Impact of microalgal feed supplementation on omega-3 fatty acid enrichment of hen eggs

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ABSTRACT

In many Western countries, the average intake of the health beneficial omega-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is below the recommended level, raising interest in food enrichment with n-3 LC-PUFA. To that end, the impact of feed supplementation with EPA rich autotrophic microalgal biomass on n-3 LC-PUFA enrichment of eggs was studied. Hens were divided in three groups receiving different diets for 28 days: a standard diet (C) for laying hens, (C) supplemented with 5.0% spray dried *Nannochloropsis gaditana*, and (C) to which 10.0% of these microalgae were added. Microalgal EPA was hardly accumulated in yolk lipids, but preferentially converted to DHA and deposited in yolk phospholipids. The efficiency of deposition of microalgal n-3 LC-PUFA to eggs was rather low. Switching back to standard feed ensured that the n-3 LC-PUFA level obtained in enriched eggs decreased back to that of the control eggs. Moreover, the colour of egg yolk shifted from yellow to more orange-red, which is presumably due to transfer of microalgal carotenoids to egg yolk. Thus, the use of autotrophic microalgae as supplement for standard feed offers an alternative to current sources for the production of DHA enriched eggs.

KEYWORDS

Omega-3 polyunsaturated fatty acids

Microalgae

Nannochloropsis

Eggs

Enrichment

Efficiency of deposition of n-3 LC-PUFA

1. Introduction

It is generally accepted that omega-3 polyunsaturated fatty acids (n-3 PUFA) have potential in the prevention and treatment of several diseases aside from their important role in neuronal development (Gogus & Smith, 2010; Jordan, 2010; Yashodhara et al., 2009). The health benefits are mainly ascribed to the long chain (LC) n-3 PUFA eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), rather than to the shorter chain n-3 PUFA α -linolenic acid (ALA, 18:3 n-3). The conversion of ALA to EPA and further to DHA is very limited and inefficient in the human body (Komprda, 2012), and especially in infants and elderly people (Lagarde, 2008; Nitsan, Mokady, & Sukenik, 1999; Trautwein, 2001). Various governments and health organizations recommend an average dietary intake of EPA plus DHA ranging from 140 to 600 mg per day (Komprda, 2012; Molendi-Coste, Legry, & Leclercq, 2011). Unfortunately, in many Western countries, the recommended dietary intake of n-3 LC-PUFA is rarely met by the majority of consumers (Sioen et al., 2006; Welch, Shakya-Shrestha, Lentjes, Wareham, & Khaw, 2010). To that end, there is clearly a need for direct intake of EPA/DHA in the human diet, raising interest in food enrichment with these n-3 LC-PUFA. Hence, their inclusion into foods and specialty products has been in the forefront of research and development (Shahidi, 2009).

Eggs are an integral part of our diet either as food but also as food ingredient in many applications. Hence, eggs form an interesting food product to enrich with n-3 LC-PUFA. Moreover, it has been shown that the level and type of PUFA in eggs can be modified through dietary supplementation with n-3 PUFA (Baucells, Crespo, Barroeta, Lopez-Ferrer, & Grashorn, 2000; Cachaldora, Garcia-Rebollar, Alvarez, De Blas, Méndez, 2008). Different sources of n-3 PUFA can be used to enrich eggs. For the interested reader we refer to the review of Fraeye et al. (2012). Briefly, when hens' diet is supplemented with a traditional ALA rich plant source, such as flaxseed, eggs are mainly enriched with ALA, and to a lesser extent with n-3 LC-PUFA. The n-3 LC-PUFA levels in such 'ALA-enriched' eggs are generally below 100 mg/egg (Aymond & Van Elswyk, 1995; Bean & Leeson, 2003; Fraeye et al., 2012). However, when hens are fed fish oil (rich in EPA/DHA), egg yolk lipids are mainly enriched with DHA, whereas EPA is rarely detected and seems to be converted to DHA before it is deposited. In such 'DHA-enriched' eggs, the level of yolk DHA is generally below 100 mg/egg (Fraeye et al., 2012; Gonzalez-Esquerra & Leeson, 2000; Lawlor, Gaudette, Dickson, & House, 2010), which is rather low since similar DHA levels, *i.e.* maximum 100 mg DHA/egg, can be obtained when hens are fed with flaxseed. These rather low DHA levels obtained when hens are fed fish oil can be explained by the fact that the dosage of fish oil for feed supplementation needs to be restricted since n-3 LC-PUFA in fish oil are highly susceptible to oxidation and the formed oxidation products cause undesirable off-flavours in the enriched eggs (Gonzalez-Esquerra & Leeson, 2000, 2001).

During the last 15 years, a number of research groups have investigated the possibility of feeding laying hens microalgae, since they are the primary natural producers of n-3 LC-PUFA. Khozin-Goldberg, Iskandarov, & Cohen (2011) and Ryckebosch, Bruneel, Muylaert, & Foubert (2012) reported an overview of LC-PUFA occurrence in microalgae species from different classes. Most reports about enrichment of eggs using microalgae as feed supplement deal with DHA rich heterotrophic microalgae which use organic compounds as a primary source of nutrition. The PUFA profile of eggs from hens fed those algae is very similar to that of eggs from hens fed fish oil (Cachaldora et al., 2008; Gonzalez-Esquerria & Leeson, 2001; Herber & Van Elswyk, 1996). However, DHA levels up to 200 mg per egg are reported by Herbert-McNeill and Van Elswyk (1998). Next to heterotrophic microalgae, also autotrophic microalgae, which are able to use CO₂ to produce organic compounds with the aid of sunlight (Nuño et al. 2012), can be used to enrich eggs with n-3 LC-PUFA. However, to the best of our knowledge, only two studies were already published in which autotrophic microalgae, i.e. *Nannochloropsis*, are used as a feed supplement for laying hens to enrich their eggs with n-3 LC-PUFA (Fredriksson, Elwinger, & Pickova, 2006; Nitsan et al., 1999). *Nannochloropsis* has an interesting fatty acid profile since it contains only EPA as n-3 LC-PUFA and relatively low levels of n-6 LC-PUFA (Khozin-Goldberg et al., 2011). When hens were fed *Nannochloropsis*, microalgal EPA was not accumulated in egg yolk, but apparently converted and deposited in egg yolk as DHA (Fredriksson et al., 2006; Nitsan et al., 1999). However, it should be mentioned that the experimental standard feed of both studies was already rich in ALA to increase the n-3/n-6 ratio of the diet and, hence, to stimulate the conversion steps of the n-3 pathway. Furthermore, autotrophic microalgae contain, next to n-3 LC-PUFA, also other nutritionally interesting components, such as carotenoids. They can act as antioxidants to preserve the relatively unstable PUFA and thus increase lipid stability (Pangestuti & Kim, 2011). However, they also have an impact on yolk colour which can shift from yellow to more orange-red (Fraeye et al., 2012; Fredriksson et al., 2006; Nitsan et al., 1999).

From the above summary, it is clear that very little research has been done about the impact of autotrophic microalgae to enrich eggs with n-3 LC-PUFA. Nevertheless, these sustainable autotrophic microalgae are an interesting alternative source to study further in this field of research. However, it is important to determine the impact of EPA supplementation by adding *Nannochloropsis* to a commercially available standard feed for laying hens instead of adding it to an ALA-enriched feed. Furthermore, since the price of autotrophic microalgae is currently still high, the efficiency of deposition of microalgal n-3 LC-PUFA in egg yolk needs to be calculated. Furthermore, it has not yet been studied to what extent n-3 LC-PUFA are still deposited in eggs after returning the hens to a standard feed, and there is also little known about the egg quality characteristics and zootechnical performances of hens fed autotrophic microalgae. Thus, the aim of this study was to investigate the impact of the EPA rich autotrophic microalga *Nannochloropsis gaditana* as a supplement to a commercially available standard diet on n-3 LC-PUFA enrichment of egg yolk. The efficiency of deposition of microalgal n-3 LC-PUFA in egg yolk was, to the best of our knowledge, for the

68 first time calculated for autotrophic microalgae. Moreover, the impact of returning to the basal feed,
69 devoid of *N. gaditana*, on the level of n-3 LC-PUFA in eggs was assessed.

70 **2. Materials and methods**

71 All used chemicals and reagents were at least of analytical grade and purchased from Sigma-Aldrich
72 (Bornem, Belgium), unless specified otherwise.

73 *2.1. Microalgal biomass*

74 Spray dried biomass from the autotrophic cultured *Nannochloropsis gaditana* was obtained from Clean
75 Algae (Las Palmas de Gran Canaria, Spain). The composition of the microalgal biomass - as given by the
76 supplier - is shown in **Table 1**. The total lipid content and the fatty acid profile of the microalgal biomass
77 were determined in our laboratory by a modified method of Folch, Lees, & Sloane Stanley (1957) which is
78 described in section 2.5. The results are added to **Table 1**.

79 *2.2. Animals and diet formulation*

80 Twelve laying hens (ISA Brown), 25 weeks of age at the start of the experiment, were individually housed in
81 cages (width: 50 cm, height: 40 cm, depth: 44 cm). The hens received 14 h light per day throughout the
82 experiment and the room temperature was controlled at 20 °C. Feed and water were supplied *ad libitum*.
83 The overall experimental period lasted 56 days: adaptation period (14 days), supplementation period with
84 microalgal biomass (28 days) and wash-out period (14 days).

85 During the first two weeks, *i.e.* the adaptation period, the hens could adapt to the new environment and to
86 the standard diet, which was a commercially available wheat-corn-soybean based diet (legkorrel Total 77,
87 AVEVE, Wilsele, Belgium) typical for laying hens. The chemical composition of the standard feed - as given
88 by the supplier - is shown in **Table 1**. Total lipid content and fatty acid profile of the standard feed were
89 determined in our laboratory according to the modified method of Folch et al. (1957) which is described in
90 section 2.5 (**Table 1**). It is important to mention that almost no n-3 PUFA were present in the standard feed.
91 After the adaptation period, the hens were randomly assigned to receive one of the three dietary
92 treatments: (1) the commercially available standard diet served as the control diet (n = 4 hens per
93 treatment), (2) the control diet in which 5.0% of the standard feed was replaced with 5.0% *N. gaditana* (n =
94 3 hens per treatment), and (3) the control diet in which 10.0% of the standard feed was replaced with
95 10.0% *N. gaditana* (n = 4 hens per treatment). It should be stressed that one hen from the 5.0% treatment
96 group stopped laying eggs at the beginning of the supplementation period with microalgae. Hence, this hen
97 was eliminated from the experiment and therefore the 5.0% treatment group had only three hens instead
98 of four. Furthermore, all hens were fed the diets for 28 days, and after this supplementation period all hens
99 again received the control diet for 14 days (*i.e.* wash-out period).

100 2.3. Zootechnical performance of laying hens

101 Feed intake, egg production and egg weight were recorded daily during the microalgal supplementation
102 and wash-out period. The body weight was measured at the start and at the end of the supplementation
103 period (day 0 and day 28, respectively).

104 2.4. Sampling and storage of eggs

105 For each dietary treatment, eggs were collected at day 0 (start of microalgal supplementation period), day
106 14 (halfway microalgal supplementation period), day 28 (end of microalgal supplementation period), and
107 day 42 (end of wash-out period). All collected eggs were stored at minus 20 °C until analysis. Lipids were
108 extracted, their fatty acid profile and lipid composition were determined, and the yolk colour of all
109 collected eggs was measured, as described in section 2.5.

110 2.5. Analytical methods

111 2.5.1. Extraction of lipids - total lipid content

112 Total lipids were extracted in triplicate from egg yolk, microalgal biomass or standard feed. A sample of yolk
113 (200 – 250 mg), biomass (300 – 400 mg) or feed (150 – 200 mg) was vortex mixed with methanol (4.5 mL)
114 and subsequently with chloroform (9.0 mL) (Boom, Meppel, The Netherlands). An internal standard [5.0 mg
115 arachidic acid C20:0 (Nu-check, Minnesota, USA) in 0.4 mL chloroform] was used to allow quantification of
116 the level of n-3 PUFA as percentage of egg, microalgal biomass or feed, respectively. The mixture was
117 homogenised (90 sec, 800 rpm) using a CAT X620 homogeniser (VWR International, Leuven, Belgium) and
118 centrifuged (20 min, 2800 g). The supernatant was removed and the remaining lipids in the residue were
119 re-extracted with chloroform/methanol (2:1, v/v; 12.0 mL). After homogenisation (60 sec, 8000 rpm) and
120 centrifugation (20 min, 2800 g), the supernatant was removed and combined with the first obtained
121 supernatant. These combined solvent layers were washed with KCl (0.88%, 6.2 mL, VWR) to remove all non-
122 lipids. After centrifugation (10 min, 2800 g), the bottom layer was filtered (Whatman filter papers, grade 1).
123 Total lipids were quantified gravimetrically after drying (under nitrogen stream at 40 °C) the obtained
124 extract. Total lipids were redissolved in chloroform/methanol (19:1, v/v; 2.0 mL), further referred as total
125 lipid extract. Half of the total lipid extract (1.0 mL) was used to determine the *fatty acid profile* (method
126 described in section 2.5.2), while the other half (1.0 mL) was used to determine *lipid composition* (method
127 described in section 2.5.3).

128 2.5.2. Fatty acid profile

129 To determine the *fatty acid profile*, half of total lipid extract obtained in section 2.5.1 (1.0 mL) was dried
130 (under nitrogen stream at 40 °C) and redissolved in toluene (1.0 mL, Boom) and sulphuric acid (1.0%) in
131 methanol (2.0 mL). The mixture was stored overnight in a sealed tube at 50 °C. Afterwards, sodium chloride

132 (5.0%, 5.0 mL) was added, and the formed fatty acid methyl esters (FAME) were extracted with hexane
133 (10.0 mL). The obtained FAMEs were separated on a EC-WAX column (length: 30 m, internal diameter: 0.32
134 mm, film: 0.25 μ m) (Grace Davison Discovery Sciences, Lokeren, Belgium) using a gas chromatograph
135 (Thermo Scientific Trace GC Ultra, Interscience, Louvain-la-Neuve, Belgium) containing a cold on-column
136 injection port and a flame ionization detector (FID). The following time-temperature program was applied:
137 50 °C – 180 °C (20 °C/min) and 180 °C – 235 °C (5 °C/min). Fatty acid identification was performed by
138 comparing the retention times with these of standards (Nu-check, Minnesota, USA) containing 35 different
139 FAMEs. Peak areas were quantified with Chromcard for Windows software (Interscience).

140 2.5.3. Lipid composition

141 Fractionation of total lipids was performed by solid-phase extraction (SPE) on a silica packed cartridge
142 column (500 mg – 6 mL, Grace Davison Discovery Sciences, Lokeren, Belgium) according to Christie (2003).
143 The silica column was conditioned with chloroform before use (6 mL). Half of total lipid extract obtained in
144 2.5.1, dried (under nitrogen stream at 40 °C) and redissolved in 200 μ L chloroform, was loaded onto the
145 column. Solvents with increasing polarity were used to separate total lipids in neutral lipid (NL) fraction,
146 glycolipid (GL) fraction and phospholipid (PL) fraction. The NL fraction was eluted with chloroform (10 mL),
147 followed by elution of the GL fraction using acetone (10 mL, Boom), and finally the PL fraction was eluted
148 with methanol (10 mL). Each collected fraction was dried under a stream of nitrogen at 40 °C, and the
149 amount of lipids per lipid class was quantified gravimetrically. Finally, the fatty acid profile of each obtained
150 fraction was determined using GC according to the method described in section 2.5.2.

151 2.6. Colour determination

152 Two different methods were used to determine the colour of egg yolk. First, the yolk colour was scored
153 visually by comparison with a Roche yolk colour fan (DSM, Basel, Switzerland). Secondly, a colorimeter (CR
154 300, Konica Minolta, Zaventem, Belgium) was used to determine the colour of egg yolk. The colour was
155 measured in a CIE 1976 L^* , a^* , b^* colour space. L^* is a measure for the brightness from black (0) to white
156 (100). The a^* value describes red-green colour with positive a^* values indicating redness and negative a^*
157 values indicating greenness. The b^* value describes yellow-blue colour with positive b^* values indicating
158 yellowness and negative b^* values indicating blueness.

159 2.7. Statistical analysis

160 The impact of dietary treatments was evaluated using Two Way Repeated Measures (ANOVA) and a post
161 hoc Tukey's test with $\alpha = 0.05$ (Sigmaplot 11, Systat Software Inc., Illinois, USA).

162 3. Results

163 3.1. Yolk lipid composition

164 The level of the n-3 PUFA ALA, EPA and DHA in egg yolk at the start (day 0), halfway (day 14), at the end
165 (day 28) of the microalgal supplementation period, and at the end of the wash-out period (day 42) are
166 shown in **Table 2**.

167 Control eggs contained on average 17.4 ($\pm$ 0.8) mg ALA, no EPA, and 26.5 ($\pm$ 6.8) mg DHA per egg at the start
168 of the experiment. The levels remained constant during 28 days of feeding standard diet. From **Table 3** it is
169 clear that ALA and DHA were present in all lipid classes, whereas EPA was not detected in any of the lipid
170 classes. When hens were fed 5.0% *N. gaditana*, the level of ALA remained constant with respect to control
171 eggs, whereas the level of EPA and DHA increased to respectively 2.1 ($\pm$ 0.3) mg and 42.9 ($\pm$ 5.6) mg per egg
172 after 14 days feeding. Furthermore, when hens were fed 10.0% *N. gaditana* for 14 days, EPA and DHA levels
173 further increased to respectively 3.3 ($\pm$ 0.8) mg and 48.7 ($\pm$ 5.7) mg per egg (**Table 4**). However, it seems
174 that a doubling of the microalgal dose from 5.0% to 10.0% did not double the amount of n-3 LC-PUFA in egg
175 yolk. The presence of EPA in eggs when hens were fed *N. gaditana* seems to be only in the PL fraction of
176 yolk lipids, while the increased DHA levels were mostly detected in the PL fraction and, to a lesser extent, in
177 the neutral lipid fraction (**Table 3**).

178 Feeding hens longer than 14 days with *N. gaditana* did not further increase EPA and DHA levels, except for
179 the EPA level of the 10.0% treatment group which slightly increased from 3.3 ($\pm$ 0.8) mg to 4.7 ($\pm$ 0.8) mg
180 per egg (**Table 4**). Hence, it seems that egg enrichment with omega-3 fatty acids increased during the first
181 14 days, reached a plateau around the 14th day, or earlier, and remained constant during further
182 supplementation.

183 When hens again received the standard diet for 14 days (wash-out period), the levels of EPA and DHA were
184 not significantly different between the three treatment groups at day 42. However, the DHA level remained
185 significantly higher for all treatment groups after the wash-out period compared to the level measured in
186 control eggs at day 0 (**Table 4**).

187 In conclusion, it can be said that microalgal EPA is hardly accumulated in egg yolk as was also observed by
188 Nitsan et al. (1999) and Fredriksson et al. (2006). It is largely converted and deposited as DHA in the
189 phospholipid, and to a lesser extent in the neutral lipid fraction of egg yolk. When hens' feed was
190 supplemented with microalgae, we were able to double the DHA level in egg yolk compared to control eggs
191 from hens fed a standard feed.

192 3.2. Egg yolk colour

193 Yolk colour values at the start (day 0), halfway (day 14), and at the end (day 28) of the microalgal
194 supplementation period, and at the end of the wash-out period (day 42) are shown in **Table 4**. After 14

195 days feeding hens *N. gaditana*, the Roche value and a^* values (measure for redness) of egg yolk increased
196 significantly with increasing dosage of *N. gaditana*, whereas the L^* value (measure for brightness)
197 significantly decreased and the b^* value (measure for yellowness) was unaffected. However,
198 supplementing the feed for longer than 14 days, *i.e.* 28 days, had no further significant impact on the colour
199 scores between the different treatment groups. As for the n-3 LC-PUFA enrichment in eggs, also the
200 intensity of the yolk colour increased during the beginning of supplementation, reached a plateau around
201 the 14th day (or earlier), and remained constant during further feeding. When all hens again received the
202 standard feed for two weeks (wash-out period), the yolk colour intensity decreased for all groups to that
203 measured at the start of the experiment.

204 3.3. Zootechnical performance of laying hens

205 The performance parameters during the microalgal supplementation period of 28 days are summarized in
206 **Table 5**. It should be stressed that these results are only an indication since an experiment with more hens
207 is required to study the impact of microalgae on performances of laying hens. The average daily feed intake
208 (g/day), egg weight (g) and egg production (egg/day) during the microalgal supplementation period of 28
209 days were not significantly affected, even not as function of time, by feeding hens *N. gaditana*. While not
210 reaching the level of significance, a trend towards lower feed intake can be observed for hens fed 10.0% *N.*
211 *gaditana*. This can be ascribed to a lower feed intake during the supplementation period by two of four
212 hens. Furthermore, the body weight of the hens measured at the start (day 0) and at the end (day 28) of
213 the microalgal supplementation period was not significantly different among the three dietary treatment
214 groups, not even as a function of time regardless of the type of diet.

215 4. Discussion

216 Various n-3 PUFA sources, such as flaxseed and fish oil, are used to enrich poultry products with these
217 health beneficial fatty acids. Since microalgae are the primary natural plant producers of n-3 LC-PUFA, the
218 autotrophic microalga *N. gaditana* was used in this study as an alternative source to enrich eggs. This
219 microalga species contains approximately 19% lipids and has an interesting fatty acid profile. It comprises a
220 single n-3 LC-PUFA, *i.e.* EPA (7 g per 100 g microalgal lipids, or 1.5 g per 100 g microalgal biomass), no DHA,
221 and relatively low levels of n-6 PUFA (**Table 1**).

222 Feeding hens EPA rich *N. gaditana* affect the fatty acid profile of egg yolk. Microalgal EPA was hardly
223 deposited in egg yolk, but largely converted to DHA and accumulated predominantly in egg phospholipids
224 (**Tables 2 and 3**). Thus, DHA, rather than EPA, was preferentially incorporated into egg lipids, as was also
225 observed by Nitsan et al. (1999) and Fredriksson et al. (2006). However, as already mentioned, they used an
226 ALA-rich diet to which *Nannochloropsis* was added as an EPA supplement. This way, the n-3/n-6 ratio of the
227 diet increased, and, hence, the conversion steps of the n-3 pathway have been stimulated. When

228 comparing the results, it seems that a slightly higher DHA enrichment in eggs can be obtained if an ALA-rich
229 diet was used for feed supplementation of n-3 LC-PUFA from autotrophic microalgae.

230 Furthermore, after feeding hens 5.0% *N. gaditana* for 28 days, eggs contained 2.3 ($\pm$ 0.6) mg EPA and 44.9
231 ($\pm$ 6.6) mg DHA per egg, while doubling the concentration of algae, *i.e.* supplementation with 10.0% *N.*
232 *gaditana*, only resulted in a slightly further increase to 4.7 ($\pm$ 0.8) mg EPA and 50.4 ($\pm$ 4.6) mg DHA per egg.
233 It was earlier reported that the retention rate for n-3 LC-PUFA is reduced with increasing dietary
234 concentration (Cachaldora et al., 2008; Herber-McNeill & Van Elswyk, 1998). In addition, efficiency of
235 deposition of n-3 LC-PUFA in yolk can be determined as the proportion of total mg n-3 LC-PUFA (*i.e.* EPA +
236 DHA in our study) enriched in eggs divided by the total mg n-3 LC-PUFA (*i.e.* EPA in our study) ingested by
237 autotrophic microalgal supplementation. For instance, when hens were fed 85 mg microalgal EPA per day
238 (supplementation of 5.0% *N. gaditana*), taking into account their daily feed intake of 114 g per day (**Table**
239 **5**), the yolk level of n-3 LC-PUFA increased after 28 days from 0 to 2.3 mg EPA and from 26.5 to 44.9 mg
240 DHA. Thus, eggs were enriched with 21 mg n-3 LC-PUFA on average. This means that the efficiency of
241 deposition was rather low, *i.e.* 25% or 20% when 5.0% or 10.0% *N. gaditana* was supplemented,
242 respectively. To the best of our knowledge, this is the first study that reports calculations for the efficiency
243 of deposition of n-3 LC-PUFA from autotrophic microalgae to eggs. The rather low efficiencies of deposition
244 can presumably be explained by the rather low digestibility of the cell wall since *Nannochloropsis* produces
245 an aliphatic, non-hydrolysable biopolymer, algaenan, which can prevent enzymatic degradation of the cell
246 wall (Gelin et al., 1999).

247 Moreover, 14 days microalgal feed supplementation appeared to be sufficient to reach a plateau for
248 incorporation of n-3 LC-PUFA in egg yolk since the results were not significant different with those obtained
249 at day 28 (**Table 4**). Similar results were reported by Herber and Van Elswyk (1996) when different diets,
250 based on heterotrophic microalgae or menhaden oil containing EPA (233 mg per day) whether or not in
251 combination with different levels of DHA (ranging from 155 mg to 400 mg per day), were fed to hens for 28
252 days. Furthermore, Nitsan et al. (1999) observed that 8 days feeding with 1% lipids from *Nannochloropsis*
253 was enough to reach maximum incorporation of n-3 LC-PUFA.

254 It is important to remark that the European Food Safety Authority (EFSA) has approved two nutrition claims
255 and three health claims concerning omega-3 LC-PUFA in food products (European Commission 2010, 2012),
256 with the exception of the claims concerning children's health. The claims may be used only for food which
257 contains a certain amount of EPA and/or DHA, depending on the claim, expressed per 100 g product and
258 per 100 kcal. If we take into account the average egg weights (**Table 5**) and that one egg contains 82 kcal,
259 the enriched eggs from hens fed *N. gaditana* contain between 79.7 mg and 97.5 mg EPA plus DHA per 100 g
260 egg (expressed per 100 kcal: between 54.9 mg and 67.2 mg EPA plus DHA), or contain between 75.9 mg and
261 89.2 mg DHA per 100 g egg (expressed per 100 kcal: between 52.3 mg and 61.5 mg DHA) depending on the

262 used levels of *N. gaditana* (5 or 10%) and the used supplementation period (14 and 28 days). Since all
263 enriched eggs contain at least 40 mg EPA+DHA per 100 kcal and per 100 g product, they meet the nutrition
264 claim 'source of omega-3 fatty acids' and the health claim 'EPA and DHA contribute to the normal function
265 of the heart' (European Commission, 2010, 2012). Moreover, the enriched eggs also meet the health claims
266 'DHA contributes to maintenance of normal brain function' and 'DHA contributes to maintenance of normal
267 vision', since the enriched eggs contain at least 40 mg DHA per 100 kcal and per 100 g product (European
268 Commission, 2012). For the health claims, it is important to inform the consumer that the health beneficial
269 effect is obtained with a daily intake of 250 mg EPA and/or DHA, depending on the claim used. However,
270 before such enriched eggs with algal n-3 LC-PUFA could be commercialized and may be labeled with
271 nutrition and health claims, more research is necessary to optimize the process of feeding hens autotrophic
272 microalgae to enrich their eggs with n-3 LC-PUFA.

273 When all hens had returned to the standard diet for 14 days, the n-3 LC-PUFA enrichment was offset and
274 decreased to the level measured in control eggs at day 42. However, it seems that the level of DHA
275 measured in control eggs at the end of the experiment was higher (on average: 34.9 mg/egg at day 42) than
276 the level measured at the start of the experiment (on average: 26 mg/egg at day 0) (**Table 2**). According to
277 Scheideler, Jaroni, & Froning (1998) and Fredriksson et al. (2006), older hens have a larger liver and thereby
278 can more effectively elongate ALA, present in standard feed, into DHA.

279 Next to the enrichment of eggs with n-3 LC-PUFA, also the colour intensity of egg yolk increased and shifted
280 from yellow to more orange-red when increased levels of microalgal biomass were supplemented to hens'
281 feed (**Table 4**). Similar results were reported by Nitsan et al. (1999) and Fredriksson et al. (2006) who also
282 used *Nannochloropsis* as microalgal feed supplement. They explained the colour shift by transfer of
283 microalgal carotenoids to egg yolk (Fredriksson et al., 2006; Ginzberg et al., 2000; Gouveia et al., 1996;
284 Nitsan et al., 1999). *Nannochloropsis sp.* contains a significant level of carotenoids such as violaxanthin,
285 zeaxanthin and β -carotene (Jeffrey, Mantoura, & Wright, 1997; Khozin-Goldberg et al., 2011). However, it
286 seems that not every carotenoid, such as β -carotene, is deposited in egg yolk (Herber-McNeill & Van
287 Elswyk, 1998; Khozin-Goldberg et al., 2011). Thus, feeding hens *N. gaditana* had an additional enhanced
288 nutritional value above current n-3 LC-PUFA sources since part of microalgal carotenoids are deposited in
289 egg yolk. Moreover, carotenoids act as antioxidants and are able to protect lipids against oxidation
290 (Pangestuti & Kim, 2011).

291 Furthermore, addition of 5.0% or 10.0% *N. gaditana* to hens' feed had no significant impact on hens body
292 weight, feed intake, egg production or egg weight (**Table 5**). This indicates that the microalgal biomass
293 seems to be well tolerated by the laying hens. However, more research is needed to confirm this
294 hypothesis.

295 **5. Conclusions**

296 EPA rich microalgal biomass of *N. gaditana* was used to enrich eggs with n-3 LC-PUFA. Microalgal EPA was
297 hardly accumulated in yolk lipids, but largely converted to DHA before being preferentially deposited in
298 yolk phospholipids. However, the efficiency to deposit n-3 LC-PUFA from *N. gaditana* to eggs was rather
299 low. In addition, the yolk colour of these DHA enriched eggs shifted from yellow to more orange-red,
300 presumably due to transfer of algal carotenoids to egg yolk. To that end, EPA rich *N. gaditana* can be used
301 as an alternative to current sources of n-3 LC-PUFA for the production of DHA enriched eggs. However,
302 further research is needed to determine the potentially interested autotrophic microalgae to enrich eggs,
303 and the added value of autotrophic microalgae over that of the current sources.

304

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308

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397 *Journal*, 85, 84-90.

400 **TABLES**

401 **Table 1:** Composition of spray dried microalgal biomass of *Nannochloropsis gaditana* (Clean Algae, Las
402 Palmas de Gran Canaria, Spain) (% dry base, db) and the standard diet (legkorrel Total 77, AVEVE, Wilsele,
403 Belgium) based on wheat, corn, soybean and palmoil (% wet base, unless specified otherwise)

	<i>N. gaditana</i>	Standard diet
Metabolisable Energy (MJ/kg)		10.8
Moisture		11.0
Crude protein	28.0	15.5
Lysine		0.67
Methionine		0.29
Crude fat ¹	18.8	4.2
α -linolenic acid (ALA) ¹ (% db)	0.02	0.08
Eicosapentaenoic acid (EPA) ¹ (% db)	1.5	0.004
Crude ash	24.0	11.7
Calcium		3.5
Phosphorus		0.57
Sodium		0.15
Carbohydrates	32.0	
Crude fiber		5.4
Starch		37.5
Sugar		3.5

404 ¹Determined values according to a modified method of Folch et al. (1957).

405

Table 2: Level of omega-3 fatty acids ALA, EPA and DHA (expressed as mg/egg) in eggs collected at the start (day 0), halfway (day 14), at the end (day 28) of the microalgal supplementation period, and at the end of the wash-out period (day 42) from hens fed graded levels of *Nannochloropsis gaditana* (0% *N. gaditana* = control, 5.0% *N. gaditana*, and 10.0% *N. gaditana*)

Day	Group	ALA (mg/egg)	EPA (mg/egg)	DHA (mg/egg)
0		17.4 ± 0.8	n.d.	26.5 ± 6.8
14	Control	16.8 ± 3.6 ^a	n.d.	27.9 ± 2.3 ^c
	5.0% <i>N. gaditana</i>	18.1 ± 3.4 ^a	2.1 ± 0.3 ^c	42.9 ± 5.6 ^a
	10.0% <i>N. gaditana</i>	18.1 ± 1.8 ^a	3.3 ± 0.8 ^b	48.7 ± 5.7 ^a
28	Control	19.7 ± 5.1 ^a	n.d.	25.1 ± 4.4 ^c
	5.0% <i>N. gaditana</i>	15.9 ± 2.0 ^a	2.3 ± 0.6 ^c	44.9 ± 6.6 ^a
	10.0% <i>N. gaditana</i>	17.0 ± 2.6 ^a	4.7 ± 0.8 ^a	50.4 ± 4.6 ^a
42	Control	19.7 ± 0.9 ^a	n.d.	34.1 ± 0.3 ^{bc}
	5.0% <i>N. gaditana</i>	17.0 ± 1.0 ^a	0.6 ± 0.4 ^d	37.9 ± 5.9 ^{ab}
	10.0% <i>N. gaditana</i>	17.5 ± 3.0 ^a	0.7 ± 0.4 ^d	32.7 ± 3.5 ^{bc}

Values with the same letter in the same column are not significantly different (P < 0.05).

n.d., not detected.

Table 3: Composition of fatty acids (expressed as percentage of total fatty acids lipid class) in the different lipid classes (neutral lipids, glycolipids, and phospholipids) in eggs measured halfway (day 14) and at the end (day 28) of the microalgal supplementation period for the different treatment groups (0% *N. gaditana* = control, 5.0% *N. gaditana*, and 10.0% *N. gaditana*)

	Day 14			Day 28		
	Control	5.0% <i>N. gaditana</i>	10.0% <i>N. gaditana</i>	Control	5.0% <i>N. gaditana</i>	10.0% <i>N. gaditana</i>
Neutral lipids (79% of total yolk lipids)						
C18:3 n-3 (ALA)	0.6 ± 0.1 ^a	0.6 ± 0.1 ^a	0.7 ± 0.1 ^a	0.7 ± 0.1 ^a	0.6 ± 0.1 ^a	0.7 ± 0.1 ^a
C22:6 n-3 (DHA)	0.1 ± 0.0 ^a	0.2 ± 0.0 ^b	0.3 ± 0.1 ^c	0.1 ± 0.0 ^a	0.2 ± 0.1 ^b	0.3 ± 0.1 ^c
Total n-3	0.8 ± 0.1 ^a	0.8 ± 0.1 ^a	0.9 ± 0.1 ^b	0.8 ± 0.1 ^a	0.8 ± 0.1 ^a	1.0 ± 0.1 ^b
Glycolipids (1% of total yolk lipids)						
C18:3 n-3 (ALA)	1.1 ± 0.9 ^a	1.6 ± 1.5 ^a	1.6 ± 1.3 ^a	2.9 ± 1.6 ^a	2.9 ± 1.2 ^a	2.7 ± 1.1 ^a
C22:6 n-3 (DHA)	1.8 ± 0.2 ^a	1.5 ± 0.5 ^a	1.4 ± 0.4 ^a	2.0 ± 0.6 ^b	1.8 ± 0.4 ^b	2.0 ± 0.5 ^b
Total n-3	2.9 ± 0.9 ^a	3.1 ± 1.5 ^a	2.9 ± 1.5 ^a	5.0 ± 2.1 ^b	4.8 ± 1.4 ^b	4.7 ± 1.3 ^b
Phospholipids (20% of total yolk lipids)						
C18:3 n-3 (ALA)	0.2 ± 0.0 ^a	0.1 ± 0.1 ^a	0.1 ± 0.0 ^a	0.2 ± 0.0 ^a	0.1 ± 0.0 ^a	0.2 ± 0.3 ^a
C20:5 n-3 (EPA)	0.0 ± 0.0 ^a	0.3 ± 0.1 ^b	0.4 ± 0.1 ^c	0.0 ± 0.0 ^a	0.3 ± 0.1 ^b	0.5 ± 0.1 ^d
C22:5 n-3	0.3 ± 0.1 ^a	0.5 ± 0.2 ^a	0.7 ± 0.3 ^b	0.4 ± 0.1 ^c	0.7 ± 0.2 ^c	0.9 ± 0.1 ^d
C22:6 n-3 (DHA)	4.4 ± 0.4 ^a	6.2 ± 0.7 ^b	7.9 ± 0.8 ^{cd}	4.2 ± 0.5 ^a	7.1 ± 1.1 ^{bc}	8.8 ± 1.0 ^d
Total n-3	4.9 ± 0.4 ^a	7.1 ± 0.8 ^b	9.2 ± 0.8 ^c	4.8 ± 0.6 ^a	8.1 ± 1.0 ^b	10.5 ± 1.1 ^d

Values with the same letter in the same row are not significantly different (P < 0.05).

Table 4: Colour values of egg yolk measured at the start (day 0), halfway (day 14), at the end (day 28) of the microalgal supplementation period, and at the end of the wash-out period (day 42) from hens fed graded levels of *Nannochloropsis gaditana* (0% *N. gaditana* = control, 5.0% *N. gaditana*, and 10.0% *N. gaditana*)

Day	Group	Roche value	CIELAB value		
			L^*	a^*	b^*
0		10 ± 0	73.0 ± 0.0	8.6 ± 1.2	62.8 ± 3.1
14	Control	10 ± 1^c	70.4 ± 2.5^a	6.1 ± 2.2^c	56.1 ± 5.4^a
	5.0% <i>N. gaditana</i>	14 ± 1^b	62.8 ± 4.2^b	18.6 ± 1.8^b	60.7 ± 4.8^a
	10.0% <i>N. gaditana</i>	15 ± 1^b	59.7 ± 2.6^b	26.2 ± 1.8^a	60.8 ± 2.3^{ab}
28	Control	11 ± 1^c	72.8 ± 2.4^a	9.8 ± 3.4^c	63.4 ± 3.4^a
	5.0% <i>N. gaditana</i>	13 ± 1^b	63.0 ± 3.8^b	18.3 ± 3.6^b	58.5 ± 2.9^a
	10.0% <i>N. gaditana</i>	15 ± 0^a	60.1 ± 0.3^b	24.7 ± 1.2^a	57.5 ± 1.9^a
42	Control	11^c	71.0^a	10.2^c	64.1^{ac}
	5.0% <i>N. gaditana</i>	11 ± 1^c	72.5 ± 1.4^a	11.1 ± 5.1^c	64.6 ± 8.5^{ac}
	10.0% <i>N. gaditana</i>	12 ± 0^c	70.3 ± 1.2^a	15.5 ± 3.2^c	71.5 ± 2.1^{bc}

Values with the same letter in the same column are not significantly different ($P < 0.05$).

L^* = lightness, a^* = red-greenness, and b^* = blue-yellowness.

Table 5: The mean feed intake (g/day), egg weight (g) and egg production (egg/day) from hens fed graded levels of *Nannochloropsis gaditana* (0% *N. gaditana* = control, 5.0% *N. gaditana*, and 10.0% *N. gaditana*) during the supplementation period of 28 days

	Control	5.0% <i>N. gaditana</i>	10.0% <i>N. gaditana</i>
Feed intake	112.4 ± 4.2 ^a	113.8 ± 4.1 ^a	100.7 ± 8.3 ^a
Egg weight	57.5 ± 2.2 ^a	56.5 ± 1.7 ^a	56.5 ± 1.1 ^a
Egg production	0.96 ± 0.02 ^a	0.99 ± 0.02 ^a	0.95 ± 0.07 ^a

Values with the same letter in the same row are not significantly different (P < 0.05).