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The effects of *Nannochloropsis limnetica* supplementation on digestibility, performance, intestinal morphometry and systemic inflammatory markers parameters in laying hens

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Summary

Microalgae can be a circular, sustainable and promising feed ingredient for poultry. Microalgae can be grown on nutrient rich side streams or discharge water, without competing for arable land. The fatty acid content of microalgae, especially the high content of omega 3 fatty acids such as eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6 n-3), could enrich eggs when fed to laying hens. The properties and bioactive components of omega 3 fatty acids could also positively influence the laying hens' health and production. This study aimed to measure the effects of increasing doses of the microalgae *Nannochloropsis limnetica*, on n-3 fatty acids level in eggs, laying hen performance, digestibility, intestinal morphometry and systemic inflammatory markers. In total, 240 H&N Super Nick laying hens (25 weeks of age) were randomly allotted to 30 pens, which were blocked (6 blocks) and 5 treatments were randomly divided within the blocks. Hens were grouped in 8 birds per pen, with 30 pens in total. Treatment A was the control group and treatments B, C and D contained the control diet with 1%, 2% and 3% microalgae respectively. In treatment E the diet included 3% microalgae and part of the soybean meal of the control diet was replaced by rapeseed meal to induce a mild nutritional challenge. All diets contained titanium dioxide as an inert marker for the digestibility measurements. Feed was provided *ad libitum* throughout the experimental period of 28 days. All pens were group weighed at the beginning and end of the experimental period. On a weekly basis, the feed intake, rate of lay and egg weight were determined. Feed conversion ratio was calculated on basis of the feed intake and egg weight. From day 22 to day 27 all eggs per pen were collected and total egg weight, scale weight, albumen weight (fresh and dry) and yolk weight (fresh and dry) were determined. Litter material was removed at day 24 and faeces were collected from day 24 to day 27. Representative samples were taken of all five diets. Feed was dried and faeces were freeze dried, grinded and analysed for dry matter content, crude ash, crude protein, crude fat, crude fibre and titanium. Of the microalgae, feed, yolk and faeces, the fatty acid composition was determined. At day 28 two laying hens per pen were sacrificed and tissue from the jejunum and colon were analysed for morphology measurements. Furthermore, blood samples were taken from these two laying hens to obtain blood plasma for IL-13 and haptoglobin determination. The data were analysed with analysis of variance (ANOVA) as a randomised block design using GenStat statistical software. Due to transport, most hens stopped laying for a few days which resulted in a very low lay percentage in week one (on average 27.3%). After one week, laying performance returned to normal (on average 91.0%, 94.0% and 96.0% resp.). The different microalgae inclusion levels did not affect digestibility. Inclusion of rapeseed meal to the diet tended to decrease the crude fat digestibility compared to all other treatments (P = 0.063). The microalgae treatments had little effects on laying hen performance. The rate of lay increased with approximately 5% (P = 0.039) when the birds were fed with 2 or 3% microalgae compared to the control group. Furthermore, inclusion of 2% and 3% microalgae resulted in a higher feed intake compared to the control group (126, 125 and 119 g/hen/d respectively; P = 0.001). In the first 3 weeks, the rate of lay tended to normalize faster upon inclusion of the microalgae in the diet which might suggest a faster recovery from stress conditions during transport (P =0.092). Body weight, egg weight and feed conversion ratio were not affected by the dietary treatments. There were no differences found on the weights of the shell, yolk or albumen upon inclusion of the microalgae in the diet. Furthermore, the ratio of these parts of the egg also did not differ. The EPA content of the eggs increased both linear and quadratically and the DHA content only linear by the increasing inclusion of microalgae (P < 0.001). A 2% inclusion of algae resulted in 58.3 and 603 mg EPA and DHA per 100g dry yolk respectively. The omega 6 content of the yolk was slightly reduced upon inclusion of 2% and 3% microalgae (P = 0.004). The haptoglobin levels of laying hens receiving 3% microalgae were almost three times lower compared to the control group (1.25 and 1.62 vs. 5.60; P < 0.001), regardless of the inclusion of rapeseed in the diet. The IL13 concentration and intestinal morphometry were not affected by the dietary treatments.

From this study it can be concluded that the inclusion of *N. limnetica* resulted in a higher rate of lay, a slightly increased feed intake and increased DHA and EPA content in the yolk and consequently the eggs. Inclusion of 3% microalgae reduced the haptoglobin levels but did not affect the IL13 concentrations and intestinal morphometry. Furthermore, the microalgae did not affect digestibility of the diets. Based

on these results, there are no indications that up to 3% inclusion of *N. limnetica* in the diet would negatively affect laying hens.
Eggs of laying hens fed with 1% algae in their diet contained 490 mg DHA + EPA / 100 gram egg enough DHA to be considered a source of omega-3 in the European Union. Even more, 3% algal inclusion resulted in 827 mg/ DHA + EPA / 100 gram egg, which is enough to claim a food high in omega-3 fatty acids.

1 Introduction

Microalgae could be a circular, sustainable and promising feed ingredient for poultry diets. Microalgae need CO_2, nitrogen and phosphorus as main components and can grow on nutrient rich side streams such as e.g. discharge water of some greenhouses. In such an approach algae can clean the water by reducing the amounts of nitrogen and phosphorus and simultaneous produce a valuable product. By selecting relevant strains, algal biomass might compete in poultry diets with oils that are currently used as fat sources (i.e. palm oil, fish oil or soya oil) or add specific functionalities. Large groups of consumers prefer table eggs, produced with feed from plant origin and some control organisations (e.g. KAT in Germany) prohibit the use of feed ingredients of animal origin. Specific photosynthetic microalgae, containing high amounts of docosahexaenoic acid (DHA, C22:6 n-3) and eicosapentaenoic acid (EPA, C20:5 n-3), could be an interesting feedstuff for poultry diets. From an animal nutrition point of view, it is important to know the extent to which the algae products can deliver digestible nutrients. In addition, it is relevant to determine the bioactive components in the algae products that can have health-promoting effects for the laying hens themselves. Microalgae contain natural pigments and include antibacterial, anti-inflammation and antioxidant properties have proven positive effects on gut health and immunity of humans and animals (Christaki et al., 2011; de Jesus Raposo et al., 2013; Noda et al., 2016; Furbeyre et al., 2017). The omega-3 fatty acids as well as some bioactive components in the algae products, like beta-glucans, might have health-promoting effects for the laying hens, as has been found in broiler chickens (Kang et al., 2013). This could improve the immune status of the hens, making them more robust against infectious diseases. Regarding the poultry products (meat and eggs), with algae in the diet of laying hens the fatty acid composition of the yolk might be altered by the omega-3 fatty acids of the algae (Ginzberg et al., 2000; Fredriksson et al., 2006; Rizzi et al., 2009; Neijat et al., 2016). An increase in omega-3 fatty acids in the yolk, leads to more healthy and balanced diet for humans. Consequently, this might decrease costs related to combat chronic diseases such as cardiovascular disease. Benefits for human health are mostly related to EPA and DHA, which are converted from alpha-linolenic acid (ALA, C18:3 n-3) (Trautwein, 2001). In humans, however, this conversion is very limited, making the direct ingestion of omega-3 (with a focus on DHA and EPA) enriched food essential (Wu et al., 2019).

We hypothesize that feeding specific microalgae to hens enhances the omega-3 content of the yolk. Furthermore, the microalgae might improve nutrient digestion, thereby reducing the negative impact of hind-gut fermentation, and gut health. Results of an in vitro study conducted at our lab have shown positive effect of microalgae on intestinal cells line (Hulst et al., submitted publication). Briefly, intestinal cells lines were incubated with microalgae and their potential effects was accessed by studying genome-wide transcriptome response. It emerged that microalgae positively effects numerous metabolic and immunological processes. However, there are many microalgae species available, and the exact properties and functions of each individual microalgae species needs to be researched before it can be (safely) used in a poultry diet. The differences in their composition and functional properties result in differences in digestibility and enrichment of the egg (Lemahieu et al., 2013). Furthermore, the optimal dosage requirement for microalgae to reach desired results, which are technically viable and economically feasible, for use as 'functional' feed-ingredients in laying hen diets under commercial setting is not known.

In this research we report the response in terms of performance, digestibility, intestinal morphometry and systemic inflammatory markers in laying hens when fed with a diet containing 3 doses of the EPA producing freshwater microalgae *Nannochloropsis limnetica*.

The effects of supplementing graded levels of the microalgae in the diet on nutrients digestibility, egg composition (fatty acid composition in the yolk), blood plasma parameters (cytokines & chemokines) and gut tissue by morphometry in the digestive tract in laying hens have been identified.

2 Material and methods

This study was conducted from August until September 2019 at the animal research facilities of Wageningen Bioveterinairy Research (Lelystad, the Netherlands), in accordance with EU directive 2010/63 and approved by the Dutch Central Committee of Animal Experiments (The Hague, the Netherlands; protocol number: AVD401002015196).

2.1 Animals and Housing

In total 240 H&N Super Nick (Agromix, The Netherlands) laying hens (25 weeks of age; 1165.9 ± 29.3 g body weight) were purchased from a commercial laying hen farm. Hens were randomly allotted to 30 pens, with a minimum group weight difference of 5% (8 birds per pen). The pens were located in two mechanically ventilated rooms and each room contained 15 pens (1.0 x 0.75 m) with flexible plastic slats. The flooring of the pens was covered with wood shavings and each pen contained a perch (0.75 m) and laying nest. Feed was provided *ad libitum* in a trough adjacent to the pen and water was provided *ad libitum* by two drinking nipples per pen. The photoperiod consisted of 16 h light (04.00 to 20.00 h) with an illumination of 20 lux. During the experiment the temperature was kept at 21°C.

2.2 Experimental diets and experimental design

In total, three experimental diets were produced (Research Diet Service, Wijk bij Duurstede):

1. CD: Control diet
2. CD3: Diet with 3% of the microalgae *N. limnetica*
3. RS3: Diet with 3% of the microalgae *N. limnetica* and 53% of soybean meal replaced by rapeseed meal to induce a mild dietary challenge.

Experimental dietary treatment A was obtained from solely diet CD, experimental treatment D from solely CD3 and experimental dietary treatment E from solely RS3. The diets for experimental dietary treatments B and C were obtained by mixing 2/3 of diet CD and 1/3 of diet CD3, and 1/3 of diet CD and 2/3 of diet CD3, respectively. The experimental diets were isocaloric and isonitrogenous and based on commercial guidelines. Dietary ingredients and nutrient compositions of the diets can be found in Appendix 1. Feed was provided as a mash and was fed ad libitum during the complete experimental period of 28 days.

A completely randomized block design was used to arrange the treatments. Both rooms contained 3 blocks in which the treatments were randomly allotted, resulting in 6 replicates per treatment. Dietary treatments consisted of 4 inclusion levels (0, 1, 2 and 3%) of the microalgae *N. limnetica,* and a dietary challenging treatment with 3% microalgae and inclusion of rapeseed meal (Table 1). Treatment A was considered the reference diet.

Table 1 *Experimental dietary treatments with increasing inclusion of microalgae N. limnetica.*

Diet	algae inclusion (%)	RSM inclusion[1]	# replicates	# total birds
A	0	N	6	48
B	1	N	6	48
C	2	N	6	48
D	3	N	6	48
E	3	Y	6	48

[1] Rapeseed meal exchanged part of the soybean meal to induce a mild dietary challenge

2.3 Measurements

All pens were group weighed at the beginning and end of the experimental period (days 0 and 28). On a weekly basis, the feed intake, laying percentage and egg weight were determined. Feed conversion ratio was calculated based on the feed intake and egg weight. From day 22 to day 27 all eggs per pen were collected. Per collection day, 5 random eggs were collected and pooled per three days (days 22, 23, 24 and days 25, 26, 27). Total egg weight, scale weight, albumen weight (fresh and dry) and yolk weight (fresh and dry) were determined. The pooled yolk was dried to obtain the dry matter content. An external laboratory determined the fatty acid composition in the yolk (NutriControl BV, Veghel). Litter material was removed at day 23 and faeces were collected from day 24 to day 27. Representative samples were taken of all five provided diets. Feed was dried and faeces were freeze dried and analysed for dry matter content, crude ash, crude protein, crude fat, crude fibre, fatty acid composition and titanium by an external laboratory (NutriControl BV, Veghel).
At day 28 two laying hens per pen were sacrificed and 1 cm of tissue from the middle of the intestine part, both from the jejunum and colon, were collected. Samples were analysed for villi length and crypt depth by an external laboratory (Gezondheidsdienst voor Dieren, Deventer). Furthermore, blood samples were taken from these two laying hens to obtain blood plasma for IL-13 and haptoglobin determination.

2.4 Calculations

The rate of lay was calculated by dividing the total number of eggs in one week by the production days (number of birds multiplied by the number of days where eggs are produced). Feed conversion ratio was calculated by dividing the total feed intake by the total egg mass of one pen.
Based on the analysed content in the experimental diets and excreta, the faecal digestibility of DM, ash, organic matter (=DM – ash), crude fat and crude fibre were calculated using equation I.

$$\text{Digestibility (\%)} = 100 - [100 \times (M_{diet} \times \text{Nutrient}_{faeces}) / (M_{faeces} \times \text{Nutrient}_{diet})] \quad \text{(I)}$$

With

- M_{diet} and M_{faeces} are the analysed concentrations of marker (TiO_2) in the diet and faeces (g/kg DM).
- Nutrient_{diet} and Nutrient_{faeces} are the analysed concentrations of nutrient in the diet and faeces (g/kg DM).

The amounts of fatty acids were analysed in dry egg yolk of eggs collected in the last experimental week (week 4). In the report the amounts are presented on basis of 100 g dry egg yolk as well as in mg per egg.

2.5 Statistical analyses

The data were analysed with analysis of variance (ANOVA) as a randomised block design using Genstat (19th Edition) statistical software. The pen was the experimental unit for the response parameters, and the dependent variables were expressed as average of the pen. For all variables this average consisted out of 8 laying hens, except for the blood and gut tissue related parameters, which consisted of 2 laying hens. The general model included addition of the microalgae in the diet as fixed effects and room and block (place within the room) as random effects:

$$Y_{ijk} = \mu + Room_i + Block_j + Diet_k + e_{ijk}$$

in which:

Y_{ijkl} = dependent variable,
μ = overall mean
$Room_i$ = room effect (i=1,2)
$Block_j$ = block effect (j=1, 2, 3, 4, 5, 6)
$Diet_k$ = effect of dietary treatment, (k= 1, 2, 3, 4, 5)
e_{ijk} = residual error.

This model was used to analyse the results of the performance parameters (body weight, feed intake, rate of lay, egg weight and feed conversion ratio), digestibility, egg characteristics (shell, yolk and albumin weights and ratio) and fatty acid compositions. Parameters were tested for normal distribution before analyses. For the performance parameters, digestibility, egg characteristics and fatty acid composition, analyses were performed to determine linear and quadratic effects of dietary inclusion of the microalgae (dose-response). A Fisher unprotected t-test has been used for comparison of treatment means. Pairwise differences are marked with superscripted indices when significant differences ($P < 0.05$) were observed.
Because of the different objectives (effect of microalgae on performance vs. influence on intestinal morphometry and systemic inflammatory markers) and dietary differences, treatment B, C and E were incomparable. Due to the influence treatment E has on the averages, variances and analysis, treatment E was excluded from the analyses of performance egg characteristics and fatty acid composition. The means of treatment E can be found in Appendix 4. Intestinal morphometry and systemic inflammatory markers were only analysed comparing treatments A (reference diet), D and E.
The statistics of the measured systemic inflammatory blood concentration levels of IL13 and haptoglobin and the intestinal morphometry were analysed in GraphPad Prism (v8.2.1; GraphPad Software, San Diego, California, USA). Briefly, normality test i.e. Shapiro-Wilk and Kolmogorov-Smirnov test were carried out in the blood concentration levels of IL13 and haptoglobin and the intestinal morphometry in each treatment. To find the significant differences, we separately compared treatment D and E with the reference diet i.e. treatment A using t-test and nonparametric tests. Parametric t-test were performed when both the treatments passed the normality test, but when the compared treatments failed the normality test then nonparametric i.e. Mann-Whitney test were performed. P value < 0.05 was considered significant.

3 Results

3.1 General

The experiment was conducted according to protocol without any deviations. The laying hens arrived healthy and were distributed with a maximum of 5% average group body weight deviation between pens. Total mean body weight of the hens in one pen was 9327 g (average of 1166 g per hen). Due to transport, most hens stopped laying for a few days which resulted in a very low laying percentage in week one (27.3%). After this one week of adaptation, laying performance returned to normal. The first week was therefore excluded from analyses. Laying percentage in week 2, 3 and 4 (91.0%, 94.0% and 96.0% resp.) did not differ from H&N Super Nick performance objectives. During the experiment, no veterinary treatments have been executed and mortality was very low (0,42%, one bird was found dead).

3.2 Diets & Microalgae

3.2.1 Contents of the diets

The calculated and analysed contents of the experimental diets are presented in Table 2. The analysed values for DM, crude protein, crude ash and starch were close to the calculated values of the diets. The analysed crude fat content of the A diet was close to the calculated value, however the analysed crude fat content for diets B, C, D and E was respectively 4, 5, 5 and 5 g/kg lower than calculated. Crude fibre content of the D and E diets were close to the calculated values, whereas the analysed values for A, B and C were 3 g/kg, 2 g/kg and 2 g/kg higher than the calculated value, respectively. The analysed values for sugar were higher in all five diets. Furthermore, the titanium content of the diets was 0.4, 0.3, 0.3, 0.3 and 0.2 g/kg lower than the calculated content of A, B, C, D and E respectively.

Table 2 *Calculated (Calc) and analysed (Ana) nutrient content of experimental diets (g/kg) and their differences (%).*

Diet[1]	A			B			C			D			E		
	Calc	Ana	*Diff*	Calc	Ana	*Diff*	Calc	Ana	*Diff*	Calc	Ana	*Diff*	Calc	Ana	*Diff*
Dry matter	891	900	+1.0	891	901	+1.0	891	901	+1.0	891	902	+1.0	892	904	+1.4
Crude protein	161	157	-2.4	161	159	-1.0	161	160	+0.0	161	162	+0.5	161	159	-1.2
Crude ash	128	133	+4.0	128	131	+3.0	128	129	+1.0	127	127	-0.2	127	131	+3.1
Crude fat	58	56	-4.0	58	54	-6.0	57	52	-8.0	56	51	-9.9	69	64	-6.9
Crude fibre	37	40	+8.6	37	39	+7.0	37	39	+6.0	36	38	+4.1	43	44	+1.5
Starch	379	384	+1.3	379	387	+2.0	379	391	+3.0	379	394	+4.0	354	362	+2.1
Sugar	30	34	+14.7	29	33	+13.0	29	32	+10.0	29	32	+8.3	33	37	+11.2
TiO_2	5.0	4.6	-7.4	5.0	4.7	-7.0	5.0	4.7	-6.0	5.0	4.7	-5.6	5.0	4.8	-3.6

[1] Diets: A = control diet 0% microalgae; B = control diet + 1% microalgae; C = control diet + 2% microalgae; D = control diet + 3% microalgae; E = exchange of soybean by rapeseed + 3% microalgae

The DM, crude protein, crude ash and crude fibre content of the faeces did not differ amongst treatments. However, the faeces of the birds that were fed the rapeseed + 3% microalgae diet (treatment E) had a higher crude fat content compared to the other treatments (P = 0.048). Furthermore, the titanium content of the faeces from birds fed treatment E differed from birds fed the control diet, and the diet containing 1% microalgae (P = 0.043).

3.2.2 Fatty acid composition in microalgae and dietary treatments

The levels of DHA, EPA and major fatty acids groups of the dried microalgae and the treatments are presented in Table 3, the full fatty acid composition of the microalgae can be found in appendix 1. In neither the dry material of the microalgae, nor the dietary treatments DHA was found. The EPA level in the diets increased in accordance with the supplementation of the microalgae.

Table 3 *Fatty acid composition (g/kg) of dry N. limnetica biomass and the dietary treatments.*

Treatment[1]	*N. limnetica*	A	B	C	D	E
DHA	0	0	0	0	0	0
EPA	35.8	0	0.28	0.47	0.71	0.70
Monounsaturated	64.9	17.9	17.9	16.0	15.7	21.3
Poly unsaturated	54.0	14.3	15.0	13.9	14.3	15.9
Saturated	38.8	20.2	19.7	17.0	16.1	21.6
Omega 3	36.2	0.67	0.95	1.04	1.26	1.54
Omega 6	16.7	13.6	14.0	12.8	12.9	14.2
Omega 9	21.7	17.3	17.1	15.0	14.3	19.3

[1] Treatment: A = control diet + 0% microalgae; B = control diet + 1% microalgae; C = control diet + 2% microalgae; D = control diet + 3% microalgae; E = exchange of soybean by rapeseed + 3% microalgae

3.3 Performance & digestibility

3.3.1 Faecal digestibility of the diets

Table 4 shows the faecal digestibility coefficients of the diets with increasing inclusion of microalgae. The inclusion of microalgae did not significantly affect digestibility. Inclusion of rapeseed tended to decrease the crude fat digestibility compared to all other treatments (P = 0.063). Inclusion of rapeseed numerically decreased all digestibility coefficients.

Table 4 *Digestibility (%) of diets with increasing inclusions (0, 1, 2, 3%) of microalgae.*

Treatment[1]	A	B	C	D	E	SEM[2]	*P* - value
Dry matter	69.1	67.2	70.6	70.1	60.6	4.3	0.16
Crude protein	50.3	46.4	52.5	51.5	40.7	6.9	0.44
Crude ash	46.9	39.0	48.7	45.3	31.2	7.8	0.20
Crude fat	71.8	69.2	68.7	67.9	57.2	4.9	0.063
Crude fibre	7.3	-9.7	8.2	8.1	-14.0	13.7	0.31

[1] Treatment: A = control diet + 0% microalgae; B = control diet + 1% microalgae; C = control diet + 2% microalgae; D = control diet + 3% microalgae; E = exchange of soybean by rapeseed + 3% microalgae

[2] SEM = average standard error of the mean

3.3.2 Laying hen performance

Because of the different objectives (effect of microalgae on performance vs. influence on intestinal morphometry and systemic inflammatory markers) and dietary differences, treatment B, C and E were incomparable. Due to the influence treatment E has on the averages, variances and analysis, treatment E was excluded from the analyses of performance egg characteristics and fatty acid composition. The means of treatment E can be found in Appendix 4.

Table 5 represents the effects of the inclusion of microalgae on laying hen performance. The average increase in body weight of the individual hens was not affected by the treatments. Furthermore, the feed conversion ratio and the average egg weight was not affected by the inclusion of the microalgae. The egg weight increased over time for all treatments, but inclusion of algae in the diet did not affect or strengthen the effect on egg weight.
The average feed intake per hen per day was affected by the dietary treatments. Inclusion of 2% and 3% microalgae resulted in a higher feed intake compared to the control group (P = 0.001). The rate of lay of birds fed 2 or 3% microalgae was respectively 5.8% and 4.4% (P =0.018) higher compared to the control group. In the first 3 weeks, the rate of lay tended to normalize faster upon inclusion of the microalgae in the diet which might suggest a faster recovery from stress conditions during transport (P = 0.092). Although not significant, the numerical difference in feed intake between week 1 and week 2 agree to a faster recovery due to the supplementation of the microalgae.

Table 5 *Effects of increasing inclusions (0, 1, 2, 3%) of microalgae on laying hen performance.*

Treatment[1]	A	B	C	D	Average	SEM[2]	P-value				
							Treatment			Week	Week* Treatment
								Linear	Quadratic		
Average body weight per hen (g)											
Start	1175	1162	1161	1171		16.8	0.80	0.82	0.34		
End (28 days)	1495	1464	1483	1487		18.8	0.42	0.92	0.20		
Growth	321	302	322	316		33.3	0.70	0.92	0.64		
Feed intake (g/hen/day)											
wk 1	106	106	109	106	107						
wk 2	119^{B}	126^{A}	125^{A}	126^{A}	124	2.0	0.007	0.004	0.034		
wk 3	117^{B}	121^{AB}	126^{A}	124^{A}	122	2.7	0.051	0.019	0.16		
wk 4	122	120	126	124	123	2.3	0.06	0.043	0.96		
Average[3]	119^{C}	122^{BC}	126^{A}	125^{AB}		1.6	0.004	0.001	0.08	0.10	0.24
Rate of lay (%)											
wk 1	31.3	24.4	30.4	27.1	28.30						
wk 2	86.6	89.6	97.3	92.9	91.6^{b}	4.32	0.12	0.07	0.24		
wk 3	90.5	96.7	97.0	93.2	94.3^{ab}	2.62	0.07	0.33	0.016		
wk 4	94.8	95.8	94.8	98.9	96.1^{a}	2.23	0.24	0.13	0.34		
Average[3]	90.6^{B}	94.1^{AB}	96.4^{A}	95.0^{A}		1.83	0.039	0.018	0.083	0.013	0.092
Average egg weight per egg (g)											
wk 1	47.6	48.0	48.4	46.9	47.7						
wk 2	54.2	54.7	54.2	53.7	54.2	0.74	0.61	0.42	0.35		
wk 3	57.8	57.7	57.5	56.8	57.4	0.77	0.58	0.22	0.56		
wk 4	57.3	57.1	57.5	56.4	57.1	0.79	0.54	0.36	0.48		
Average[3]	56.4	56.5	56.4	55.6		0.65	0.53	0.24	0.39	<0.001	0.77
Feed conversion ratio (%)											
wk 1	7.77	10.33	8.08	8.66	8.71						
wk 2	2.60	2.60	2.40	2.50	2.52^{c}	0.13	0.57	0.56	0.58		
wk 3	2.30	2.20	2.20	2.30	2.25^{b}	0.08	0.26	0.26	0.11		
wk 4	2.20	2.20	2.30	2.20	2.61^{a}	0.08	0.37	0.53	0.70		
Average[3]	2.47	2.43	2.44	2.49		0.059	0.75	0.71	0.31	<0.001	0.211

[1] Treatments: A = control diet 0% microalgae; B = control diet + 1% microalgae; C = control diet + 2% microalgae; D = control diet + 3% microalgae;

[2] SEM = average standard error of the mean. Week 1 is excluded from the average

[3] Due to severe drop in production, week 1 is excluded from the average and analysis

[AB] Means within a row with no common superscript differ ($p < 0.05$)

[ab] Means within a column with no common superscript differ ($p < 0.05$)

3.4 Eggs

3.4.1 Egg characteristics

Egg characteristic results during the experimental period are presented in Table 6. Inclusion level of microalgae had no effect on the weight of the shell, yolk or albumen neither on the ratio of these parts of the egg.

The EPA content of the eggs was both linear and quadratically affected by the increasing inclusion of microalgae ($P < 0.001$). With each increasing step of the level of microalgae in the diets, the yolk content of EPA increased as well. The DHA content was only linear affected by the increasing inclusion of the microalgae ($P < 0.001$). For both EPA and DHA, all the groups differed from each other; thus, for EPA the quadratic effects indicate the plateau is not yet reached. For the EPA content, the highest increase is between the inclusion of 0% and 1% microalgae. Between 1%, 2% and 3% the content increases, however it increases with a slope that gets smaller, indicating the quadratic effect towards a slope (Figure 1A). The DHA content seems to increase linear with every inclusion step (Figure 1B).

Table 6 *Effects of increasing inclusions (0, 1, 2, 3%) of microalgae on egg characteristics.*

Treatment[1]	A	B	C	D	SEM[2]	P-value	
						Linear	Quadratic
Fresh weights (g per egg)							
Shell	7.7	7.6	7.8	7.6	0.1	0.30	0.55
Yolk	15.1	14.6	15.0	14.6	0.3	0.16	0.84
Albumin	34.4	34.4	34.5	34.1	0.7	0.76	0.58
Ratio (%)							
Shell	13.4	13.4	13.5	13.3	0.1	0.57	0.85
Yolk	26.5	25.9	26.3	26.1	0.3	0.46	0.38
Albumin	60.3	61.0	60.5	60.8	0.4	0.42	0.51
Fatty acids in egg (mg per 100g dry yolk)							
EPA	10.0[d]	41.7[c]	58.3[b]	74.2[a]	2.15	<0.001	<0.001
DHA	277[d]	448[c]	603[b]	753[a]	12.5	<0.001	0.24

[1] Treatment: A = control diet 0% microalgae; B = control diet + 1% microalgae; C = control diet + 2% microalgae; D = control diet + 3% microalgae;

[2] SEM = average standard error of the mean

[abc] Means within a row with no common superscript differ ($p < 0.05$)

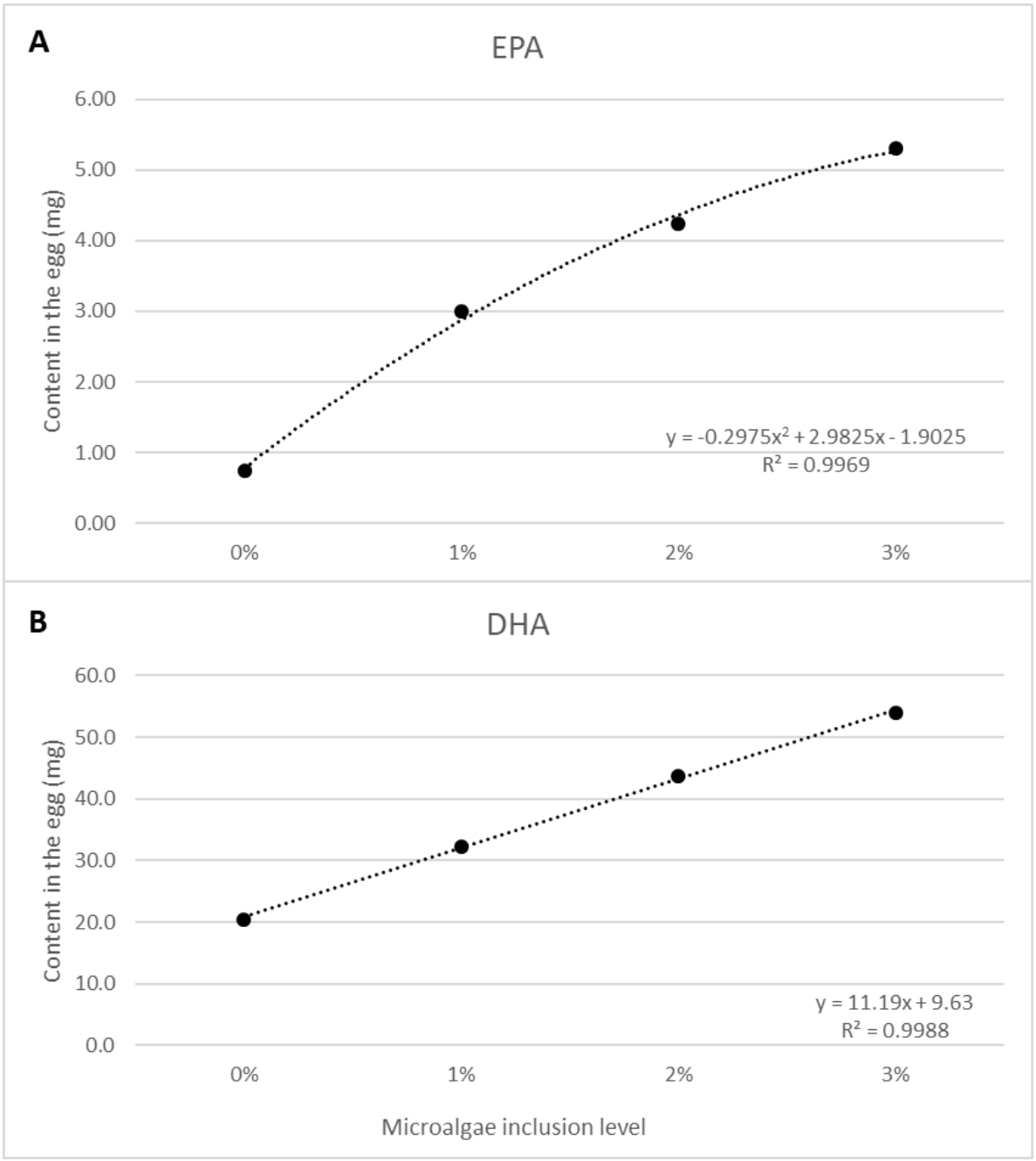

Figure 1 *Relation between microalgae inclusion level in the feed and the EPA (A) and DHA (B) content in the egg.*

3.4.2 Fatty acid composition

The effects of the experimental diets on the monounsaturated, polyunsaturated, saturated, omega 3, omega 6 and omega 9 fatty acids of the yolk are presented in Table 7. The complete overview of the fatty acid content of the yolk per treatment can be found in appendix 2. The different dietary treatments did not affect the monounsaturated fatty acids, polyunsaturated fatty acids and omega 9 fatty acids. However, the omega 3 fatty acid content was both linearly and quadratically affected by the dietary treatments ($P < 0.001$). While the inclusion level increased, the omega 3 content increased as well. All the treatments differed from each other. The increase however, declined in small steps when more microalgae were included. Thus, the plateau of the effects of including microalgae on the omega 3 content of the yolk is higher than 3%. The omega 6 content of the yolk was negatively affected by the 2% and 3% inclusion of microalgae ($P = 0.004$). The saturated fatty acid content was approximately 230 mg higher when 2% and 3% microalgae were included, compared to no supplementation of microalgae ($P = 0.022$).

Table 7 *Effects of increasing inclusions (0, 1, 2, 3%) of microalgae on yolk fatty acid composition (g/100g dry yolk).*

Treatment[1]	A	B	C	D	SEM[2]	p-value	
Fatty acids						Linear	Quadratic
Monounsaturated	26.50	26.64	26.49	26.78	0.174	0.22	0.57
Polyunsaturated	8.11	8.27	8.11	8.26	0.174	0.61	0.97
Saturated	20.17^{b}	20.35^{ab}	20.42^{a}	20.39^{a}	0.090	0.022	0.126
Omega 3	0.43^{d}	0.67^{c}	0.87^{b}	1.06^{a}	0.014	<0.001	0.022
Omega 6	7.56^{a}	7.47^{ab}	7.13^{bc}	7.09^{c}	0.166	0.004	0.84
Omega 9	22.9	22.80	22.77	22.66	0.198	0.60	0.95

[1] Treatment: A = control diet 0% microalgae; B = control diet + 1% microalgae; C = control diet + 2% microalgae; D = control diet + 3% microalgae;

[2] SEM = average standard error of the mean

[abc] Means within a row with no common superscript differ (p < 0.05)

3.5 Intestinal morphometry and systemic inflammatory markers

To investigate whether an inclusion level of 3% microalgae in laying hen diets resulted in any changes in systemic immunity, IL13 and haptoglobin in blood plasma of hens from dietary group D and E were measured and compared with the levels measured in hens fed the reference diet A (Figure 2). The blood plasma concentration of haptoglobin was low (P < 0.05) in both microalgae supplemented groups i.e. treatment D and E compared to treatment A. One notable finding was that hens which received the rapeseed meal as a mild dietary challenge alongside the 3% inclusion of microalgae (i.e. diet E) recorded no significant difference (P > 0.05) in blood plasma haptoglobin levels compared to hens fed 3% microalgae (i.e. diet D). No difference was found on IL13 blood plasma concentrations.
The intestinal morphometry of both the jejunum and colon was not affected by the dietary challenge nor by inclusion of 3% microalgae in laying hen diets (Figure 3).

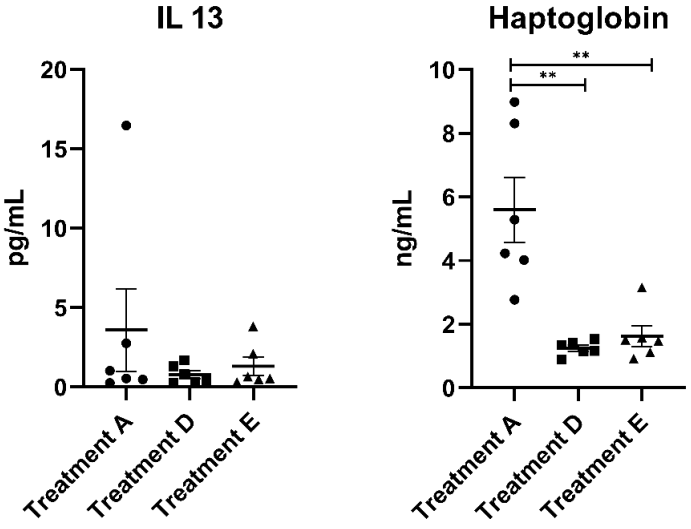

Figure 2 *Boxplot based on the effects of 3% microalgae inclusion (treatment D) and 3% microalgae inclusion + mild dietary rapeseed challenge (treatment E) compared to the reference diet (treatment A) on systemic inflammatory parameters. Each dot, square and triangle represents the average per pen (two birds per replicate). ** represent a significance of P < 0.01 between the treatments indicated by the horizontal lines.*

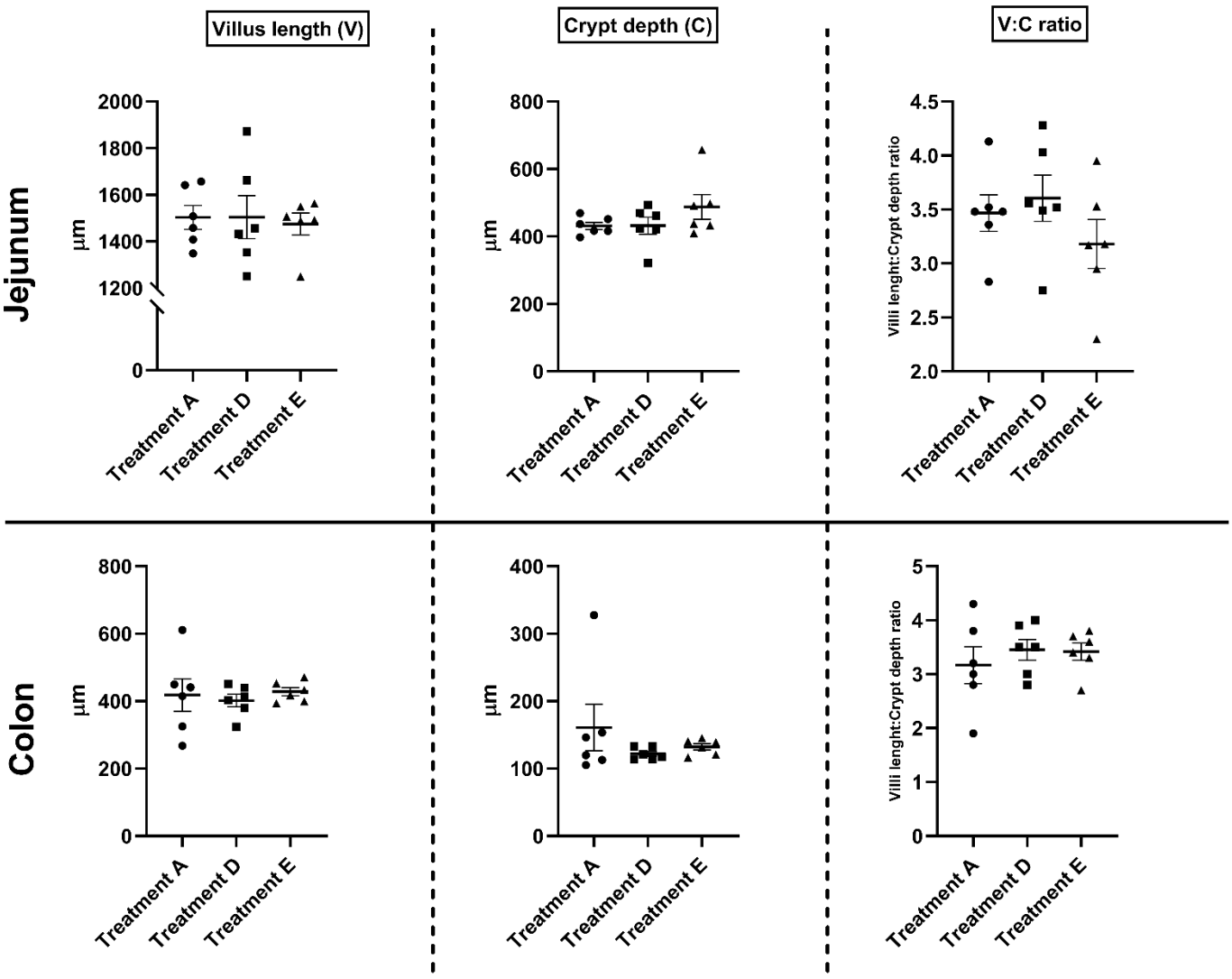

Figure 3 *Boxplot based on the effects of 3% microalgae inclusion (treatment D) and 3% microalgae inclusion + mild dietary rapeseed challenge (treatment E) compared to the reference diet (treatment A) on gut morphometry in the jejunum and colon. Each dot, square and triangle represents the average per pen (two birds per replicate).*

4 Discussion

4.1 Diets & digestibility

The diets for this study were produced in agreement with the calculated nutrient content. Although the sugar content was slightly higher in all five diets, no influences on the different treatments were expected. The fat content was relatively more than 5% lower in all diets, but a bit more expressed in both diets containing microalgae (for ingredients of diets CD3 and RS3 see appendix 1), which probably resulted in lower fat content in all the treatments with microalgae inclusions (treatments B, C, D and E). This could result in the higher feed consumption as was found in this study, since laying hens regulate their feed intake based on energy requirements. Furthermore, the fibre content of the A, B and C treatment diets was higher, due to the higher fibre content of the control diet (CD). Potentially, the fibre content of the diet could positively influence the feed intake and total digestibility of the feed. However, as total fat and fibre content of each diet is not affected much no influences of these differences are expected. This is confirmed by the nutrient content of the faeces, in which no differences were found in fat and fibre content between treatments A, B, C and D. The difference found in fat content between treatment E and the other treatments is probably due to the inclusion of rapeseed meal. The differences found in titanium are common and probably due to the analysis methods. When calculating the digestibility of the diets, the titanium content of the diet and the faeces are used for corrections on the calculation.

The nutrient digestibility of the diets was not affected by the inclusion of *N. limnetica*. Only the digestibility of fat tended to be lower in treatment E, indicating that rapeseed meal negatively affects the fat digestibility, which cannot be counteracted for by the inclusion of 3% microalgae. Overall, the faecal digestibility found in this experiment was considered normal. The faecal digestibility of crude fibre is very variable and can result in negative values.
Studies researching the digestibility of other microalgae in chickens found contradictory effects. Choi et al. (2017) and (Abdelnour et al., 2019) supplemented broiler diets with *Chlorella vulgaris* and found a higher energy digestibility, but no differences in dry matter digestibility. On the other hand, Park et al. (2018) found a linear increase in apparent total tract digestibility of dry matter and nitrogen of broilers fed with diets with increasing inclusion of *Arthrospira platensis (Spirulina)*. Similar results were found in weaned piglets, fed with *Schizochytrium spp.* supplementation (Kibria and Kim, 2019) and piglets fed with *Spirulina* or *C. vulgaris* (Furbeyre et al., 2017), whom also found an increase in total tract digestibility for gross energy. *In vitro* work on several microalgae (*Spirulina, C. vulgaris, Tetraselmis suecia* and *Phaeodactylum tricornutum*) did not show any differences in digestibility (Batista et al., 2017).

4.2 Performance

Table 9 shows the comparison between the results of this study and other studies researching the influence of microalgae on laying hen performance. As different microalgae have their own structure, components and function, the overall outcomes of this and other studies are not conclusive for microalgae in general.
In our study the rate of lay was higher when the microalgae was provided. Most other studies found either no effects, or also an improvement on the egg production (Park et al., 2015; Choi et al., 2018). Only Kulshreshtha et al. (2014) found a decrease with a low supplementation and an increase with a higher supplementation of microalgae. Their results indicate supplementation of that particular microalgae should be at least 2% or higher to initiate any influence on egg production. However, none of the above studies, nor our study was designed to determine the mode of action to explain an improvement of egg production. It might be speculated that the essential amino acid content or other health beneficial microalgal compounds have a positive influence since a healthier animal is better able

to produce. Another possibility is the influence of the microalgae on the microbiome (Janczyk et al., 2009). The microbiome composition and the metabolites provided by the microbiome could influence the egg production process.

Inclusion of microalgae in the diet did not affect the body weight of the laying hens. This is in line with most of the results found in other studies (Bruneel et al., 2013; Kulshreshtha et al., 2014; Ao et al., 2015; Ekmay et al., 2015; Neijat et al., 2016; Moran et al., 2019). Only Lemahieu et al. (2013) found a 5% decrease in body weight when feeding two levels of *N. limnetica* between 2.5% and 8.6% supplementation. However, no explanation was given for this decrease.

In our work, the feed intake was higher in the treatments with 2% and 3% supplementation of *N. limnetica*. This is in contrast with other studies, where either no effect or a decrease in feed intake has been found (Halle et al., 2009; Ao et al., 2015; Ekmay et al., 2015; Neijat et al., 2016). The feed intake in this study was, compared to the reference diet, increased 7 or 6 g per hen per day when fed the C or D diets respectively. In practice, this difference results in a serious increase of the required feed and increased feeding costs. However, the rate of lay is improved in the C and D diets as well thus, although a detailed cost-benefit analysis was not included, the revenues for the farmer will also be higher. Furthermore, laying hens usually have a lower feed intake at the beginning of the production period, which is usually lower than required for maintenance and production. Thus, a voluntary increased feed intake is positive for the short term (increase of production) and the long term (persistency). This study did not find any effects of the microalgae on the feed conversion ratio nor on the average egg weight. Similar results have been found by other studies (Halle et al., 2009; Ao et al., 2015; Neijat et al., 2016; Wu et al., 2019).

Table 8 *Comparison of experiments studying the influence of microalgae on laying hen performance.*

Study	Algae researched	Body weight	Feed intake	Rate of lay	Egg weight	Feed conversion ratio
Current study	1%, 2%, 3% of *N. limnetica*	No effect	Higher with 2% and 3% supplementation	Higher with 2% and 3% supplementation	No main effect, interaction with week	No effect
Wu et al. (2019)	1%, 2%, 4%, 8% *Nannochloropsis* spp.	n.d.	No effect	No effect	No effect	No effect
Neijat et al. (2016)	0.20%, 0.40%, 0.60% of microalgal DHA	No effect	Decreasing with increasing MA	No effect	No effect	n.d.
Park et al. (2015)	0.5%, 1% *Schizochytrium*	n.d.	n.d.	Increasing with increasing supplementation	No effect	n.d.
Ekmay et al. (2015)	11.7% *Desmodesmus* spp. (defatted) or 11.7% *Staurosira* spp. (full-fatted)	No effect	Lower with supplementation	No effect	No effect	n.d.
Ao et al. (2015)	1%, 2%, 3% Whole-cell microalgae (All-G-Rich)	No effect	Lower with supplementation	No effect	No effect	No effect
Lemahieu et al. (2013)	125 mg, 250 mg per 100g *Phaeodactylum tricornutum, Nannochloropsis oculata, Isochrysis galbana, Chlorella fudca*	Lower when supplemented with *N. oculata*	No effect	No effect	No effect	n.d.
Bruneel et al. (2013)	5%, 10% *Nannochloropsis gaditana*	No effect	Tended to be lower with 10% supplementation	No effect	No effect	n.d.
Zheng et al. (2012)	1 mg, 2 mg *C. vulgaris* per kg	n.d.	No effect	Higher with supplementation	No effect	n.d.
Halle et al. (2009)	2.5g 5.0g, 7.5g per kg; spray dried (SD) powder or bullet milled and spray dried (BM) powder *C. vulgaris*	n.d.	Lower when supplemented with inclusion of 7.5g BM powder	No effect	No effect	No effect
Ginzberg et al. (2000)	5%, 10% *Porphyridium*	No effect	10% reduction	No effect	No effect	n.d.

4.3 Eggs

4.3.1 Egg characteristics

In this study, no effects were found of the inclusion of microalgae on the shell, yolk or albumin weights or the ratio of these egg components. This is in line with other egg characteristics studies where no differences were found (Fredriksson et al., 2006; Lemahieu et al., 2013; Ao et al., 2015; Ekmay et al., 2015; Neijat et al., 2016; Wu et al., 2019). Even though it has not been recorded in this study, the colour of the yolk was affected by the inclusion of the microalgae (Figure 4). Some of the yolks were visually observed to be green or have some green hints and it may be that, without proper information, egg consumers will be hesitant to buy and consume such green eggs. In this work we did not study which level of *N. limnetica* and which specific compound caused the yolk colouring. It could be possible to use other natural colourings in the diet (i.e. carotene) to counteract the green colouring. Other studies also found differences in yolk colour when providing microalgae (Ginzberg et al., 2000; Halle et al., 2009; Zheng et al., 2012; Bruneel et al., 2013; Wu et al., 2019). For example, Wu et al. (2019) observed big differences in yolk colour in all treatments receiving feed supplemented with *Nannochloropsis* sp. However, these authors did not report green colours but only a colour shift from yellow to orange-red. Lemahieu et al. (2014) did report a red-greenness yolk colour after supplementation with 4 different microalgae species. The yolk colour is important for sale targets, whereas the favourable colouring differs per country. A green colouring may not be favourable for sale purposes, or might need another way of branding, and should therefore be researched further.

Figure 4: *Observation of green colour of the yolk.*

4.3.2 Fatty acid composition

This study showed clear effects of the inclusion of *N. limnetica* on the EPA and in particular the DHA content in the yolk. Consequently, the total omega 3 content of the egg is affected by the microalgae inclusion levels as well. Results reported by Nitsan et al. (1999) showed that addition of 1.0% *Nannochloropsis spp.* to the diet elevated the DHA content of the eggs by almost 25%. Wu et al. (2019) also studied *Nannochloropsis spp.* supplementation (1%, 2%, 4% and 8%) in laying hens' diets. Similar to the results of current study, they also reported increasing DHA and EPA content while increasing the supplementation in the diet. It should be noted that the study of Wu et al. (2019) did not find any EPA within the 1% and 2% supplementation. Increasing levels of DHA levels are also found in the study of Ao et al. (2015) upon supplementing increasing levels of a commercial microalgae. Similar results were found by Lemahieu et al. (2013), who tested 4 microalgae (*Phaeodactylum tricornutum, Nannochloropsis oculata, Isochrysis galbana, Chlorella fudca*) with 2 inclusion levels (0.125% and 0.250%). Three out of the four microalgae resulted in higher DHA in the eggs while the dose increased. Only the *I. galbana* did not differ between the two doses but was higher than the control group without

microalgae. Furthermore, the EPA content increased with increasing doses of microalgae included in the diets, for three of the tested microalgae. Only in the *C. fudca* treatment EPA was not found at all. Furthermore, that study reported DPA in all microalgae enriched eggs. DPA is an intermediate in the conversion process of EPA to DHA. According to Nitsan et al. (2019) and Lemahieu et al. (2013), it seems that microalgal omega 3 fatty acids are first converted to DHA, before those fatty acids are deposited in the egg yolk, due to the much higher amounts of DHA compared to EPA found in the eggs. This hypotheses and results are confirmed by Bruneel et al. (2013), who also found low levels of EPA and high levels of DHA. The results of the current study contribute to this hypothesis as well, since also in this study the DHA levels were much higher than the EPA levels. However, the levels in eggs found in this study (DHA: 20.5 – 53.9 mg/egg and EPA: 0.7 – 5.3 mg/egg), are a bit lower, but comparable to levels found by Lemahieu et al. (2013) (approx. DHA: 48 - 67 mg/egg and approx. EPA: 3.1 – 6.2 mg/egg). On basis of dry yolk our results are considerably higher than the levels found in the studies by Wu et al. (2019) (average DHA: 42.0 – 111.6 mg/g yolk and average EPA: 7.75-14.92 mg/yolk).

As figure 1 shows, the DHA and EPA content did not plateau yet upon increases algae content. Thus, the optimum of *N. limnetica* inclusion in laying hen diets to increase the DHA and EPA content in the yolk is considerably higher than 3%, based on the amount of algal omega-3 fatty acids included in the feed. Wu et al. (2019) supplemented the diets with 1%, 2%, 4% and 8% of *Nannochloropsis spp*., and saw the DHA content plateau in time for each group but on different levels, dependent on the initial level that was provided in the diet. The study of Wu et al. (2019) did not report quadratic testing of the doses. However, the results show that inclusion of 4% and 8% is possible and indicate that higher levels of microalgae result in similar dynamics, but at a different level. Nevertheless, the effects on performance are not properly researched when including even higher amounts of microalgae, thus these possible consequences should be studied before using high inclusions of microalgae in practice. Furthermore, high inclusion is also dependent on the amount and bioavailability of omega 3 fatty acids in the microalgae. Based on the average intake of feed and the amount of eggs per time period, the estimated efficiency of algal EPA + DHA deposition in the eggs was about 35% for all algal inclusion levels (data not shown). This value indicates a quite well bioavailability of the algal biomass which was only dried before inclusion in the feed and is higher than the efficiency reported by Lemahieu et al. (2013), who found a 20% efficiency of *Nannochloropsis.*

4.4 Intestinal histomorphometry and systemic inflammatory markers

To screen for any detrimental effects due to the experimental diets, in this study the intestinal histomorphometry properties and changes in concentration levels of APP (haptoglobin) and cytokines (interferon-γ; data not shown as the measured concentrations were lower than detection limit; and IL-13) in blood of laying hens fed with the experimental diets were recorded and compared with the reference dietary group. This approach was undertaken because (intestinal) tissue injury induces inflammatory reactions consisting of a series of complex physiological events occurring in the host to negate the offend. A salient purposes of these events is to prevent further tissue damage and restore the homeostasis of the host organism. Studying the changes in the intestinal histomorphometry provide the direct evidence of any tissue damage imparted by the dietary treatments. Whereas the measurement of changes in concentration levels of APP and IL13 is an indirect effect of intestinal injury. Moreover, the early sets of reactions that occur immediately after tissue damage are known as the acute phase response. Acute phase response includes pro-inflammatory cytokines and acute phase protein production. Acute phase response includes pro-inflammatory cytokines and acute phase protein (APP) production (Petersen et al., 2004). The APP are blood proteins primarily synthesized by the liver, in which the concentration of APP change to infection, inflammation, surgical trauma, or stress (Murata et al., 2004; Gruys et al., 2005). Many advances in monitoring the APP response in animals for clinical and experimental purposes have been achieved (Eckersall and Bell, 2010). On exposure to various inflammatory conditions, white blood cells release pro-inflammatory cytokines, which are the major mediators of APP synthesis in the liver and are essential for the recruitment of neutrophils to the site of inflammation (Ananian et al., 2005). One of the major pro-inflammatory cytokines is interferon-γ (IFN-γ), which activate macrophages and promote cell-mediated immune responses against invasive

intracellular pathogens (Rich et al., 2012). In addition, IL-13 is suspected to be the central mediator of the physiologic changes induced by (allergic) inflammation in many tissues and known to suppresses the production of pro-inflammatory cytokines and other cytotoxic substances by macrophages, fibroblasts, and endothelial cells (Minty et al., 1993). Chickens, similar to other animals, produce APP during inflammation and diseases. Several research studies have been published about APP and their changes due to different inflammatory and non-inflammatory conditions in birds (Nielsen et al., 1999; Henry et al., 2000; Koutsos and Klasing, 2001; Barnes et al., 2002; Juul-Madsen et al., 2003). However, the importance of and sensitivity towards the evaluation of APP and inflammatory cytokines in the screening of feed-ingredients or -additives for hazardous effect mounted in the host as a response of dietary inclusion are not clearly known.

In this study, neither the rapeseed challenge, nor the microalgae affected the morphology of the jejunum or the colon. Usually, it is considered that the gut morphometry influences the utilization of the feed and it is believed that increased intestinal villi length and crypt depth could result in an better feed utilization (Uni et al., 1999; Sklan, 2019). Other studies did find improvements of the gut morphology with inclusion of microalgae in chicken feed. Increased jejunum villus heights and crypt depth were reported in broilers fed with a basal diet supplemented with *Chlorella* by-products included at 1%, (Mirzaie et al., 2020) or at 2.5%, 5% and 7.5% (Kang et al., 2017). However, Mirzaie and colleagues observed no significant difference in the intestinal histomorphometry in the birds fed with a diet that included 2% *Chlorella* by-products (Mirzaie et al., 2020). By combining the results from this study with the previous studies it seems that the impact of microalgae on intestinal histomorphometry is dependent on the type and inclusion levels of microalgae. However, further research is warranted with graded levels of microalgae included in chicken diets to verify and validate the observed results.

We didn’t measure concentration levels of IFN-γ cytokine above the detection limit in blood of birds that received treatment A, D or E diet. For IL-13 no significant differences were recorded for treatment D and E, as compared to reference diet A. These observation of both cytokine & chemokine measure confirms that the dietary treatments neither promoted cell-mediated immune response nor elicited any physiologic changes induced by inflammation in tissues. Furthermore, we did not record any signs of inflammation or signs of any patho-physiology events in the histomorphometry that further stand in line with the observation made for cytokines & chemokines. Additionally, we observed significant lower concentration level of haptoglobin in the treatment group D and E compared to the reference diet i.e. treatment A. Although the variability between individual samples was high for treatment A, the results indicate that treatment D and E resulted in a different inflammatory response than treatment A. Higher concentration levels of haptoglobin reflects the inflammatory or infectious status due to microbes (pathogenic bacterial, protozoal or viral loads) in broilers (Garcia et al., 2009; Georgieva et al., 2010; Asasi et al., 2013). However, quails infected with fungal (aspergillosis) infection recorded lower haptoglobin levels compared to the non-infected groups (Goetting et al., 2013). This suggest that in birds, discrepancies in the direction of haptoglobin change might depend on the type of infection.
Although the direction of haptoglobin change of this study matches to that of fungal infection study in quail (Goetting et al., 2013), in our study we didn’t encountered heavy mortality or any other signs that are indicative of any negative impact on health or productive performance of the layers. Taken together, the results observed in histomorphometry, IL13 and haptoglobin suggest that the experimental diets most likely do not contain hazardous substances that could negatively impact the intestinal tissue or markers of inflammation at such alarming levels that it would compromise the health or productive performance in layers during the 4-weeks period of the experiment.

5 Conclusions

From this study, in which the effects of increasing inclusion of *N. limnetica* biomass in laying hen diets on performance, digestibility, fatty acid composition of the yolk, intestinal morphometry and systemic inflammatory markers were determined, it can be concluded that:

- The rate of lay was higher when 2% or 3% *N. limnetica* was provided.
- Feed intake was increased in the treatments with 2% and 3% supplementation of the microalgae. This was possibly caused by the lower crude fat content of the algae diets.
- Diet digestibility was not affected by the inclusion of the microalgae.
- Inclusion of 3% microalgae resulted in lower haptoglobin levels in both the control and rapeseed diets.
- DHA and EPA content of the yolk increase with increasing supplementation of the microalgae.
- Although there are no official guidelines for the intake of long-chain omega-3 fatty acids for humans yet, a minimum of 160- 250 mg and a maximum of 3,000 mg of combined EPA and DHA per day is indicated for human consumption. Consumption of omega-3 enriched eggs can contribute to such an intake.

In this study we did not reach the optimal inclusion level of *N. limnetica* in laying hen diets to affect the omega 3 fatty acids in the yolk. According to this study, inclusion levels of *N. limnetica* up to 3% can be used in laying hen diets, since no negative effects are found in digestibility or morphometry of the gut. However, the literature available on these topics is scarce, thus further research confirming these results are necessary. It seems that including *N. limnetica* in the diet, resulted in a greenish yolk colour which might be unfavourable from a consumers' perspective. Further research should focus on the yolk colour, consumer willingness and possible counteracts (i.e. by supplementing orange pigments).

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Appendix 1 Fatty acids composition microalgae and dietary treatments

Table 9 *Fatty acid composition of N. Limnetica (g/kg) and dietary treatments (g/kg fat).*

Treatment[1]	*N. Limnetica*	A	B	C	D	E	Treatment[1]	*N. Limnetica*	A	B	C	D	E
Fatty acid							*Fatty acid (continued)*						
anteiso-C15:0	<0.1	0	0	0	0	0	C20:3n3	0	0	0	0	0	0
anteiso-C16:0	<0.1	0	0	0	0	0	C20:3n6	0.6	0	0	0	0	0
anteiso-C17:0	<0.1	0	0	0	0	0	C20:4n3	0.1	0	0	0	0	0
C10:0	0.2	0	0	0	0	0	C20:4n6	9.1	0	1.5	3	4	3
C10:1	0	0	0	0	0	0	C20:5n3 EPA	35.8	0	5	9	14	11
C11:0	<0.1	0	0	0	0	0	C21:0	0	0	0	0	0	0
C12:0	0.3	3.5	3.5	3	3.5	3	C22:0	<0.1	1.5	1.0	1.0	1.5	1.0
C12:1	0	0	0	0	0	0	C22:1n11	0	0	0	0	0	0
C14:0	4.8	7.0	7.5	7.5	8.0	8.0	C22:1n9	0	0	0	0	0	0
C14:1n5	<0.1	0	0	0	0	0	C22:2n6	0	0	0	0	0	0
C14:1n9	<0.1	0	0	0	0	0	C22:3n3	<0.1	0	0	0	0	0
C15:0	0.8	0	0	0	0	0	C22:4n6	0	0	0	0	0	0
C15:1	<0.1	0	0	0	0	0	C22:5n3	<0.1	0	0	0	0	0
C16:0	30.9	308	300.5	278.5	270	288.5	C22:5n6	<0.1	0	0	0	0	0
C16:1n7	41.5	2.0	6	11	17	14	C22:6n3 DHA	<0.1	0	0	0	0	0
C16:1n9	0.1	0	0	0	0	0	C23:0	<0.1	0	0	0	0	0
C16:3	0	0	0	0	0	0	C24:0	<0.1	1	1	1	0.5	1
C16:4	0.1	0	0	0	0	0	C24:1n9	0	0	0	0	0	0
C17:0	0.3	1	1	1	1	0.5	C8:0	0	0	0	0	0	0
C17:1	0.8	0	0	0	0	0	C9:0	0	0	0	0	0	0
C18:0	0.4	33	32	29.5	29	30	CLA 10trans 12cis	<0.1					
C18:1 trans	0.1	0	0	0	0	0	CLA 9cis 11trans	0					
C18:1n other	0.7	8	8	7	8	15	Monounsaturated	64.9	319.0	320.5	308.5	310.5	333.0
C18:1n9	21.5	306.0	302.0	285.5	281	299	Iso C14:0	<0.1	0	0	0	0	0
C18:2 trans	0.9	0	1.5	0	1.5	2	iso-C15:0	<0.1	0	0	0	0	0
C18:2n6	5.9	242.0	248.5	243.5	251	218.5	iso-C16:0	0	0	0	0	0	0
C18:3n3	0	11	11	11	11	12	iso-C17:0	<0.1	0	0	0	0	0
C18:3n6	0.9	0	0	0	0	0	iso-C18:0	0.3	0	0	0	0	0
C18:4n3	<0.1	0	0	0	0	0	Polyunsaturated	54.0	256	268.5	267.5	282.5	248.0
C19:0	<0.1	0	0	0	0	0	Omega 3	36.2	12	17	20	25	24
C20:0	<0.1	4.0	3.5	3.5	3.5	3.5	Omega 6	16.7	243	250.5	246.5	256.0	222.0
C20:1n11	0	0	0	0	0	0	Omega 9	21.7	308.5	305.0	288.5	284.0	302.0
C20:1n9	0	2	2	2	2	1.5	Saturated	38.8	360.5	352.5	327	319.5	338.0
C20:2n6	0.1	0	0	0	0	0	Trans	1.2	0	2	0.5	2	2.5

[1] Treatment: A = control diet + 0% microalgae; B = control diet + 1% microalgae; C = control diet + 2% microalgae; D = control diet + 3% microalgae

Appendix 2 Experimental diets

Table 10 *Dietary ingredients, and calculated nutrients of the experimental diets (g/kg, as-fed basis).*

Diet[1]	CD	CD3	RS3
Ingredient			
Maize	400.0	400.0	400.0
Wheat	205.5	204.7	151.0
Sunflower meal	125.0	125.0	71.0
Soybean meal	115.2	96.2	45.4
Limestone	73.0	73.4	71.8
Palm oil	36.1	27.3	38.8
Chalk	20.0	20.0	20.0
Monocalcium phosphate	5.0	4.2	3.3
Premix[2]	5.0	5.0	5.0
Titanium dioxide	5.0	5.0	5.0
Salt	2.6	2.0	2.2
Phytase 1	2.0	2.0	2.0
L-Lysine HCl	2.0	2.1	1.8
Sodium-Bicarbonate	1.5	0.9	0.6
DL-Methionine	1.4	1.5	1.2
Phytase 2	0.5	0.5	0.5
L-Threonine	0.2	0.2	0.0
Rapeseed meal	0.0	0.0	150.0
L-Isoleucine	0.0	0.1	0.4
N. limnetica	0.0	30.0	30.0
Total	1000.0	1000.0	1000.0
Calculated content[3]			
AME_n (MJ/kg)	11.51	11.52	11.45
DM	891	891	892
Crude ash	128	127	127
Crude protein	161	161	161
Crude fat	58	56	69
Crude fibre	37	37	43
Starch	379	379	354
Sugar	30	29	33
NDF	113	112	129
NSP	153	157	172
Dig. Lys	6.90	6.90	6.90
Dig. Met+Cys	6.10	6.10	6.10
Dig. Thr	4.80	4.80	4.80
Dig. Trp	1.50	1.50	1.50
Na	1.5	1.5	1.5
K	6.7	6.6	6.3
Cl	2.5	2.5	2.5
DEB (mEq/kg)	168	164	157
Ca	38.0	38.0	38.0
Total phosphorus	4.9	4.9	5.3
Available phosphorus	2.8	2.8	2.8

[1] Diets: CD = control diet 0% microalgae; CD3 = control diet + 3% microalgae; RS3 = exchange of soybean by rapeseed + 3% microalgae.

[2] Provided per kilogram of complete diet: vitamin A 10.000 IE; vitamin D_3 2.000 IE; vitamin E 25 mg; vitamin K_3 1.5 mg; vitamin B_1 1.0 mg; vitamin B_2 3.5mg; vitamin B_6 1.0 mg; vitamin B_{12} 15 µg; niacin 30 mg; D-pantothenic acid 12 mg; choline chloride 350 mg; folic acid 0.8 mg; biotin 0.1 mg; iron 50 mg; copper 10 mg; manganese 60 mg; zinc 54 mg; iodine 0.7 mg; selenium 0.1 mg.

[3] CVB matrix values (CVB, 2011) were used for diet formulations.

Appendix 3 Fatty acid composition of the egg yolk

Table 11 *Fatty acid composition of the yolk (mg g dry yolk) of eggs in treatments with increasing addition of microalgae.*

Treatment[1]	A	B	C	D	Treatment[1]	A	B	C	D
Fatty acid					*Fatty acid (continued)*				
anteiso-C15:0	<0.1	<0.1	<0.1	<0.1	C20:3n3	<0.1	<0.1	<0.1	<0.1
anteiso-C16:0	<0.1	<0.1	<0.1	<0.1	C20:3n6	1.1	1.1	1.0	1.0
anteiso-C17:0	<0.1	<0.1	<0.1	<0.1	C20:4n3	<0.1	<0.1	<0.1	<0.1
C10:0	<0.1	<0.1	<0.1	<0.1	C20:4n6	9.5	9.4	9.2	9.0
C10:1	<0.1	<0.1	<0.1	<0.1	C20:5n3 EPA	0.1	0.4	0.6	0.7
C11:0	<0.1	<0.1	<0.1	<0.1	C21:0	<0.1	<0.1	<0.1	<0.1
C12:0	<0.1	<0.1	<0.1	<0.1	C22:0	<0.1	<0.1	<0.1	<0.1
C12:1	<0.1	<0.1	<0.1	<0.1	C22:1n11	<0.1	<0.1	<0.1	<0.1
C14:0	2.2	2.3	2.3	2.2	C22:1n9	<0.1	<0.1	<0.1	<0.1
C14:1n5	0.7	0.7	0.7	0.7	C22:2n6	<0.1	<0.1	<0.1	<0.1
C14:1n9	<0.1	<0.1	<0.1	<0.1	C22:3n3	<0.1	<0.1	<0.1	<0.1
C15:0	0.3	0.3	0.4	0.4	C22:4n6	<0.1	<0.1	<0.1	<0.1
C15:1	<0.1	<0.1	<0.1	<0.1	C22:5n3	0.3	0.5	0.8	0.9
C16:0	151.0	152.5	152.5	152.9	C22:5n6	3.6	3.0	2.3	1.9
C16:1n7	22.6	22.9	24.0	25.2	C22:6n3 DHA	2.8	4.5	6.0	7.5
C16:1n9	2.9	2.9	2.8	2.9	C23:0	<0.1	<0.1	<0.1	<0.1
C16:3	<0.1	<0.1	<0.1	<0.1	C24:0	0.3	0.1	0.1	0.1
C17:0	0.7	0.7	0.7	0.7	C24:1n9	<0.1	<0.1	<0.1	<0.1
C16:4	<0.1	<0.1	<0.1	<0.1	C8:0	<0.1	<0.1	<0.1	<0.1
C17:1	0.4	0.5	0.5	0.5	C9:0	<0.1	<0.1	<0.1	<0.1
C18:0	46.4	46.7	47.6	46.9	CLA 10trans 12cis	0.3	0.3	0.3	0.2
C18:1 trans	1.0	1.0	0.9	0.9	CLA 9cis 11trans	0.2	0.2	0.2	0.3
C18:1n other	12.1	12.2	12.0	12.6	Monounsaturated	265.0	266.4	264.9	267.8
C18:1n9	223.4	224.5	222.2	223.4	Iso C14:0	<0.1	<0.1	<0.1	<0.1
C18:2 trans	0.7	0.7	0.6	0.6	iso-C15:0	<0.1	<0.1	<0.1	<0.1
C18:2n6	60.0	60.0	57.5	57.8	iso-C16:0	<0.1	<0.1	<0.1	<0.1
C18:3n3	1.2	1.2	1.2	1.3	iso-C17:0	0.2	0.2	0.1	0.1
C18:3n6	0.6	0.6	0.6	0.6	iso-C18:0	<0.1	<0.1	<0.1	<0.1
C18:4n3	<0.1	<0.1	<0.1	<0.1	Polyunsaturated	81.1	82.7	81.1	82.6
C19:0	0.1	0.1	0.1	0.1	Omega 3	4.3	6.7	8.7	10.6
C20:0	0.2	0.2	0.2	0.2	Omega 6	75.6	74.7	71.3	70.9
C20:1n11	0.2	0.3	0.2	0.2	Omega 9	228.0	229.0	226.6	227.7
C20:1n9	0.2	0.2	0.3	0.3	Saturated	201.7	203.5	204.2	203.9
C20:2n6	1.7	1.6	1.5	1.5	Trans	2.2	2.2	2.0	2.0

[1] Treatment: A = control diet + 0% microalgae; B = control diet + 1% microalgae; C = control diet + 2% microalgae; D = control diet + 3% microalgae

Appendix 4 Mean performance and egg characteristics of treatment E

Variable	Mean treatment E
Laying hen performance	
Average body weight per hen (g)	
Start	1161
End (28 days)	1463
Growth	302
Feed intake (g/hen/day)	
wk 1	104
wk 2	130
wk 3	125
wk 4	131
Average	122
Rate of lay (%)	
wk 1	23.2
wk 2	88.7
wk 3	92.6
wk 4	95.8
Average	75.1
Average egg weight per egg (g)	
wk 1	48.1
wk 2	54.0
wk 3	57.3
wk 4	57.0
Average	54.1
Feed conversion ratio (%)	
wk 1	9.92
wk 2	2.73
wk 3	2.35
wk 4	2.79
Average	4.45
Egg characteristics	
Fresh weights (g per egg)	111
Shell	7.4
Yolk	13.9
Albumin	35.0
Ratio (%)	
Shell	13.0
Yolk	24.8
Albumin	62.4
Fatty acids Egg (g per 100 g dry yolk)	
EPA	0.08
DHA	0.81
Monounsaturated	27.12
Polyunsaturated	8.85
Saturated	20.03
Omega 3	1.16
Omega 6	7.57
Omega 9	23.40

To explore the potential of nature to improve the quality of life

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