski and Sacchi 1987). Liver tissue (60 mg) was homogenized in 500 µl Trizol reagent by syringe and needle, and incubated at room temperature for 5 min. A volume of 200 µl chloroform was added to the homogenised sample and mixed thoroughly by gently shaking and incubated at room temperature for 10 min. The mixture was centrifuged at 12,000 g for 15 min at 4°C. The upper aqueous phase was transferred to a new tube and its RNA was precipitated by adding 500 µl isopropanol, gently shaking. The sample was incubated at room temperature for 10 min and then centrifuged at 12,000 g for 10 min at 4°C. The supernatant was removed and the RNA pellet was washed with 1 ml ethanol (70%) and then centrifuged for 5 min. The supernatant was removed, the RNA pellet was air-dried for 10 min and dissolved in 50 µl RNase-free water and stored at -80°C for further use.
To remove the DNA residue, the RNA was treated with DNase. The reaction contained a mixture of 20 µl RNA, 4 µl RQ1 buffer, 7.5 µl RQ1 DNase, 1 µl RNase inhibitor and 7.5 µl RNase-free water. The mixture was incubated at 37°C for 1 hour and then purified by using RNeasy Mini Kit. The RNA concentration was measured by a spectrophotometer at 260 nm and 280 nm with the best ratio of RNA of approximately 1.7-1.8. RNA quality was also checked on 1.2% FA (Formaldehyde agarose) gel. The purified RNA was used for synthesizing cDNA.

### 3.5.2 cDNA synthesis

First strand cDNA synthesis was performed in a volume of 20 µl containing 1.2 µg of total RNA (5µl), 1 µl oligo d(T)12 (500 µg/ml) and 1µl random primers (500 µg/ml) were heated at 70°C for 5 min and immediately chilled on ice. A mixture containing 4.5 µl 10mM dNTP mix, 4 µl 5 x first strand buffer, 2.5 µl 0.1M DTT, 1 µl RNasin (40 u/l) and 1 µl SuperScript™II (200 units) was added. The reaction was incubated at 42°C for 90 min and at 70°C for 15 min. The cDNA was diluted with 80 µl RNase free water and stored at -20°C as a template for further use.
The SMART RACE cDNA Amplication kit (Clontech) was used for cDNA synthesis following the manufacture's protocol.

### 3.5.3 DNA isolation

DNA isolation from liver tissue

Liver tissue was cut into small pieces and digested with 700 µl digestion buffer, 70 µl of 10% SDS and 18 µl of proteinase K. The mix was incubated in the shaking incubator at 37°C overnight (for 12 – 24 hrs). A volume of 700 µl phenol-chloroform (1:1) was added, gently shaken and then centrifuged for 10 min at 10,000 g. The upper phase was transferred into a new tube and 700 µl chloroform was added. The solution was mixed by gently shaking and then centrifuged for 10 minutes at 10,000 g. The upper phase was again transferred into a new tube and 700 µl isopropanol with 70 µl of 3M natrium acetate (with 1:10 ratio) was added to precipitate the DNA and then gently shaken and centrifuged for 5 minutes. The upper phase was discarded, the pellet was washed with 700 µl ethanol (70%) and centrifuged for 5 min. The supernatant was removed and the DNA pellet was dried for 20 min. The DNA pellet was re-suspended in 500 µl 1xTE buffer. The DNA concentration was measured by taking 20 µl DNA stock into a total volume of 700 µl 1xTE (dilution factor = 35, 1 OD = 50 ng/µl and DNA concentration (µg/ml) = $OD_{260}$ x 50 x dilution factor). The DNA stock was diluted to a final concentration of 50 ng/µl, which is used further as working solution.

DNA purification from agarose gels for cloning and sequencing of PCR fragments

The band of interest was excised from 1% agarose gel, placed in an 1.5 ml tube and frozen at -20°C for 30 min. The frozen gel was ground by an 1 ml pipette tip. A volume of 500 µl 1 x TE buffer was added and homogenized by needle and syringe. Phenol-chloroform (1:1) was added in equal volume (500 µl). Needle and syringe were used to homogenise the solution. This mixture was centrifuged for 20 min at 10,000 g at 20°C. The supernatant was transferred into a new tube. A volume of 500 µl chloroform was added, mixed and centrifuged for 15 min. The upper phase was transferred into a new tube. The DNA was precipitated by adding 3M natrium acetate (pH=5.2) with a ratio $V_{sample}/V_{NaOAc}$=0.1 and was chilled in 1ml ethanol (100%). The solution was gently mixed, incubated at -20°C overnight or at -80°C for 1hr and then centrifuged for 30 min at 14,000 g at 4°C. The supernatant was removed. The DNA pellet was washed by 1ml ethanol (70%) and centrifuged for 10 min. The supernatant was removed, the pellet dried for 5-10 min and then dissolved in 7 µl millipore water and kept at 4°C for further use.

DNA purification by Qiagen mini-kits for sequencing on CEQ8000

The band of interest was excised from 1% agarose gel. The gel slice was weighed and 3 volumes of QG buffer to 1 volume of gel was added. This was incubated in the hot water bath until the gel was completely dissolved in QG buffer. An equal volume of isopropanol was added and homogenized by shaking up and down. The whole liquid was transferred to the spin column and then centrifuged for 1 min at 14,000 g at 19-20°C. The flow-through was discarded, a volume of 500 µl QG buffer added and centrifuged for 1 min at 14,000 g at 19-20°C. The flow-through was discarded, 750 µl PE buffer added and centrifuged for 1 min at 14,000 g at 19-20°C. The flow-through was discarded and the column was centrifuged for 1 min at 14,000 g at 19-20°C. The spin column was transferred to an 1.5 ml tube, 50 µl millipore water added and incubated at room temperature for 5-10 min. The column was centrifuged and the sample was dried in speed vacuum machine for 30 min at 50°C. Finally, the DNA was dissolved in 10 µl water as template for PCR.

### 3.5.4 Ligation, transformation, plasmid isolation and sequencing

Ligation

The purified PCR product was ligated into the pGEM®-T vector or pGEM®-T easy vector. The reaction was performed in 6 µl total volume which contained 2.5 µl of 2 x rapid ligation buffer, 0.5 µl of T4 DNA ligase (3 units/µl), 0.5 µl vector (50 ng/µl) and 2.0 µl of purified DNA template. The reaction was incubated at room temperature for 1 hr or at 4°C overnight.

Cloning and transformation

A volume of 3.5 µl ligation reaction was transferred to a sterile 15 ml tube containing 70 µl competent E.coli DH5α and incubated on ice for 20 min. The competent cells were heat shocked in a water bath at 42°C for 90 seconds and immediately cooled on ice for 2 min. The tube was removed from ice, 650 µl nutrient medium (LB-Broth or SOC medium) with ampicillin (10 mg/ml) added and incubated in the shaking incubator at 37°C at 100 rpm for 90 min. The transformed bacterial culture was platted in duplicate on the LB-Broth agar-ampicillin petri dishes, which contained 20 µl of X-gal (50 mg/ml) and 20 µl of 0.1M IPTG. These plates were incubated at 37°C overnight until colonies

are visible. The colonies containing plasmid with DNA insert were white, while the other colonies were blue. For each sample 4 white and 1 blue colonies were picked up and suspended into 30 µl of 1 x PCR buffer. The white colonies were also immersed into 500 µl LB-Broth-Ampicillin medium and incubated in the shaking incubator at 37°C at 100 rpm for further plasmid isolation after the inserted fragment was confirmed.

The suspension was heated at 95°C for 15 min and 10 µl of its lysate was used as template for M13 PCR. The M13 reaction was performed in a total volume of 20 µl containing 1 x PCR buffer, 50 µM of each dNTP, 0.5 unit of Taq DNA polymerase and 0.2 µM of M13 primer (F: 5'-TTGTAAAACGACGGCCAGT-3'; R:5'-CAGGCCACAGCTATGACC-3'). The PCR was as follows denaturion at 95°C for 3 min, followed by 35 cycles (95°C for 30 seconds, 56°C for 30 seconds, 70°C for 30 seconds) and a final extension at 70°C for 5 min. The extension time was set to a longer time for longer fragments. A total volume of 5 µl PCR product was electrophoresed on a 2% agarose gel. The positive colonies were moved slower than non-insert colonies and were used for sequencing.

Plasmid isolation

From M13 results, the cultured bacteria were transferred into a 15 ml tube containing 5 ml culture medium with ampicillin at 37°C overnight. These cultured bacteria were used for plasmid isolation.

Plasmid with DNA insert was isolated by using GenElute™ plasmid mini kit (Sigma). The cultured bacteria were centrifuged for 1min at 14,000 g and the supernatant was removed. The cells were resuspended in 200 µl resuspension solution and homogenised by pipetting until it completely dissolved. The cells were transferred to a 2ml tube and 200 µl lysis solution added. The tube was gently inverted for 8-10 times until the mixture became clear and viscous and then incubated at room temperature for 4 min. The cell debris was precipitated by adding 350 µl neutralized solution into the tube and mixed by gently inversion 4-6 times. The cell tube was centrifuged at 14,000 g for 10 min. The GenElute miniprep binding column was prepared by adding 500 µl solution, with short centrifugation and discarding the flow-through. Subsequently, the clear supernatant was transferred to the GenElute column, centrifuged for 1 min and the flow-through discarded. The GenElute column was washed by adding 750 µl wash solution, centrifugation for 1 min and discarding the flow-through. The column was centrifuged again to eliminate excess ethanol. The column was transferred to a fresh collection tube, 50 µl elution solution or milli-pore water added and centrifuged at

14,000 g for 5 min. A volume of 5 µl plasmid DNA with 2 µl loading buffer was electrophoresed on a 2% agarose gel. The plasmid DNA quality was measured at 260 nm and 280 nm by taking 7µl of plasmid DNA to 693 µl milli-pore water (dilution factor = 100) in a spectrophotometer (UV/Visible Spectrophoto, Biosciences). The plasmid DNA concentration (ng/µl = OD260 x dilution factor x 50) was converted into number of copies (molecules) using the following program http://www.molbiol.ru/eng/scripts/01_07.html. The plasmid DNA was diluted to be similar concentration with serial dilutions of linearized plasmid DNA from $10^{10}$ to $10^{1}$ copy number in 45 µl volume.

Sequencing by using LI-COR sequencer

The positive colony was subsequently sequenced by the dideoxy chain-termination method (Sanger et al. 1977) using the SequiTherm Excell TM II DNA sequencing kit. For each of the four terminators (ddATP, ddCTP, ddGTP and ddTTP) 1µl (0.25 µmol) was added into marked tubes as well as 2 µl of the mixture, which contained 3.6 µl of 3.5 x sequencing buffer, 0.25 µl (2.5 pmol) of each primer (700-IRD labelled SP6 primer 5'-TAAATCCACTGTGATATCTTATG-3' and 800-IRD labelled T7 primer (5'-ATTATGCTGAGTGATATCCCGCT-3'), 0.5 µl of SequiTherm Excel II DNA polymerase (5 units/µl) and 3.5 µl of the plasmid DNA elute. The PCR was as follows: denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 70°C for 30 seconds and a final extension at 70°C for 5 min. The reaction was stopped by adding 1.5 µl stop solution, denaturing at 95°C for 5 min and chilling on ice. The PCR sequence was loaded on 6% Sequagel XR. The electrophoresis was performed on a LI-COR model 4200 automated DNA sequencer in 1xTBE buffer at 50°C, 50 W and 1200 V. Sequence results were analyzed by using the Image Analysis program, version 4.1 (LI-COR).

### 3.5.5 Clean-up PCR and sequencing on CEQ 8000

A volume of 5 µl each amplified fragment was subjected to purification adding 1 µl ExoSAP-IT and incubating at 37°C for 45 min, with following inactive action of the enzymes at 80°C for 15 min.

The clean DNA template was subsequently used for the sequencing PCR which contained 8 µl of milli-pore water, 2 µl of 1 M primer giving 1pmole per reaction and 4 µl of DTCS Quick Start Master Mix (Beckman Coulter). The PCR reaction was performed

with 30 cycles (96 °C for 20 sec, 50 °C for 20 sec, 60 °C for 4 min). The stop solution was prepared in a volume of 2.0 µl of 3M NaOAc (pH = 5.2), 2.0 µl of 100 mM EDTA (pH = 2.0) and 1.0 µl of glycogen (20 mg/ml). The PCR product was transferred to a 1.5 ml sterile tube and mixed with 5 µl stop solution. A volume of 60 µl cold ethanol (98%) was added and mixed by vortex and then centrifuged for 15 min at 4°C. The supernatant was removed and the pellet washed 2 times with 200 µl cold ethanol (70%) and centrifuged for 5 min at 4°C. The pellet was dried by the speed vacuum machine at 35°C and resuspended in 40 µl SLS (Sample loading solution). The sample was loaded in to plates and sequenced using the CEQ8000 Genetic Analysis System.

## 3.6 Identification of the candidate genes FADS1 and FADS2 in divergent lines of Japanese quails

### 3.6.1 Sample collection

Collection of liver tissue in quails

According to the selection experiment of the high and low lines, the birds from the S5, S6 and S7 generation were slaughtered at the end of the trial and the livers were collected for further RNA and DNA isolation.

Collection of liver tissue in Vietnamese local chickens

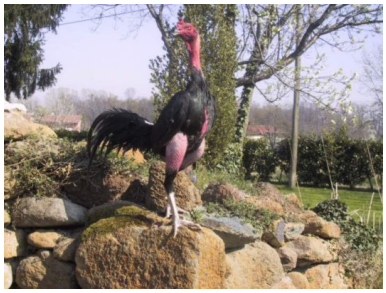
Unrelated laying hens of the breeds Ri (n=3), H'mong (n=3), Te (n=3) were collected from the National Institute of Animal Husbandry, Hanoi, Vietnam. Noi (n=6) and Ac (n=6) chickens were collected from Cantho City, Vietnam. The European breed, selected Lohman light (SLS) (n=4) was used (Figure 6).
From these samples RNA was isolated (see section 3.5.1) and cDNA generated (see section 3.5.2) for further studies.

Ri chicken

H'mong chicken

Ac chicken

Te chicken



Noi chicken

Lohman selected light

Figure 6: Different Vietnamese local chicken breeds and European chicken

### 3.6.2 Characterisation of FADS1 and FADS2 genes

Sequence identification of cDNA FADS1 and FADS2 genes in quails

FADS1 gene

The primers for the FADS1 gene were designed based on the sequence of the chicken FADS1 gene (Genbank accession number BM491157) and by comparing the sequences of the human FADS1, FADS2 and FADS3 gene (Genbank accession number NM_013402, NM_004265 and NM_021727, respectively) in order to avoid cross amplification between these family members. The nucleotide sequences for the forward and reverse primers were 5'-AGCCACTACGCCGGGCAGGA-3' and 5'-AGCCAGCCAGCCTGGGCCTG-3'. The PCR amplification was performed in a 20 µl reaction volume (95°C for 4 min, followed by 40 cycles was at 95°C for 30 seconds, 63°C for 30 seconds, 72°C for 30 seconds), a final extension at 72°C for 5 min. The PCR products were subjected and purified by using ExoSAP-IT (Amersham Biosciences) and DTCS Quick Start Master Mix (Beckman Coulter) (see section 3.5.5) and sequenced on a CEQ8000 Genetic Analysis System (Beckman Coulter). In order to obtain the whole cDNA sequence 5'- and 3'- RACE were performed using the gene specific primers designed from the new Japanese quail sequence of FADS1 (Table 8).

The 5'- RACE_FADS1 primer was performed by denaturation at 95°C for 3 min, and touchdown PCR followed by 10 cycles (95°C for 30 seconds, 55-45°C for 30 seconds, 72°C for 1 min) and further 30 cycles (95°C for 30 seconds, 45°C for 30 seconds, 72°C for 1 min); the final extension was 72°C for 10 min.
The 3'- RACE_FADS1 primer for PCR amplification was performed by denaturation at 95°C for 3 min, and then followed by 40 cycles (95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 min), the final extension was 72°C for 10 min.
Another pair of FADS1 primer (Table 8), FADS1p, were also amplified by denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 min and a final extension at 72°C for 10 min. The PCR products were purified by using ExoSAP-IT (Amersham Biosciences) and DTCS Quick Start Master Mix (Beckman Coulter) and sequenced on CEQ8000 Genetic Analysis System (Beckman Coulter) (see section 3.5.5).

FADS2 gene

The primers for the FADS2 gene were designed based on the sequence of the chicken FADS2 gene (Genbank accession number BG709923) and by comparing the sequences of the human FADS1, FADS2 and FADS3 gene (Genbank accession number NM_013402, NM_004265 and NM_021727, respectively) in order to avoid cross amplification between these family members. The nucleotide sequences for the forward and reverse primers were 5'-GGCAAGAAGAAGCTGAA-3' and 5'-AGAAACAGGGATCCCAGAAT-3', respectively. A touch down PCR was performed in a total reaction volume of 20 µl containing 25 ng cDNA as template, beginning with 95 °C for 5 min, followed by 20 cycles (95 °C for 30 seconds, 61-51 °C (-0.5 °C/cycle) for 30 seconds, 72 °C for 1 min) and further 20 cycles (95 °C for 30 seconds, 51 °C for 30 seconds, 72 °C for 1 min), the final extension was 72 °C for 5 min. The amplified fragments were subjected to electrophoresis in a 1% agarose gel with a lambda DNA *Bst*EII marker as a reference for fragment size and visualized under UV transilluminator. The gel containing the DNA fragment of interest was cut out and isolated with phenol-chloroform (see section 3.5.3). The extracted DNA was cloned (pGEM-T Vector System I) and sequenced on a LI-COR Model 4200 automated sequencer (see section 3.5.4). To obtain the whole cDNA sequence 5'- and 3'- RACE was performed. Specific primers were designed in Table 8.

Table 8: Specific primers for RACE PCR on quail FADS1 and FADS2 genes

| Amplicon | 5'- RACE (5'→3') | Ann. temp. (°C) |
|---|---|---|
| FADS1 | AGTGGATGACCCAAAGTACCAGAT | TD 55-45 |
| FADS2 | TACCTGCCTTACAACCACCAGCACG | 60 |

| Amplicon | 3'- RACE (5'→3') | Ann. temp. (°C) |
|---|---|---|
| FADS1 | CAACCCAGCTTTGAACCCAGCAAGAAT | 55 |
| FADS1p | F: TCGACACAATTACTGGAAGGTGGC | 55 |
| | R: ATCGTACACCTTCCTATCGACCAC | |
| FADS2 | GGATCCCAGAATGCCATAGAATGGG | 60 |

The 5'- RACE_FADS2 and 3'- RACE_FADS2 primers (Table 8) were amplified in a volume of 20 µl by denaturation at 95°C for 3 min, and then touchdown PCR followed by 10 cycles (at 95°C for 30 seconds, 57-62°C for 30 seconds, 72°C for 2 min); 30 cycles (at 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 2 min); and a final extension at 72°C for 10 min. The amplified fragments were purified for cloning and subsequently sequencing (see section 3.5.3).

Sequence identification of FADS1 and FADS2 cDNA in chicken

The primers characterizing the FADS1 and FADS2 genes in quail were applied in chickens.

### 3.6.3 Identification of polymorphisms of the FADS1 and FADS2 genes

Animals

Eight representative animals of the 6th generation of each of the high and low lines (n= 16) were selected for detecting polymorphisms of the FADS1 and FADS2 genes. RNAs were isolated as described in section 3.5.1.

Identification of polymorphisms of the FADS1 gene

The first pair primer of the FADS1 gene (Table 9), FADS1a, was performed in 20µl reaction volume containing 25 ng cDNA as template by 95°C for 3 min, followed by 40 cycles (95°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds) and a final extension at 72°C for 5 min.

The second pair primer of the FADS1 gene (Table 9), FADS1b, was amplified. A touch down PCR was performed in 20 µl reaction volume containing 25 ng cDNA as template, beginning with 95 °C for 3 min, followed by 14 cycles (95 °C for 30 seconds, 51-45 °C (-0.5 °C/cycle) for 30 seconds, 72 °C for 30 seconds) and further 26 cycles (95 °C for 30 seconds, 45 °C for 30 seconds, 72 °C for 30 seconds) and 72 °C for 5 min.

The PCR products were purified by using ExoSAP-IT (Amersham Biosciences) and DTCS Quick Start Master Mix (Beckman Coulter) and sequenced on a CEQ8000 Genetic Analysis System (Beckman Coulter) (see section 3.5.5).

Comparative sequencing of animals from the eight divergent lines of quail was performed using the CEQ8000 Genetic Analysis System.

Table 9: Primer sequences used for screening the SNPs in quail FADS1 gene

| Amplicon | Sequence (5'→3') | Length (bp) | Ann. temp. (°C) |
|---|---|---|---|
| FADS1a | CACGGATCCTTTCGTAGCAT | 455 | 50 |
| | GCTGGAAGTGGAGGTGGTTC | | |
| FADS1b | TCTGCTTCCGAAAGGACCCTGAT | 539 | TD51-45 |
| | GCCACCTTCCAGTAATTGTGTCGA | | |
| FADS1-1a | ACTGGTAGAAGATTTCCGTGAGC | 185 | 50 |
| | TGCCCAGAAGCAACGCAGAGAAG | | |

Identification of polymorphisms of the FADS2 gene

To identify polymorphisms in the coding region, two pairs of primers (FADS2a and FADS2b) for PCR amplification were designed based on the total length of the quail *FADS2* genes (Table 10). The two amplified fragments of the FADS2 gene were 120 bp overlapping. Touch down PCR was performed in 20 µl reaction volume containing cDNA as template, beginning with 95 °C for 5 min, followed by 8 cycles (95 °C for 30 seconds, 64-60 °C (-0.5 °C/cycle) for 30 seconds, 72 °C for 1.5 min) and 32 cycles (95 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 1.5 min) and 72 °C for 5 min. Comparative sequencing of animals from the eight divergent lines of quail was performed using the Image Analysis program, version 4.1 (LI-COR).

Table 10: Primers used for PCR sequencing and single base extension

| Amplicon | Sequence (5'→3') | Length (bp) | Ann. temp. (°C) |
|---|---|---|---|
| FADS2a | AAGACAGCAGAGGACATGAACTTG | 420 | TD 64-60 |
| | CAGGTACTTCAGCTTCTTCTTGCC | | |
| FADS2b | CTTCCAACATCACGCTAAGCC | 527 | TD 64-60 |
| | GGCATTGTTGGGAACAAGGTG | | |
| FADS2-1 | AAGACAGCAGAGGACATGAACTTG | 201 | 55 |
| | GTGTCCAATCACAAACTTGTG | | |
| FADS2-2 | CCTTACAACCACCAGCACGA | 77 | 55 |
| | ATTTGGATTTGGAAGTACAC | | |
| FADS2-3 | GATTGTAGAAGCACAAAAGA | 110 | 55 |
| | TCAATTTGGAAGTTCAGGTG | | |

TD: Touch down

### 3.6.4 Genotyping approach for the FADS1 and FADS2 genes

Genotyping of the FADS1 gene

In total, 347 quails (including 85 males and 262 females) of the divergent high (n= 150) and low (n= 197) lines from the 4$^{th}$, 5$^{th}$ and 6$^{th}$ generation were genotyped for the SNPs within the FADS1 gene by using SSCP method.

The DNA samples were used for genotyping of two SNPs at position 391 (C to A) and 468 (C to T), a pair of primer generating a 185 bp fragment was designed (Table 9). The forward primer was 5'-ACTGGTAGAAGATTTCCGTGAGC-3' and the reverse primer was 5'-TGCCCAGAAGCAACGCAGAGAAG-3'. The target fragments were amplified in a total reaction volume of 20 µl containing 100 ng genomic DNA as template by 95 °C for 3 min, 40 cycles (95 °C for 15 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds) and 72 °C for 5 min. The PCR products were subjected to electrophoresis in a 2% agarose gel and visualized under UV transilluminator. Furthermore, PCR products were diluted with an equal volume of loading buffer (1:1), this solution was denatured at 95°C for 5 min, chilled on ice and resolved on a 12% polyacrylamide gel (49:1 acrylamide: bis-acrylamide). The electrophoresis was run in vertical gel (20 x 20.5 x 0.04 cm) in 0.5 x TBE buffer at 12 W for 3hrs. The DNA bands were visualized by silver staining

Silver staining of nucleic acid

After electrophoresis, the glass-polyacrylamide gels were fixed and stained by silver (Anolle's and Gresshoff 1994) with following steps. First, the glass gel was fixed in 10% acetic acid for 15 min, shortly washed with distilled water for 2 times and fixed again in 1% nitric acid for 10 min and again washed two times with distilled water for 4 min. The gel was impregnated with 0.2% silvernitrate (2 g/l) and 0.056% formaldehyde (37%) for 10 min, quickly rinsed with distilled water and developed using the developed solution (30g natrium carbonate/1l, 0.056% formaldehyde (37%) and 120 µl 0.1N $Na_2S_2O_3$) until the optimal image contrast was obtained. The image development was stopped in 10% acetic acid for 1 min. The gel was washed with distilled water and dried at room temperature or transferred to the filter paper (Whatmann 3MM) and dried in the gel dryer at 80 °C for 2 hours.

Genotyping of the FADS2 gene

In total, 160 quails (including 39 males and 121 females) of the divergent high (n=68) and low (n=92) lines from the 4$^{th}$ and 5$^{th}$ generation were genotyped for the SNPs within the FADS2 gene by single base extension (SBE) (Hirschhorn et al. 2000) using the CEQ8000 Genetic Analysis System.

For genotyping three pairs of primers (FADS2-1, FADS2-2 and FADS2-3) for amplification of the genomic DNA were designed from the cDNA sequences (Table 10). Specific primers for SBE are shown in Table 11. The target fragments were amplified in a total reaction volume of 20 µl containing 100 ng genomic DNA as template by 95 °C for 3 min, 40 cycles (95 °C for 15 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds), and 72 °C for 5 min. The PCR products were mixed and purified (see section 3.2.2). SBE was performed as a multiple PCR in reaction volume of 10 µl containing 0.1 pmole of the synthetic clean DNA templates, 1 pmole multi-primer mix and 0.2 µM SNP primer extension premix (Beckman Coulter) by 25 cycles (96 °C for 10 sec, 50 °C for 10 sec, 72 °C for 30 Sec). The multiple SBE products were purified (2.5 µl SBE product added to 1 µl SAP [Amersham Biosciences]) by 37 °C 30 min, 80 °C 10 min. The genotypes were determined using the CEQ8000 Genetic Analysis System.

Table 11: Specific primers used for single base extension reaction PCR

| Amplicons | Extension primer sequence (5'→3') |
|---|---|
| FADS2-SNP1 | GTGTCCAATCACAAACTTGTGGAC |
| FADS2-SNP2 | $(T)_{14}$CCTTACAACCACCAGCACGA |
| FADS2-SNP3 | $(T)_{24}$ATTTGGATTTGGAAGTACAC |
| FADS2-SNP4 | $(T)_{34}$GATTGTAGAAGCACAAAAGA |
| FADS2-SNP5 | $(T)_{44}$TCAATTTGGAAGTTCAGGTG |

## 3.7 Expression of the FADS1 and FADS2 genes

### 3.7.1 Animals

Quail

RNA pools of five or six animals of each family of the four high and four low lines were prepared to obtain in total eight pools of 44 liver samples represent. Pooled RNAs were reverse transcribed using oligo d(T) and SuperScript II reverse transcriptase (see section 3.5.2) from 1μg of total RNA. In addition, RNA samples of the 16 animals used to identify polymorphisms (see section 3.6.3) were prepared.

Chicken

A total of 26 samples from the Ri, H'mong, Te, Choi, Ac and Germany chicken breeds (see section 3.6.1) were used for comparison the different expression of the FADS2 and FADS1 genes.

### 3.7.2 Quantitative by real-time PCR

Gene expression experiments were performed to evaluate whether the gene is differentially expressed in the high and low quail lines and among different chicken breeds. Real-time RT-PCR was optimized as described by Simpson et al. (2000) with slight modification and performed in an iCycler iQ detection system (BioRad). Primers showed in Table 12 were used for the real-time PCR. The target fragments were amplified in a total reaction volume of 20 μl containing 20 ng cDNA by initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1min. PCR products were monitored by using the RealMasterMix/SYBR solution (Eppendorf). Plasmids harbouring inserts of the FADS1, FADS2 and 18S fragments

were constructed and serial dilutions of linearized plasmid DNA were used to obtain standard curves for each gene. The house keeping gene 18S was used to normalize and Real–time PCR products were verified for their specificity by melting curve analysis (iCycler iQ software programm), agarose gel electrophoresis and sequencing.

Table 12: Primers used for the quantitative Real-time PCR

| Amplicon | Sequence (5'→3') | Ann. temp. (°C) |
|---|---|---|
| FADS1 | AGCCACTACGCCGGGCAGGA | 60 |
| | AGCCAGCCAGCCTGGGCCTG | |
| FADS2 | GGCAAGAAGAAGCTGAA | 60 |
| | AGAAACAGGGATCCCAGAAT | |
| 18S | GAGCGAAAGCATTTGCCAAG | 60 |
| | GGCATCGTTTATGGTCGGAAC | |

## 3.8 Statistical analysis

### 3.8.1 Genetic evaluation based on selection of the eight divergently selected lines of Japanese quails

The fatty acid profiles in the egg yolks and the different generations were analyzed between the high and low lines. The differences between the lines were estimated for each generation with the following model by analysis of variance using the General Linear Models (GLM) produce, Least Square Means (LSM) of SAS (Version 9.1).

$$Y_{ijk} = \mu + (\text{generation x line})_{ij} + e_{ijk}$$

Where $Y_{ijk}$ is the individual observation for fatty acid profiles, $\mu$ is the overall mean, (generation x line) is the fixed effect of the jth line within generation, $e_{ijk}$ is the random residual effect.

The genetic distance between sires was defined. The breeding values were estimated by the BLUP (best linear unbiased prediction) using the PEST package. The variance components were obtained from REML analysis considering an animal model with the lines within generation as fixed effect, and animals and replication as random effects. The heritability was estimated from parent-offspring by using the following formula

$$h^2 = \frac{\delta^2_a}{\delta^2_a + \delta^2_e}$$

Where $\delta^2_a$ is variance component of the measured fatty acid trait by animal record, $\delta^2_e$ is variance component of the random residual effect.

### 3.8.2 Genotype analysis

Allelic frequencies, genotype frequencies and Hardy-Weinberg equilibrium

Allele frequencies of candidate genes FADS1 and FADS2 within lines were calculated and Hardy-Weinberg equilibrium was tested by Chi-square test. Genotype frequencies at the five SNPs were separately estimated and the influence of the generations was tested by Chi-square test. Both analyses were performed by using the procedure FREQ (SAS). Furthermore, the haplotypes of the FADS2 and FADS1 genes, based on the family structure of the parents and their offspring from the high and low lines of quail, were estimated using Merlin (Abecasis et al. 2002).

Association analysis of the candidate genes FADS2 and FADS1 and fatty acid profiles in yolk of Japanese quails

Association analysis between FADS2, FADS1 and fatty acid profiles was using the statistical linear model comprising the effects of line and genotypes as well as their interaction. This analysis was performed with the procedure “PROC GLM” of the SAS software package (SAS System for Windows, release 8.02). Multiple mean comparisons were conducted by using Ryan-Einot-Gabriel-Welsch (REGW) F-Test.
In addition, family-based association test was done using the FBAT program, providing a test for linkage as well as association and by this avoiding spurious association caused by admixture of populations. With this analysis, it was tested whether higher and lower fatty acid contents go together with excess transmission of a particular allele from parents and offspring.

# 4 Results

## 4.1 Fatty acid composition in egg yolk

### 4.1.1 Composition of fatty acids in egg yolk of the high and low lines of the 5th, 6th and 7th generation

The egg yolk fatty acid composition of the high and low line of the 5th, 6th and 7th generation is shown in Table 13. Myristic acid (C14:0) and palmitic acid (C16:0) were significantly different between high and low lines in each generation ($P<0.01$), whereas stearic acid (C18:0) was significantly different among the three generations of the high and low line ($P<0.01$). Oleic acid (C18:1 ω-9) was the main monounsaturated fatty acid present in the egg yolk being significantly different between high and low lines ($P<0.01$) in each generation as well as among three generations within the low and high lines ($P<0.01$). The amount of linoleic acid (LA; C18:2 ω-6) in the egg yolk was significantly reduced compared to the feed and this fatty acid was significantly higher in the high line than in the low line ($P<0.01$) as well as significantly lower ($P<0.01$) in generation 7 for both high and low lines compared to the other generations. The feed contained α-linolenic acid (ALA; C18:3 ω-3) about 3.5%, however egg yolk contained less than 0.1% and eicosapentanoic acid (EPA; C20:5 ω-3) was present only in small amounts in both feed and egg, therefore ALA and EPA were not considered for analysis. Arachidonic acid (AA; C20:4 ω-6) and docoxahexaenoic acid (DHA; C22:6 ω-3), were not detected in the diet but were found in the yolk. The present data (Table 13) demonstrate that AA and DHA content were significantly lower in the high line than in the low line ($P<0.01$). Significant differences were observed for the low and high lines in the total amount of saturated fatty acids (SFA) ($P<0.01$) and monounsaturated fatty acids (MUFA) contents ($P<0.05$). Also the amounts of ω-3 PUFA and ω-6 PUFA in the low and high lines were significantly different ($P<0.01$). Comparison of the ratio of the ω-6 and ω-3 fatty acids shows that the ratio of the ω-6 and ω-3 fatty acids were significantly different ($P<0.01$) between the low and high lines, however, there was no significant difference in the 7th generation in the high and low lines.

Table 13: Fatty acid composition in egg yolk of the low and high Japanese quail lines of the 5th, 6th and 7th generation

| Fatty acid (%) Generation | Feed[1] | Low | | | High | | | F-Test (P) |
|---|---|---|---|---|---|---|---|---|
| | | 5 | 6 | 7 | 5 | 6 | 7 | |
| Myristic (C14:0) | 1.36 | 0.46±0.01$^{c}$ | 0.38±0.01$^{d}$ | 0.51±0.01$^{b}$ | 0.52±0.01$^{b}$ | 0.45±0.01$^{c}$ | 0.58±0.01$^{a}$ | ** |
| Palmitic (C16:0) | 14.42 | 26.57±0.10$^{b}$ | 25.26±0.09$^{d}$ | 25.89±0.10$^{c}$ | 27.09±0.10$^{a}$ | 26.21±0.09$^{c}$ | 26.81±0.10$^{ab}$ | ** |
| Palmitoleic (C16:1ω-7) | 0.30 | 3.43±0.08$^{e}$ | 3.64±0.07$^{de}$ | 4.77±0.07$^{b}$ | 3.88±0.08$^{d}$ | 4.15±0.07$^{c}$ | 5.37±0.07$^{a}$ | ** |
| Stearic acid (C18:0) | 4.96 | 9.46±0.10$^{a}$ | 9.04±0.10$^{bc}$ | 8.76±0.10$^{c}$ | 8.87±0.11$^{bc}$ | 9.21±0.10$^{ab}$ | 8.65±0.10$^{c}$ | ** |
| Oleic acid (C18:1 ω-9) | 29.48 | 43.88±0.25$^{a}$ | 40.98±0.23$^{c}$ | 41.50±0.24$^{c}$ | 42.64±0.26$^{b}$ | 38.55±0.24$^{e}$ | 40.02±0.24$^{d}$ | ** |
| Linoleic (C18: 2 ω-6) | 45.97 | 13.02±0.13$^{b}$ | 11.58±0.12$^{c}$ | 9.57±0.12$^{d}$ | 14.31±0.13$^{a}$ | 12.77±0.12$^{b}$ | 9.94±0.12$^{d}$ | ** |
| ALA (C18:3 ω-3) | 3.53 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | |
| AA (C20:4 ω-6) | - | 2.22±0.06$^{b}$ | 2.50±0.05$^{a}$ | 2.41±0.06$^{ab}$ | 1.94±0.06$^{c}$ | 2.45±0.06$^{ab}$ | 2.24±0.06$^{b}$ | ** |
| DHA (C22:6 ω-3) | - | 0.95±0.03$^{b}$ | 1.08$^{a}$±0.03 | 0.77±0.03$^{c}$ | 0.75±0.03$^{c}$ | 0.93±0.03$^{b}$ | 0.69±0.03$^{c}$ | ** |
| Σ SFA | 20.74 | 36.49±0.13$^{a}$ | 34.68±0.13$^{c}$ | 35.16±0.13$^{c}$ | 36.48±0.14$^{a}$ | 35.87±0.13$^{b}$ | 36.04±0.13$^{ab}$ | ** |
| Σ MUFA | 29.78 | 47.31±0.25$^{a}$ | 44.62±0.24$^{d}$ | 46.27±0.24$^{bc}$ | 46.52±0.27$^{ab}$ | 42.70±0.24$^{c}$ | 45.39±0.25$^{cd}$ | * |
| Σ PUFA ω-3 | 3.53 | 0.95±0.03$^{b}$ | 1.08$^{a}$±0.03 | 0.77±0.03$^{c}$ | 0.75±0.03$^{c}$ | 0.93±0.03$^{b}$ | 0.69±0.03$^{c}$ | ** |
| Σ PUFA ω-6 | 45.97 | 15.24±0.15$^{b}$ | 14.08±0.14$^{c}$ | 11.98±0.15$^{d}$ | 16.25±0.16$^{a}$ | 15.22±0.15$^{b}$ | 12.18±0.15$^{d}$ | ** |
| ω6:ω3 | 13.02 | 20.70±0.43$^{b}$ | 13.73±0.41$^{d}$ | 12.52±0.42$^{d}$ | 25.56±0.45$^{a}$ | 17.63±0.41$^{c}$ | 13.41±0.42$^{d}$ | ** |

∑ SFA, total saturated fatty acids including C14:0, C16:0 and C18:0. ∑ MUFA, total unsaturated fatty acids including C16:1 and C18:1. ∑ PUFA ω-3, total ω-3 polyunsaturated fatty acids including C18:3 ω-3 and C22:6 ω-3. ∑PUFA ω-6, total ω-6 polyunsaturated fatty acids including C18:2 and C20:4, ω-6. The ω-6:ω-3 PUFA ratio is given by (C18:2 ω-6 + C20:4 ω-6):(C18:3 ω-3 + C22:6 ω-3). F-Test p: significance of line effect; * P<0.05; ** P<0.01. $^{a, b, c, d}$ differ significantly means in a line.

### 4.1.2 Fatty acid profiles in Ri chicken

The fatty acid profile of the Vietnamese local chicken (Ri) is presented in Table 14. Oleic acid was the most prevalent fatty acid in the egg yolk (39.92 %). The DHA content was 1.0 % compared to AA (3.11 %). Averages of MUFA, PUFA and ω-6:ω-3 PUFA were 42.64%, 17.24 % and 13.17 %, respectively.

Table 14: Fatty acid profiles in the Ri local chicken breed

| | (n=11) | Mean ±SD | Range | |
|---|---|---|---|---|
| | | (%) | Minimum | Maximum |
| Myristic acid | (C14:0) | 0.33±0.06 | 0.26 | 0.47 |
| Palmitic acid | (C16:0) | 24.43±1.28 | 22.39 | 26.32 |
| Palmitoleic acid | (C16:1 ω-7) | 2.73±0.97 | 1.87 | 5.28 |
| Stearic acid | (C18:0) | 8.95±0.59 | 8.26 | 9.85 |
| Oleic acid | (C18:1 ω-9) | 39.91±1.89 | 37.03 | 42.50 |
| Linoleic acid | (C18:2 ω-6) | 12.90±1.94 | 8.09 | 14.59 |
| α-linolenic acid | (C18:3 ω-3) | 0.22±0.03 | 0.16 | 0.28 |
| Arachidonic acid | (C20:4 ω-6) | 3.11±0.28 | 2.78 | 3.72 |
| Docosahexaenoic acid | (C22:6 ω-3) | 1.00±0.12 | 0.77 | 1.23 |
| Σ SFA | | 33.72±1.03 | 31.80 | 35.04 |
| Σ MUFA | | 42.64±2.13 | 39.91 | 47.12 |
| Σ PUFA ω-3 | | 1.22±0.13 | 0.99 | 1.42 |
| Σ PUFA ω-6 | | 16.02±2.02 | 10.91 | 17.86 |
| ω6:ω3 PUFA | | 13.16±1.56 | 10.20 | 16.24 |

## 4.2 Heritability

The heritability estimates were based on mean records for the C22:6 (ω-3) and ω-6:ω-3 PUFA ratio (Table 15). The C22:6 (ω-3) trait showed low to moderate heritability in the high line from 0.19 to 0.29 for the 5$^{th}$, 6$^{th}$ and 7$^{th}$ generation. For the low line, the heritabilities varied from 0.18 to 0.33.

The ω-6:ω-3 PUFA ratio had a moderate heritability in both high and low lines ranging from 0.38 to 0.40 for the high line, from 0.36 to 0.37 for the low line.

The low line tended to show higher heritability than the high line for the C22:6 (ω-3), whereas the ω-6:ω-3 PUFA ratio was more heritable in the high line than in the low line.

Table 15: Heritability estimates for the C22:6 (ω-3) and the ω-6:ω-3 PUFA ratio in different generations of the high and low lines of quail

| Generation | No. record | | C22:6 (ω-3) | | | | ω-6:ω-3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | High | | Low | | High | | Low | |
| | high | low | $h^2$ | SE | $h^2$ | SE | $h^2$ | SE | $h^2$ | SE |
| S1-S5 | 618 | 556 | 0.19 | ne | 0.18 | 0.03 | 0.38 | ne | 0.36 | 0.03 |
| S1-S6 | 785 | 771 | 0.21 | 0.03 | 0.22 | 0.03 | 0.35 | 0.03 | 0.36 | 0.03 |
| S1-S7 | 944 | 891 | 0.29 | 0.02 | 0.33 | 0.03 | 0.40 | 0.02 | 0.37 | 0.03 |

ne: not estimated; S1-S5: accumulated data from generation 1 to generation 5; S1-S6: accumulated data from generation 1 to generation 6; S1-S7: accumulated data from generation 1 to generation 7

The genetic correlations between the traits are presented in Table 16. The correlation between the ω-6:ω-3 PUFA ratio and C22:6 (ω-3) fatty acid tended to decrease over generations and was lowest in the 7$^{th}$ generation.

Table 16: Genetic correlation between the fatty acid traits for the high and low lines in quail

| Generation | Traits | ω-6:ω-3 | |
|---|---|---|---|
| | | High | Low |
| S1-S5 | C22:6 (ω-3) | -0.978 | -0.985 |
| S1-S6 | | -0.832 | -0.854 |
| S1-S7 | | -0.399 | -0.612 |

## 4.3 Cloning and characterizations of the FADS1 and FADS2 genes

The nucleotide sequence of the cDNA of the FADS1 gene was obtained from ESTs homologous with human, mouse, rat and chicken by cloning and sequencing. A cDNA of 1797 bp of the total length was obtained for the FADS1. Aligment of the quail FADS1 cDNA sequence showed that there is higher homology of 81%, 82%, 82% and 95% with human, rat, mouse and predicted chicken, respectively (Table 17).

The deduced protein comprised 1334 nucleotides encoding 445 amino acids. Alignment of the amino acid sequences of quail FADS1 gene with human, rat, mouse and predicted chicken is shown in Figure 7. The amino acid sequence of the quail FADS1 gene has 76% similarity with FADS1 of human, 78% with FADS1 of rat and mouse, and 95% homology to the predicted chicken sequence (XP_421052) (Figure 7). The amino acid sequence of the FADS1 gene shared 60%, 59% and 58% identity with FADS2 of human, rat and mouse, respectively.

Similar to FADS1, the FADS2 gene (Genbank accession number DQ336389) was obtained by cloning and sequencing. A total length of 1350 bp was obtained, including the poly (A) tail, along with the polyadenylation site, AATAAA, in the corresponding 3' UTR region. Sequence comparison of quail FADS2 with the chicken orthologue (Genbank accession number XM_421053) showed an identity of 95% and with mammalian orthologues (including human, mouse, rat and cow) an average of 83% (Table 17). The translated protein (position 10 to 1223 bp) is 404 amino acids in size. A comparison of the quail amino acid sequence with that from other species is shown in Figure 8, indicating that the nucleotides encoding the first 40 amino acids of the quail protein were not covered by the cloned and sequenced fragment. Nevertheless, high similarity was found with the chicken protein (93%), whereas compared to human, mouse, rat and cow the amino acid sequence showed 75% identity.

Comparison of quail FADS1 and FADS2 cDNA sequences shared high sequence identity (76%) between the two genes.

The exon/intron organization of FADS2 and FADS1 was deduced by aligning the cDNA to the human genomic sequences (NT_0033903.7 from 6901108 to 6941020 for FADS*2*; and from 6889822 to 6890258 for FADS1). These alignments showed that quail FADS2 cDNA analyzed cover exon 3 to exon 8, while quail FADS1 cDNA consists of 12 exons as in human (Figure 9).

The amino acid sequence comparisons of quail FADS1 and FADS2 shows that both genes present the cytochrome $b_5$-heme function and also the conserved three histidine motifs, HDFGH, HFQHH and QIEHH that are essential for enzymic activity of membrane-bound desaturase (Shankline et al. 1994). However, the cytochrome $b_5$-like

heme represented in both FADS1 and FADS2 is “HPGA” which is different compared to the conserved sequence motif “HPGG” in human, mouse and rat (Figure 8).

Table 17: Percentage of nucleotide sequence identities of quail FADS1 and FADS2 genes with other species using the BLAST algorithm

| Gene | Species | bp homology | % identity | Genbank Acc.No |
|---|---|---|---|---|
| FADS1 | Chicken (1561 bp) | 1363 | 96 | |
| quail | Chicken (2847 bp) | 1665 | 95 | XM_421052 |
| (1797 bp) | Human (1335 bp) | 900 | 79 | AF199596 |
| | Human (4213 bp) | 737 | 81 | NM_013402 |
| | Chicken (1704 bp) | 325 | 83 | XM_426408 |
| | Chicken (1308 bp) | 659 | 82 | XM_421051 |
| | Mouse (3408 bp) | 490 | 83 | NM_146094 |
| | Dog (1519 bp) | 471 | 83 | XM_540914 |
| | Chimpanzee (1932 bp) | 656 | 82 | XM_508481 |
| | Cow (1831 bp) | 574 | 82 | XM_612398 |
| | Rat (3413 bp) | 453 | 82 | NM_053445 |
| | Pig (418 bp) | 248 | 82 | AY512560 |
| FADS2 | Chicken (1380 bp) | 983 | 96 | |
| quail | Chicken (2001 bp) | 1180 | 95 | XM_421053 |
| (1350 bp) | Human (3149 bp) | 558 | 83 | NM_004265 |
| | Cow (1630 bp) | 578 | 83 | XM_600092 |
| | Dog (1869 bp) | 571 | 83 | XM_540913 |
| | Pig (461 bp) | 266 | 85 | AY512561 |
| | Rat (1706 bp) | 506 | 83 | NM_031344 |
| | Mouse (1508 bp) | 505 | 82 | NM_019699 |

```
          301                                                       360
  Quail                                                GRAMEERGA E-PGMRRFTW
Chicken* VELRREGRVL ASRRANERRA SGAARNRRLR PSAAGGLGPA VGRAMEERGA E-PEQRRFTW
  Human                                     MAPD PVAAE--TAA QGPTPRYFTW
    Rat                                     MAPD PVQTPDPASA QLRQMRYFTW
  Mouse                                     MAPD PVPTPGPASA QLRQTRYFTW

          361                                                       420
  Quail   EEIPQRTGRG PAADERWLVI DRKVYDISRF HRRHPGAARV ISHYAGQDAT DPFVAFHLDK
Chicken* EEIAQRTGRG PAADERWLVI DRKVYDISRF HRRHPGGSRV ISHYAGQDAT DPFIAFHLDK
  Human   DEVAQRSGC- --E-ERWLVI DRKVYNISEF TRRHPGGSRV ISHYAGQDAT DPFVAFHINK
    Rat   EEVAQRSGR- --EKERWLVI DRKVYNISDF SRRHPGGSRV ISHYAGQDAT DPFVAFHINK
  Mouse   EEVAQRSGR- --EKERWLVI DRKVYNISDF SRRHPGGSRV ISHYAGQDAT DPFVAFHINK

          421                                                       480
  Quail   TLVKKYMSPL LIGELAPDQP SFEPSKNKKL VEDFRELRAT VEKMGLLKPN RAFFLLHLCH
Chicken* TLVKKYMSPL LIGELAPDQP SFEPSKNKKL VEDFRELRAT VEKMGLLKPN RTFFLLHLCH
  Human   GLVKKYMNSL LIGELSPEQP SFEPTKNKEL TDEFRELRAT VERMGLMKAN HVFFLLYLLH
    Rat   GLVRKYMNSL LIGELAPEQP SFEPTKNKAL TDEFRELRAT VERMGLMKAN HLFFLFYLLH
  Mouse   GLVRKYMNSL LIGELAPEQP SFEPTKNKAL TDEFRELRAT VERMGLMKAN HLFFLVYLLH

          481                                                       540
  Quail   ILVLDVVAWL TIWYFGSSTV PFLFSALLLG TVQAQAGWLQ HDFGHLSVFS ESKWNHWVHK
Chicken* ILALDVAAWL TIWYFGSSTV PFLFSALLLG TVQAQAGWLQ HDFGHLSVFS ESKWNHWVHK
  Human   ILLLDGAAWL TLWVFGTSFL PFLLCAVLLS AVQAQAGWLQ HDFGHLSVFS TSKWNHLLHH
    Rat   ILLLDVAAWL TLWIFGTSLV PFTLCAVLLS TVQAQAGWLQ HDFGHLSVFS TSTWNHLVHH
  Mouse   ILLLDVAAWL TLWIFGTSLV PFILCAVLLS TVQAQAGWLQ HDFGHLSVFG TSTWNHLLHH

          541                                                       600
  Quail   FVIGHLKGAP ASWWNHLHFQ HHAKPFCFRK DPDVNMHPLF FALGKKLSVE LGDQKKKFMP
Chicken* FVIGHLKGAP ASWWNHLHFQ HHAKPNCFRK DPDVNMHPLF FALGKKLSVE LGEQKKKFMP
  Human   FVIGHLKGAP ASWWNHMHFQ HHAKPNCFRK DPDINMHPFF FALGKILSVE LGKQKKKYMP
    Rat   FVIGHLKGAP ASWWNHMHFQ HHAKPNCFRK DPDINMHPLF FALGKVLSVE LGKEKKKHMP
  Mouse   FVIGHLKGAP ASWWNHMHFQ HHAKPNCFRK DPDINMHPLF FALGKVLPVE LGREKKKHMP

          601                                                       660
  Quail   YNHQHKYFFI IGPPALVPLY FQWYIFYFAV QRKQWVDLAW MLTFYIRFFL TYLPLLGVKG
Chicken* YNHQHKYFFI IGPPALVPLY FQWYIFYFVV QRKQWVDLAW MLTFYIRFFL TYLPLLGVKG
  Human   YNHQHKYFFL IGPPALLPLY FQWYIFYFVI QRKKWVDLAW MITFYVRFFL TYVPLLGLKA
    Rat   YNHQHKYFFL IGPPALLPLY FQWYIFYFVV QRKKWVDLAW MLSFYVRVFF TYMPLLGLKG
  Mouse   YNHQHKYFFL IGPPALLPLY FQWYIFYFVV QRKKWVDLAW MLSFYARIFF TYMPLLGLKG

          661                                                       720
  Quail   ILGLHLLVRF IESNWFVWVT QMNHIPMHID YDKNVDWFST QLQATCTCSS VLQFNDWFSG
Chicken* ILGLHLLVRF IESNWFVWIT QMNHIPMHID YDKNVDWFST QLQATCNVRQ SL-FNDWFSG
  Human   FLGLFFIVRF LESNWFVWVT QMNHIPMHID HDRNMDWVST QLQATCNVHK SA-FNDWFSG
    Rat   LLCLFFIVRF LESNWFVWVT QMNHIPMHID HDRNVDWVST QLQATCNVHQ SA-FNNWFSG
  Mouse   FLGLFFIVRF LESNWFVWVT QMNHIPMHID HDRNVDWVST QLQATCNVHQ SA-FNNWFSG

          721                                                       780
  Quail   HLNFQIEHHL FPTMPRHNYW KVAPLVKSLC AKHGVEYQCK PLLTAFADIV YSLKDSGELW
Chicken* HLNFQIEHHL FPTMPRHNYW KVAPLVKSLC AKHGIEYHCK PLLTAFADIV YSLKDSGELW
  Human   HLNFQIEHHL FPTMPRHNYH KVAPLVQSLC AKHGIEYQSK PLLSAFADII HSLKESGQLW
    Rat   HLNFQIEHHL FPTMPRHNYH KVAPLVQSLC AKYGIKYESK PLLTAFADIV YSLKESGQLW
  Mouse   HLNFQIEHHL FPTMPRHNYH KVAPLVQSLC AKYGIKYESK PLLTAFADIV YSLKESGQLW

          781
  Quail   LDAYLHK
Chicken* LDAYLHK
  Human   LDAYLHQ
    Rat   LDAYLHQ
  Mouse   LDAYLHQ
```

Figure 7: Alignment of the FADS1 amino acid sequences of quail, human (Genbank Accession No. NP_037534), rat (Genbank Accession No. BAB69054), mouse (Genbank Accession No. NP_666206) and the predicted chicken (Genbank Accession No. XP_421052)*. Three conserved histidine motifs (HDxGH, HFQHH and QIEHH) are underlined. The conserved histidine of N-terminal cytochrome $b_5$ of the desaturase domains is indicated by arrows. HPGG motif is in box. The position of the polymorphisms in the FADS2 are indicated by arrows


Figure 8: Aligment of the FADS2 amino acid sequences of Japanese quail, cow (Genbank Accession No. XP_600092), human (Genbank Accession No. NP_004256), mouse (Genbank Accession No. NP_062673), rat (Genbank Accession No. NP_112634) and the predicted chicken (XP_421053). Three conserved histidine motifs (HDxGH, HFQHH and QIEHH) are underlined. The conserved histidine of N-terminal cytochrome $b_5$ of the desaturase domains is indicated by arrows. HPGG motif is in box. The position of the polymorphisms in the FADS2 are indicated by black arrows

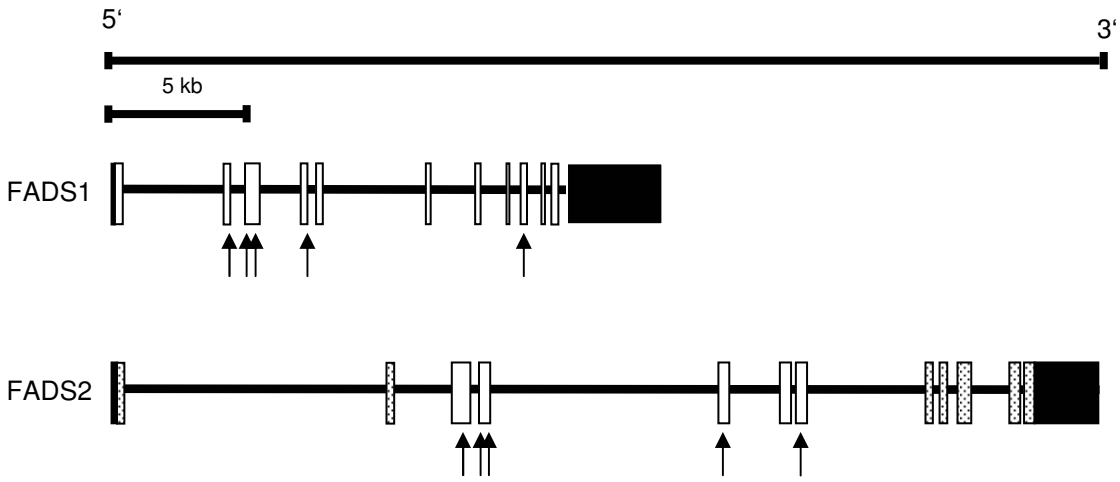



Figure 9: Exon/intron structure of the FADS1 and FADS2 genes in quail
Exons of FADS1 and FADS2 are indicated by white box. The 5'- and 3'- untranslated regions are in black box. The dotted boxes indicate the exons that are not found in quail sequence. Five SNPs of FADS1 and FADS2 genes are indicated by arrows

The nucleotide sequence of the cDNA of the FADS2 and FADS1 genes in chicken were obtained with heterologous primers matching with conserved and gene-specific regions, and subsequently 5´- and 3´- RACE. A total length of 1380 bp and 1561 bp were achieved for the chicken FADS2 and FADS1 genes, respectively. Sequence comparison of chicken FADS2 obtained with the available sequence XM_421053 showed an identity of 99% and 96% with quail (Genbank accession number DQ336389) and with mammalian orthologues (including human, mouse, rat and cow) an average of 83%. The chicken FADS1 was homologues for 97% with the predicted chicken sequence (Genbank accession number XM_421052) and for 96% with quail, 84% orthologues with mouse, 83% with rat and 81% with human (Table 18).
The deduced protein sequence of FADS2 (position 202 to 1251 bp) is 332 amino acids in size. A comparison of the quail amino acid sequence with those from other species indicates that the first 186 amino acids of the chicken protein are not yet identified. Nevertheless, high similarity was found with the quail protein (95%), whereas compared to human, mouse and rat the amino acid sequence showed 79% identity.
The deduced protein sequence of FADS1 (position 65 to 1396 bp) is 444 amino acids in size. A comparison of the chicken amino acid sequence with those from other species is shown in Figure 10. High similarity was found with the quail protein (97%), 79% identity was found in mouse, 78% in rat, and human and dog the amino acid sequence showed 77% identity.

Table 18: Percentage of sequence identities of chicken FADS1 and FADS2 genes with other species using the BLAST algorithm

| Gene | Species | bp homology | % identity | Genbank Acc.No |
|---|---|---|---|---|
| FADS1 | Chicken (2847 bp) | 1345 | 97 | XM_421052 |
| (1561 bp) | Human (1335 bp) | 785 | 81 | AF199596 |
| | Human (4213 bp) | 785 | 81 | NM_013402 |
| | Chicken (1704 bp) | 300 | 84 | XM_426408 |
| | Chicken (1308 bp) | 584 | 83 | XM_421051 |
| | Mouse (3408 bp) | 473 | 84 | NM_146094 |
| | Dog (1519 bp) | 594 | 83 | XM_540914 |
| | Chimpanzee (1932 bp) | 704 | 81 | XM_508481 |
| | Cow (1831 bp) | 549 | 82 | XM_612398 |
| | Rat (3413 bp) | 461 | 83 | NM_053445 |
| | Pig (418 bp) | 249 | 82 | AY512560 |
| FADS2 | Chicken (2001 bp) | 1104 | 99 | XM_421053 |
| (1380 bp) | Human (3149 bp) | 545 | 83 | NM_004265 |
| | Cow (1630 bp) | 556 | 83 | XM_600092 |
| | Dog (1869 bp) | 556 | 83 | XM_540913 |
| | Pig (461 bp) | 264 | 84 | AY512561 |
| | Rat (1706 bp) | 518 | 82 | NM_031344 |
| | Mouse (1508 bp) | 513 | 83 | NM_019699 |
| | Atlantic salmon (2119 bp) | 235 | 85 | AY458652 |

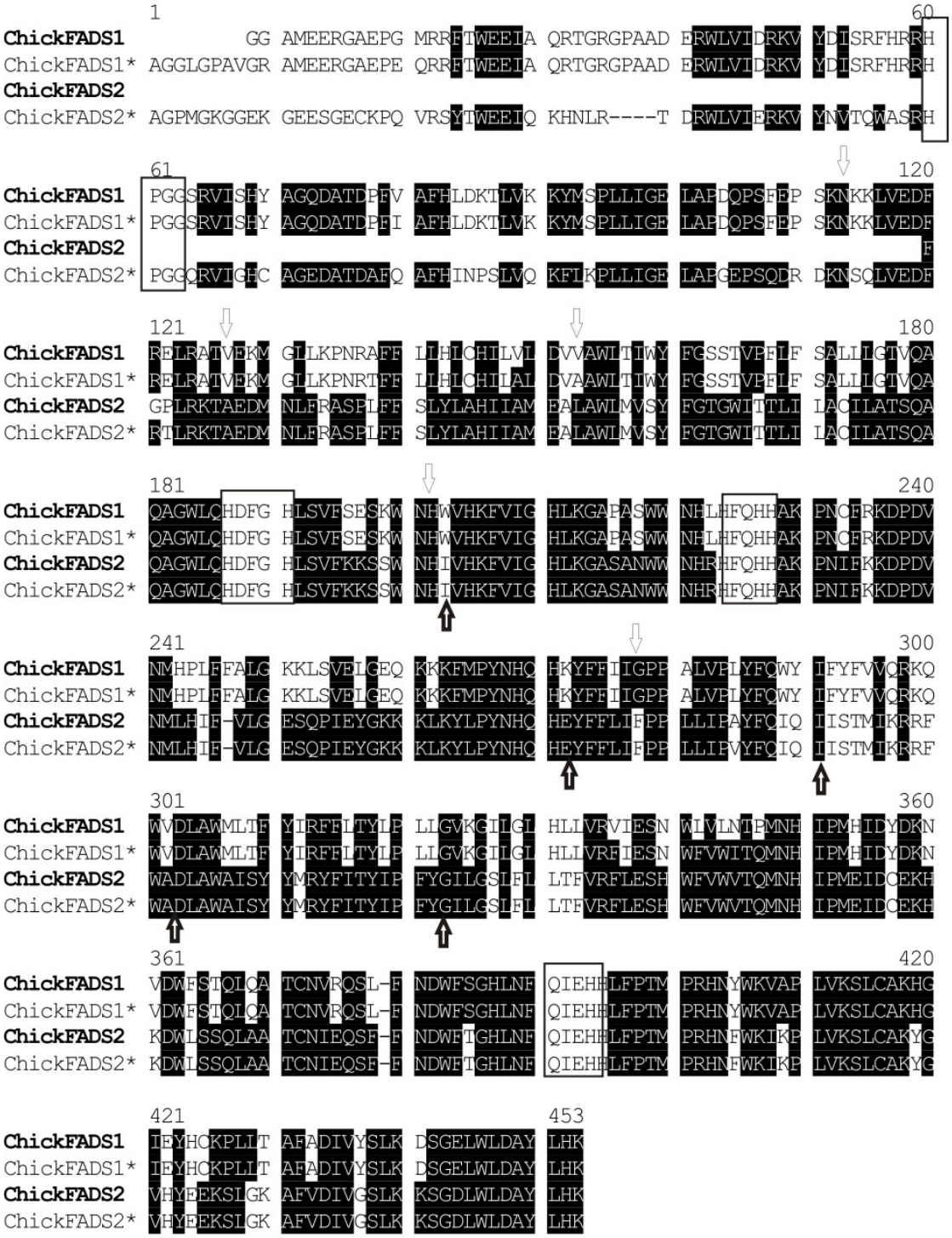



Figure 10: Aligment of FADS1 and FADS2 amino acid sequences of the present chicken and predicted chicken FADS1 (Genbank Accession No. XP_421052), FADS2 (Genbank Accession No. XP_421053) respectively. Three conserved histidine motifs (HDxGH, HFQHH and QIEHH) are in boxes. The conserved histidine of N-terminal cytochrome $b_5$ of the desaturase domains is HPGG motif is in box. The positions of the polymorphisms in the FADS2 are indicated by the up arrows while the FADS1 is indicated by the down arrows

The exon/intron boundaries in chicken FADS2 and FADS1 were determined the same way as in the quail sequences. These alignments showed that the chicken FADS2 cDNA consists of seven exons starting from exon 3 to exon 9, while FADS1 cDNA consists of 12 exons as in human (Figure 11).

The comparison of the deduced amino acid sequences of FADS2 and FADS1 genes have revealed the existence of three conserved His-box motifs: HDFGH, HFQHH and QIEHH. However, the heme-binding residue in the cytochrome $b_5$ superfamily HPGG motif on chicken FADS2 sequence was not found (Figure 10).

The cDNA sequences of FADS2 and FADS1 of the Genbank predicted sequences in chicken { XM_421052 (2847 bp) for FADS1 and XM_421053 (2001 bp) for FADS2} were compared to the new sequences in quail chicken were compared. The results showed that the new sequences in both quail and chicken were shorter than the Genbank sequences of FADS2 and FADS1 cDNA. For example, the incomplete sequence of quail FADS2 cDNA missed about 400 bp at the 5'- end and 370 bp at the 3'- end while in chicken about 600 bp and 280 bp at the 5'- and 3'- ends was missing. The FADS1 sequence was missing about 1,000 bp at the 5'- end and about 600 bp and 450 bp at the 3'- end for quail and chicken, respectively. Therefore, the missing fragments were sequenced with the primers derived from the 5'- and 3'- ends based on the overlap between the new sequences of FADS2 and FADS1 and the chicken predicted sequences (XM_421053 and XM_421052, respectively) were amplified on both cDNAs of quail and chicken. Nevertheless, no longer sequence was obtained.

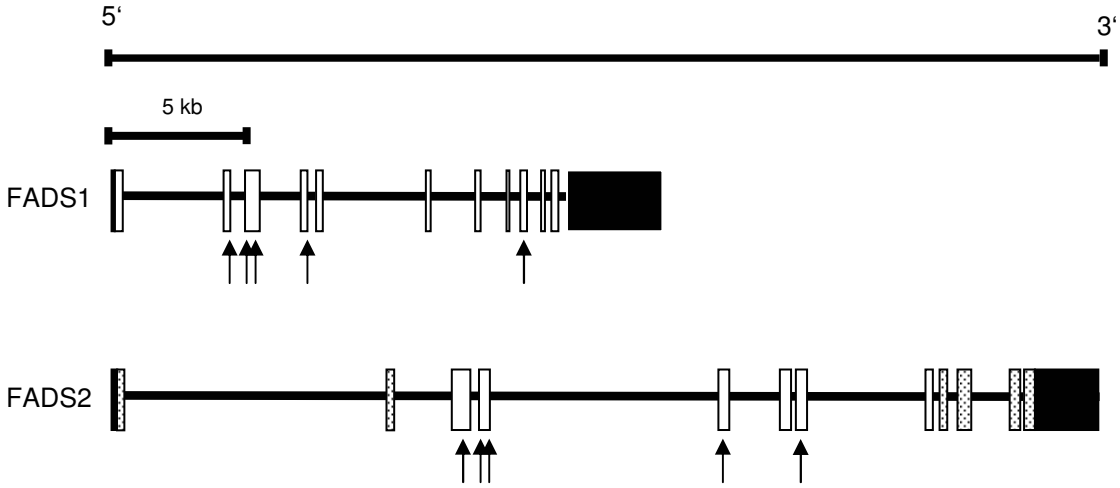



Figure 11: Exon/intron structure of the FADS1 and FADS2 genes in chicken

Exons of FADS1 and FADS2 are indicated by white box. The 5'- and 3'- untranslated regions are in black box. The dotted boxes indicate the exons that are not found in chicken sequence. Five SNPs of FADS1 and FADS2 genes are indicated by arrows

## 4.4 Expression of the FADS1 and FADS2 genes in the high and low lines of quails and in chicken

Comparing the mRNA expression between the FADS2 and FADS1 genes showed that quail FADS2 tended to be expressed at a higher level than quail FADS1, but there was no significant difference between these genes (P>0.05). The expression of quail FADS2 tended to be lower in the high line than in the low line, however, no significant differences were observed (P>0.05). While FADS1 was expressed at a similar level in both high and low lines.

The expression analysis of chicken FADS2 and FADS1 genes indicated that FADS2 was expressed stronger than FADS1, but no significant difference was found between the two genes (P>0.05). Comparing the expression among the different local chicken breeds showed that Te and European chicken breeds were significantly higher in both genes than Ac, Noi, Ri and H'mong chicken breeds (P<0.01) (Figure 12).

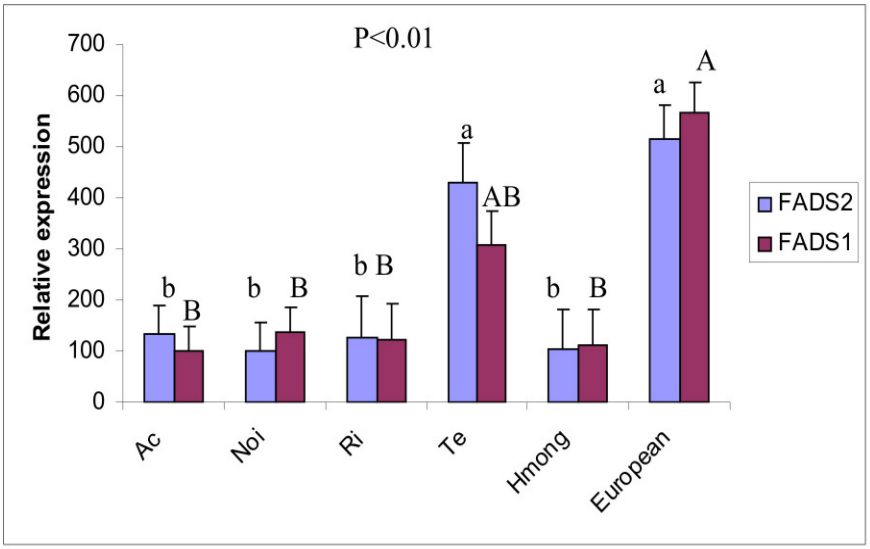



Figure 12: The expression of the FADS1 and FADS2 genes in the different local chicken breeds

a,b; A, B: P<0.01 between breed within gene

## 4.5 Screening for the polymorphisms in the FADS1 and FADS2 genes

The FADS1 polymorphisms were also screened by comparing the cDNA sequences of eight different animals of the high and low quail lines. Five polymorphisms were found at 348 bp (A to G), 391 bp (C to A), 468 bp (C to T), 570 bp (C to T) and 1075 bp (C to T). The mutations were either in purine-purine or pyrimidine-pyrimidine transitions or pyrimidine-purine transversion. All SNPs are located in the coding region, and two of five SNPs at 348 bp (A to G) and 468 bp (C to T) positions are found to change amino acid substitutions; asparagine-serine and valine-alanine, respectively. The alignment of the quail FADS1 cDNA to the genomic sequence of human FADS1 (Genbank accession number NT_033903.7 position 68890505-6872498) indicated the exon positions (Table 19).

Table 19: Polymorphic positions in the coding region of the quail FADS1 gene

| Locus | Exon | Position in cDNA | Nucleotide exchange | Amino acid |
|---|---|---|---|---|
| SNP1 | 3 | 348 | A/G | Asparagine/Serine |
| SNP2 | 4 | 391 | C/A | Valine |
| SNP3 | 4 | 468 | C/T | Valine/Alanine |
| SNP4 | 5 | 570 | C/T | Histidine |
| SNP5 | 10 | 1075 | C/T | Isolecine |

Two overlapping DNA fragments (in total 824 bp) of the FADS2 gene were used to screen for polymorphisms by comparative sequencing of eight animals of the divergent quail lines. This resulted in the identification of five SNPs at 477 bp (C to T), 681 bp (G to A), 717 bp (C to T), 953 bp (C to T) and 1023 bp (G to A) showing only purine-purine or pyrimidine-pyrimidine transitions (Table 20). All SNPs are located in the coding region, but none of them changed the amino acid sequence of the protein. Exon positions as indicated in Table 19 were estimated by aligning the cDNA sequence of the quail FADS2 gene to the human genomic FADS2 sequence (Genbank accession number NT_0339037 position 6901108-6941020).

Table 20: Polymorphic positions in the coding region of the quail FADS2 gene

| Locus | Exon | Starting position in cDNA | Nucleotide exchange |
|---|---|---|---|
| SNP1 | 3 | 477 | C/T |
| SNP2 | 4 | 681 | G/A |
| SNP3 | 4 | 717 | C/T |
| SNP4 | 5 | 953 | C/T |
| SNP5 | 7 | 1023 | G/A |

### 4.5.1 Allele frequencies of the FADS1 and FADS2 genes in the Japanese quail population

For FADS1 gene, a total of 347 animals from the 4$^{th}$, 5$^{th}$ and 6$^{th}$ generation were genotyped at position 391 bp (C to A) and 468 bp (C to T). The allelic frequencies of the SNPs within the high and low lines of the quail population are shown in Table 21. The Hardy-Weinberg equilibrium was calculated for each polymorphic position, however, none of these positions was found in Hardy-Weinberg equilibrium. Furthermore, the FADS1 genotype frequencies of the high and low lines were compatible among different generations. Table 22 shows that homozygote AA was predominantly higher in both high and low lines at position 391. Heterozygote CT and homozygote CC were predominantly in the high line compared to CC genotype in the low line at position 468 (Table 23).

Table 21: Allele frequencies of the FADS1 gene in the high and low quail lines

| Line | No. of animals | Allele frequencies | | | | | |
|---|---|---|---|---|---|---|---|
| | | Val391 | | | Val468Ala | | |
| | | C | A | P | C | T | P |
| High | 150 | 0.22 | 0.78 | 0.61 | 0.71 | 0.29 | 0.41 |
| Low | 197 | 0.24 | 0.76 | | 0.76 | 0.24 | |

Table 22: Genotype frequencies of the FADS1 Val391 genotypes in the different generations of the high and low lines

| Generation | High | | | Low | | |
|---|---|---|---|---|---|---|
| | CC | CA | AA | CC | CA | AA |
| S4 | 0.07 (2) | 0.34 (11) | 0.59 (19) | 0.07 (3) | 0.48 (19) | 0.45 (18) |
| S5 | 0.08 (4) | 0.30 (16) | 0.62 (33) | 0.05 (3) | 0.44 (31) | 0.51 (36) |
| S6 | 0.07 (5) | 0.28 (18) | 0.65 (42) | 0.08 (7) | 0.22 (19) | 0.70 (61) |

Number of animals per genotype and generation are shown in parentheses

Table 23: Genotype frequencies of the FADS1 Val468Ala genotypes in the different generations of the high and low lines in quails

| Generation | High | | | Low | | |
|---|---|---|---|---|---|---|
| | CC | CT | TT | CC | CT | TT |
| S4 | 0.45 (13) | 0.48 (14) | 0.07 (2) | 0.49 (21) | 0.44 (19) | 0.07 (3) |
| S5 | 0.58 (30) | 0.37 (19) | 0.05 (3) | 0.50 (35) | 0.46 (32) | 0.04 (3) |
| S6 | 0.49 (33) | 0.42 (28) | 0.09 (6) | 0.69 (58) | 0.23 (19) | 0.08 (7) |

Number of animals per genotype and generation are shown in parentheses

A total of 160 animals have been genotyped for the five SNPs of the FADS2 gene and the allelic frequencies are given in Table 24. All genotype distributions were tested for Hardy-Weinberg equilibrium. There only genotypes at SNP5 was to the Hardy-Weinberg equilibrium while the other four were not in Hardy-Weinberg equilibrium.

Table 24: Allele frequencies of the FADS2 SNPs in the high and low quail lines in quails

| | SNP1 | | SNP2 | | SNP3 | | SNP4 | | SNP5 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | T | G | A | C | T | C | T | G | A | P |
| Low (n=92) | 0.61 | 0.39 | 0.93 | 0.07 | 0.57 | 0.43 | 0.28 | 0.72 | 0.86 | 0.14 | <0.05 |
| High (n=68) | 0.59 | 0.41 | 0.89 | 0.11 | 0.59 | 0.41 | 0.25 | 0.75 | 0.78 | 0.22 | |

### 4.5.2 Genotype frequencies of the FADS2 and FADS1 genes in chicken

In the previous described experiments in quail, five SNPs were found in both quail FADS2 as well as FADS1. These SNPs in both FADS2 and FADS1 were used to compare the different variances among the different local chicken breeds. Four of five SNPs of the FADS2 were monomorphic among these breeds, except SNP4 that segregated in five out of the six breeds (Table 25). The SNP4 genotype in Table 25 showed that the genoptype CC was predominant in Ac, Ri, Te and SLS chickens, while the CT genotype was in Noi and H'mong chickens. The two SNPs within the FADS1 gene at position 391 (C to A) and 468 (C to T) segregated in Te, Noi, Ri and SLS (Table 25).

Table 25: Frequencies of the FADS1 and FADS2 genotypes in the different local chicken breeds

| Breed | No. of animal | FADS2 SNP4 | | FADS1 391Val | | | FADS1 Val468Ala | | |
|---|---|---|---|---|---|---|---|---|---|
| | | CC | CT | CC | CA | AA | CC | CT | TT |
| Ac | 6 | 0.70 | 0.30 | 0.50 | 0.50 | - | 0.50 | 0.50 | - |
| Noi | 6 | - | 1.00 | 0.80 | - | 0.2 | 0.80 | - | 0.20 |
| Ri | 3 | 0.70 | 0.30 | 0.30 | 0.70 | - | 0.30 | 0.70 | - |
| Te | 3 | 0.70 | 0.30 | 1.00 | - | - | 1.00 | - | - |
| H'mong | 3 | 0.30 | 0.70 | 1.00 | - | - | 1.00 | - | - |
| SLS | 5 | 0.80 | 0.20 | 0.80 | 0.20 | - | 0.80 | 0.20 | - |

## 4.6 Functional roles of FADS1 and FADS2 on PUFA in the yolk of Japanese quail

FADS1

The associations of the genotype FADS1 with fatty acid profiles were established as a result in Table 26. The associations were found between the Val391 genotypes with the polyunsaturated fatty acid linoleic acid (C18:2 ω-6) ($P<0.05$). No association was observed either with the Val391 or with the Val468Ala genotype with the other long chain polyunsaturated fatty acids C20:4 (ω-6), C22:6 (ω-3) and the ω-6:ω-3 PUFA ratio ($P>0.05$).

Table 26: FADS1 Val391 genotype effects on the fatty acid profiles in the quail yolk

| Position | Genotype | C18:2 (ω-6) | C20:4 (ω-6) | C22:6 (ω-3) | ω-6: ω-3 |
|---|---|---|---|---|---|
| Val391 | CC | 13.73±0.34[a] | 2.56±0.14 | 1.06±0.07 | 17.83±1.32 |
| | CA | 13.36±0.18[ab] | 2.40±0.07 | 1.00±0.04 | 18.13±0.68 |
| | AA | 12.94±0.12[b] | 2.36±0.05 | 1.00±0.03 | 17.57±0.46 |

[*]Results are expressed as means (%) ± SD.

[a,b] P<0.05 in a column

The association between genotypes and fatty acids were further strengthened by haplotype analysis. The infered haplotypes of FADS1 revealed three combinations, CT, AC and AT. However, only two haplotypes, CT and AC, were most common. There was significant association between the haplotypes and C18:2 ω-6 (P<0.05) (Table 27).

Table 27: The FADS1 haplotypes associate with PUFA in the quail yolk

| Haplotype | C18:2 (ω-6) | Frequency[1] |
|---|---|---|
| CT | 13.53±0.18[a] | 0.29 (100) |
| AC | 12.97±0.11[b] | 0.66 (230) |

Results are expressed as means (%) ± SD.

[1]haplotype with a frequency of <0.05 not included.

[a,b] P<0.05 in a column

FADS2

The association between single SNPs and the ω-6:ω-3 fatty acid concentration of the egg yolk was tested. According to the analysis of variance, the SNP3 genotypes were found to be significantly associated with mean of C20:4 (ω-6) and C22:6 (ω-3) fatty acids (P<0.05). Furthermore, a significant effect was also found on the ω-6:ω-3 PUFA ratio (P<0.05) with genotype CC giving the lowest value of the ω-6:ω-3 PUFA ratio compared to genotype TT giving the highest value of ω-6:ω-3 PUFA ratio (Table 28). Also for the SNP4 genotypes significantly different means of the ω-6:ω-3 PUFA were found (P<0.05). No significant effects were found for the SNP1, SNP2 and SNP5 genotypes on the polyunsaturated fatty acids.

Individual fatty acid traits were also analysed with FBAT. Using the additive genetic model, the SNP3 association on the C14:0, C18:2 (ω-6), C20:4 (ω-6), C22:6 (ω-3) and the ω-6:ω-3 PUFA ratio (P<0.05) were combined (Table 29). Similar results were found for the SNP4 genotypes showing association with the same fatty acids (P<0.05) except

the ω-6:ω-3 PUFA ratio (Table 29). No significant associations were observed among the SNP1, SNP2 and SNP5 genotypes and the phenotypic fatty acids under this model.

Table 28: Genotype effects of the FADS2 gene on polyunsaturated fatty acid profiles in egg yolk

| | SNP3 | | |
|---|---|---|---|
| Genotype | TT | CT | CC |
| Trait (%) | | | |
| C18:2ω-6 | 14.16±0.98 | 13.91±0.21 | 13.22±0.48 |
| C20:4ω-6 | 1.81$^{b}$±0.33 | 2.37$^{a}$±0.07 | 2.44$^{a}$±0.17 |
| C22:6ω-3 | 0.71$^{b}$±0.18 | 1.02$^{a}$±0.04 | 1.07$^{a}$±0.09 |
| ω-6:ω-3 | 25.31$^{a}$±3.10 | 19.78$^{b}$±0.65 | 16.49$^{b}$±1.53 |

| Genotype | SNP4 | | |
|---|---|---|---|
| Trait | TT | CT | CC |
| ω-6:ω-3 | 18.21$^{ab}$±0.83 | 21.05$^{b}$±0.94 | 13.60$^{a}$±3.53 |

*Results are expressed as means (%) ± SD

$^{a,b}$ P<0.05 in a line

Table 29: The association between the FADS2 SNPs and the fatty acids profiles by FBAT analysis

| Locus | SNP3 (C-T) | | SNP4 (C-T) | |
|---|---|---|---|---|
| Traits | Z | p-value | Z | p-value |
| C14:0 | 2.355 | 0.019$^{*}$ | 2.105 | 0.035$^{*}$ |
| C18:2 ω-6 | 2.177 | 0.029$^{*}$ | 2.173 | 0.030$^{*}$ |
| C20:4 ω-6 | 2.589 | 0.010$^{*}$ | 2.590 | 0.010$^{*}$ |
| C22:6 ω-3 | 2.602 | 0.009$^{**}$ | 2.566 | 0.010$^{*}$ |
| ω-6:ω-3 | 2.089 | 0.037$^{*}$ | 1.840 | 0.066$^{ns}$ |

* P<0.05; ** P<0.01; ns: not significant

## 4.7 Function of FADS1 and FADS2 on MUFA and SFA in the yolk of Japanese quail

FADS1

The associations of the genotype FADS1 with fatty acid profiles were established as a result in Table 30. The associations were found between the Val391 genotypes and the saturated fatty acids myristic acid (C14:0), palmitic acid (C16:0) and the monounsaturated fatty acid palmitoleic acid (C16:1 ω-7) ($P<0.05$). The genotypes at position 468 (C to T) were not significantly associated with any fatty acid profile except palmitic acid (C16:0) ($P<0.05$).

Table 30: FADS1 genotype effects on the fatty acid profiles in the quail yolk

| Position | Genotype | C14:0 | C16:0 | C16:1 (ω-7) | C18:1 (ω-9) |
|---|---|---|---|---|---|
| Val391 | CC | 0.45±0.01[b] | 25.72±0.27[b] | 3.83±0.17[ab] | 40.11±0.66 |
| | CA | 0.49±0.01[a] | 26.49±0.14[a] | 4.05±0.09[a] | 41.03±0.34 |
| | AA | 0.45±0.01[b] | 26.21±0.10[ab] | 3.60±0.06[b] | 41.48±0.23 |
| Val468Ala | CC | 0.46±0.01 | 26.33±0.10[a] | 3.70±0.06 | 41.43±0.25 |
| | CT | 0.47±0.01 | 26.23±0.12[ab] | 3.76±0.07 | 41.44±0.31 |
| | TT | 0.45±0.02 | 25.61±0.27[b] | 3.73±0.15 | 40.15±0.65 |

Results are expressed as means (%) ± SD

[a,b] $P<0.05$ in a column

The inferred haplotypes of the FADS1 gene revealed the significant differences in C16:1 (ω-7) and C18:1 (ω-9) ($P<0.05$) among these two haplotypes were found. The AC haplotype was significantly lower in C16:1 (ω-7) and higher in C18:1 (ω-9) than the CT haplotype (Table 31).

Table 31: The FADS1 haplotypes associate with fatty acid profiles in the quail yolk

| Haplotype | C16:1 (ω-7) | C18:1 (ω-9) | Frequency[1] |
|---|---|---|---|
| CT | 3.96±0.09[a] | 40.39±0.33[b] | 0.29 (100) |
| AC | 3.73±0.06[b] | 41.62±0.23[a] | 0.66 (230) |

Results are expressed as means (%) ± SD.

[1]haplotyped with a frequency of <0.05 not included.

[a,b] significantly different at $P<0.05$ in a column

FADS2

The analysis of the other fatty acids revealed significant associations between the FADS2 SNP2 genotypes and the saturated fatty acid myristic acid (C14:0) ($P<0.05$) (Table 32).

Table 32: Genotype effects of SNP2 of the FADS2 gene on fatty acid profiles in egg yolk

| SNP2 | GA | GG |
|---|---|---|
| C14:0 | $0.46\pm0.02^{b}$ | $0.51\pm0.01^{a}$ |

Results are expressed as mean (%) ± SD of high and low lines

$^{a,b}$ significantly different ($P<0.05$) in a line

The haplotypes of FADS2 with a frequency of at least 5% are shown in Table 32. The most common haplotype was CGCTG (24.71%) and the CACCG haplotype gave the lowest frequency (5.17%). The FADS2 haplotypes were significantly associated with C16:0 ($P<0.05$) (Table 33).

Table 33: Haplotype frequencies and the association of the haplotypes of FADS2 with C16:0

| Haplotype[1] | Frequencies (n) | C16:0 |
|---|---|---|
| CGCTG | 0.25 (43) | $26.59\pm0.16^{ab}$ |
| TGTTG | 0.14 (24) | $25.85\pm0.26^{b}$ |
| TGCTG | 0.13 (22) | $27.28\pm0.35^{a}$ |
| CGTTG | 0.09 (16) | $26.93\pm0.28^{ab}$ |
| CGCCA | 0.08 (14) | $26.91\pm0.33^{ab}$ |
| CGCCG | 0.07 (13) | $26.34\pm0.38^{ab}$ |
| CACCG | 0.05 (9) | $26.61\pm0.38^{ab}$ |

[1] haplotypes with a frequency of <0.05 not included

$^{a,b}$ $P<0.05$ in a column

## 4.8 Interaction between the FADS1 and FADS2 genes in the high and low lines on fatty acid profiles in yolk of quail

Significant interaction were found between lines and FADS1 Val391 genotypes for the C14:0, C16:0, C16:1 (ω-7) and C18:2 (ω-6) fatty acids ($P<0.05$) (Table 34). The

interaction between the lines and Val468Ala genotypes was significant for the C18:1 (ω-9) ($P<0.05$) (Table 35).

Table 34: The FADS1 Val391 genotypes interaction with high and low lines in fatty acid profiles

| | Position | Val391 | | |
|---|---|---|---|---|
| Phenotype | Genotype | CC | CA | AA |
| C14:0 | High line | $0.49\pm0.03^{ab}$ | $0.54\pm0.02^{a}$ | $0.47\pm0.01^{b}$ |
| | Low line | $0.40\pm0.02^{c}$ | $0.44\pm0.01^{c}$ | $0.43\pm0.01^{c}$ |
| C16:0 | High line | $26.48\pm0.41^{ab}$ | $27.04\pm0.23^{a}$ | $26.35\pm0.14^{b}$ |
| | Low line | $24.96\pm0.36^{c}$ | $25.94\pm0.16^{bc}$ | $26.06\pm0.13^{b}$ |
| C16:1 (ω-7) | High line | $4.26\pm0.27^{ab}$ | $4.54\pm0.15^{a}$ | $3.65\pm0.09^{c}$ |
| | Low line | $3.39\pm0.24^{bc}$ | $3.56\pm0.11^{c}$ | $3.55\pm0.09^{c}$ |
| C18:1 (ω-9) | High line | $37.58\pm0.99^{c}$ | $39.00\pm0.56^{c}$ | $40.84\pm0.34^{bc}$ |
| | Low line | $42.65\pm0.89^{ab}$ | $43.06\pm0.40^{a}$ | $42.12\pm0.31^{a}$ |
| C18:2 (ω-6) | High line | $15.00\pm0.51^{a}$ | $14.32\pm0.29^{a}$ | $13.82\pm0.17^{a}$ |
| | Low line | $12.46\pm0.45^{b}$ | $12.40\pm0.20^{b}$ | $12.07\pm0.16^{b}$ |

Results are expressed as means (%) ± SD.

$^{a,b,c,d}$ $P<0.05$ in a line

Table 35: The FADS1 Val468Ala genotype interaction with high and low lines in fatty acid profiles

| | Position | Val468Ala | | |
|---|---|---|---|---|
| Phenotype | Genotype | CC | CT | TT |
| C18:1 (ω-9) | High line | $40.75\pm0.32^{b}$ | $39.83\pm0.46^{bc}$ | $37.66\pm0.94^{c}$ |
| | Low line | $42.12\pm0.32^{a}$ | $43.06\pm0.40^{a}$ | $42.64\pm0.89^{ab}$ |

Results are expressed as means (%) ± SD of each line.

$^{a,b,c}$ $P<0.05$

The interaction between FADS1 haplotypes and the lines was significantly different in C16:1 (ω-7), C18:1 (ω-9) and C22:6 (ω-3) ($P<0.01$) (Table 36). The AT haplotype was only present in the high line, and the AC haplotype of the low line was lower in C16:1 (ω-7) and higher in C18:1 (ω-9) and C22:6 (ω-3) compared to the high line.

Table 36: The FADS1 haplotype interaction with lines on the mono- and polyunsaturated fatty acids

| Phenotype | Haplotype | AT | CT | AC |
|---|---|---|---|---|
| C16:1(ω-7) | High line | 2.92±0.20$^{c}$ | 4.44±0.14$^{a}$ | 3.89±0.09$^{b}$ |
| | Low line | | 3.49±0.11$^{bc}$ | 3.56±0.07$^{b}$ |
| C18:1 (ω-9) | High line | 41.32±0.77$^{ab}$ | 37.89±0.52$^{c}$ | 40.91±0.37$^{b}$ |
| | Low line | | 42.90±0.43$^{a}$ | 42.33±0.28$^{a}$ |
| C20:4 (ω-6) | High line | 2.19±0.17 | 2.52±0.11 | 2.21±0.08 |
| | Low line | | 2.48±0.09 | 2.48±0.06 |
| C22:6 (ω-3) | High line | 0.89±0.08$^{ab}$ | 1.02±0.06$^{ab}$ | 0.87±0.04$^{b}$ |
| | Low line | | 1.06±0.05$^{a}$ | 1.10±0.03$^{a}$ |
| ω-6:ω-3 | High line | 19.30±1.53 | 19.19±1.04 | 20.79±0.71 |
| | Low line | | 16.36±0.86 | 15.09±0.56 |

Results are expressed as means (%) ± SD of each line.

$^{a,b,c}$ P<0.05 in a line

The interactions of polymorphisms of the FADS2 gene with the divergently high and low lines (Table 37) were examined. Results of the SNP2 genotypes showed significant interaction with the lines for C14:0 (P<0.01). Likewise, the interaction between lines and SNP3 genotypes were significant for C16:0 fatty acid (P<0.05).

Table 37: Effect of the SNP2 and SNP3 genotypes of the FADS2 gene on the fatty acid profiles in the high and low lines

| SNP2 | | GA | GG | |
|---|---|---|---|---|
| C14:0 | High line | 0.46±0.02$^{b}$ | 0.56±0.01$^{a}$ | |
| | Low line | 0.47±0.02$^{b}$ | 0.46±0.01$^{b}$ | |
| SNP3 | | TT | CT | CC |
| C16:0 | High line | 26.75±0.69$^{cd}$ | 26.82±0.16$^{cd}$ | 27.88±0.37$^{c}$ |
| | Low line | 26.12±0.69$^{cd}$ | 26.43±0.13$^{d}$ | 26.18±0.31$^{d}$ |

$^{*}$Results are expressed as means (%) ± SD of each line.

$^{a,b}$ P<0.01

$^{c,d}$ P<0.05

# 5 Discussion

Nowadays, new insights into the relationship between food and prevention of diseases are considered to be important for human being. In addition to fatty acids from the fish source, fatty acids especially ω-3 PUFA from non-fish foods such as eggs have been paid attention to. This study emphasized on the genetic variations between the high and low lines of Japanese quail to improve the nutritive value of eggs, with respect to the ω-3 and ω-6:ω-3 PUFA ratio, through understanding the metabolic pathways particular in the desaturation of these fatty acids. This involved genomic approaches including the identification of genes and their alleles contributing to the increase of the ω-3 fatty acids as well as the reduction of the ω-6:ω-3 PUFA ratio to match the human nutritional recommendation.

## 5.1 Fatty acid profiling

As previously mentioned, the fatty acid composition of the egg yolk can be changed by modifying the hen's diet (Watkins 1991, Cherian et al. 1992, 1993, Menicken et al. 2005). The yolk fatty acid profiles generally depend on the dietary fatty acid composition and the deposition rate differs for different fatty acids. The fatty acid profiles of the high and low line in Table 13 revealed that yolk fat was relatively higher in unsaturated fatty acids, especially in oleic acid and followed by palmitic acid than in the diet (Leskanich et al. 1997). The amount of SFA and MUFA were significantly different between high and low lines in each generation with significant higher SFA and lower MUFA in the high line than in the low line. Moreover, these data also indicated that MUFA content was present at a higher level than SFA. The former fatty acids are located on sn2 position whereas the latter are located on sn1 position (Leskanich et al. 1997). From the phenotypic data, it is clearly shown that the maternal fatty acids of C18:2 (LA, ω-6) and C18:3 (ALA, ω-3) were higher in the diet than in the yolk deposition, while the polyunsaturated fatty acids as C20:4 (AA, ω-6) and C22:6 (DHA, ω-3), were the most predominant ω-6 and ω-3 fatty acids in the yolk, were converted into the egg yolk although they were not detected in the feed supply. The fatty acid profiles of the present study were in agreement with those of Mennicken et al. (2000, 2005), who showed that AA and DHA were higher in the yolk than in the diet. The increase of these fatty acids in the egg yolk can be obtained in another way than directly from the dietary fatty acids, i.e. they can be synthesized in the liver by desaturation and elongation of LA and ALA, respectively (Watkins et al. 1987). In this

synthesis, Δ6- and Δ5- desaturase enzymes, which create long chain polyunsaturated fatty acid (LC-PUFA), are involved. Furthermore, the conversion of LA to AA was relatively higher than that of ALA to DHA indicating the competition between the ω-6 and ω-3 PUFA for the enzymes involved in fatty acid elongation and desaturation.

Comparing the deposition rate between high and low lines indicated higher DHA and AA in the low line than in the high line during the selection process. These line results indicate genetic differences in utilization and storage of dietary fatty acids, with the low line having most likely a more efficient utilization of dietary fat than the high line. This could also be related to lower desaturase enzyme activities in the high line than in the low line. This result is in agreement with Mennicken et al. (2005), who have shown that the low line could be affected by divergent selection.

The increase in ω-3 fatty acids, particularly DHA, and decrease in ω-6 fatty acids especially LA in the yolk of the low line leads to a reduced the ω-6:ω-3 PUFA ratio (Table 13). Selection for the ω-6:ω-3 PUFA ratio after 6 generations in the high and low lines resulted significantly lower. However, the ratio of ω-6:ω-3 PUFA among the three generations of the two selected lines showed a similar selection response in the 7$^{th}$ generation when compared to the 5$^{th}$ and 6$^{th}$ generation. It is suggested that the divergent selection for the ω-6:ω-3 PUFA ratio has been efficient after 6 generations. In a previous study (Mennicken et al. 2005), at generation 4 the ω-6:ω-3 PUFA ratio was significantly different between the high and low lines (14.93 and 12.35, respectively). At the 7$^{th}$ generation, the ratio of ω-6:ω-3 PUFA was 13.41 and 12.52 for the high and low lines, respectively. The selection has become effective with increasing generations, even though there was a slightly increase of the ω-6:ω-3 PUFA ratio at the 7$^{th}$ generation in the low line. This result was in agreement with Zaky et al. (1996) who showed that the fatty acid composition i.e. a higher ratio for PUFA:SFA had improved over eight to nine generations by selection. In another experiment (Minvielle et al. 2002), selection on egg production increased yolk content and the ω-6:ω-3 PUFA ratio.

Little information has been reported on the estimated heritabilities for the egg quality related to the ω-6:ω-3 PUFA ratio. Genetic variance was estimated by REML (Falconer 1984) for the differential selection of the C22:6 (DHA, ω-3) and the ω-6:ω-3 PUFA ratio. Moderate heritabilities were found in the C22:6 (DHA, ω-3) and shown a higher heritability for the ω-6:ω-3 PUFA ratio. In addition, the low line is more efficient than the high line. Minvielle et al. (2002) found that selection for egg quality had moderate heritability and strong genetic correlation with yolk component, particular in the ω-6:ω-3 PUFA ratio. This study and Mennicken et al. (2000, 2005) demonstrated that the

divergent selection for the ω-6:ω-3 PUFA ratio was significantly higher in yolk weight, proportion and the soluble fat content in the low line compared to the high line while there was no significant difference between selected high and low lines as well as no effect on fertility and hatchability. It is interesting to note that the heritability for the abdominal fat of the female quail at 58 days was also moderate (0.33) (Sadjadi and Becker 1980). There may be a close relationship between the abdominal fat trait and yolk fat content. However, Suk et al. (1998) demonstrated that there was no correlation between the yolk cholesterol and yolk fat content and the abdominal fat deposition.

In Table 15, the estimated heritabilities increased for both fatty acid traits during the selection process suggesting that the breeding model can change the yolk fatty acids. Mennicken et al. (2005) also recorded the heritability, which was higher when compared to the results of this study but in agreement with the earlier results (Mennicken et al. 2000). Moreover, negative genetic correlation between the C22:6 (DHA, ω-3) and the ω-6:ω-3 PUFA ratio indicates that selecting for higher ω-6:ω-3 PUFA ratio cooperates with a lower level of C22:6 (DHA, ω-3) fatty acid. This can be illustrated by comparing the mean of C22:6 (ω-3) and the ω-6:ω-3 PUFA ratio between high and low lines (Table 13). Mennicken et al. (2005) found a similar phenotypic correlation between the C22:6 (DHA, ω-3) and the ω-6:ω-3 PUFA ratio.

As expected, the estimated heritablities for the ω-6:ω-3 PUFA ratio of both high and low lines increased over generations whereas the phenotypic selection decreased. These results indicate that selection can genetically improve DHA and change the ω-6:ω-3 PUFA ratio in the egg yolk.

## 5.2 Characterisation of the FADS1 and FADS2 genes in quail

In this study, the quail FADS2 gene was cloned and a sequence of 1350 bp obtained, which comprised of 1227 bp coding sequence (CDS), the stop codon (nt1228-1230) and the 3' UTR region with the polyadenylation site. However, the 5' end of CDS with the start codon was not identified. Compared to the human cDNA FADS2 sequence (3121 bp; Genbank accession number NM_004265.2) (Marquardt et al. 2000) the CDS of the quail FADS2 gene is 120 bp shorter, which corresponds to the missing 40 amino acids of the quail FADS2 protein (Figure 8).

In addition to the FADS2 gene, the quail FADS1 was also cloned and had a partial cDNA of 1797 bp in length that comprised 1334 nucleotides encoding 445 amino acids with the stop codon (nt1367-1369). However, the quail FADS1 cDNA was incomplete with missing the start codon like the FADS2.

The quail FADS2 and FADS1 nucleotide sequences showed high identity (77%) as did the deduced protein. Moreover, both FADS2 and FADS1 showed high identity and are closely related to the FADS2 and FADS1 of human, rat and mouse. Thus, quail FADS2 and FADS1 have also acquired as the functional characterization of fatty acid desaturase activities. Human FADS1 and FADS2 together with FADS3 are clustered genes, which are located on chromosome 11q12–q13.1. The first two desaturases are oriented head to head and arose by duplication of the other (Marquardt et al. 2000, Nakamura et al. 2004). By following the chicken genome project (NCBI, genome project, chicken ID 10804), it is shown that FADS2 and FADS1 in chicken are located head to head on chromosome 5. Based on the comparative map between chicken chromosome 5 and quail chromosome 5 (Kayang et al. 2006) the FADS2 and FADS1 genes are most likely located on quail chromosome 5 as well. Therefore, it is possible that quail FADS2 and FADS1 genes arose also from gene duplication as in human (Marquardt et al. 2000, Nakamura et al. 2004) and mouse (Nakamura et al. 2004).

The deduced amino acid sequences of quail FADS2 and FADS1 contain two domains; a N-terminal catalytic domain which is involved in the electron transport and acts as an electron donor in the desaturation reaction (Michell et al. 1995, Marquardt et al. 2000) and the C-terminal cytochrome $b_5$-like domain containing three highly conserved histidine motifs, HDXGH, HFQHH and QIEHH, which are characteristic for membrane-bound desaturase (Jump et al. 1999, Marquardt et al. 2000) and essential for desaturase activity. These structural features characteristic for the desaturase genes were found in human (Cho et al. 1999 a, b, Marquardt et al. 2000), rat (Aki et al. 1999) and mouse (Cho et al. 1999 a). Despite these similarities with other species, the heme-binding residue in the cytochrome $b_5$ superfamily “HPGG” motif, which is present in human, rat, mouse and *C. elegans* FADS2 (Marquardt et al. 2000), is replaced by a “HPGA” motif in both quail FADS2 and FADS1. This change from “G” to “A” in the hem-binding iron in quail suggests that the cytochrome $b_5$ of FADS2 and FADS1 containing the “HPGA” motif may be characteristic in quail species and may not change the protein expression and catalytic activities. As Guillou et al. (2004) found that the deletion of the “HPGG” motif or the substitution of histidine for an alanine residue did not change Δ6-desaturase expression in rat. Moreover, recent study in humans demonstrated that front-end desaturases may accept some differences at the methyl end of their substrates and require only an appropriate carboxylic end where the new double bonds are introduced (Domergue et al. 2002).

In this study, the comparative sequencing of eight animals of the divergent quail lines revealed five SNPs for both FADS2 and FADS1. As described in Figure 6, the last three of five synonymous SNPs (SNP3, SNP4 and SNP5) of FADS2 were observed

between the second and the third histidin box. They revealed highly conserved regions across human, it could mean that these SNPs are ancestral amino acids of the FADS gene cluster. Similarly, the identified five SNPs found for FADS1 included two amino acid substitutions from asparagine to serine at position 348 and from valine to alanine at position 468, which may have an important role on the enzyme function. Alignment of the deduced amino acid sequences (Figure 7 and 8) indicated that the former amino acid substitution and amino acid at SNP position 570 were highly conserved across the three human FADS family, whereas the latter amino acid substitution together with the other two SNPs at position 391 and 1075 were highly represented amino acids of FADS1.

It is interesting that the five SNPs of quail FADS2 are located within the histindine rich boxes of the desaturase enzyme, while the SNPs of FADS1 are near to the N-terminal cytochrome $b_5$ like domain. This may be related to the localization of both FADS2 and FADS1 genes on the chromosome and possibly affected the transcription sequences of FADS2 and FADS1 within this region. In human, the Northern-blot result indicated that FADS2 and FADS1 genes are in closer relationship than to any other desaturase (Cho et al. 1999 a).

This study additionally addressed the expression of the two genes in quail. In humans FADS2 and FADS1 are expressed higher in liver compared to other tissues (Brenner 1989, Scott et al. 1989, Cho et al. 1999 a,b). Each desaturase displayed similar activities toward the different substrates and the increment in FADS2 activity may have masked an increase in FADS1 activity. Furthermore, the activity of both FADS2 and FADS1 enzymes is mainly controlled by nutritional and hormonal factors (Wakil et al. 1983, Brenner 1989).

Quantitative Real-time-PCR results showed that quail FADS2 mRNA has a similar expression compared to the quail FADS1 mRNA, though the FADS2 mRNA expressed higher than that of FADS1. This result is in agreement with Leonard et al. (2000) who showed that the expression of both FADS2 and FADS1 varied in different tissues and was highest for FADS2. It is known that FADS2 is the rate-limiting enzyme in the biosynthesis of the long chain PUFA and acts not only on C18- PUFA but also on C20-PUFA (Sprecher et al. 1995) suggesting that FADS2 may require an appropriate higher level for the transcription at both sites than FADS1. Besides, Hasting et al. (2001) showed that the FADS2 gene is more active towards Δ6-desaturase substrates than Δ5-desaturase substrates and prefers to convert ω-3 fatty acids rather than ω-6 fatty acids.

In comparison, the expression of the FADS genes in the divergent selection of the high and low lines showed that the low line expressed FADS2 at a higher level, about two

fold of that in the high line, whereas the expression of FADS1 was the same level in both high and low lines. These results confirm and extend the phenotypic data which showed a high deposition rate of DHA and AA and led to a low ratio of the ω-6:ω-3 PUFA in the low line. Haugaard et al. (2006) showed that there is a negative correlation between the ω-6:ω-3 PUFA ratio and the Δ6-desaturase activity, i.e. increase in Δ6-desaturase activity was parallel to a decrease in the ω-6:ω-3 PUFA ratio while the Δ5-desaturase activity did not increase.

## 5.3 The expression of the FADS1 and FADS2 genes in different chicken breeds

Birds are able to synthesize the long chain ω-3 PUFA that are important source of EPA and DHA for human health. However, a wide variation in fatty acids among the avian species has been found (Leskanich et al. 1997, Surai et al. 1999). Molecular understanding of the different species in their abilities to synthesis of EPA and DHA may contribute to a biotechnological solution for production of EPA and DHA sources which have been demonstrated in plant (Galili et al. 2002). In quail, the functional FADS2 and FADS1 genes have been cloned and characterized as well as the association between their SNPs with the fatty acid composition in the egg yolk. The FADS2 and FADS1 genes in chicken were also identified and characterized. The SNPs that were detected in quail were studied to evaluate their contributions to synthesize AA and DHA by FADS2 and FADS1 desaturase activities in different Vietnamese local chicken breeds.

Fatty acid profiles in Ri chicken yolk contained a higher level of oleic acid and palmitic acid, which is consistent with the results in quail. The interesting findings in this study are the levels of AA and DHA, the predominant polyunsaturated fatty acids in the yolk. Compared with other results (Ahn et al. 1995, Leskanich et al. 1997) the low amount of DHA and high AA in Ri chicken yolk, then consequently, the ratio of ω-6:ω-3 PUFA in Ri chicken yolk was higher than in chicken eggs enriched with ω-3 fatty acids. Of course, many studies illustrated that yolk fatty acid composition reflected dietary fat for the laying hens, thus resulted in the increase of the proportion of the fatty acids that deposit into the egg (Leskanich et al. 1997, Raes et al. 2002). An explanation for the lower ω-3 fatty acids as well as the higher ω-6 fatty acids in Ri chicken may be because the hens were fed a normal diet with a lower ω-3 fatty acid content. However, the level of DHA content in Ri chicken compared with other species like turkey, goose and duck (Surai et al. 1999) indicates that Ri chicken is also more efficient in converting to longer chain ω-3 PUFAs than the above species. Therefore, studies on the different local

chicken breeds are usefull regarding the improvement of the ω-3 fatty acid sources for human consumption as well as the management of genetic resource populations and their utilisation in future improvement programmes.

Regarding the different yolk fatty acids in relation to enzyme activities involved in the conversion of long chain PUFAs, chicken FADS2 and FADS1 were identified and characterized. A total length of 1380 bp and 1561 bp were obtained for the chicken FADS2 and FADS1 genes, respectively. The coding sequence of FADS2 comprised 969 bp encoding 332 amino acids and the 1332 bp chicken FADS1 cDNA contained an open reading frame of 444 amino acids. Both FADS genes share high similarity to the predicted chicken genes, FADS2 (XM_421053) and FADS1 (XM_421052) and about 79% identities with their human analogues (Marquardt et al. 2000). However, the alignment of FADS2 amino acid sequences in Figure 10 shows the small difference between the present and predicted chickens, e.g. at position 121-122 and 285, it could be potential polymophisms of this gene in chicken.

The structural characteristics of the FADS2 and FADS1 revealed the typical features of membrane bound desaturases, HDFGH, HFQHH and QIEHH, and a cytochrome $b_5$ like domain that is similar to human, rat, mouse and especially quail, except for the deduced protein of FADS2 that was lacking of the cytochrome b5 like domain “HPGG”. Aligned sequences reveal that the orthologous chicken FADS2 and FADS1 genes have retained their intron/exon structure, indicating that the common ancestor of chicken FADS genes may have similar functions to other mammalian desaturases reported.

Despite similarities between quail and chicken (Sadjadi and Becker 1980), both of these species have some definite differences including the cytochrome $b_5$ domain containing the highly conserved sequence motif “HPGG”, which is found in chicken, human and mouse, but changing to “HPGA” in quail. In addition, comparing the length of the coding sequence and the deduced protein sequences of FADS2 and FADS1 in chicken and quail showed that chicken sequences were shorter than those of quail. However, both chicken and quail FADS2 and FADS1 sequences obtained shorter length than the predicted molecular sizes, 2021 bp and 2847 bp for Gallus gallus species of FADS2 (XM_421053) and FADS1 (XM_421052). One possible explanation is the alternative transcripts of these genes in both quail and chicken that could be a reason for getting shorter transcripts as described by Stöhr et al. (1998) for the transcripts of 3,000 and 4,000 bp.

Consistent with previous expression results in quail, the mRNA expression of FADS2 and FADS1 genes in chicken showed no significant difference between both genes, though expression was higher in FADS2 than FADS1. This could be because the FADS2 and FADS1 are oriented in inverse sequence to each other on human

chromosome 11 and the transcription of the FADS2 and FADS1 genes are co-ordinately governed by regulator sequences within the 11,000 bp sequences (Cho et al. 1999 b, Marquardt et al. 2000).

Although the different avian species with different fatty acid profiles have been reported by Leskanich et al. (1997) and Surai et al. (1999), this is the first study on the local genetic variability of chicken breeds regarding the expression of FADS2 and FADS1. The expression of FADS2 and FADS1 mRNA in six different local chicken breeds showed that the expression of Te chicken and European chicken is significantly (4.5 – 5 fold) higher than that in the other chicken breeds, Ac, Noi, Ri and H'mong (Figure 12). As the expression in quail showed, the ω-6:ω-3 PUFA ratio depends on the relative expression of the FADS2 activity (Haugaard et al. 2006). It is therefore suggested that the endogenous metabolism of Te and European chicken breeds may be more efficient in the incorpation of the higher ω-3 PUFA into the egg yolk than others. In human, hormones and other factors have been implicated in the control of expression of FADS2 and FADS1. Here, genetic variation, another factor is hypothesized to increase the activities of the desaturase enzymes. Also, the existence of genetically distinct geographical populations or phylogenetic species could be concerned.

The differences among the diverse local chicken breeds were displayed furthermore by the frequencies of FADS2 genotypes (Table 25). Interestingly, different genotypes of FADS2 were observed for SNP4 position which showed significant association to ω-3 and ω-6 PUFAs in quail, whereas the other FADS2 SNPs were homologous. The genotype CC was predominant for Ac, Ri, Te and European chicken breeds, whereas for the Noi and H'mong chickens the CT genotype was predominant. The difference between quail and chicken for the FADS2 gene on the ω-3 and ω-6 PUFA is that the genotypes at SNP3 position were the most important in quail while in chicken the SNP4 position was most important.

The genotype frequencies of FADS1 are shown in Table 25. The genotype CC at 391 and 468 positions was predominant on Te, H'mong, European and Noi chickens; and the genotypes CA and CT were predominant for Ri chicken.

## 5.4 Function of FADS1 and FADS2 on the fatty acids of the yolk

The FADS2 gene is addressed as a functional candidate gene for traits related to ω-6 and ω-3 PUFA concentration in egg yolk. Five SNPs were identified that segregated among experimental divergently selected lines of quails. The SNPs were synonymous, i.e. changing amino acids. The association analysis comprising analysis of variance and family based association test (FBAT), revealed significant effects for SNP3 and

SNP4 of FADS2 on the egg yolk fatty acid profiles, especially on the ω-6 and ω-3 PUFAs. These results indicate close linkage and linkage disequilibrium, i.e. association, of the two SNPs with a causal polymorphism within or very close to FADS2. No effects of the other SNPs were found, indicating that these are not in linkage disequilibrium with the causal polymorphism. Comparisons of means depending on genotypes indicate that the effect of the hypothesed causative polymorphism is towards a more efficient desaturation, elongation and deposition of ω-3 PUFA than ω-6 PUFA in the genotype favourised by selection for low ω-6:ω-3 PUFA ratio, i.e. linoleic (LA) and α-linolenic acid (ALA) are precursors of long polyunsaturated fatty acids that were available to the bird via the diet with the first being metabolized to arachidonic acid (AA) and the second to docosahexaenoic acid (DHA).

In addition to the significant association between the FADS2 SNPs and the yolk ω-6 and ω-3 fatty acids, association was also found with the SFA myristic acid (C14:0). This result may explain the fact that C14:0 increases Δ6-desaturase activity significantly (Jan et al. 2004). Besides, Shappell et al. (2001) demonstrated that Δ6-desaturase preferably receives C18 substrates for the catalytic mechanism and also allows preferential desaturation of C14:0.

The haplotype analyses also showed significant association with an SFA, C16:0. The TGTTG haplotype was associated with a significantly lower level of C16:0 compared to the TGCTG haplotype. The different analyses indicate that the possible change of fatty acid profile is mostly influenced by allele “C” or “T” at the SNP3 position. Miyazaki et al. (2002) and Guillou et al. (2004) found that FADS2 acts on C16:0. Therefore, the association between the FADS2 and the saturated fatty acids in quail indicate that it may be correlated to saturated fatty acids that are effective in the *de novo* fatty acid synthesis. Further studies on this aspect should be considered.

Furthermore, the other functional candidate gene FADS1, whose encoded protein is specifically for the conversion of DGLA (C20:3 ω-6) to AA (C20:4 ω-6) or ETA (C20:4 ω-3) to DHA (C22:6 ω-3) in egg yolk (Sprecher et al. 1995), was addressed. Although ω-6 and ω-3 C20 PUFA as DGLA and ETA are preferred substrates for the FADS1 in other organisms, the association between FADS1 genotypes at position 391 and 468 with phenotypic fatty acid profiles in the yolk indicated that these polymorphisms were neither significant associated with C20:3 (ω-6) nor C20:4 (ω-3). Even though, the phenotypic fatty acid data strongly indicated the presence of FADS1 converting highly specific the C20 – C22 PUFA. It is because the egg yolk contained too small amounts of C20:3 (ω-6) and C20:4 (ω-3) fatty acids, which are the substrate for FADS1 in the biosynthesis pathway, were therefore not available for analysis.

In contrast, the association between FADS1 genotypes and saturated C14:0 and C16:0 fatty acids were significant. In animals, saturated C16:0 and C18:0 fatty acids are desaturased to C16:1 (ω-7) and C18:1 (ω-9) fatty acids by stearoyl-CoA Δ9-desaturase. Recently, a study in *Bacillus subtilis* showed that FADS1 desaturase can introduce a double bond in saturated fatty acids (Aguilar et al. 1998, Altabe et al. 2003). Additionally, the results continuously showed that associations were significant not only with monounsaturated fatty acids but also with C18:2 (ω-6). These results were further strengthened by the haplotype analysis which showed significant association with both monounsaturated and C18:2 (ω-6) fatty acids. It is known that animals lack Δ12 and Δ15 fatty acid desaturases, which are responsible for converting oleic acid (C18:1, ω-9) into C18:2 (ω-6) and C18:3 (ω-3). Plants normally can produce these essential fatty acids by desaturase enzyme at C12 and C15 positions, but a recent study showed that Δ5- desaturase also acts on C18:1 (ω-9) (Kajikawa et al. 2006). In yeast, it is found that the endogenous substrate oleic acid could be converted to C20:4 (ω-6) by the coexpression of Δ12-, Δ6- and Δ5- desaturases (Parker-Barnes et al. 2000). Another example showed that the gene encoded for Δ5-desaturase catalyses monoenoic and dienoic C16 – 20 fatty acids instead of trienoic and tetraenoic C20 fatty acids (Cahoom et al. 2000). The question remains whether quail FADS1 may have a similar desaturase reaction like in plant and in yeast. It is known that different acyl carriers are used by different enzymes involved in the fatty acid metabolism, e.g., in yeast acyl-lipid desaturase is used while in animals acyl co-enzyme A (CoA) desaturase (Tocher et al. 1998, Nakamura et al. 2004) is active.

An explanation for the association of FADS1 to C18:2 (ω-6) may be that the alterations in fatty acid desaturase activities are not specific to any fatty acid series. Maeda et al. (1978) illustrated that the metabolism of PUFA altered in Chang cells and presented activities of both Δ6 and Δ5 on C18:3 (ω-3) and C20:4 (ω-3) substrates. Moreover, Domergue et al. (2002) demonstrated that the indiscriminate use of ω-6 and ω-3 fatty acids for several Δ5- and Δ6-desaturases in different organisms may be a general feature of front-end desaturases. These findings led to hypothesize that the FADS1 desaturase enzyme might be different from that of saturated or monounsaturated specific enzymes.

# 6 Conclusions

This study is the first report on the effect of genetic factors on the fatty acid profiles, which were found to be associated with the polymorphisms of the FADS1 and FADS2 genes coding for the enzymes catalysing the synthesis of essential fatty acids.

The selection experiment on quail demonstrated that modern breeding can genetically improve DHA and the ω-6:ω-3 PUFA ratio in the yolk by breeding.

Sequencing of the FADS1 and FADS2 genes was done to elucidate the molecular structure and gene expression of these genes and their encoded proteins in the biosynthesis pathway of the ω-6:ω-3 PUFA, as well as their association with the fatty acid profiles in the egg yolk. The genetic identification of the FADS1 and FADS2 is expected to provide beneficial effects in chicken.

Studying both FADS1 and FADS2 genes in the different local chicken breeds contributed to the existing knowledge on local chicken genetics regarding the production of key nutrients for human health. The management of genetic resource populations and their utilisation in future breeding programmes should be considered. The selection on both FADS genes provides information for future breeding and selection strategies using molecular information and gives an opportunity for the commercial egg producer to minimize the ω-6:ω-3 PUFA in egg yolk. However, in further studies more birds should be used to validate the current findings.

Finally, this study clearly showed that the results found in quail can be used in chicken. Therefore, the quail is an excelent model organism for studies in other bird species.

# 7 Summary

It is hypothesized that genetic effects have an influence on the PUFA content in egg yolk, especially in the ability to the synthesis of long chain PUFAs from the modified diet of laying hen by carbon chain elongation and desaturation and the deposition of these substances into the yolk. Therefore, divergent quail lines of the 5th, 6th and 7th breeding generation, continuously selected for a high and low ω-6: ω-3 PUFA ratio in the yolk, were used as an animal model to estimate genetic effects on the yolk fatty acid profiles.

The objectives of this study were to elucidate the genetic divergence of these high and low quail lines by estimating the genetic parameters, to clone and characterize the direct candidate genes, FADS1 and FADS2 and to analyse the effects of polymorphisms within these genes on the ω-6 and ω-3 fatty acid contents in egg yolk. Furthermore, the expressions of the FADS1 and FADS2 genes, as well as their polymorphisms were examined in different European (LSL) and Vietnamese chicken (Ac, Noi, H'mong, Ri und Te) breeds.

The fatty acid profiles of the high and low lines showed that AA (C20:4 ω-4) and DHA (C22:6 ω-3) content were significantly lower in the high line than in the low line ($P<0.01$). Furthermore, the ratio of the ω-6 and ω-3 PUFA was significantly reduced ($P<0.01$) between the low and high lines, however, no significant difference was found between the high and low lines of the 7th generation. Moderate heritabilties were found in the C22:6 (ω-3) and ω-6:ω-3 PUFA ratio. The low line seems to be higher heritable for the ω-6:ω-3 PUFA ratio and is more efficient than the high line. Moreover, negative genetic correlation between the C22:6 (ω-3) and the ω-6:ω-3 PUFA ratio indicate that selecting for a higher ω-6:ω-3 PUFA ratio coincided with lower C22:6 (ω-3) fatty acid. It is suggested that breeding can genetically improve DHA and the ω-6:ω-3 PUFA ratio in the yolk.

The nucleotide sequences of the FADS1 and FADS2 cDNA were obtained by cloning and sequencing PCR products, resulting from heterologous primers matching conserved and gene-specific regions of the FADS family of human, mouse, rat and subsequently 5´- and 3´- RACE.

The quail cDNA sequences of FADS1 and FADS2 consisted of 1797 bp and 1350 bp, which encoded 445 amino acids and 404 amino acids, respectively. The deduced amino acid sequences of quail FADS1 and FADS2 contain two domains; the N-terminal catalytic domain and C-terminal cytochrome $b_5$-like domains which encoded protein of both FADS1 and FADS2 and presented three highly conserved histidine motifs,

HDXGH, HFQHH and QIEHH which are characteristic for membrane-bound desaturase and essential for desaturase activity.

Comparing the mRNA expression between the quail FADS1 and FADS2 genes, in the quail, FADS2 was expressed at a higher level than quail FADS1, but no significant difference was found between the FADS1 and FADS2 genes between the high and low lines ($P>0.05$).

The FADS1 and FADS2 genes were screened for polymorphisms by comparative sequencing of eight animals from the high and low lines resulting in five SNPs in the coding region of both *FADS* genes. The five SNPs of the FADS1 gene were found at 348 bp (A to G), 391 bp (C to A), 468 bp (C to T), 570 bp (C to T) and 1075 bp (C to T), giving either purine-purine or pyrimidine-pyrimidine transitions or pyrimidine-purine transversion. The two SNPs at position 348 and 468 resulted in an amino acid substitution, asparagine-serine and valine-alanine, respectively. The FADS2 SNPs were synonymous and found at 477 bp (C to T), 681 bp (G to A), 717 bp (C to T), 953 bp (C to T) and 1023 bp (G to A), showing only purine-purine or pyrimidine-pyrimidine transitions.

Single-base-extension (SBE) was used for genotyping the five SNPs of the FADS2 gene and single strand conformation polymorphism (SSCP) was applied for genotyping two SNPs of FADS1 at 391 bp and 468 bp position. Allelic frequencies of both FADS2 and FADS1 polymorphisms showed that only SNP5 of the FADS2 was in Hardy-Weinberg equilibrium.

The chicken cDNA sequences of FADS1 and FADS2 were obtained, resulting in a length of 1380 bp and 1561 bp, respectively. The quail SNPs were identified within both genes and used to compare the different variances among the different local chicken breeds. The FADS2 SNPs were monomorphic among these breeds except SNP4 that segregated in five out of the six breeds. The two SNPs of the FADS1 gene at position 391 (C to A) and 468 (C to T) segregated in Te, Noi, Ri and LSL.

The mRNA expression of the FADS2 gene was stronger than of the FADS1 gene and the significant highest levels for both genes were observed in Te and European chicken breeds ($P<0.01$).

The association between single SNPs and fatty acid profiles in the egg yolk was analyzed. The association results of the FADS1 showed that genotypes at position 391 were significantly associated with saturated C14:0, C16:0 ($P<0.05$), monounsaturated C16:1 (ω-7) ($P<0.05$), as well as with the polyunsaturated fatty acid C18:2 (ω-6) ($P<0.05$). The genotypes at position 468 were only significantly associated with C16:0 ($P<0.05$). The infered haplotypes from these two SNPs show that haplotypes C/T and A/C were significantly associated with C16:1 (ω-7), C18:1(ω-9); C18:2 (ω-6) ($P<0.05$).

For FADS2 SNPs, means of SNP3 genotypes were found to be significantly associated with C20:4 (ω-6), C22:6 (ω-3) and the ω-6:ω-3 PUFA ratio ($P<0.05$) while SNP4 genotypes were significant associated to the ω-6:ω-3 PUFA ratio ($P<0.05$). The comprising analysis of variance and family based association test (FBAT) revealed significant effects of SNP3 and SNP4 genotypes on the egg yolk fatty acid profiles, especially the ω-6 and ω-3 PUFAs ($P<0.05$). The results strongly promote FADS2 as a functional candidate gene for traits related to ω-6 and ω-3 PUFA in eggs.

# 8 Zusammenfassung

Es wird angenommen, dass genetische Effekte einen Einfluss auf den PUFA Gehalt im Eidotter, besonders für die Fähigkeit der Synthese von langkettigen PUFAs aus der modifizierten Fütterung von Legehennen mit langkettigen und gesättigten Kohlenstoffketten und die Verlagerung dieser Substanzen in das Eidotter haben. Daher wurden divergente Wachtellinien der fünften, sechsten und siebten Zuchtgeneration durchgängig für ein hohes und niedriges ω-6: ω-3 PUFA Verhältnis selektiert und als Tiermodell zur Messung des erwarteten genetischen Effekts auf das Fettsäureprofil im Eidotter verwendet.

Ziel der Vorliegenden Arbeit war es, die genetische Divergenz in Wachtellinien, die auf hohes („high line“) bzw. niedriges („low line“) ω-6:ω-3 PUFA Verhältnis selektiert waren, zu eruieren. Die direkten Kandidatengene FADS1 und FADS2 wurden geklont und charakterisiert, sowie der Einfluss von Polymorphismen in diesen Genen auf den ω-6 und ω-3 Fettsäuregehalt in Eidotter untersucht. Weiterhin wurden Expression und Polymorphismen von FADS1 und FADS2 in verschieden europäischen (LSL) und vietnamesischen Hühnerrassen (Ac, Noi, H’mong, Ri und Te) verglichen.

Das Fettsäureprofil der hohen und niedrigen Linien zeigte, dass die AA (C20:4 ω-4) und DHA (C22:6 ω-3) Gehalte in den hohen Linien signifikant niedriger waren als in den niedrigen Linien ($P<0.01$). Weiterhin war das Verhältnis der ω-6 und ω-3 PUFA zwischen den niedrigen und hohen Linien signifikant verringert ($P<0.01$), es konnte jedoch kein signifikanter Effekt zwischen den hohen und niedrigen Linien der siebten Generation gefunden werden. Moderate Heritabilitäten konnten für das C22:6(ω-3) und für das ω-6:ω-3 PUFA Verhältnis geschätzt werden, wobei die “low” Linie höhere $h^2$ als die “high” Linie erbrachte. Zusätzlich ergab die negative genetische Korrelation zwischen dem C22:6 (ω-3) und dem ω-6:ω-3 PUFA Verhältnis, dass eine Selektion für ein höheres ω-6:ω-3 PUFA Verhältnis mit einem niedrigeren C22:6 (ω-3) Fettsäureverhältnis übereinstimmt. Es wird angenommen, dass die Zucht das DHA und das ω-6:ω-3 PUFA Verhältnis im Eidotter verbessert.

Die Nukleotidsequenzen der FADS1 und FADS2 cDNA wurden durch Klonierung und Sequenzierung festgestellt. Dazu wurden heterologe Primer verwendet, die in den konservierten und genspezifischen Regionen der FADS Familie von Mensch, Maus und Ratte lagen, um die Sequenzen abschnittsweise durch 5’- und 3’ -RACE zu identifizieren.

Die Wachtel cDNA Sequenzen von FADS1 und FADS2 bestehen aus 1797 bp und 1350 bp, die für 445 und 404 Aminosäuren kodieren. Die Aminosäuresequenz dieser

FADS1 und FADS2 Gene in Wachteln bestehen aus zwei Domainen; der N-Terminus katalytischen Region und der C-Terminal cytochromen $b_5$-ähnlichen Region. Diese kodiert Proteine für beide Gene und stellt drei höher konservierte Histidin Motive dar, HDXGH, HFQHH und QIEHH welche charakteristisch für membrangebundene Desaturasen und notwendig für die Desaturase Aktivität sind.

Beim Vergleich der mRNA Expression der FADS1 und FADS2 Gene bei Wachteln wurde festgestellt, dass FADS2 zu einem höheren Level exprimiert war als FADS1. Es konnten jedoch keine signifikanten Unterschiede der FADS1 und FADS2 Gene zwischen den hohen und niedrigen Linien ($P<0.05$) gefunden werden.

Die FADS1 und FADS2 Gene wurden durch vergleichende Sequenzierung von acht Tieren der hohen und niedrigen Linien auf Polymorphismen durchsucht. Es konnten fünf SNPs in den kodierenden Regionen beider FADS Gene gefunden werden. Die fünf SNPs des FADS1 Gens wurden bei 348 bp (A zu G), 391 bp (C zu A), 468 bp (C zu T), 570 bp (C zu T) und 1075 bp (C zu T) gefunden und führten zu einer Purin-Purin oder Pyrimidin-Pyrimidin Transition oder zu einer Pyrimidin-Purin Transversion. Die beiden SNPs an den Positionen 348 und 468 führten zu einer Aminosäuresubstitution, entweder Asparagin-Serin oder Valin-Alanin. Die FADS2 SNPs waren synonym und wurden bei 477 bp (C zu T), 681 bp (G zu A), 717 bp (C zu T), 953 bp (C zu T) und 1023 bp (G zu A) gefunden, diese führten ausschließlich zu Purin-Purin oder Pyrimidin-Pyrimidin Transitionen.

*Single base extension* (SBE) Methode wurden zur Genotypisierung der fünf SNPs des FADS2 Gens verwendet. Die *single strand confirmation polymorphism* (SSCP) Methode wurde zur Genotypisierung der zwei SNPs im FADS1 Gen bei 391 bp und 468 bp gewählt. Die Allelfrequenzen der Polymorphismen der beiden Gene FADS1 und FADS2 zeigten, dass sich nur der SNP5 im FADS2 Gen im Hardy-Weinberg Gleichgewicht befindet.

Die cDNA Sequenzen der FADS1 und FADS2 Genen bei Hühnern wurden untersucht, diese ergaben Längen von 1380 bp und 1561 bp. Die Wachtel SNPs wurden innerhalb der beiden Gene identifiziert und dazu verwendet, die verschiedenen Varianzen zwischen den verschiedenen lokalen Hühnerrassen zu vergleichen. Die FADS2 SNPs waren monomorphisch zwischen diesen Rassen, eine Ausnahme war der SNP4, der in fünf dieser sechs Rassen segregierte. Die beiden SNPs des FADS1 Gens an den Positionen 391 (C zu A) und 468 (C zu T) segregierten in den Hühnerrassen Te, Noi, Ri und LSL.

Die mRNA Expression des FADS2 Gens war stärker als die des FADS1 Gens und die signifikant höchsten Level wurden für beide Gene in den Hühnerrassen Te und den europäischen Rassen gefunden ($P<0.01$).

Die Assoziation zwischen einzelnen SNPs und den Fettsäureprofilen im Eidotter wurde analysiert. Die Ergebnisse der Assoziationsanalysen des FADS1 Gens zeigten, dass Genotypen an der Position 391 signifikant assoziiert mit den gesättigten C14:0, C16:0 ($P<0.05$), ungesättigten C16:1 (ω-7) ($P<0.05$), ebenso wie mit den mehrfach ungesättigten Fettsäuren C18:2 (ω-6) ($P<0.05$) waren. Die Genotypen an der Position 468 waren nur mit C16:0 assoziiert ($P<0.05$). Der gebildete Haplotyp dieser zwei SNPs zeigte, dass die Haplotypen C/T und A/C signifikant mit C16:1 (ω-7), C18:1(ω-9); C18:2 (ω-6) ($P<0.05$) assoziiert waren.

Für die FADS2 SNPs waren die Durchschnitte der SNP3 Genotypen signifikant mit C20:4 (ω-6), C22:6 (ω-3) und dem ω-6:ω-3 PUFA Verhältnis ($P<0.05$) assoziiert während die SNP4 Genotypen signifikant mit dem ω-6:ω-3 PUFA Verhältnis assoziiert war ($P<0.05$). Die sich anschließende Analyse der Varianzen und der Familien-basierte Assoziationstest (FBAT) ergaben signifikante Effekte der SNP3 und SNP4 Genotypen auf die Fettsäureprofile im Eidotter, besonders die ω-6 and ω-3 PUFAs ($P<0.05$). Diese Ergebnisse bestätigen stark, dass FADS2 ein funktionelles Kandidatengen für Merkmale, die in Zusammenhang zu den ω-6 und ω-3 PUFA in Eiern stehen, ist.

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