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30 hp E-nivå

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Nyckelord: Laying hen, egg yolk, fat, cholesterol, phospholipids, fatty acid, nutrition, genotype, age, hemp

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Preface

This study was conducted as a Master's thesis of Animal Science at the Dept. of Animal Nutrition and Management, Swedish University of Agriculture Sciences (SLU), Uppsala. It was funded by Fresenius-Kabi and The Research Council for Environment, Agricultural Sciences and Spatial Planning (Formas).

My warmest gratitude to Helena Wall, Klas Elwinger and Ragnar Tauson for all the assistance and support given during this period.

The author would like to gratefully acknowledge all help given by the staff at the Division of Food Chemistry, Department of Food Science (SLU) in the laboratory part of this thesis. Special thanks to Professor Paresh Dutta and laboratory assistant Samanthi Madawala.

My biggest appreciation to the staff at Funbo-Lövsta Research Centre for their help with egg collection.

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SAMMANFATTNING

Ett ägg från en kommersiell värphönshybrid består till cirka 27 % av gula, 64 % vita och 9 % skal. Gulan fungerar som näringsreservoar för kycklingembryot och innehåller därför alla näringsämnen som är nödvändiga för att embryot ska utvecklas till en kyckling. Eftersom det inte finns möjlighet för embryot att avlägsna slaggprodukter måste näringsämnena i gulan vara av ett högt biologiskt värde. Detta innebär att äggula är ett utmärkt livsmedel för human konsumtion. Äggula som råvara eller i förädlad form är även en vanligt förekommande ingrediens i livsmedels-, läkemedels- och kosmetikaproduktion. Äggula innehåller 50 % vatten, resterande ämnen är främst lipider och proteiner. Lipider upptar cirka 65 % av torrsubstansen i gulan. Lipiderna består till största del av triacylgycerider (65 %), fosfolipider (33 %) och kolesterol (5 %). Möjligheten att selektera ägg med en speciell fettsammansättning eller manipulera denna med hjälp av foder skulle vara av stor ekonomisk betydelse för industrin.

Denna studie syftade till att undersöka om hönans ålder, genotyp eller foder påverkar andelen fettkomponenter i äggula. Fettkomponenterna som undersöktes i detta försök var totalt fettinnehåll, andelen fosfolipider, kolesterol samt fettsyrasammansättning. I försöket användes 12 grupper med värphöns, inhysta i burar med sittpinne, värprede och sandbad. Sex grupper var av kommersiella värphybriden Lohmann Selected Leghorn (LSL) och de övriga 6 grupperna bestod av djur av hybriden Lohmann Brown (LB). Djuren utfodrades *ad lib.* med ett kontrollfoder eller ett foder med 20 % inblandning av hampfrökaka, dessa två foder var fördelade med 3 replikat per genotyp. Ägg samlades in vid 32, 56 och 72 veckors ålder. Gulan separerades från vitan och ägg- respektive gulevikt registrerades. Gulorna poolades sedan gruppvis och frystes in i -18 C°. Fettet extraherades ur gulorna för att sedan analyseras på kolesterolinnehåll (gaskromatografi, GC) och fosfolipidinnehåll (Solid-Phase extraktion, SPE). Även en analys av fettsyrasammansättningen utfördes med hjälp av gaskromatografi (GC).

Som förväntat ökade äggvikten med hönornas ålder, vilket även gulevikten och det totala fettinnehållet gjorde. Hönorna av hybriden LSL lade genomgående ägg av lägre vikt än LB. Sett till de enskilda fettkomponenterna visade resultaten att kolesterolet minskade med åldern samt att fodret innehållande hampfrökaka visade tendenser att sänka kolesterolmängden i gulan. Fodret påverkade även fettsyrasammansättningen i gulan. En signifikant ökning av de fleromättade fettsyrorna linolensyra och linolsyra sågs i äggulorna från hönor som ätit fodret med 20 % hampainblandning. Varken genotyp, ålder eller foder hade någon signifikant effekt på mängden fosfolipider.

Detta försök bekräftar att fettsyrasammansättningen i äggula är relativt lätt att manipulera utifrån fettsyrainnehållet i fodret. Det faktum att fodret med 20 % hampainblandning tenderade till att minska kolesterolinnehållet, samt ökade andelen nyttiga fleromättade fettsyror torde tala till dess fördel sett ur ett humannutritionellt perspektiv. Avslutningsvis kan slutsatsen dras att genotyp och ålder snarare påverkar äggets fysiska egenskaper än dess kemiska uppbyggnad, medan den kemiska sammansättningen kan påverkas via fodret.

ABSTRACT

An egg from a commercial laying hen hybrid consists of approximately 27 % yolk, 64 % albumen and 9 % shell. The yolk acts as a nutritional vessel for the chicken embryo. Therefore the nutrient must be of high biological value, due to the fact that the embryo has no possibility to get rid of metabolic waste products. Because of this egg yolk is an excellent food for human consumption. Egg yolk is also a well used raw material in food-, pharmaceutical- and cosmetic industries. About 50 % of the yolk is water; remaining parts are mainly lipids and proteins. In yolk, 65 % of the dry matter is different lipid components. The most frequently seen lipid components are triacylglycerol (65 %), phospholipids (33 %) and cholesterol (5 %). The possibility to select eggs with a certain lipid content or to manipulate the lipid content with the diet would be of great economical importance.

The aim of this study was to investigate if the hen's genotype, age and diet affect fat components in egg yolk. The fat components measured were total fat content, phospholipids, cholesterol and the fatty acid profile. This trial involved 12 groups of laying hens housed in 8-hen cages furnished cages with perches, dust baths and nests. These groups were divided into 6 groups of the genotype Lohmann Selected Leghorn (LSL) and the other 6 groups were of the genotype Lohmann Brown (LB). The hens were fed *ad lib.* with either a control diet or a diet containing 20 % hemp seed cake. The diets were divided into 3 replicates per genotype. Eggs were collected when the hens were 32, 56 and 72 weeks old. The yolk was separated from the albumen. Total egg weight and yolk weight were registered. The yolks were pooled group wise and stored at -18 C°.

As expected the egg weight, yolk weight and total fat content increased with hen age. Hens of the genotype LB laid throughout the study heavier eggs than LSL-hens. The results showed that the cholesterol decreased with hen age. The hemp seed diet showed a tendency towards cholesterol lowering properties and also an effect on the fatty acid profile in the egg yolks. A significant increase of the poly unsaturated fatty acids linolenic acid and linoleic acid was seen in egg yolks from hens that had been eating the hemp seed diet. Neither genotype nor age or diet affected the amount of phospholipids.

This study proved the fact that the fatty acid composition in egg yolk is fairly easy to manipulate by the fatty acid profile in the layer diet. Due to the positive effects of hemp seed in laying hen diets on the healthy fatty acids and its tendency towards lowering cholesterol in egg yolk, an inclusion of hemp in laying hen diets can be motivated from a human nutrition perspective. The physical properties of eggs mainly seem to be affected by hen age and genotype, whilst the chemical properties are more easily changed by diet.

INTRODUCTION

Egg yolk lipids are a valuable product seen from both a nutritional and an industrial point of view. During recent years several studies have been conducted to investigate if the different fat components in egg yolk can be manipulated to achieve different purposes.

Studies have shown that the fatty acid composition of egg yolk lipids is fairly easy to change with dietary additives (Milinsk *et al*, 2003, Baucells *et al.* 2000, Basmacioglu *et al.* 2003). Changing the fatty acid profile towards higher ω-3 fatty acid content in order to promote human health is one example of such modification. This is generally done by adding feed additives of marine origin which are high in ω-3 fatty acid content.

Due to the increasing problem with cardiovascular health problems and their correlation with high plasma cholesterol concentration producing eggs with low cholesterol levels is desirable. Egg yolk has a high natural concentration of cholesterol, with approximately 5 % cholesterol of the total lipid content. Attempts to lower the concentration of cholesterol have been done with varying results (Milinsk *et al.* 2003, Millet *et al.* 2006, El Bagir *et al.* 2006.). Overall the cholesterol level seems hard to manipulate, probably due to its importance for reproduction and other important body functions (Champe *et al.* 2008).

Egg yolk phospholipid is a product of high importance for the pharmaceutical-, cosmetic- and food industries (Rossi, 2007). In the food industry phospholipids is used as an emulsifier, viscosity reducer and as a surfactant. In infants formula egg yolk phospholipids are highly recommended over vegetable phospholipids due to its content of healthy poly unsaturated fatty acids (PUFA). In the pharmaceutical industry egg phospholipids are used as emulsifiers in parenteral lipid emulsions and drug delivery systems (Hartmann and Wilhelmson, 2001).

PURPOSE

The purpose of this study was to investigate possible effects of hen age, genotype and hemp seed inclusion in the feed on the fatty acid profile, the amount of cholesterol and phospholipids in egg yolk. Eggs analysed were from two commercial laying hen genotypes and eggs were sampled at three hen ages.

LITERATURE REVIEW

Hemp

Hemp (*Cannabis sativa* L.) is an old culture plant which has been used as a source for medicine, food and fibre fabric production (*Callaway*, 2004). Due to the narcotic properties of the plant, it has been forbidden to cultivate hemp in Sweden. Since 2003 hemp is once again allowed to be cultivated, according to the directives of the European Union. The Swedish Board of Agriculture regulates what kind of hemp that is allowed to cultivate in Sweden. The cultivation is restricted to certain types of hemp, based on the contents of the narcotic compound delta9-tetrahydrocannabinol (THC) which may not exceed 0,–20 % (*Council of Regulation* (EC) No1420/98).

Nutritional properties of hemp seed

According to the review by Callaway (2004) hemp seed is a valuable animal feed source, regarding dietary oil, fibre and protein content. Hemp seed contains about 35 % oil and 25 % protein. The oil is an excellent source of polyunsaturated fatty acids (PUFA). The content of PUFA can be as high as 80 % and contains both linoleic acid (18:2 ω-6) and linolenic acid (18:3 ω-3), which are considered as essential fatty acids. The ratio between ω-6 and ω-3 in hemp seed oil is considered to be between 2:1 and 3:1. Due to its nutritional and cultivation properties it may be of interest to use in organic poultry production. In organic production it is desirable to find high value feed ingredients that can be cultivated in Sweden and that can replace conventional feed sources, such as soy bean.

Egg components

The egg consists of four different main structures; shell, shell membranes, albumen and yolk. An average egg from domestic laying hens contains 64 % albumen, 27 % yolk and 9 % shell (Rose, 1997). The average weight of an egg is approximately 60 g. The egg weight increases with the age of the bird. The weight increases quickly in the beginning of the laying period but less at older ages and at the end of the egg laying cycle. Egg weight also varies between different genotypes of laying hens (Scheideler *et al.* 1998).

Egg yolk

In a fertilized egg the yolk provides the bird embryo with all the vital nutrients required for its development (Anton, 2007). The nutrients have to be well metabolized by the embryo and must therefore be of high biological value due to that the embryo has no possibility to get rid of metabolic waste products. The high biological value of eggs makes it an excellent nutrient in human diet. It is also used for many industrial purposes, such as food processing, pharmaceutical, cosmetic and biotechnological purposes. Egg yolk contains about 50 % of water and the remaining part is mainly lipids and proteins (Hartmann and Wilhelmson, 2001). Lipids contribute to about 65 % of the dry matter content of egg yolk and are mainly composed of triacylglycerides (62 %), phospholipids (33%) and cholesterol (5%) (Anton 2007). Lipid content in fresh egg yolk is approximately 30 % (Nielsen, 1998).

Fatty acids

Chemistry and function

In the body of both humans and animals, fatty acids can be found as free fatty acids or as fatty acyl esters (Champe *et al* 2008). The esterified fatty acids are found in complex molecules such as triacylglycerol and cholesterol ester. In form of triacylglycerol, stored in adipose tissue, fatty acids are considered to be the body's biggest energy source. Fatty acids can be used as energy source by most of the tissues in the body. In components like membrane lipids,

fatty acids most often are a part of the structure. Chemically, fatty acids are constructed by a hydrophobic hydrocarbon chain and a hydrophilic carboxyl group (see figure 1). The carboxyl group is ionized at physiological pH which gives this part of the fatty acid an affinity for water. It is this phenomenon that gives fatty acids their amphipathic characteristics.

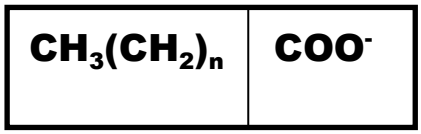

Figure 1. The chemical structure of fatty acids with the hydrophobic hydrocarbon chain and the hydrophilic carboxyl group.

When the hydrocarbon chain is very long the hydrophobic part of the acid is dominant. This makes the fatty acid insoluble in water and it must be coupled to a transport protein to be able to circulate in the blood. Thus, free fatty acids that circulate in the blood are coupled to albumin. However, more than 90 % of the fatty acids in blood plasma are esterified and serve as structures in triacylglycerols, cholesterol esters and phospholipids. These are transported in lipoproteins such as chylomicrons, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Champe *et al* 2008).

If the carbon chain of a fatty acid doesn't have a double bond, the acid is considered to be saturated (SFA). If the chain consists of one or several double bonds, the acid is unsaturated. If there is only one double bond the fatty acids is called mono unsaturated fatty acids (MUFA). If there are several double bonds they are called poly unsaturated fatty acids (PUFA).

Omega fatty acids and human health

Two different kinds of fatty acids have been highlighted a lot in science the last decades due to the increasing cardiovascular problems among humans in industrial countries. These are the essential omega (ω) -3 and ω-6 fatty acids (Simopoulos, 2002). The ω-carbon is the carbon at the end of the carbon chain, in other words the carbon in the methyl terminal of the fatty acid (Champe *et al.* 2008). A ω-3 fatty acid has the first double bond 3 carbons from the ω-carbon. In the same way a ω-6 fatty acid has the first double bond 6 carbons from the methyl terminal. The ω-3 fatty acid linolenic acid (18:3) is the precursor to the more long chained ω-3 fatty acids called eicosapentaenoic acid (EPA, 20:5) and docohexaenoic acid (DHA, 22:6) (Yannakopoulos, 2007). The ω-6 fatty acid linoleic acid (18:2) is converted to arachidonic acid (20:4) in the body. Both linolenic- and linoleic acid are considered to be essential fatty acids due to the fact that they can not be synthesized by the body. They have important physiological properties and must be supplemented to the body through the diet.

Polyunsaturated ω-3 fatty acids are most commonly found in fish and other seafood (Lewis *et al*. 2000), whereas ω-6 PUFAs are found in different oils such as maize, sunflower, soy and sesame seed oil (National Food Administration of Sweden, 2009). The balance between ω-6 and ω-3 fatty acids is considered to be important for human health (Yannakopoulos, 2007). A ω6/ω3 ratio between 1:1 and 4:1 is considered to be the ultimate balance of PUFA. When humans lived as hunters and gatherers the ratio of ω6/ω3 in food was about1:1 (Simopoulos, 2002). The feeds available were lean meat, fish, vegetables, berries and fruits. This diet is what human evolution is based upon and therefore what humans are developed to eat and utilize. In industrial countries the ratio of ω6/ ω3 in human diet are considered to be about

10:1 to 15:1. Many common diseases in western countries, such as cardiovascular problems, diabetes, cancer, autoimmune diseases are believed to be related to the disturbance of the ω6/ ω3-ratio.

Dietary effects on fatty acid composition in egg yolk
To promote human health and increase the intake of ω3-PUFA different attempts have been done to enrich foodstuffs with these fatty acids. Eggs are consumed all over the world and studies have shown that the fatty acid profile of egg is fairly easy to manipulate (Baucells *et al.* 2000, Milinsk *et al*, 2003, Basmacioglu *et al.* 2003)

Normally ordinary table eggs contain a high amount of ω-6 fatty acids and very low amounts of ω-3 fatty acids (Yannakopoulos, 2007). To change the composition of PUFA to high ω-3 fatty acid content in the eggs Yannakopulos (2007) mentions two different approaches. The first one is to add linolenic acid (18:3 ω-3) to the hen feed. Linolenic acid is then converted to DHA in the body of the hen. Flaxseed and linseed are two feedstuffs with high content of linolenic acid. The other approach is to add feed stuffs rich in EPA and DHA to the diet like e.g., feedstuffs of marine origin. Bavelaar & Beyen (2004) showed that the amount of DHA can be altered either by adding purified DHA to the feed or by the addition of linolenic acid. The level of EPA in eggs can be increased only by adding feedstuffs rich in EPA like e.g. fish oil to the diet.

Effects of genotype on fatty acid composition in egg yolk
To investigate if the genotype of hens affects fatty acid composition in yolk, Scheideler *et al.* (1998) fed three different genotypes with diets containing flax seed. The genotypes used were Babcock B300, DeKalb Delta and Hy-Line W-36. Significant effects of genotype were found for palmitic acid (16:0 SFA), stearic acid (18:0 SFA) and oleic acid (18:1 ω-9) but no effects were found on the content of linoleic acid (18:2 ω-6), arachidonic (20:4 ω-6) or DHA (22:6 ω-3).

Effects of the hen's age on fatty acid composition in egg yolk
Nielsen (1998) investigated if the age of laying hens had an effect on the composition of fatty acids in egg yolk. Eggs from white Lohmann hens were collected at the ages 21 and 51 weeks. The proportion of arachidonic acid (20:4 ω-6) and DHA (22:6 ω-3) was higher in egg yolk lipid from young hens compared with older hens. However Scheideler *et al.* (1998) found that eggs laid by the genotypes Babcock B300, DeKalb Delta and HyLine W-36 hens at the age of 36 weeks had less DHA than at 58 weeks, which contradicts the findings of Nielsen (1998).

Cholesterol
Chemistry and function
Cholesterol is a naturally occurring sterol and is considered to be one of the most important sterols in the body (Akoh and Min, 1998). Cholesterol consists of four hydrocarbon rings, called the steroid nucleus, and a hydrocarbon tail (see figure 2). It is a very hydrophobic compound. Most of the cholesterol found in plasma is in form of cholesteryl ester (see figure 2), which is even more hydrophobic than free cholesterol.

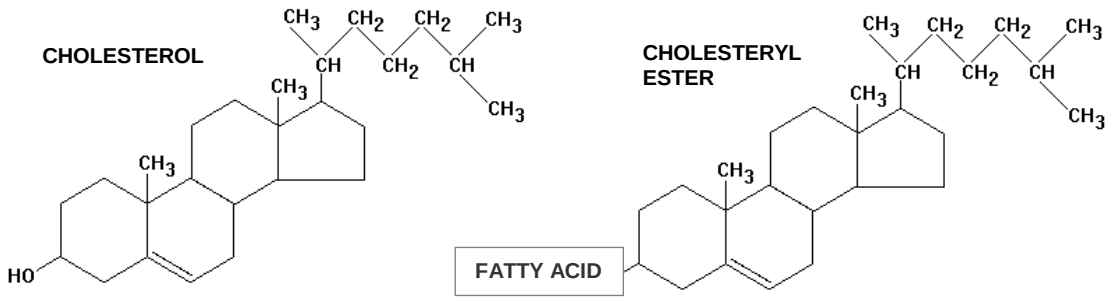

Figure 2. Chemical structures of cholesterol and cholesteryl ester. The fatty acid part of cholesteryl ester makes it even more hydrophobic than free cholesterol

Cholesterol acts as a structural component of biological membranes (Champe *et al* 2008). The liver plays an important role in the synthesis and metabolism of cholesterol. Cholesterol can enter the body in form of dietary cholesterol, which is transported to the liver by the lipoprotein chylomicrons.

The synthesis and metabolism of cholesterol in humans is not precisely regulated and the homeostasis can easily be disturbed (Champe *et al.* 2008). If the intake of cholesterol exceeds the requirement, cholesterol can increasingly gather in the endothelial linings of blood vessels. This can lead to plaque formation and causes narrowing of the vessels, called atherosclerosis, which may cause cardiovascular diseases. The plasma lipoproteins of interest are low-density lipoprotein (LDL) and high-density lipoprotein (HDL). LDL is connected to what is commonly known as "bad cholesterol" and vice versa HDL is called "good cholesterol".

Dietary effects on cholesterol levels in egg yolk

Due to the health risks connected to high cholesterol plasma levels attempts to minimize the concentration of cholesterol in egg yolk has been done. Cobos *et al.* (1995) found no significant differences in cholesterol concentration in egg yolk in their study where 4 different diets, with different fatty acid compositions, were fed to two different strains of laying hens. The results from Cobos *et al.* (1995) suggest that the fatty acid composition in the diet does not affect the cholesterol level in yolk. These findings agrees with several other studies (An *et al.* 1997, Ferrier *et al.* 1995, Milinsk *et al.* 2003, Millet *et al.* 2006) where no dietary effects were found on the cholesterol level in egg yolk.

However, dietary effects on egg yolk cholesterol were found by El Bagir *et al.* (2006). Hens fed a diet containing 20g or 30g Black Cumin seeds per kilo feed for three months showed a significant decrease compared with the control diet by 34 and 45 % respectively in cholesterol level in egg yolk. The blood plasma cholesterol level also decreased with 20 %.

Effects of genotype on cholesterol levels in egg yolk

In a comparison between the two commercial laying hen strains Single Comb White Leghorn and Brown Leghorn no significant differences were found in cholesterol levels in egg yolk (Cobos *et al.* 1995). Significant differences in cholesterol concentrations were however found between the Creole hen, which is used in small scale egg production in Mexico, and Plymouth Rock x Rhode Island cross hens (García-López *et al.* 2007). The Creole hens had less cholesterol in the yolk compared to the other strain. Millet *et al.* (2006) also found differences between genotypes. They compared the commercial genotypes Lohmann Selected Leghorn (LSL) and ISA Brown (IB) with the not so commonly used strain called Araucana. The results

show that eggs from Araucana hens had significant higher concentration of cholesterol than the other two genotypes. There were no differences between the commercial strains.

Effects of age on cholesterol levels in egg yolk
When comparing the cholesterol content in egg yolk between Isa-White laying hens at the age 38 weeks and 42 weeks, Basmacioglu *et al.* (2003) found that the concentration of cholesterol decreased with increasing age.

Phospholipids
Chemistry and function
Phospholipids are common lipids in animal cell membranes (Akoh and Min, 1998). Phospholipids consist of a backbone of glycerol or sphingosyl, Attached to the backbone are fatty acids, phosphate and an alcohol (see figure 3). The most characteristic feature of phospholipids is their amphipathic nature which is due to the presence of a hydrophilic head group and the hydrophobic fatty acid region. This amphipathic property is the main reason for the importance of phospholipids in cell membranes. Due to their amphipatihic character phospholipids play an important commercial role as an emulsifying factor.

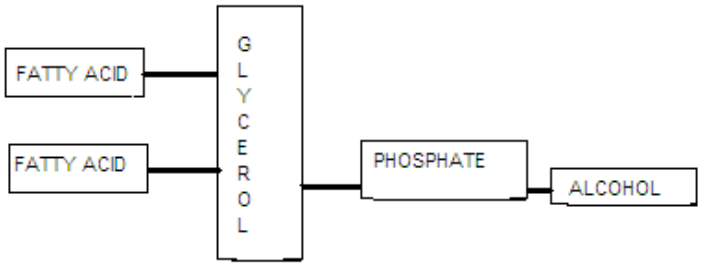

Figure 3. A general composition of a phospholipid which is composed by fatty acids attached to a glycerol backbone and a phosphate/alcohol group

Phospholipids are divided into two different classes depending on the structure of the backbone (Champe *et al.* 2008). If the lipid is based on a glycerol backbone it is called glycerophospholipid. The glycerophospholipids are the most frequently existent phospholipids.

The second class of phospholipids are the sphingophospholipids (Champe *et al.* 2008). There is only one sphingophospholipid which is important in humans and it is called sphingomyelin. Instead of a backbone of glycerol, sphingomyelin has a backbone made of an amino alcohol called sphingosine. Sphingomyelin serves as an important component of the myelin of nerve cells.

Egg yolk phospholipids
Egg yolk is a common origin for phospholipids which are used as emulsifying agents in the food industry and pharmaceutical industry. Soy phospholipids have an antioxidative effect on different dietary oils. Sugino *et al.* (1997) investigated if egg yolk phospholipids would have the same antioxidative effect. The results shows that egg yolk lipids containing phospholipids prevented oxidation of PUFA-oils and other oxidation sensitive lipids used in functional foods.

To increase the levels of phospholipids would be of high economical and nutritional value to the industry. Different attempts to change the phospholipids concentration in the yolk have been done in recent years. Hens which were fed a diet containing Black Cumin seeds showed a decrease in phospholipid concentration in egg yolk (El Bagir *et al.* 2006). An *et al.* (1997) found no significant differences in phospholipid concentration in yolk from hens fed diets with safflower phospholipids. A difference in the ratio phosphatidylcholine: phosphatidylethanolamine (PC: PE) were however found. The ratio decreased in yolk from hens fed crude safflower phospholipids (50 % phospholipids and 50 % triglycerides) compared with purified safflower phospholipids.

MATERIALS AND METHODS

Birds, diets and experimental design

The study was approved by the Uppsala Local Ethics Committee. Two commercial strains of laying hens were used, Lohmann Selected Leghorn (LSL) and Lohmann Brown (LB). The study was conducted at Funbo-Lövsta Research Station in Uppsala. The hens were housed in 8-hen cages furnished with perches, dust baths and nests, all in accordance with the Swedish Animal Welfare Directives. Feed and water was available *ad lib.* Eggs laid were collected and recorded daily during the production cycle.

This study used a total of 96 hens housed in groups of 8 in 12 furnished cages. There were six groups with LSL hens and six with LB hens. Each group of hens was fed either a diet without hempseed seed - a control diet - or a diet with an inclusion of 20 % hempseed cake, resulting in three replicates per combination of diet and genotype. The composition of experimental diets and their calculated nutrient content are presented in table 1. The analyzed fatty acid composition of the diets is shown in table 2. At 32, 56 and 72 weeks of age one days' egg production was collected group wise for analysis of fat components. The yolk was separated from the albumen, pooled group wise and stored at -18 C°. Before storage, egg weight and yolk weight were registered.

Table 1. Composition of experimental diets and calculated nutrient content

Ingedients %	**Control**	**Hemp**
Wheat	45.01	32.33
Oats	10	10
Wheat middlings	4	4.17
Lucerne meal	3.46	3.46
Hemp seed cake	0	20
Peas	10	10
Potatoe protein	7.31	0
Rape seed cake	10	10
Calcium carbonate	8.18	8.22
Monocalcium phosphate	0.77	0.55
NaCl	0.26	0.27
Vitamine premix	1	1
Calculated nutrient content, %		
Fat	49.72	73.55
Linoleic acid	12.84	27.82
Starch	367.5	300.62
Sugar	46.38	36.98
Crude fibre	43.27	79.92

Table 2. Analyzed fatty acid composition of control- and hemp diet, respectively.

Fatty acid	Control diet (%)	Hemp diet (%)
Myristic acid 14:00 SFA[1]	0.26	0.11
Palmitic acid 16:00 SFA	15.94	9.39
Palmitoleic acid 16:1 MUFA[2]	0.26	0.20
Stearic acid 18:0 SFA	2.87	2.14
Oleic acid 18:1 ω-9 PUFA[3]	34.06	27.95
Vaccenic acid 18:1 ω-11 PUFA	2.54	2.24
Linoleic acid 18:2 ω-6 PUFA	35.66	42.65
Linolenic acid 18:3 ω-3 PUFA	6.65	11.55
Gadoleic acid 20:1 MUFA	0.68	0.69
Eicosadienoic acid 20:2 ω-6 PUFA	0	0
Eicosatrienoic acid 20:3 ω-3 PUFA	0	0
Arachidonic acid 20:4 ω-6 PUFA	0	0
EPA 20:5 ω-3 PUFA	0	0
Behenic acid 22:0 SFA	0.23	0.28
Erucic acid 22:1 ω-9 MUFA	0	0
DHA 22:6 ω-3 PUFA	0	0

[1]Saturated Fatty Acid, [2]Mono Unsaturated Fatty Acid, [3]Poly Unsaturated Fatty Acid

Lipid analysis

Extraction

Lipid extractions were made according to the methods presented by Folch *et al.* (1957) and Bligh and Dyer (1959) with some adjustments.

Frozen samples were thawed at room temperature. A sample of 2.75 g of egg yolk was mixed with a chloroform:methanol:water solvent in the proportion 25:25:21 (ml). First the chloroform and methanol part of the solvent were mixed with the sample. The mixture was homogenized for three 10 seconds periods with a short break between each 10 second interval. Thereafter water was added and the mixture was shaken properly and filtered in a Büchner funnel. The sample was then added to a separatory funnel and left standing for 30 minutes allowing separation into two different layers. The top layer contained the alcohol part with non-lipid substrates and the lower layer contained chloroform and the lipids. The chloroform layer was transferred to an evaporatory flask and dried in a rotary evaporator. The

remaining lipid fraction was dried one more time in nitrogen gas for 10 minutes to be certain that no solvent residues was left in the sample.

Fatty acid analysis
Determination of fatty acid composition was done by preparing fatty acid methyl esters (FAME) and gas chromatograph (GC) analysis.

Approximately 10 mg of egg yolk lipids was dissolved in 0.5 ml hexane. Thereafter 2 ml of 0.01 M NaOH in dry methanol was added to catalyse the reaction and the sample was placed in a water bath at 60 °C for 10 minutes with continuous shaking. To complete the formation of fatty acid methyl esters (FAME) 3 ml of BF_3 –reagent was added and the sample was once again put in a water bath at 60 °C for 10 minutes with continuous shaking. The sample was then cooled under running water. Two ml 20% NaCl and 1 ml hexane was added and the tube was shaken vigorously. After approximately 15 minutes the layers had separated and the upper hexane layer, which contained the FAME, was transferred to another test tube. The FAME fraction was then analyzed in a gas chromatograph.

Cholesterol analysis
A three steps analyses including saponification, preparation of trimethylsilyl ether (TMS) derivatives of sterols and a gas chromatograph (GC) analysis, was conducted according to Savage *et al.* (1997) with some adjustments.

For the saponification 50 mg egg yolk lipids were mixed with 1 ml 2M potassium oxide (KOH) in ethanol and then placed in boiling water with intermittent shaking for 10 minutes. This step hydrolyzes the lipids. After 10 minutes the sample was cooled under running water to stop the reaction. To the sample 1 ml water, 2 ml hexane containing 80 µg 5α-cholestane (internal standard) and 200 µl absolute ethanol were added. The sample tube were shaken vigorously and then centrifuged at 2500 rpm for 3 minutes. Non-saponifiable components, such as cholesterol, are separated from water soluble components in the upper hexane layer. This layer was transferred to another glass tube and dried under nitrogen gas.

In the second step the sterols are derivatized to TMS derivates. To the non-saponifiable components 100 µl of a Sil-reagent were added and the tube was vortexed briefly. The sample was dispersed in an ultrasonic bath and then incubated at 60 °C for 45 minutes. After the incubation the reagent was dispersed on the ultrasonic bath again and then the solvent was evaporated under nitrogen gas. The TMS-derivates were dissolved in 300 µl hexane, dispersed in ultrasonic bath and centrifuged at 2500 rpm for 3 minutes. There after a GC analyse was performed.

Phospholipid analysis
The phospholipid fraction of the egg yolk samples was derived using solid-phase extraction (SPE) with a diol-column for the SPE of phospholipids (Carelli *et al.,* 1997). In a comparison of different columns the diol SPE-column proved to be the most efficient one (Chua *et al.,* 2008). Some adjustments were made to adapt the analysis to egg yolk lipids. The amount of sample was lowered from the recommended 100 mg to 50 mg, due to the high phospholipid content in egg yolk. The adjustment was confirmed to be suitable by analysis on thin-layer-chromatography (TLC) plates.

The diol-column was pre-conditioned with 2 ml methanol, 2 ml chloroform and 4 ml hexane. Then 50 mg of egg yolk lipid was added to the column. This fraction was eluted with 2.5 ml

chloroform and collected in a pre-weighed glass tube. The chloroform released the triacylglycerol from the sorbent bed. The column was then eluted with 7 ml methanol (containing 0.5 ml 25% ammonia (NH_3) in 100 ml methanol) to recover the phospholipids from the sorbent bed. This fraction was also collected into a pre-weighed glass tube. The phospholipid fraction was dried under nitrogen and then weighed.

Statistical analysis

Each cage with 8-hens constituted an experimental replicate and before statistical analyses mean values for egg weight and yolk weight were calculated for each experimental unit and age.

Statistical analyses were carried out with the general linear model procedure of SAS software with diet and genotype considered as fixed effects and with adjustment for repeated measurements. Two-way interactions between fixed effects and age were included in all analyses.

RESULTS

Effects on egg weights and yolk lipid parameters are presented in table 3. Egg weight and yolk weight increased with age of hens. A significant difference in egg weight was found between the genotypes, with LB having heavier eggs than LSL hens. No significant effects were found of diet on egg weight or yolk weight. Genotype and age had significant effects on the total lipid content in egg yolk. LB had higher lipid content than LSL and the lipid content increased with hen age. Diet did not affect total lipid content.

The concentration of cholesterol decreased with increasing age of hens ($p<0.001$), ranging from 27 to 19 %. No differences were found between the genotypes, but there was a tendency ($p<0.06$) of lower concentration of cholesterol with the hemp seed diet. There were no significant effects of any of the variables on the percentage of phospholipids in egg yolk lipid, which averaged 25 %.

Results of fatty acid analysis are shown in table 4. Diet affected the concentration of four of the analysed fatty acids. The hemp diet gave a lower percentage of palmitoleic acid 16:1(MUFA) (1.43 % vs. 2.0 %, respectively) and oleic acid18:1 ω-9 (MUFA) (36.4 % vs. 40.6 %, respectively) compared to the control diet. There was a tendency towards significant ($p<0.06$) lower levels on myristic acid 14:0 (SFA) in hemp eggs compared with control eggs. For linoleic acid 18:2 ω-6 (PUFA) and linolenic acid 18:3 ω-3 (PUFA) the percentage was higher in egg yolks from hens fed the hemp diet in comparison to the control diet ($p<0.003$ and $p<0.0019$, respectively).

Egg yolks from LSL hens had significantly higher levels of Eicosatrienoic acid
20:3 (PUFA) (0.26 vs. 0.09 %, respectively, $p<0.03$) than LB, although the percentage was very small for both LSL and LB. An almost significant effect of genotype was found for palmitic acid 16:0 (SFA), with LSL having a higher level than LB.

The only significant effect of age was found for stearic acid 18:0 (SFA), which decreased with hen age ($p<0.003$), from 8.1 % to 6.6 %.

Table 3. Effects of genotype, diet and age of hen on egg weight and yolk lipid parameters.

Parameter[1]	**Genotype**		**Diet**		**Age, weeks**			**Statistical analysis p-value**		
	LSL	*LB*	*Control*	*Hemp*	*32*	*56*	*72*	*Genotype*	*Diet*	*Age*
Egg weight, g	63.81	67.91	65.28	66.44	62.83	66.52	68.33	0.03	0.44	0.0002
Yolk weight, %	25.98	25.03	25.48	25.52	24.34	26.12	26.16	0.09	0.75	0.0014
Total lipid, %	29.98	32.15	31.75	30.47	27.36	30.46	35.40	0.03	0.21	0.0008
Cholesterol, mg/g	23.49	22.56	24.07	22.01	26.98	22.56	19.45	0.38	0.06	< 0.0001
Phospholipids, %	25.0	25.6	23.9	26.7	27.6	25.5	22.9	0.78	0.22	0.24

[1] Yolk weight is presented as percentage of egg weight. Total lipid content and phospholipids are presented as percentage of the yolk in this table. Cholesterol is presented as mg cholesterol / g yolk. All values are means.

Table 4. Effects of genotype, diet and age of hen on the fatty acid composition in egg yolk lipids. Values are in % of total lipid content.

								Statistical analysis		
Fatty acid	**Genotype**		**Diet**		**Age, weeks**			**p-value**		
	LSL	*LB*	*Control*	*Hemp*	*32*	*56*	*72*	*Genotype*	*Diet*	*Age*
Myristic acid 14:0 SFA	0.27	0.25	0.27	0.24	0.26	0.26	0.26	0.15	0.06	0.82
Palmitic acid 16:0 SFA	23.43	21.06	22.41	22.03	22.87	22.76	21.05	0.06	0.69	0.29
Palmitoleic acid 16:1 MUFA	1.72	1.69	2.00	1.43	1.46	1.88	1.79	0.61	0.008	0.24
Stearic acid 18:0 SFA	7.56	7.35	7.32	7.58	8.05	7.76	6.56	0.60	0.59	0.05
Oleic acid 18:1 ω-9 MUFA	39.13	37.79	40.61	36.38	38.16	36.73	40.28	0.45	0.03	0.17
Vaccenic acid 18:1 ω-11MUFA	2.38	4.23	4.29	2.42	3.34	4.72	2.05	0.24	0.11	0.13
Linoleic acid 18:2 ω-6 PUFA	12.64	13.53	9.85	16.17	11.32	13.23	14.75	0.40	0.003	0.17
Linolenic acid 18:3 ω-3 PUFA	1.59	1.54	1.10	1.20	1.44	1.79	1.48	0.97	0.0009	0.59
Gadoleic acid 20:1 MUFA	0.01	0.004	0.01	0.001	0.000	0.01	0.01	0.48	0.26	0.36
Eicosadienoic acid 20:2 ω-6 PUFA	0.12	0.16	0.14	0.14	0.10	0.2	0.12	0.45	0.94	0.25
Eicosatrienoic acid 20:3 ω-3 PUFA	0.26	0.09	0.13	0.21	0.18	0.23	0.11	0.02	0.18	0.53
Arachidonic acid 20:4 ω-6 PUFA	1.09	1.19	1.08	1.20	1.12	1.02	1.27	0.34	0.29	0.47
EPA 20:5 ω-3 PUFA	0	0	0	0	0	0	0	0	0	0
Behenic acid 22:0 SFA	0.00003	0.001	0.001	0.000	0.000	0.002	0.000	0.43	0.32	0.37
Erucic acid 22:1 ω-9 MUFA	0.005	0.002	0.004	0.002	0.000	0.007	0.003	0.38	0.47	0.22
DHA 22:6 ω-3 PUFA	0.1	0.95	0.09	1.04	1.10	0.95	0.87	0.76	0.31	0.13

DISCUSSION

Egg weight, yolk weight and total yolk lipid

The increase in egg weights and yolk weights with hen age is considered to be common fact (Rose, 1997; Basmacioglu *et al .2003*). Genotype differences in weight of eggs and yolks are also a well known effect (Scheideler *et al.* 1998). The result in the present study also corresponds well with the information given by the breeding company (Lohmann Tierzucht, 2009) predicting a higher egg weight for LB than LSL hens. This can be partly explained by the higher body weight and higher feed consumption of LB compared with LSL. As where seen in this study the total lipid content in egg yolk increased with increasing age, which corresponds with the findings of Pisarski and Cytawa-Koniceczny (2004). This phenomenon may be correlated to the increase of yolk weight as the hens get older. That could also be an explanation to why LB, that throughout the study laid heavier eggs than LSL, also produced eggs with a higher amount off egg yolk lipids.

In this study no effects of the hemp diet were found either in egg weight, yolk weigtht or total lipid content. This agrees with the findings by Collins *et al.* (1997) where laying hens were fed diets containing pearl millet (*Pennisetum glaucum)*. However, there are also examples of studies where the composition of the diet affected different egg parameters, such as egg- and yolk weight. Elwinger and Inborr (1999) found that eggs from hens fed a diet with fish oil had a lower weight compared with eggs from hens which were fed diets containing vegetable oils. This was probably due to an increasing weight effect of linoleic acid (18:2 ω-6 PUFA) in vegetable oils and a decreasing effect of the fish oil, in agreement with Loh *et al.* (2009), Schreiner *et al.* (2004) and Gonzalez-Esquerra and Leeson (2000). The latter authors suggested that ω -3 fatty acids in fish oil leads to a decrease in circulating triacylglycerols in blood plasma which may have a limiting effect on the availability of lipids for yolk formation.

Cholesterol

The observed changes towards decreasing cholesterol levels with increasing age of the hens may be connected to the physiological importance of cholesterol in the body. Cholesterol is a precursor for bile acids and steroid hormones, such as progesterone, testosterone, estradiol, cortisol and vitamin D (Champe *et al* 2008). The fertility of hens decreases with age, which may also be the case for cholesterol production in the body.

Several approaches have been made to manipulate the cholesterol concentration in egg yolk to present a healthier alternative to ordinary table eggs for human consumption (Elkin, 2006). The major route to do this has been by addition of different nutrients in the diets. In this study the results suggests that hemp may have a lowering influence on the cholesterol levels in egg yolk. Diet has in several studies (Cobos *et al.* 1995, Ferrier *et al.* 1995, An *et al.* 1997, Milinsk *et al* 2003, Millet *et al.* 2006) shown no effect at all on the levels of cholesterol. In contrast there are also a number of studies (El Bagir *et al.* 2006, Ayerza and Coates 2000) that supports the findings in this study, showing effects of diet on the cholesterol level in egg yolk. In Elkin's (2007) review, different nutrients which can have effect on the cholesterol level in yolk are presented. A high amount of fats and oils in the diets seems to increase the cholesterol level, but the effect is depending on the type of fat. For example a diet with safflower oil gave a higher cholesterol level than a diet with animal fat (Elkin, 2007). Other feed additives, like ω -3 fatty acids, cupper and garlic, which all may have cholesterol lowering properties are presented in the review by Elkin (2007). None of the additives

presented in the literature gave consistent results, which seems to be the over all conclusion when it comes to dietary manipulation of cholesterol levels in egg yolk.

Phospholipids

The proportion of phospholipids in egg yolks in this study were at an average of 25 %, which do not correspond to the directions given by the literature (Anton 2007). According to Anton (2007) the amount of phospholipids in egg yolk should be approximately 33 %. This difference may partly be explained by the analyse method chosen in this experiment; solid-phase extraction (SPE) with a diol-column (Carelli *et al.*, 1997). This method is supposed to be the most efficient method to analyse phospholipids (Chua *et al.*, 2008). In the case with egg yolk the method had to be modified, due to the high amount of phospholipids in egg yolk. Many of the steps in the process is sensitive when it comes to accurate results, such as weight elements, the objective interpretation of TLC-plates and nitrogen drying of the samples. These mentioned steps in the analyse method may all be sources of error which may explain the difference between the results in this study and the literature

A few studies have been made to establish the possibility to manipulate the phospholipid content in egg yolk. Some attempts to change the fatty acid composition in the phospholipid fraction have been done, e.g., by Chojnacka *et al.* 2009, who enzymatically changed the fatty acid profile of phosphatidylcholine in egg yolk. According to Chojnacka *et al.* 2009 egg yolk phospholipids mainly contains saturated fatty acids and no ω-3 fatty acids. In the present study it would had been interesting to investigate if the hemp diet had any effect on the fatty acid composition of phospholipids. The hemp seed affected the total fatty acid composition in this study, towards a higher amount of linolenic acid (18:3 ω-3 PUFA), which suggests that hemp in the diet would affect the fatty acid composition in the phospholipid fraction.

In this study no effects were found on the amount of phospholipids in egg yolk. Effects of diet on the amount of phospholipids have in contrast to this study and An *et al.* (1997) been found by ChangChun et al. 2010. ChangChun et al. 2010 fed laying hens a diet containing soy-lecithin (phosphatidylcholine) and found a significant increasing effect on the amount of total phospholipids in egg yolk. The biological mechanism behind manipulation of total phospholipids needs further investigation before an explanation for the varying results can be presented. To use soy lecithin in laying hen diets could be a way to refine phospholipids from soy to the more desirable phospholipids in egg yolk.

Fatty acids

The results of this study, where both the percentage of linoleic acid (18:2 ω-6 PUFA) and linolenic acid (18:3 ω-3 PUFA) were higher and palmitic acid (16:0 SFA) lower for the hemp diet compared to the control agrees with the findings of Silversides *et al.* (2005). Silversides *et al.* (2005) came to the conclusion that hemp seed had an effect on the fatty acid composition of egg yolk, when feeding hens with diets containing 0, 5, 10 and 20 % hemp seed meal (HSM). In that study an increased amount of HSM increased the amount of linoleic acid (18:2 ω-6 PUFA) and linolenic acid (18:3 ω-3 PUFA) and the amount of palmitic acid (16:0 SFA) decreased. These findings suggest that hemp seed (meal or cake) inclusion in laying hen diets can be motivated with the result of healthier eggs for human consumption. This is due to the fact that both linoleic acid and linolenic acid are precursors to the healthy ω-3 fatty acids ARA, EPA and DHA in the human body.

Yannakopoulos (2007) mentioned that one approach to enrich eggs with DHA is to add linolenic acid in the hen diet. If the fatty acid profiles of the control diet and the hemp diet,

table 4, in this study are compared with the results of the fatty acid composition in the egg yolks, table 6, the effect of Yannakopoulos (2007) statement can be seen. In the diets no DHA at all is present, but the amount of linolenic acid is fairly high. In the egg yolks on the other hand, some amount of DHA is present, implying that some of the linolenic acid have been converted to DHA in the body of the hen. The fact that no EPA is found either in the two diets or in the egg yolks is confirming the report made by Bavelaar & Beyen (2004), which declares that the level of EPA only can be increased in egg yolk by adding pure EPA or feed stuffs rich in EPA in birds diet. In this study no EPA was found in either the control diet or the hemp diet.

CONCLUSIONS

The conclusion of this study is that hen age and genotype affect the physical properties of the eggs. Some of the chemical properties are more easily manipulated with diet, as seen in the case of fatty acids. Both phospholipids and cholesterol are closely connected to important bodily functions and embryo development, making them more difficult to manipulate.

Hemp seed in the laying hen diet has positive effects on the fatty acid profile in egg yolk. This in combination with the tendency towards a cholesterol lowering effect speaks for its advantage, implying healthy table eggs for human consumption.

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