Impact of feed supplementation with different omega-3 rich microalgae species on enrichment of eggs of laying hens

Charlotte Lemahieu^{a,*}, Charlotte Bruneel^a, Romina Termote-Verhalle^a, Koenraad Muylaert^b,
Johan Buyse^c, and Imogen Foubert^a

^aResearch unit Food and Lipids, KU Leuven Kulak, Etienne Sabbelaan 53, 8500 Kortrijk,
Belgium.

Leuven Food Science and Nutrition Research Centre (LFoRCe), KU Leuven, Kasteelpark
Arenberg 20, 3001 Leuven, Belgium.

* Corresponding author: Charlotte Lemahieu
Phone: +32 (0) 56 24 61 72
Fax: +32 (0) 56 24 69 99
E-mail: charlotte.lemahieu@kuleuven-kulak.be

^bResearch unit Aquatic Biology, KU Leuven Kulak, Etienne Sabbelaan 53, 8500 Kortrijk,
Belgium.

^cDivision of Livestock-Nutrition-Quality, KU Leuven, Kasteelpark Arenberg 30, 3001
Leuven, Belgium.

ABSTRACT

Four different omega-3 rich autotrophic microalgae, *Phaeodactylum tricornutum*, *Nannochloropsis oculata*, *Isochrysis galbana* and *Chlorella fusca*, were supplemented to the diet of laying hens in order to increase the level of omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) in egg yolk. The microalgae were supplemented in two doses: 125 mg and 250 mg extra n-3 PUFA per 100 g feed. Supplementing these microalgae resulted in increased but different n-3 LC-PUFA levels in egg yolk, mainly docosahexaenoic acid enrichment. Only supplementation of *Chlorella* gave rise to mainly α -linolenic acid enrichment. The highest efficiency of n-3 LC-PUFA enrichment was obtained by supplementation of *Phaeodactylum* and *Isochrysis*. Furthermore, yolk color shifted from yellow to a more intense red color with supplementation of *Phaeodactylum*, *Nannochloropsis* and *Isochrysis*, due to transfer of carotenoids from microalgae to eggs.

This study shows that besides *Nannochloropsis* other microalgae offer an alternative to current sources for enrichment of hen eggs.

34 **KEYWORDS**

35 Omega-3 polyunsaturated fatty acids

36 Microalgae

37 *Phaeodactylum triconutum*

38 *Nannochloropsis oculata*

39 *Isochrysis galbana*

40 *Chlorella fusca*

41 Eggs

42 Enrichment

43 Carotenoids

1. Introduction

Health benefits associated with omega-3 fatty acids are generally accepted. However, it is less known that omega-3 polyunsaturated fatty acids (n-3 PUFA) can be divided in short and medium chain ($\leq C_{18}$) PUFA, such as α -linolenic acid (ALA, C18:3 n-3) and stearidonic acid (SDA, C18:4 n-3), and long chain (LC, $\geq C_{20}$) PUFA, such as eicosapentaenoic acid (EPA, C20:5 n-3), docosapentaenoic acid (DPA, C22:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3) and that the health benefits are related to the n-3 LC-PUFA, EPA and DHA, rather than to ALA (Trautwein, 2001). The link between n-3 LC-PUFA and the reduction and prevention of several cardiovascular diseases is well-documented (Trautwein, 2001; Gogus & Smith, 2010). Moreover, n-3 LC-PUFA also play an important role in the prevention and treatment of several chronic diseases, such as neurological disorders, cancer, obesity, inflammatory diseases and diabetes mellitus. Furthermore, maternal intake of n-3 LC-PUFA lowers the risk of preterm birth and low birth weight. Finally, especially DHA is critical to fetal growth and development of visual and cognitive functions in fetus and children (Gogus et al., 2010).

The recommended daily intake of n-3 LC-PUFA by several governments and health organizations is ranging from 140 to 667 mg/day (Kris-Etherton, Grieger & Etherton, 2009; Molendi-Coste, Legry & Leclercq, 2011). However, intake of 250 mg n-3 LC-PUFA per day already provides primary protection against cardiovascular diseases (Kris-Etherton et al., 2009). Inhabitants of only a few countries worldwide including Japan, Korea, the Philippines, Finland, Iceland, Norway and Sweden reach the intake of 250 mg n-3 LC-PUFA per day. The intake in all other countries is below 250 mg n-3 LC-PUFA per day (Sioen, De Henauw, Van Camp, Volatier & Leblanc, 2009). Hence, a growing interest to enrich food products with n-3 LC-PUFA has emerged. Enrichment of eggs is a potential way to increase the n-3 LC-PUFA content in our diet. The level and type of PUFA in the egg yolk can be modified by the feed of

the laying hens, whereas the saturated and monounsaturated fatty acids in egg yolk are hardly affected by the feed (Baucells, Crespo, Barroeta, López-Ferrer & Grashorn, 2000). A lot of research has been carried out in order to increase the n-3 PUFA level in eggs by dietary supplementation of n-3 PUFA rich sources to the diet of laying hens. Flaxseed, as source of ALA, gives mainly rise to enrichment of ALA in the egg yolk. However, also a slight increase in EPA and DHA is observed. This can be explained by the quite inefficient conversion of ALA to EPA and DHA in laying hens (Fraeye, Bruneel, Lemahieu, Buyse, Muylaert & Foubert, 2012). A second source of n-3 LC-PUFA that can be supplemented to the hens' feed is fish oil, rich in EPA and/or DHA. It provides an enrichment of EPA and especially DHA in egg yolk (Van Elswyk, 1997). However, the declining fish stocks, the presence of pollutants such as heavy metals and PCBs in fish, and the off-flavours detected in eggs when more than 1.5% fish oil is supplemented, implies that alternative sources of n-3 LC-PUFA must be found (Fraeye et al., 2012). Microalgae can be promising, as the n-3 PUFA found in fish are originating from microalgae. Mainly heterotrophic microalgae, rich in DHA and obtained by fermentation processes, have been supplemented to laying hens' feed (Fraeye et al., 2012). The enrichment of n-3 LC-PUFA in the egg yolk is very similar to the enrichment observed when fish oil is supplemented: their DHA content is markedly increased (Rizzi, Bochicchio, Bargellini, Parazza & Simioli, 2009). Only a few studies have been performed using autotrophic microalgae, which produce organic compounds by using CO₂ and sunlight. To the best of our knowledge, only the species *Nannochloropsis* has been supplemented to laying hens' feed to raise the level of n-3 LC-PUFA in the egg yolk (Nitsan, Mokady & Sukenik, 1999; Fredriksson, Elwinger & Pickova, 2006; Bruneel et al., 2013). *Nannochloropsis* has an interesting fatty acid composition, since it contains EPA as the sole n-3 LC-PUFA. When *Nannochloropsis* is added to hens' feed, the level of DHA in eggs significantly increased. It seems that microalgal EPA is first converted to DHA before it is stored in the egg yolk

93 (Nitsan et al., 1999; Fredriksson et al., 2006; Fraeye et al., 2012; Bruneel et al., 2013). Not
94 only the fatty acid profile changed considerably, also the redness of the egg yolk increased by
95 feed supplementation with *Nannochloropsis*. This can be explained by transfer of carotenoids
96 from microalgae to the egg yolk (Nitsan et al., 1999; Fredriksson et al., 2006; Fraeye et al.,
97 2012). Other microalgae, like *Chlorella* and *Porphyridium*, also gave rise to a transfer of
98 carotenoids from the microalgae to the egg yolk. Especially upon supplementation with the
99 red alga *Porphyridium*, a significantly darker yellow egg yolk color was observed (Gouveia,
100 Veloso, Reis, Fernandes, Novai & Empis, 1996; Ginzberg, Cohen, Sod-Moriah, Shany,
101 Rosenshtrauch & Arad, 2000).

102 However, next to *Nannochloropsis*, Ryckebosch, Bruneel, Muylaert & Foubert (2012b)
103 suggested other autotrophic microalgae as an alternative n-3 LC-PUFA source based on total
104 lipid content, n-3 LC-PUFA content, and ease of cultivation. They reported *Phaeodactylum*,
105 next to *Nannochloropsis*, as the most promising EPA source, and *Isochrysis* as the most
106 promising DHA source. To that end, the objective of this study was to investigate the
107 influence of using other autotrophic microalgae species for n-3 LC-PUFA enrichment in eggs
108 of laying hens. Four different autotrophic microalgae were selected for supplementation to the
109 laying hens' feed: *Phaeodactylum tricornutum*, *Isochrysis galbana* and *Nannochloropsis*
110 *oculata* because of their high n-3 LC-PUFA content and *Chlorella fusca* because of its high
111 ALA content. The four autotrophic microalgae were supplemented in two different
112 concentrations to the laying hens' feed. The fatty acid profile, the carotenoid and sterol
113 content, and the color of egg yolk were measured.

2. Materials and methods

All the chemicals used were at least of analytical grade or high-performance liquid chromatography (HPLC) grade and purchased from Sigma-Aldrich (Bornem, Belgium), unless specified otherwise.

2.1. Microalgal biomass

The following four n-3 PUFA rich microalgae, which were commercially available in high quantities, were selected for this study: *Phaeodactylum tricornutum* (freeze dried biomass, SBAE, Belgium), *Nannochloropsis oculata* (freeze dried biomass, SBAE, Belgium), *Isochrysis galbana* (freeze dried biomass, Algaenergy, Spain) and *Chlorella fusca* (dried biomass, Ingrepro, The Netherlands).

The proximate composition (total lipids, n-3 PUFA, carotenoids and sterols) of the selected microalgae was determined (in triplicate) and expressed on dry matter (DM) biomass. The **moisture content** of all microalgae was determined by drying 0.5 g microalgal biomass at 103°C till constant weight was achieved (24 hours) (Wrolstad et al., 2005). The **total lipids** were extracted from the microalgae according to the method described by Ryckebosch, Muylaert & Foubert (2012a). At the start of the extraction, an internal standard (C20:0; Nu-check, Elysian, USA) was added to calculate the level of n-3 LC-PUFA in g per 100 g DM biomass. After gravimetric determination of the total lipid content, methylation was performed, as described by Ryckebosch et al. (2012a), to form fatty acid methyl esters (FAMES). These FAMES were separated by gas chromatography (GC) with cold on-column injection and flame ionization detection (FID) (Trace GC Ultra, Thermo Scientific, Interscience, Louvain-la-Neuve, Belgium) using an EC Wax column (length: 30m, ID 0.32mm, film: 0.25µm) (Grace, Lokeren, Belgium). The used time-temperature program was:

70 °C – 180 °C (5 °C/min), 180 °C – 235 °C (2 °C/min), 235 °C (9.5 min). Fatty acid identification was performed using standards containing a total of 35 different FAMES (Nutcheck). Peak areas were quantified with Chromcard for Windows software (Interscience).

The **carotenoid composition** was determined by dissolving 2 mg of the total lipid extract in 10 mL methanol. This solution and the dilution 1/10 were analyzed by high performance liquid chromatography (HPLC) with a photodiode array detector (PAD) (Alliance, Waters, Zellik, Belgium) as described by Wright et al. (1991). Calibration curves were composed for alloxanthin, diadinoxanthin, diatoxanthin, lutein, neoxanthin, violaxanthin, zeaxanthin (all DHI, Hørsholm, Denmark), fucoxanthin and β -carotene to express the results as $\mu\text{g/g}$ DM biomass.

For determination of the **sterol composition** of the algal biomass, first an internal standard, 5β -cholestan- 3α -ol, 200 μg), was added to the microalgal biomass (40 mg) and used to quantify the level of sterols in microalgae. Saponification was performed according to Abidi (2004), with some slight modifications. Saponification of the algal biomass was performed by overnight stirring with potassium hydroxide in ethanol (1 M, 4 mL) (VWR International, Leuven, Belgium). Then, water (4 mL) was added to the reaction mixture followed by three sequential extractions with hexane (8 mL) (Boom, Meppel, The Netherlands). The hexane extracts were combined and the solvent was removed by rotary evaporation at 40 °C. The sterol components were silylated, as described in Toivo, Lampi, Aalto, & Piironen, (2000). Anhydrous pyridine (200 μL) and derivatisation reagent (200 μL) containing bis(trimethylsilyl)trifluoroacetamide (BSTFA) (99%) and trimethylchlorosilane (TMCS) (1%) were added to the nonsaponifiable fraction. The solutions were incubated at 60 °C for 1 hour to complete the silylation. After incubation, the solutions were cooled to room temperature and diluted with 600 μL hexane. The silylated sterols were separated by GC with on-column

injection and FID. An Rtx-5 column (length 30 m, ID 0.25 mm, film 0.25 µm) (Restek, Interscience) was used with the following time-temperature program: 200-340 °C (15 °C/min), 340 °C (10 min). Peak areas were quantified with Chromcard for Windows software (Interscience).

2.2. Animals, housing and diets

Seventy-two ISA Brown laying hens ('t Munckeni, Wingene, Belgium) of 29 weeks of age, were housed in battery cages (two hens/cage) maintained in an environmentally controlled room where temperature was set to 20 °C. The hens received 16 hours light per day. Feed and water were supplied *ad libitum*. The experimental trial lasted 42 days: 14 days of adaptation and 28 days of supplementation with microalgae. During the adaptation period, the hens could adapt to the new environmental conditions and the new commercially available control diet (Legmeel Total 277, AVEVE, Willebe, Belgium). After the adaptation period, the hens were randomly assigned to one of the nine treatment diets (n = 8 hens per treatment diet): a control diet, without supplementation of microalgae, and the other eight diets were supplemented with one of the four microalgae (*Phaeodactylum tricornutum*, *Nannochloropsis oculata*, *Isochrysis galbana* or *Chlorella fusca*) in two different doses. The amount of each supplemented microalgae was calculated as to obtain two levels of extra microalgal ALA+EPA+DHA being 125 mg and 250 mg per 100 g feed. To achieve these concentrations, the added levels of microalgae varied between 2.5% and 8.6%.

2.3. Zootechnical performance of laying hens

Feed intake, egg production, mortality and morbidity were registered on a daily basis and this during the entire trial of 42 days. Moreover, the body weight of the laying hens was

determined at the start (day 0) and at the end (day 28) of the supplementation period with microalgae.

2.4. Sample collection and storage of eggs

Eggs were collected daily and stored at -20 °C until further analyses. The eggs at the end of the supplementation period were analyzed for several egg quality parameters as described in 2.5. Furthermore, the total lipid content of those eggs and the content and composition of n-3 PUFA, carotenoids and sterols were determined in triplicate, as described in 2.6, 2.7 and 2.8. To calculate the enrichment of n-3 LC-PUFA in eggs by dietary supplementation of microalgae, the fatty acid profile of the eggs at the start (day 0) of the supplementation period was also analyzed.

2.5. Egg quality parameters

At the end of the supplementation period several quality parameters of the eggs were determined. First of all, the total egg weight, the shell weight, the albumen weight and the yolk weight were determined. Secondly, the quality of the shell was determined by observing the shell thickness and shell strength and by detecting the cracks. The shell thickness was measured by a micrometer, whereas the shell strength and cracks were determined by the acoustic egg tester (InDuct, Leuven, Belgium) (De Ketelaere, Coucke & De Baerdemaeker, 2000). The final egg quality parameter was the color of the egg yolk which was measured by using the Hoffman-La Roche color scale (range 1 – 15; DSM, Basel, Switzerland) on the one hand and the Minolta colorimeter (CR 300, Konica Minolta, Zaventem, Belgium) on the other hand (Bruneel et al., 2013).

2.6. Total lipid content and fatty acid profile of eggs

The total lipids from all egg yolks obtained at the start and the end of the supplementation period were extracted according to the method described by Bruneel et al. (2013), with slight modifications. Briefly, an internal standard (C20:0, Nu-check) was added to the yolk sample (200-250 mg) and after addition of methanol (4.5 mL), the yolk sample was homogenized (30 sec, 8000 rpm), using a CAT X620 homogenizer (VWR International). Subsequently chloroform (9 mL) (Boom) was added and the mixture was again homogenized (60 sec, 8000 rpm). After homogenization, the mixture was centrifuged (10 min, 2800 g) (Thermo Scientific, Aalst, Belgium). The supernatant was removed and the remaining lipids in the residue were re-extracted with chloroform:methanol (2:1, v/v, 12 mL). After homogenization (60 sec, 8000 rpm) and centrifugation, the supernatant was removed and combined with the earlier obtained supernatant. The combined chloroform:methanol extracts were washed with KCl (0.88%, 6.2 mL) and after centrifugation, the bottom layer was filtered over anhydrous sodium sulphate (VWR international). The solvents were removed by rotary evaporation (Buchi Qlab, Vilvoorde, Belgium). After gravimetric determination of the total lipid content, the lipids were redissolved in chloroform:methanol (2:1, v/v, 2 mL) for further analysis.

To determine the fatty acid profile, the lipids were methylated as described by Ryckebosch et al. (2012a). The separation and quantification of the FAMES by GC analysis was the same as described for the microalgae in 2.1.

2.7. Carotenoid composition in eggs

To determine the carotenoid composition of the egg yolks obtained at the end of the supplementation period, 1 mL of the lipid extract, obtained in 2.6, was dried under nitrogen and redissolved in 1 mL methanol in the presence of butylated hydroxytoluene (0.1%). The carotenoids were determined by HPLC as described in 2.1. The results were expressed as $\mu\text{g/egg}$.

2.8. Sterol composition in eggs

The sterol content in the egg yolks obtained at the end of the supplementation period was determined by direct saponification of egg yolk. Van Elswyk, Schake & Hargis (1991) showed that direct saponification gave rise to a higher cholesterol content than saponification after lipid extraction. Saponification was performed according to Van Elswyk et al. (1991), with slight modifications. Briefly, egg yolk (200 mg) was mixed with 50% KOH in water (1.6 mL), ethanol (8 mL) (VWR International) and an internal standard (5 β -cholestan-3 α -ol, 500 μ L, 2 mg/mL) using a CAT X620 homogenizer (8000 rpm). The mixture was kept for 1 hour at 60 °C. After cooling to room temperature, 8 mL water was added and the sterols were obtained after three sequential extractions with hexane (12 mL). The combined hexane extracts were filtered (Whatmann filter papers, grade 1) over anhydrous sodium sulphate. The solvent was evaporated using a rotary evaporator at 40 C. The nonsaponifiable fraction was redissolved in ethanol:hexane (2:3 v/v, 2 ml) and sterol components of this fraction (40 μ L) were silylated according to the method described in 2.1. The silylated sterols were separated in cholesterol and phytosterols by GC using standards as described in 2.1.

2.9. Statistical analysis

Results were statistically evaluated by two-way analysis of variance (ANOVA) and a post-hoc Tukey's test with $\alpha=0.05$ (Sigmaplot 11, Systat Software, Inc., Illinois, USA).

3. Results and discussion

3.1. Proximate composition of n-3 PUFA rich autotrophic microalgae

The composition (total lipids, n-3 PUFA, carotenoids, and sterols) of *Phaeodactylum*, *Nannochloropsis*, *Isochrysis* and *Chlorella* biomass is summarized in **Table 1**.

Isochrysis contained the highest lipid content (31.6%, expressed as g/100 g DM biomass), followed by *Nannochloropsis* (25.1%) and *Phaeodactylum* (24.1%). The lowest lipid content was measured in *Chlorella* (14.7%). Furthermore, they all contained a different n-3 PUFA profile. *Chlorella* mainly contained ALA (2.8 g/ 100 g biomass) and could thus function as an alternative for flaxseed. *Nannochloropsis* mainly contained EPA (4.0 g/ 100 g biomass), whereas *Phaeodactylum* and *Isochrysis* contained next to EPA (2.8 g and 3.5 g per 100 g biomass, respectively) also DHA (0.3 g and 0.8 g per 100 g biomass, respectively). Next to EPA and DHA, *Isochrysis* also contained a significant level of SDA (1.1 g/ 100 g biomass), which was hardly observed in the other microalgae.

Due to the photosynthetic pathway, autotrophic microalgae are also rich in pigments, such as chlorophylls and carotenoids. **Table 1** shows that *Phaeodactylum* and *Isochrysis* mainly contained fucoxanthin and, to a lesser extent, beta-carotene (135.1 µg and 106.7 µg/ g DM, respectively). However, the fucoxanthin content in *Isochrysis* (1464.0 µg/ g DM) was three times lower than that measured in *Phaeodactylum* (4381.8 µg/ g DM). *Nannochloropsis* mainly contained violaxanthin (2286.8 µg/ g DM), and a four to five times higher amount of beta-carotene (581.3 µg/ g DM) in comparison with *Phaeodactylum* and *Isochrysis*. In the microalga *Chlorella*, only a low level of carotenoids was measured. It contained no beta-carotene or fucoxanthin, only lutein (97.7 µg/ g DM), neoxanthin (39.4 µg/ g DM) and violaxanthin (21.6 µg/ g DM).

The sterols of microalgae can be divided into two groups: cholesterol and phytosterols. A low level of cholesterol was detected in *Nannochloropsis* (2.6 mg/ g DM) and *Isochrysis* (1.1 mg/ g DM). All four microalgae contained phytosterols varying in amount between 1.1 and 13.5 mg per g DM biomass (**Table 1**). The highest level of phytosterols was measured in *Isochrysis*.

3.2. Zootechnical performance of laying hens

The average daily feed intake, egg production, mortality and morbidity were not significantly affected by supplementation of microalgae to the laying hens (detailed results not shown). The body weight of the laying hens remained constant, except for hens supplemented with *Nannochloropsis* (in both doses), which displayed a slight significant decrease (< 5%) in body weight. This is in contrast with the results of Bruneel et al. (2013) who also supplemented *Nannochloropsis* to the laying hens' feed, in two doses (5% and 10% *Nannochloropsis*) and observed no significant impact on the zootechnical parameters, also not on the body weight. The reason for this discrepancy is not clear but it should be taken into account that we only used 8 hens per dietary treatment as the focus was on enrichment of microalgal components in eggs.

3.3. Egg parameters

The egg parameters, such as total egg weight, shell weight, albumen weight, yolk weight and shell quality were not significantly affected by supplementation of different microalgae in different doses to the laying hens (**Table 2**).

3.4. Enrichment of egg yolk

3.4.1. N-3 PUFA enrichment

Figure 1 shows the level of the omega-3 fatty acids ALA, EPA, DPA and DHA in eggs obtained at the end of the microalgal supplementation period. It should be mentioned that SDA, although present in *Isochrysis*, was not present in the egg yolks.

A significant increase of ALA in egg yolk was observed when *Chlorella*, source of ALA, was supplemented to the laying hens' feed, compared with the ALA content in eggs of the control group. Supplementation of hens with the other three selected microalgae, rich in EPA and/or DHA, did not significantly influence the level of ALA in eggs. Similar results were observed for fish oil, which is also rich in EPA and DHA (García-Rebollar, Cachaldora, Alvarez, De Blas & Méndez, 2008). The EPA content in the egg yolks increased significantly when hens were fed with *Phaeodactylum*, *Nannochloropsis* and *Isochrysis*, whereas EPA was not detected in eggs of hens fed *Chlorella*. It should also be noted that an extra omega-3 fatty acid (DPA) was detected in all microalgal enriched eggs, compared to the control group. DPA is an intermediate in the conversion process of EPA to DHA. This may indicate that not only the conversion of ALA to n-3 LC-PUFA is a rate limiting step, but also the conversion of EPA to DHA. Cachaldora, García-Rebollar, Alvarez, De Blas & Méndez (2006) supplemented fish oil, with a high amount of EPA, to the laying hens' feed and also observed a significant increase in the DPA content in the egg yolk. The most striking increase of n-3 PUFA in egg yolk was observed for DHA. The highest enrichment of DHA was obtained by supplementation with *Phaeodactylum* and *Isochrysis*, followed by *Nannochloropsis*, whereas the lowest DHA enrichment was observed for supplementation with *Chlorella*. Since DHA, rather than EPA, was preferentially incorporated into the egg yolk lipids, it seems that microalgal n-3 PUFA are first converted to DHA, before being deposited in egg yolk lipids. This was also observed by Nitsan et al. (1999), Fredriksson et al. (2006) and Bruneel et al. (2013) for the microalga *Nannochloropsis*.

In **Table 3**, the total n-3 LC-PUFA (EPA+DPA+DHA) enrichment of eggs for the four different microalgae used, in two doses, is shown. The enrichment of the n-3 LC-PUFA was calculated from the n-3 LC-PUFA content in eggs obtained at the end of the supplementation period minus the n-3 LC-PUFA content at the start of the supplementation period, for the respective groups. Furthermore, the conversion factors were calculated to determine the efficiency of the n-3 LC-PUFA incorporation in the egg yolk (**Table 3**). The conversion factors were obtained by taking the ratio of the enrichment of n-3 LC-PUFA (EPA+DPA+DHA) in the egg yolk to the supplemented n-3 PUFA in the feed and multiplied by 100. The total amount of supplemented n-3 PUFA/100 g feed was corrected for the mean feed intake of the respective groups. For supplementation with *Phaeodactylum*, *Isochrysis* and *Chlorella*, also the SDA content was taken into account. SDA could also be converted to n-3 LC-PUFA, but was not taken into consideration for the experimental design.

The enrichment of n-3 LC-PUFA by supplementation with *Nannochloropsis* was significantly lower in comparison with *Phaeodactylum* and *Isochrysis* (**Table 3**), while the same doses of microalgal ALA+EPA+DHA were supplemented. The highest enrichment efficiencies ($\pm$ 42%) were obtained by supplementation of *Phaeodactylum* and *Isochrysis* in the lowest dose. The efficiency of the n-3 LC-PUFA deposition in the egg yolk by supplementation with *Nannochloropsis* was rather low, approximately 20%. This corresponds to the results obtained by Bruneel et al. (2013), who supplemented *Nannochloropsis* in two doses, 5% (76 mg ALA+EPA+DHA per 100 g feed) and 10% (152 mg ALA+EPA+DHA per 100 g feed), and observed an efficiency of 25% and 20% for the two doses respectively. Two possible explanations may exist for the lower enrichment observed with *Nannochloropsis*. First of all, *Phaeodactylum* and *Isochrysis* contain a certain amount of DHA, which can be incorporated into the egg yolk without any conversion steps. *Nannochloropsis* on the other hand, contains

only EPA. As already mentioned, EPA has to be converted first to DHA before it is stored in the yolk. So, the need for a conversion from EPA to DHA can cause a lower enrichment in the egg yolk. Cachaldora et al. (2006) supplemented fish oils with different EPA/DHA ratios to the laying hens' feed. They observed the highest n-3 LC-PUFA enrichment with supplementation of fish oil with the lowest EPA/DHA ratio, so with mainly DHA. This indicates that the EPA/DHA ratio can play a role in the n-3 LC-PUFA enrichment in the yolk. The second explanation for the lower enrichment with *Nannochloropsis* could be the reduced digestibility of the cell wall. *Nannochloropsis* produces an aliphatic, non-hydrolysable biopolymer, algaenan, which can prevent enzymatic degradation of the cell wall by the hen (Gelin, Volkman, Largeau, Derenne, Sinninghe Damsté & De Leeuw, 1999). Another explanation for the lower enrichment by supplementation with *Nannochloropsis* could also be a combination of the conversion of EPA to DHA and the reduced digestibility of the cell wall. The lowest total enrichment of n-3 LC-PUFA was observed by supplementation with *Chlorella* (**Table 3**). Only an n-3 LC-PUFA enrichment efficiency of approximately 10% was observed. *Chlorella* only contained ALA and, in contrast to the other microalgae, especially ALA enrichment was observed in the egg yolk (**Figure 1**). Several studies already mentioned that the conversion of ALA to EPA and DHA in the hen is rather limited (Aymond & Van Elswyk, 1995; Van Elswyk, 1997). This can presumably explain the rather low enrichment of n-3 LC-PUFA. Addition of flaxseed to the laying hens' feed also caused mainly ALA enrichment (Fraeye et al., 2012), which means that *Chlorella* could potentially serve as an alternative for flaxseed.

The microalgae were supplemented in two doses, chosen as to obtain 125 and 250 mg extra ALA+EPA+DHA per 100 g feed. Doubling the supplemented dose of n-3 PUFA in the feed did not result in a much higher increase of the n-3 LC-PUFA content in the egg yolk (**Table**

3). Comparing the conversion factors for the different doses for the same microalgae, the conversion factor for the highest dose was much lower. Other studies also mentioned decreasing efficiency of retention of the dietary n-3 PUFA in the yolk with increasing dietary concentration (Cachaldora, García-Rebollar, Alvarez, De Blas & Méndez, 2008; Bruneel et al., 2013). This indicates that the optimal supplementation dose of the microalgae is presumably below 250 mg ALA+EPA+DHA per 100 g feed.

3.4.2. Yolk color and carotenoid enrichment

Table 4 summarizes the color values (Roche value and CIELAB value) of the egg yolks obtained at the end of the supplementation period. Generally, supplementation of microalgae to the laying hens' feed gave rise to a significantly increased Roche value, except for supplementation with *Chlorella*. An increased Roche value corresponds with an increased a^* value, which is a measure for the redness of egg yolk, whereas the L^* value and b^* value decreased or remained more or less the same. **Figure 2** shows the carotenoid content of the enriched egg yolks using four different microalgae in two doses compared to control eggs.

The most striking result on yolk color was obtained when hens were fed *Phaeodactylum*. Feed supplementation with graded levels of *Phaeodactylum* (0.125% and 0.250%) increased the Roche value and a^* value, whereas the L^* value and b^* value decreased in comparison to the values of control eggs. A similar significant increase of the yolk redness was observed when *Isochrysis* was supplemented, especially in the highest dose. The increase of the a^* value can presumably be explained by the relative high fucoxanthin content, which has a typical brown color, in these two microalgae (**Table 1**). However, **Figure 2** shows that fucoxanthin derivatives, rather than fucoxanthin itself, were deposited into the egg yolk. This can be ascribed to enzymatic modifications of fucoxanthin occurring in the laying hens (Strand, Herstad & Liaaen-Jensen, 1998). Supplementation with *Nannochloropsis* resulted in only a

slight increase of the Roche and a^* values. Similar results were reported by Nitsan et al. (1999), Fredriksson et al. (2006) and Bruneel et al. (2013), who also supplemented *Nannochloropsis* to the laying hens' feed. Violaxanthin and β -carotene are present in the species *Nannochloropsis* (**Table 1**). However, these carotenoids were not detected in the egg yolk. It is generally recognized that β -carotene is hardly deposited in egg yolk because laying hens are able to efficiently convert β -carotene to vitamin A (Schlatterer & Breithaupt, 2006).

In contrast to the results obtained for *Phaeodactylum*, *Isochrysis* and *Nannochloropsis*, the Roche value and the a^* value remained constant when *Chlorella* was supplemented to the feed. Only a slight decrease of the a^* value was observed for the highest supplementation dose of *Chlorella*. The measured carotenoid content in *Chlorella* was also much lower (**Table 1**), which can explain the limited enrichment of carotenoids in the egg yolk. Only microalgal lutein from *Chlorella*, was transferred to the egg yolk, which corroborates the findings of Gouveia et al. (1996). Lutein has a typical yellow color, which gave rise to a decrease of the yolk redness.

The species and dose of the microalgae supplemented to the diet thus affect the yolk color, which can presumably be explained by transfer of carotenoids from microalgae to the egg yolk. Algal carotenoids are fat soluble and are deposited in the egg yolk in a similar way as the LC-PUFA (Nitsan et al., 1999). The transfer of carotenoids from the microalgae to the egg yolk provides an additional benefit for microalgal supplementation, next to the enrichment with n-3 LC-PUFA. The high carotenoid content in the egg yolk may protect lipids, especially LC-PUFA, against oxidation because of their antioxidant properties.

Nowadays, carotenoids are usually supplemented to the laying hens' feed to obtain the preferred yolk color, because animals do not have the ability to synthesize carotenoids by themselves (Gouveia et al., 1996). However, carotenoids are easily degraded by oxidation, so

encapsulation of the carotenoids is investigated to increase the stability (Gouveia et al., 1996).
Supplementation of microalgae to the laying hens' feed is a possible way of transferring
carotenoids in an encapsulated form. But, it should certainly be taken into account that not
always the desired yolk color was obtained, especially due to the enrichment of fucoxanthin
derivatives. The desired yolk color for domestic use varies with the geographical location. In
Belgium, deeply colored yolks with a definite orange tinge, corresponding with a Roche value
of 12-13, are preferred (Gouveia et al., 1996). Enrichment of certain carotenoids however
gave rise to higher Roche values, which could lead to a decreased consumers' acceptability.

3.4.3. Yolk sterol enrichment

A negative connotation is linked to eggs, and especially the egg yolks, because of the
presence of cholesterol (200 - 300 mg/ egg) (Spence, Jenkins & Davignon, 2010). As
microalgae contain significant levels of health beneficial phytosterols (**Table 1**), which may
on the one hand also be transferred to egg yolk in a similar way as n-3 LC-PUFA and
carotenoids and may on the other hand decrease the amount of cholesterol in the egg yolk,
giving the cholesterol lowering properties of phytosterols (Mackay & Jones, 2011).
Unfortunately, in this study no phytosterols were transferred from the microalgae to the egg
yolk (results not shown). Only cholesterol was detected in the egg yolk and the amount
remained more or less constant when microalgae were supplemented to hens (results not
shown), even though some microalgae, *Nannochloropsis* and *Isochrysis*, contained a low level
of cholesterol in their biomass (**Table 1**) and despite the presence of phytosterols in all the
biomass. Research has already shown that reducing the cholesterol content in the egg yolk is
rather impossible. The inability to markedly reduce the yolk cholesterol content is possibly
due to natural selection pressures to maintain a certain level of cholesterol for the use by the
developing embryo (Hargis, 1988).

4. Conclusion

Four different microalgae, *Phaeodactylum tricornutum*, *Nannochloropsis oculata*, *Isochrysis galbana* and *Chlorella fusca*, were supplemented to the laying hens' feed. All four microalgae gave rise to increased levels of n-3 LC-PUFA in the egg yolk. The highest enrichment of n-3 LC-PUFA, mainly DHA, was achieved by supplementation of *Phaeodactylum* and *Isochrysis*, both rich in EPA and DHA. A lower enrichment was obtained by supplementation with *Nannochloropsis*, probably because of the hardly digestible cell wall and/or the necessary conversion of EPA to DHA. *Chlorella* gave rise to the lowest enrichment, presumably due to the quite inefficient conversion of ALA to DHA. Not only an enrichment of n-3 LC-PUFA was observed, also an increase of the carotenoid content was detected by supplementation of *Phaeodactylum*, *Isochrysis* and *Nannochloropsis*. This gave rise to an increased red color of the yolk. On the other hand, the sterol content of the egg yolk was hardly influenced.

Thus, several omega-3 rich autotrophic microalgae offer an alternative to current sources of n-3 LC-PUFA to increase the level of these fatty acids in the eggs. However, the carotenoid enrichment should be taken into account since the color shift of the egg yolk could decrease the consumers' acceptability.

452 **ACKNOWLEDGEMENTS**

453 This publication has been produced by the financial support of Flanders' Food –IWT
454 (OMEGA-EI). We wish to thank everybody who has contributed to this project, especially the
455 companies of the Flanders' Food OMEGA-EI project, ILVO (for making the different
456 experimental diets), Stefaan Verhelle (for delivery of the laying hens), Marcel Samain and
457 André Respen (for taking care of the laying hens) and Prof. dr. Hans Pottel (for helping us
458 with the experimental design).

REFERENCES

- Abidi, S. L. (2004). Capillary electrochromatography of sterols and related sterol esters derived from vegetable oils. *Journal of Chromatography. A*, 1059(1-2), 199-208.
- Aymond, W. M., & Van Elswyk, M. E. (1995). Yolk thiobarbituric acid reactive substances and n-3 fatty acids in response to whole and ground flaxseed. *Poultry Science*, 74, 1388-1394.
- Baucells, M. D., Crespo, N., Barroeta, A.C., López-Ferrer, S., & Grashorn, M. A. (2000). Incorporation of different polyunsaturated fatty acids into eggs. *Poultry Science*, 79(1), 51-59.
- Bruneel, C., Lemahieu, C., Ryckebosch, E., Fraeye, I., Muylaert, K., Buyse, J., & Foubert, I. (2013). Impact of microalgal feed supplementation on omega-3 fatty acid enrichment of hen eggs. *Journal of Functional Foods*, 5(2), 897-904.
- Cachaldora, P., García-Rebollar, P., Alvarez, C., De Blas, J. C., & Méndez, J. (2006). Effect of type and level of fish oil supplementation on yolk fat composition and n-3 fatty acids retention efficiency in laying hens. *British Poultry Science*, 47(1), 43-49.
- Cachaldora, P., García-Rebollar, P., Alvarez, C., De Blas, J. C., & Méndez, J. (2008). Effect of type and level of basal fat and level of fish oil supplementation on yolk fat composition and n-3 fatty acids deposition efficiency in laying hens. *Animal Feed Science and Technology*, 141(1-2), 104-114.
- De Ketelaere, B., Coucke, P. & De Baerdemaeker, J. (2000). Eggshell crack detection based on acoustic resonance frequency analysis. *Journal of Agricultural Engineering Research*, 76, 157-163.

479 Fraeye, I., Bruneel, C., Lemahieu, C., Buyse, J., Muylaert, K. & Foubert, I. (2012). Dietary
 480 enrichment of eggs with omega-3 fatty acids: A review. *Food Research International*, 48,
 481 961-969.

482 Fredriksson, S., Elwinger, K., & Pickova, J. (2006). Fatty acid and carotenoid composition of
 483 egg yolk as an effect of microalgae addition to feed formula for laying hens. *Food Chemistry*,
 484 99(3), 530-537.

485 García-Rebollar, P., Cachaldora, P., Alvarez, C., De Blas, C., & Méndez, J. (2008). Effect of
 486 the combined supplementation of diets with increasing levels of fish and linseed oils on yolk
 487 fat composition and sensorial quality of eggs in laying hens. *Animal Feed Science and*
 488 *Technology*, 140(3-4), 337-348.

489 Gelin, F., Volkman, J., Largeau, C., Derenne, S., Sinninghe Damsté, J., & De Leeuw, J.
 490 (1999). Distribution of aliphatic, nonhydrolyzable biopolymers in marine microalgae.
 491 *Organic Geochemistry*, 30(2-3), 147-159.

492 Ginzberg, A., Cohen, M., Sod-Moriah, U. A., Shany, S., Rosenshtrauch, A., & Arad, S. M.
 493 (2000). Chickens fed with biomass of the red microalga *Porphyridium* sp. have reduced blood
 494 cholesterol level and modified fatty acid composition in egg yolk. *Journal of Applied*
 495 *Phycology*, 12, 325-330.

496 Gogus, U., & Smith, C. (2010). n-3 Omega fatty acids: a review of current knowledge.
 497 *International Journal of Food Science & Technology*, 45(3), 417-436.

498 Gouveia, L., Veloso, V., Reis, A., Fernandes, H., Novais, J., & Empis, J. (1996). *Chlorella*
 499 *vulgaris* used to colour egg yolk. *Journal of the Science of Food and Agriculture*, 70, 167-
 500 172.

501 Hargis, P. S. (1988). Modifying egg yolk cholesterol in the domestic fowl - A review. *World's*
502 *Poultry Science Journal*, 44, 17-29.

503 Kris-Etherton, P. M., Grieger, J. A., & Etherton, T. D. (2009). Dietary reference intakes for
504 DHA and EPA. *Prostaglandins, Leukotrienes, and Essential Fatty Acids*, 81(2-3), 99-104.

505 MacKay, D. S., & Jones, P. J. H. (2011). Phytosterols in human nutrition: Type, formulation,
506 delivery and physiological function. *European Journal of Lipid Science and Technology*, 113,
507 1427-1432.

508 Molendi-Coste, O., Legry, V. & Leclercq, I.A. (2011). Why and how meet n-3 PUFA dietary
509 recommendations? *Gastroenterology Research and Practice*, doi: 10.1155/2011/364040.

510 Nitsan, Z., Mokady, S., & Sukenik, A. (1999). Enrichment of poultry products with omega-3
511 fatty acids by dietary supplementation with the alga *Nannochloropsis* and mantur oil. *Journal*
512 *of Agricultural and Food Chemistry*, 47(12), 5127-5132.

513 Rizzi, L., Bochicchio, D., Bargellini, A., Parazza, P., & Simioli, M. (2009). Effects of dietary
514 microalgae, other lipid sources, inorganic selenium and iodine on yolk n-3 fatty acid
515 composition, selenium content and quality of eggs in laying hens. *Journal of the Science of*
516 *Food and Agriculture*, 89(10), 1775-1781.

517 Ryckebosch, E., Muylaert, K., & Foubert, I. (2012a). Optimization of an analytical procedure
518 for extraction of lipids from microalgae. *Journal of the American Oil Chemists' Society*, 89,
519 189-198.

520 Ryckebosch, E., Bruneel, C., Muylaert, K., & Foubert, I. (2012b). Microalgae as an
521 alternative source of omega-3 long chain polyunsaturated fatty acids. *Lipid Technology*,
522 24(6), 128-130.

523 Schlatterer, J., & Breithaupt, D. E. (2006). Xanthophylls in commercial egg yolks:
 524 quantification and identification by HPLC and LC-(APCI)MS using a C30 phase. *Journal of*
 525 *Agricultural and Food Chemistry*, 54(6), 2267-2273.

526 Sioen, I., De Henauw, S., Van Camp, J., Volatier, J. L., & Leblanc, J. C. (2009). Comparison
 527 of the nutritional-toxicological conflict related to seafood consumption in different regions
 528 worldwide. *Regulatory Toxicology and Pharmacology*, 55(2), 219-228.

529 Spence, J. D., Jenkins, D. J., & Davignon, J. (2010). Dietary cholesterol and egg yolks: Not
 530 for patients at risk of vascular disease. *Canadian Journal of Cardiology*, 26(9), 336-339.

531 Strand, A., Herstad, O., & Liaaen-Jensen, S. (1998). Fucoxanthin metabolites in egg yolks of
 532 laying hens. *Comparative Biochemistry and Physiology Part A*, 119(4), 963-974.

533 Toivo, J., Lampi, A., Aalto, S., & Piironen, V. (2000). Factors affecting sample preparation in
 534 the gas chromatographic determination of plant sterols in whole wheat flour. *Food Chemistry*,
 535 68, 239-245.

536 Trautwein, E.A. (2001). n-3 Fatty acids – physiological and technical aspects for their use in
 537 food. *European Journal of Lipid Science and Technology*, 103, 45-55.

538 Van Elswyk, M. E. (1997). Comparison of n-3 fatty acid sources in laying hen rations for
 539 improvement of whole egg nutritional quality: a review. *The British Journal of Nutrition*, 78,
 540 S61-69.

541 Van Elswyk, M. E., Schake, L. S., & Hargis, P. S. (1991). Research note: Evaluation of two
 542 extraction methods for the determination of egg yolk cholesterol. *Poultry Science*, 70, 1258-
 543 1260.

544 Wright, S. W., Jeffrey, S. W., Mantoura, R. F. C, Llewellyn, C. A., Bjoernland, T., Repeta,
545 D., & Welschmeyer, N. (1991). Improved HPLC method for the analysis of chlorofylls and
546 carotenoids from marine phytoplankton. *Marine Ecology Progress Series*, 77(2-3), 183-196.

547 Wrolstad, R. E., Acree T. E, Decker, E. A., Penner, M. H., Reid, D. S., Schwartz, S. J.,
548 Shoemaker, C. F., Smith, D. M., & Sporns, P. (2005). *Handbook of Food Analytical*
549 *Chemistry*. Hoboken: John Wiley & Sons, Inc, (Chapter 1).

550

Figure Captions

Figure 1: Level of omega-3 fatty acids ALA (A), EPA (B), DPA (C) and DHA (D) (in mg/egg; mean $\pm$ SD; n = 8) in eggs measured at the end of the supplementation period with four microalgae supplemented in 2 doses (125 mg and 250 mg microalgal ALA+EPA+DHA per 100 g feed, respectively 0.125% and 0.250%). Standard feed for laying hens served as control (dose: 0.000%). Results with the same letter for the same microalga species, in comparison with the control group, are not significant different ($p < 0.05$). Results with the same number for the same dose are not significant different ($p < 0.05$).

Figure 2: Carotenoids in egg yolk [Fucoxanthin derivatives (A-B) – lutein (C) – zeaxanthin (D) – canthaxanthin (E); in $\mu\text{g/egg}$; mean $\pm$ SD; n = 8] obtained at the end of microalgal supplementation period with four microalgae supplemented in 2 doses (125 mg and 250 mg microalgal ALA+EPA+DHA per 100 g feed, respectively 0.125% and 0.250%). Standard feed for laying hens served as control (dose: 0.000%). Results with the same letter for the same microalga species, in comparison with the control group, are not significant different ($p < 0.05$). Results with the same number for the same dose are not significant different ($p < 0.05$).

569 Table 1: Proximate composition of *Phaeodactylum tricornutum*, *Nannochloropsis oculata*,
570 *Isochrysis galbana* and *Chlorella fusca*: the lipid content (in g/100 g DM; mean $\pm$ SD; n = 3),
571 n-3 PUFA content (in g/100 g biomass; mean $\pm$ SD; n = 3), carotenoid content (in μ g/g DM;
572 mean $\pm$ SD; n = 3) and sterol content (in mg/g DM; mean $\pm$ SD; n = 3).

	<i>Phaeodactylum</i>	<i>Nannochloropsis</i>	<i>Isochrysis</i>	<i>Chlorella</i>
Lipid	24.1 $\pm$ 2.8	25.1 $\pm$ 1.5	31.6 $\pm$ 1.4	14.7 $\pm$ 1.5
n-3 PUFA				
ALA	0.048 $\pm$ 0.009	0.022 $\pm$ 0.002	0.156 $\pm$ 0.006	2.750 $\pm$ 0.290
SDA	0.037 $\pm$ 0.043	-	1.135 $\pm$ 0.020	0.264 $\pm$ 0.038
EPA	2.755 $\pm$ 0.026	4.010 $\pm$ 0.178	3.520 $\pm$ 0.072	0.020 $\pm$ 0.028
DHA	0.306 $\pm$ 0.036	-	0.832 $\pm$ 0.018	-
Carotenoids				
β -carotene	135.1 $\pm$ 28.6	581.3 $\pm$ 161.8	106.7 $\pm$ 26.4	-
Fucoxanthin	4382.8 $\pm$ 124.1	-	1464.0 $\pm$ 103.6	-
Lutein	-	-	-	97.7 $\pm$ 5.3
Neoxanthin	-	-	-	39.5 $\pm$ 1.3
Violaxanthin	-	2286.8 $\pm$ 418.0	-	21.6 $\pm$ 3.5
Sterols				
Cholesterol	-	2.59 $\pm$ 0.05	1.08 $\pm$ 0.09	-
Phytosterols	2.58 $\pm$ 0.07	1.10 $\pm$ 0.04	13.50 $\pm$ 0.79	4.12 $\pm$ 0.22

573

574

575 Table 2: Egg quality parameters at the end of microalgal supplementation period: egg weight, shell weight, albumen weight, yolk weight (all
576 expressed in g; mean $\pm$ SD; n = 8), shell thickness (in mm; mean $\pm$ SD; n = 8) and shell strength (in N/m; mean $\pm$ SD; n = 8).

	Egg weight	Shell weight	Albumen weight	Yolk weight	Shell quality	
					Shell thickness	Shell strength
Control	59 $\pm$ 1 ^a	9 $\pm$ 1 ^a	31 $\pm$ 4 ^a	14 $\pm$ 1 ^a	0.51 $\pm$ 0.07 ^a	129 $\pm$ 45 ^a
<i>Phaeodactylum</i>						
0.125%	61 $\pm$ 1 ^a	9 $\pm$ 1 ^a	35 $\pm$ 3 ^a	15 $\pm$ 1 ^a	0.53 $\pm$ 0.07 ^a	135 $\pm$ 15 ^a
0.250%	59 $\pm$ 1 ^a	10 $\pm$ 2 ^a	33 $\pm$ 2 ^a	14 $\pm$ 1 ^a	0.51 $\pm$ 0.06 ^a	131 $\pm$ 24 ^a
<i>Nannochloropsis</i>						
0.125%	60 $\pm$ 1 ^a	9 $\pm$ 1 ^a	35 $\pm$ 3 ^a	15 $\pm$ 1 ^a	0.56 $\pm$ 0.06 ^a	137 $\pm$ 27 ^a
0.250%	61 $\pm$ 1 ^a	9 $\pm$ 1 ^a	34 $\pm$ 2 ^a	15 $\pm$ 1 ^a	0.53 $\pm$ 0.05 ^a	122 $\pm$ 8 ^a
<i>Isochrysis</i>						
0.125%	60 $\pm$ 1 ^a	9 $\pm$ 1 ^a	31 $\pm$ 5 ^a	15 $\pm$ 2 ^a	0.49 $\pm$ 0.07 ^a	123 $\pm$ 28 ^a
0.250%	60 $\pm$ 1 ^a	9 $\pm$ 2 ^a	32 $\pm$ 5 ^a	15 $\pm$ 1 ^a	0.52 $\pm$ 0.06 ^a	120 $\pm$ 29 ^a
<i>Chlorella</i>						
0.125%	61 $\pm$ 1 ^a	10 $\pm$ 1 ^a	35 $\pm$ 2 ^a	15 $\pm$ 1 ^a	0.55 $\pm$ 0.05 ^a	134 $\pm$ 17 ^a
0.250%	61 $\pm$ 2 ^a	10 $\pm$ 1 ^a	33 $\pm$ 4 ^a	16 $\pm$ 1 ^a	0.52 $\pm$ 0.08 ^a	128 $\pm$ 26 ^a

577 ^aResults with the same letter in the same column are not significant different (p<0.05).

Table 3: The supplemented n-3 PUFA (in mg/100 g feed), the enrichment of n-3 LC-PUFA (in mg/egg; mean $\pm$ SD; n = 8) and the conversion efficiency of n-3 LC-PUFA (in %; mean $\pm$ SD) for the four microalgae supplemented in 2 doses (125 mg and 250 mg microalgal ALA+EPA+DHA per 100 g feed, respectively 0.125% and 0.250%).

	Supplemented n-3 PUFA	Enrichment of n-3 LC-PUFA	Conversion efficiency
<i>Phaeodactylum</i>			
0.125%	127	$62 \pm 9^{a,1}$	$43 \pm 6^{a,1}$
0.250%	253	$85 \pm 26^{b,1}$	$29 \pm 9^{b,1}$
<i>Nannochloropsis</i>			
0.125%	125	$33 \pm 10^{a,2}$	$23 \pm 7^{a,2}$
0.250%	250	$56 \pm 19^{b,2}$	$19 \pm 6^{a,23}$
<i>Isochrysis</i>			
0.125%	153	$75 \pm 16^{a,3}$	$41 \pm 9^{a,1}$
0.250%	307	$81 \pm 16^{a,1}$	$23 \pm 5^{b,13}$
<i>Chlorella</i>			
0.125%	136	$19 \pm 10^{a,4}$	$12 \pm 6^{a,3}$
0.250%	272	$31 \pm 9^{b,3}$	$10 \pm 3^{a,4}$

^aResults with the same letter for the same microalga species are not significant different (p<0.05).

^bResults with the same number for the same dose are not significant different (p<0.05).

Table 4: Color values (Roche and CIELAB-value; mean $\pm$ SD; n = 8) of the egg yolk measured at the end of the supplementation period with four microalgae supplemented in 2 doses (125 mg and 250 mg microalgal ALA+EPA+DHA per 100 g feed, respectively 0.125% and 0.250%).

Treatment group	Roche	CIELAB-value		
		L^*	a^*	b^*
Control	12 ± 0^a	66 ± 4^a	12 ± 1^a	54 ± 3^{ad}
<i>Phaeodactylum</i>				
0.125%	$14 \pm 1^{b,1}$	$58 \pm 5^{b,1}$	$19 \pm 1^{b,1}$	$47 \pm 3^{b,1}$
0.250%	$> 15^{c,1}$	$48 \pm 7^{c,1}$	$25 \pm 4^{c,1}$	$36 \pm 8^{c,1}$
<i>Nannochloropsis</i>				
0.125%	$13 \pm 1^{ab,2}$	$66 \pm 3^{a,2}$	$14 \pm 3^{ab,2}$	$51 \pm 3^{a,13}$
0.250%	$13 \pm 1^{b,2}$	$65 \pm 4^{a,2}$	$16 \pm 3^{b,2}$	$51 \pm 3^{a,2}$
<i>Isochrysis</i>				
0.125%	$13 \pm 1^{a,2}$	$65 \pm 3^{a,2}$	$15 \pm 1^{a,2}$	$58 \pm 3^{a,2}$
0.250%	$14 \pm 1^{b,2}$	$58 \pm 4^{b,3}$	$19 \pm 4^{b,3}$	$52 \pm 7^{bd,2}$
<i>Chlorella</i>				
0.125%	$11 \pm 1^{a,3}$	$68 \pm 2^{a,2}$	$9 \pm 2^{ab,3}$	$53 \pm 2^{a,23}$
0.250%	$12 \pm 1^{a,3}$	$69 \pm 2^{a,2}$	$8 \pm 1^{b,4}$	$52 \pm 4^{a,2}$

^aResults with the same letter for the same microalga species, in comparison with the control group, are not significant different (p<0.05).

^aResults with the same number for the same dose are not significant different (p<0.05).

^b L^* = lightness, a^* = red-greenness, and b^* = yellow-blueness.