Retention of carotenoids in egg yolks of laying hens supplemented with heterotrophic *Chlorella*

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ABSTRACT: The present study investigated the effect of 1 and 2% feed supplementation with biomass of *Chlorella* grown through heterotrophic fermentation on the concentration of total and individual carotenoids in egg yolks. A total of twenty-four Hisex Brown laying hens aged 56 weeks were included in the experiment. The layers were kept individually in cages. They were divided into three groups of eight birds and fed a diet typical for laying hens. Control birds (C) received only a basal diet, while experimental diets (P1 and P2) were supplemented with 1 and 2% (i.e. 10 and 20 g/kg) dry disintegrated *Chlorella* biomass. Egg yolk deposition of total carotenoids was significantly ($P < 0.01$) increased by 46% (P1) and 119% (P2). The rising curves of total carotenoids reached their plateau during the fourth experimental week. The respective values oscillated around 25 mg (P1) and 40 mg (P2) per g of yolk during the following weeks. Lutein and zeaxanthin were equally deposited and they represented more than 90% of total carotenoids in yolk. The deposition of carotenoids significantly ($P < 0.01$) increased the colour characteristics of yolks measured using the Roche Yolk Colour Fan scale. Supplementation with *Chlorella* biomass significantly decreased the egg yolk weight of P2 in comparison with P1 ($P < 0.05$) and C ($P < 0.01$). These effects were probably related to lower feed consumption in these hens. The daily feed intake per hen, as well as its consumption per egg, was lower by 5–7 g in both supplemented groups. Recalculation of the diet consumption per kg of egg and yolk mass eliminated these differences. There were no differences among laying hens in plasma concentrations of triacylglycerol and cholesterol.

Keywords: lutein; zeaxanthin; enriched eggs; *Chlorella* algae; layers

Modulation of egg yolk colour characteristics by using diet components or feed additives has long been employed in farming laying hens. Synthetic carotenoids have extensively been used in many European countries since the 1990s in order to meet the requirements of consumers preferring eggs with colourful yolks. The current aim is to achieve the same effects using natural feed additives. Both carotenoids and oxycarotenoids, such as lutein and zeaxanthin, have been received more attention. Apart from the colouring effects, these "yellow" pigments have important biological functions. For example, lutein has antioxidant and immunomodulatory functions and positively influences the developing embryo as well as the hatched chick (Surai and Sparks, 2000; Koutsos et al., 2003). Higher concentrations of lutein in the diet of laying hens enhance the immune response of birds vaccinated against infectious bronchitis (Bedecarrats and Leeson, 2006). Lutein can also influence male reproduction parameters because its addition significantly increases sperm viability (Pizzei and Bedecarrats, 2007).

Data on the importance for human health attracted interest in both pigments. Evidence has accumulated gradually since the end of the 1990s that both xanthophylls belong to the so-called macular pigments that protect the retina by selective light filtration. The majority of studies evaluated the

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association between the nutritional intake of lutein and protection of vision against age-related macular degeneration (Landrum et al., 1997; Granado et al., 2003; Resnikoff et al., 2004; Vishwanathan et al., 2009). Importantly, both pigments are known to bear significant antioxidant effects in relation to cardiovascular diseases (Ribaya et al., 2004). The high content of lutein and zeaxanthin in egg yolks can increase their nutritional value due to their good bioavailability (Handelmann et al., 1999) and eggs can then be considered functional foods, the so-called "designer eggs" (Bedecarrats and Leeson, 2006). Typical diets for laying hens, in particular those based on wheat, do not provide adequate levels of lutein and zeaxanthin. Various additives containing these substances are therefore used. Natural sources employed for this purpose include, for example, microalgae (Frederiksson et al., 2006), plant extracts (Karadas et al., 2006), and different species of carrots (Hammershoj et al., 2010).

In the present study, we used the freshwater algal species *Chlorella* as a source of carotenoids for laying hens. It is known that chlorococcal algae grown in an autotrophic way (i.e. outdoors) concentrate many plant pigments. They store not only chlorophyll but also a wide range of carotenoids including β-carotene. In the feed industry, they are donors of provitamin A as well as carriers of organic-bound trace elements such as selenium and iodine (Kotrbáček et al., 2004; Skřivan et al., 2006; Doucha et al., 2009; Svoboda et al., 2009). Under conditions of heterotrophic culture (i.e. without sunlight) they synthesize less chlorophyll but some strains store more xanthophylls, including lutein (Shi et al., 1997), zeaxanthin, and astaxanthin (Sun et al., 2008).

The aim of the present study was to evaluate the possible use of algae grown heterotrophically as a natural source of xanthophylls in commercial diets for laying hens. We were interested in the dynamics of deposition of algal pigments into the yolk mass, their total concentration and spectrum following supplementation of birds with different levels of *Chlorella* in feeds. Experiments also determined the effects of supplementation on selected parameters of lipid metabolism of laying hens and on their production parameters.

MATERIAL AND METHODS

A total of twenty-four Hisex Brown laying hens aged 56 weeks were divided into three groups per eight birds and fed a standard diet for laying hens (Table 1). Control (C) received only a basal diet, while experimental diets (P1 and P2) were supplemented with 10 and 20 g/kg of dry *Chlorella* biomass. Table 1 presents the nutritional composition of basal and supplemented diets, including the total content of carotenoids. The *Chlorella* biomass was produced in the Institute of Microbiology (Academy of Sciences of the Czech Republic, Třeboň). Heterotrophic

Table 1. Ingredients and chemical composition of basal diet[a]

Ingredient	(g/kg)
Wheat	330
Maize	300
Soybean meal	240
Soybean oil	20
Dicalcium phosphate	10
Sodium chloride	3
Limestone	93
DL-Methionine	2
Vitamin-mineral premix[b]	2
Analyzed nutrient contents	
Dry matter	89.6
Crude protein	163.7
Crude fat	46.3
Crude fibre	18.9
Ash	116.6
Calcium	40.3
Phosphorus	5.9
Total carotenoids (mg/kg)	5.1
ME_N (calculated) (MJ/kg)	15.5

[a]experimental diets were supplemented with 10 (P1) and 20 (P2) g/kg of dry *Chlorella* biomass. *Chlorella* contained 248 mg/kg of lutein and 241 mg/kg of zeaxanthin. First and second experimental groups contained 11.0 and 16.8 mg/kg of total carotenoids, respectively
[b]vitamin-mineral premix provided per kg of diet: vitamin A 3200 IU, vitamin D_3 1000 IU, α-tocoferol 20 mg, vitamin K_3 0.6 mg, vitamin B_1 1.6 mg, vitamin B_2 2.6 mg, vitamin B_6 1.2 mg, vitamin B_{12} 6 μg, biotin 60 μg, folic acid 1 mg, vitamin B_3 12 mg, calcium pantothenate 5 mg, betaine 100 mg, ethoxyquin 1.08 mg, manganese oxide 32 mg, zinc oxide 32 mg, copper sulphate 3.4 mg, potassium iodide 0.4 mg, sodium selenite 60 μg, cobalt sulphate 0.1 mg, glucanase 48 BGU, xylanase 2200 EXU

fed-batch cultivation (i.e. without sunlight) was used in accordance with Doucha and Lívanský (2001). The microalgal biomass was harvested by centrifugation, disintegrated, spray-dried, and stored in the dark at room temperature until use. Laying hens were kept individually in cages in a room with a 16-hour light-cycle. Feed and water were supplied *ad libitum*. Feed consumption was measured in individual groups at the end of the first experimental month and on day 54, i.e. at the end of the experiment. Live weights were measured individually at the start, after one month, and at the end of the experiment. The number of eggs and hens and their health status were observed daily.

Laboratory analyses

Individual laying hens were examined with regard to the egg weights and the egg yolk weights. Colour characteristics of egg yolks were evaluated according to the colour scale of Roche Yolk Colour Fan (RYCF). Egg yolks were then lyophilized and mixed with the others from the weekly egg production of individual hens in order to determine the content of total and individual carotenoids with the high performance liquid chromatography (HPLC) using the Agilent 1100 Series system equipped with a UV-VIS diode-array detector (Agilent Technologies, Santa Clara, USA). Pigments were separated using a modified method of Van Heukelem and Thomas (2001). The peak assignment was based on comparison of spectral characteristics with the known retention behaviour of photosynthetic pigments in reverse phase system and confirmed by characteristic mass spectral ions.

Prior to the experiment, hens had blood samples taken from the *vena basilaris* for the determination of triacylglycerols (TAG) and cholesterol (CL). These plasma values were also measured using an automatic analyser Konelab 20 (Thermo Electron Corporation, Vantaa, Finland) in samples obtained after one month and at the end of the experiment.

The feed dry-matter was determined by oven drying at 105°C to constant weight. The ash content of feed was determined by ashing at 550°C in a muffle furnace (AOAC, 1997). The fat content of feed was analyzed by extraction with petroleum ether in a Soxtec 1045 apparatus (Tecator Comp., Hoganas, Sweden). The crude protein content of feed was measured using a Kjeltec Auto 1030 Analyzer (Tecator Comp., Hoganas, Sweden). The calcium content of feed was analyzed in ashed samples by atomic absorption spectrometry (Solaar M-6; JTA Solutions, Cambridge, UK), and the phosphorus content was colourimetrically analyzed using a molybdate reagent (Huxtable and Bressler, 1973). The determination of the content of total carotenoids of feed was similar to that of egg yolks, described above.

Statistical analyses

Restricted sample sizes made it necessary to use non-parametric statistical methods. Differences between controls and experimental groups in individual parameters were tested using the Kruskal-Wallis test together with post-hoc tests according to Steel-Dwass. The development of values during the experiment was evaluated with the Friedman test for One-way repeated measures analysis of variance and post-hoc Wilcoxon-Nemenyi-McDonald-Thompson test (Hollander and Wolfe, 1999) using the code by Galili (http://www.r-statistics.com/2010/02/post-hoc-analysis-for-friedmans-test-r-code).

RESULTS

As shown in Figure 1A, the mean total carotenoids in egg yolks were already significantly higher in laying hens after one week of supplementation. The differences between controls (C) and groups P1 and P2 gradually increased during the next three weeks. Compared with controls, total carotenoids in egg yolks from groups P1 and P2 increased 1.7- and 2.4-fold, respectively by the end of the first month. These differences remained during the second month of the experiment with no considerable shifts in groups P1 and P2. The significant increase in total carotenoids was due to all pigments measured (Table 2), with lutein and zeaxanthin contributing most (Figure 1B, 1C). Including a small proportion of their *cis* forms, these pigments represented more than 90% of all carotenoids deposited. The proportion of β-carotene in egg yolks was low despite its dynamic rise dependent on the supplementation with *Chlorella* during the experiment. Figure 1D shows that there was a significant increase in β-carotene of the P2 group after one week, while three weeks of supplementation were necessary for the concentration rise in the P1 group.

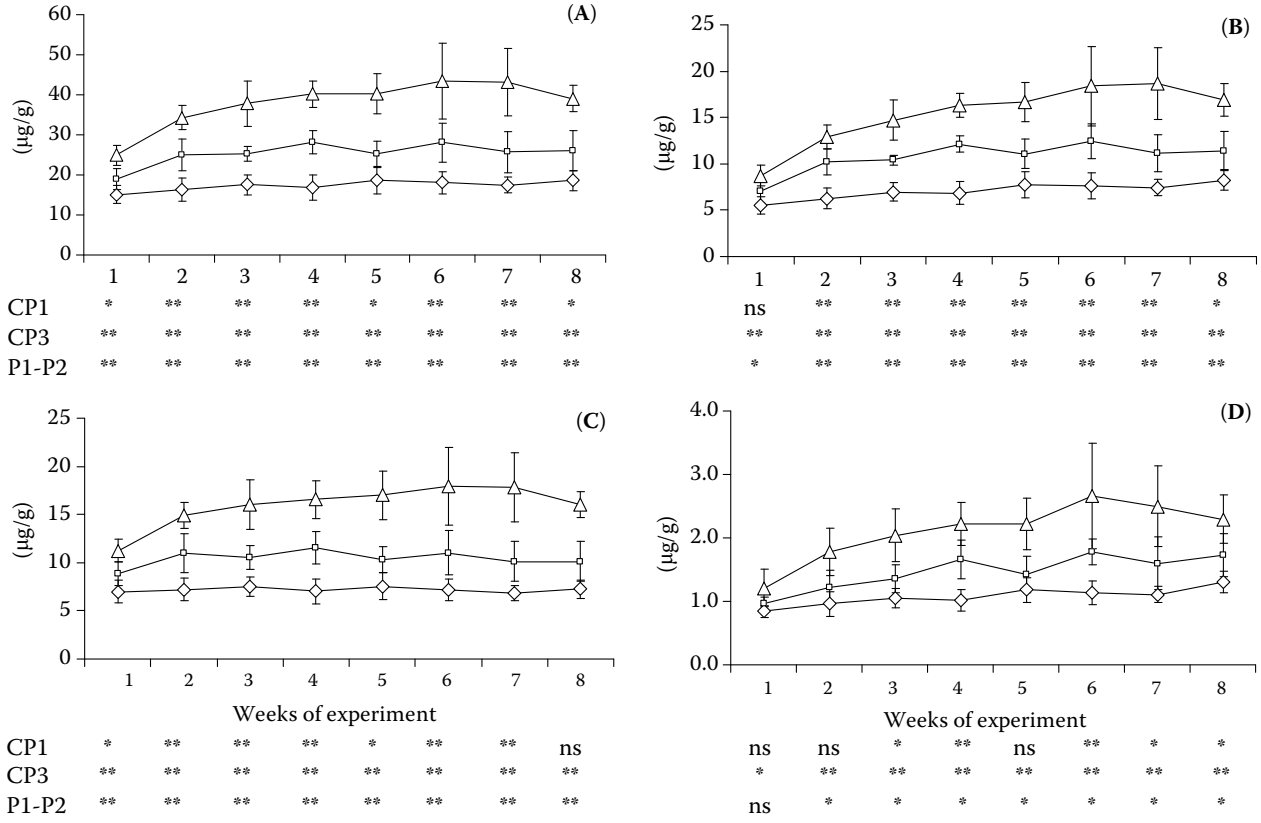

Figure 1. Means ± SD of carotenoids in egg yolk as a function of feeding time in control (◇), P1 (□ 10 g/kg of *Chlorella*), and P2 (Δ 20 g/kg of *Chlorella*) group: (**A**) total carotenoids, (**B**) lutein, (**C**) zeaxanthin, (**D**) β-carotene

ns = not significant

symbols indicate significant differences between groups in each week of experiment

$^{*}P < 0.05$, $^{**}P < 0.01$

There were no differences in plasma TAG and CL concentrations between hens supplemented with *Chlorella* and controls (Figure 2A, 2B). The rise in both parameters was observed at the end of the experiment in all groups of hens. Although it was higher in supplemented hens, differences were not of statistical significance due to data variability in these groups.

Table 2. Mean egg yolk carotenoids content (mean ± SD; μg/kg) during the whole experiment

Characteristic	C	P1	P2	Probability		
				C : P1	C : P2	P1 : P2
Total carotenoids	17.33 ± 1.218^{c}	25.30 ± 2.827^{b}	37.90 ± 6.008^{a}	< 0.001	< 0.001	< 0.001
Lutein	7.09 ± 0.861^{c}	10.72 ± 1.686^{b}	15.43 ± 3.289^{a}	< 0.001	< 0.001	< 0.01
Zeaxanthin	7.09 ± 0.247^{c}	10.44 ± 0.819^{b}	15.94 ± 2.145^{a}	< 0.001	< 0.001	< 0.001
β-carotene	1.07 ± 0.142^{c}	1.47 ± 0.281^{b}	2.12 ± 0.453^{a}	< 0.01	< 0.001	< 0.01
cis-Zeaxanthin	0.69 ± 0.030^{c}	1.00 ± 0.139^{b}	1.89 ± 0.358^{a}	< 0.001	< 0.001	< 0.001
cis-Lutein	0.85 ± 0.170^{c}	1.20 ± 0.159^{b}	1.55 ± 0.161^{a}	< 0.001	< 0.001	< 0.001
X carotenoids	0.46 ± 0.034^{c}	0.77 ± 0.092^{b}	0.97 ± 0.130^{a}	< 0.001	< 0.001	< 0.01

C = control group, P1 and P2 = treatment groups

^{abc}values in rows with different letters differ significantly

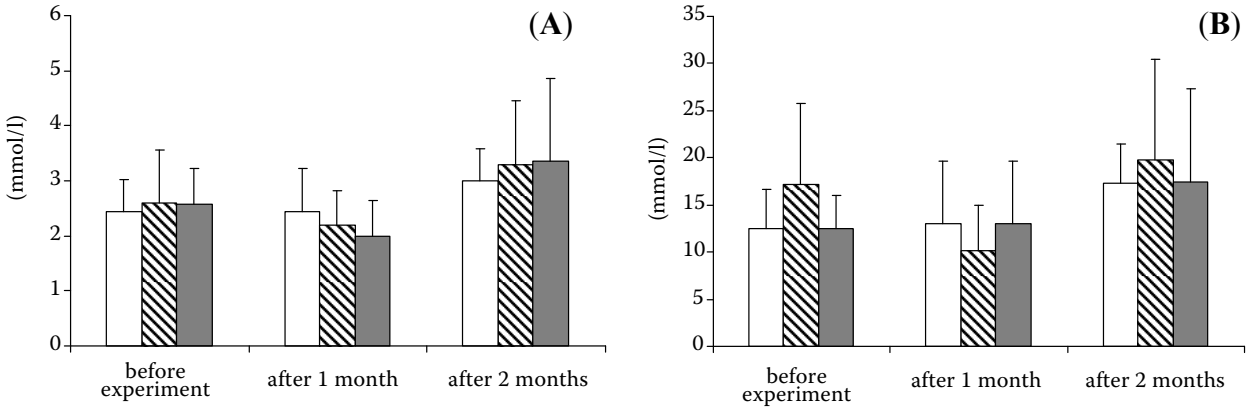

Figure 2. Means ± SD of blood parameters as a function of feeding time in control (□), P1 (▨ 10 g/kg of *Chlorella*), and P2 (■ 20 g/kg of *Chlorella*) group: (**A**) triacylglycerols, (**B**) cholesterol

Table 3 demonstrates that the colour of egg yolks increased significantly with the rising concentration of carotenoids ($P < 0.001$). While the mean RYCF grade reached 4.1 in controls, groups P1 and P2 were characterized by values 5.0 and 6.1, respectively. Table 3 also presents the egg weight and the yolk weight. The mean egg weight was higher in the control group than in both experimental groups P1 and P2. The differences were, however, not significant. Interestingly, weight of egg yolk was significantly lower in P2 birds when compared with controls ($P < 0.01$) and P1 birds ($P < 0.05$). A reduction in feed consumption, although not statistically significant, was observed in hens fed with *Chlorella*. Significant differences among C, P1, and P2 groups were not found in feed consumption per kg of egg mass (2.00, 2.02, and 2.05 kg, respectively) and per egg yolk (7.65, 7.47, and 7.68 kg, respectively). Throughout the experiment, the mean live-weight of hens was the highest and the lowest in the groups C and P2, respectively. Differences between groups were, however, not significant. Significant body weight changes during the experiment were found in hens from groups C and P2. While control birds were heavier at the end of the experiment (2.04 kg; $P < 0.05$), in P2 hens body weights decreased at the mid-period of the experiment (1.80 kg; $P < 0.05$).

DISCUSSION

As shown in Table 1, the lower content of total carotenoids in the standard diet for hens was due to its composition, because it contained a greater proportion of wheat and less corn, i.e. the source of the yellow pigments, xanthophylls. Addition of *Chlorella* resulted in more than two- and three-fold increases of total carotenoids in the diet for P1 and P2 hens, respectively (Table 1). The significant increase in total carotenoids of egg yolks produced by supplemented hens was, therefore, not surprising (Table 2). Changes in the present study occurred more slowly compared with effects of synthetic carotenoids on the colour characteristics of egg yolks and the speed of pigment deposition (Leeson and Caston, 2004). The effects are, however, comparable with those due to other natural sources of carot-

Table 3. Egg traits (mean ± SD) and statistical differences between groups

Characteristics	C	P1	P2	Probability		
				C : P1	C : P2	P1 : P2
Egg weight (g)	65.8 ± 1.07	64.2 ± 1.15	63.8 ± 1.50	ns	ns	ns
Yolk weight (g)	17.4 ± 0.30	17.3 ± 0.44	16.6 ± 0.48	ns	< 0.01	< 0.05
Yolk colour (RYCF value)	4.1 ± 0.55[a]	5.0 ± 0.62[b]	6.1 ± 0.86[c]	< 0.001	< 0.001	< 0.001

C = control group, P1 and P2 = treatment groups, ns = not significant
[abc]values in rows with different letters differ significantly

enoids; for example, after supplementation of 2 g/kg marigold extract or 20 g/kg alfalfa concentrate into wheat-barley diets fed to quails (Karadas et al., 2006). Use of the marigold extract for 23 days resulted in a total carotenoid content of 39 mg/g in egg yolks. This corresponds with findings of the present study in P2 hens. The supplement of alfalfa concentrate produced lower values, i.e. 22.4 mg/g of egg yolk. This result is comparable with our P1 group (Figure 1A). There were, however, differences in the composition of the carotenoids. While the above authors mention the prevailing content of lutein amounting to 80% of total carotenoids, our hens deposited lutein and zeaxanthin equally (Figure 1B, 1C). Different varieties of carrots were also used as a natural source of carotenoids in laying hens (Hammershoj et al., 2010). A two-week supplementation of commercial diets by Purple Haze carrots at the dose of 70 g per hen per day resulted in a maximum carotenoid value of 21 mg/g of egg yolk. Comparable results were achieved in this experiment in P1 birds early during the second week of the experiment and in P2 hens after one week of supplementation by algae. Figure 1A demonstrates that the rising curve reached the plateau level after about three weeks of supplementation. Compared with controls, in P1 and P2 hens the mean values of total carotenoids increased by 46 and 119%, respectively.

The efficacy of transfer of algal pigments into egg yolks was not decreased when using a diet supplemented with higher levels of *Chlorella*. Experiments with lutein showed that this pigment can accumulate with a wide range of values depending on its content in feeds (Leeson and Caston, 2004; Leeson et al., 2007).

The proportion of β-carotene in egg yolks of our control laying hens was not high (Figure 1D). The increase of this provitamin after supplementation with *Chlorella* was, however, rather dynamic. Its mean levels increased by one-third and even two-fold in groups P1 and P2, respectively, when compared with controls. A similar increase was reported after adding carrots rich in β-carotene, i.e. orange and purple ones (Hammershoj et al., 2010). In agreement with the above reports, we assume that the increase was enabled by the sufficient addition of vitamin A into the standard diet (Table 1). β-carotene supplied with *Chlorella* is, therefore, not converted into vitamin A and can be deposited in egg yolks.

High intake of both yellow pigments in *Chlorella*-supplemented laying hens resulted in colour changes of yolks (Table 3). Significant increases on the Roche Yolk Colour Fan were noted both in groups P1 and P2 when compared with controls as well as with each other ($P < 0.01$). Regarding commercial requirements, mean values from 5 to 6 were relatively low but they were comparable with the colour characteristics following addition of the same amount of alfalfa concentrate (Karadas et al., 2006), and exceeded values obtained when providing various carrots (Hammershoj et al., 2010). Values of TAG and CL in controls and experimental laying hens were statistically insignificant (Figure 2). The rise in both parameters at the end of the experiment occurred in all groups. However, the rise in these parameters was more marked in supplemented birds and, moreover, there was a high variability of both TAG and CL. Some birds showed signs of lipomobilization, including a body mass drop in P2 hens ($P < 0.05$), as opposed to the body weight gain in controls ($P < 0.05$).

As feed consumption was lower in P1 and P2 groups than in controls, feed conversion per egg was higher in experimental birds. These differences disappeared, however, after recalculating the data on feed consumption per kg of egg and yolk mass. The drop in feed consumption after addition of *Chlorella* was rather surprising. Its association with the intake of carotenoids can be excluded because even several-times higher doses of lutein did not influence feed intake by laying hens (Leeson et al., 2007). Lower feed consumption was commonly reported after addition of voluminous components in feed for hens. For example, laying hens supplementation with 108 g of carrots daily decreased standard diet consumption by 16 g per day (Steenfeld et al., 2007). Clearly, there are volume limits of the gastrointestinal tract. Addition of *Chlorella* powder to birds in this experiment, however, did not change the diet volume. It is well known that the dry disintegrated biomass can bind water and its content of microcrystalline and amorphous cellulose may slow down the passage of digesta through the gastrointestinal tract and influence the diet consumption in this way (Lipstein et al., 1980). Group P2 birds consumed the least amount of diet and showed significantly lower egg yolk mass when compared with controls ($P < 0.01$) as well as P1 hens ($P < 0.05$). These findings correspond with data on lower diet consumption and a drop in egg yolk mass after supplementation of hens with 70 g of carrots (Hammershoj et al., 2010).

CONCLUSION

It can be concluded that diet supplementation with the heterotrophic *Chlorella* biomass significantly increases the deposition of total carotenoids in egg yolks. Both nutritional and biological valuable pigments, lutein and zeaxanthin, accumulated equally in egg yolks and represented 90% of all carotenoids deposited. Their maximum concentrations were achieved after four weeks of supplementation and exceeded 25 and 40 mg per g of yolk after addition of 10 and 20 g of *Chlorella* per kg of diet, respectively. Deterioration of some parameters in birds with a higher level of supplementation was probably due to drop in feed consumption.

REFERENCES

AOAC (1997): Official Methods of Analysis. 16th Ed. Association of Official Analytical Chemists, Gaithersbourg, USA.

Bedecarrats G.Y., Leeson S. (2006): Dietary lutein influences immune response in laying hens. Journal of Applied Poultry Research, 15, 183–189.

Doucha J., Lívanský K. (2001): Method of controlled cultivation of algae in heterotrophic mode of nutrition. Czech Patent 288638/2001.

Doucha J., Lívanský K., Kotrbáček V., Zachleder V. (2009): Production of *Chlorella* biomass enriched by selenium and its use in animal nutrition: a review. Applied Microbiology and Biotechnology, 83, 1001–1008.

Frederiksson S., Elwinger K., Pickova J. (2006): Fatty acids and carotenoids composition of egg yolk as an effect of microalgae addition to feed formula to laying hens. Food Chemistry, 99, 530–537.

Granado F., Olmedilla B., Blanco I. (2003): Nutritional and clinical relevance of lutein in human health. British Journal of Nutrition, 90, 487–504.

Hammershoj M., Kidmose U., Steenfeldt S. (2010): Deposition of carotenoids in egg yolk by short-term supplement of coloured carrot (*Daucus carota*) varieties as forage material for egg-laying hens. Journal of the Science of Food and Agriculture, 90, 1163–1171.

Handelmann D., Nightingale A.H., Lichtenstein E.J., Schaefer E.J., Blumberk J.B. (1999): Lutein and zeaxanthin concentrations in plasma after dietary supplementation with egg yolk. American Journal of Clinical Nutrition, 70, 247–251.

Hollander M., Wolfe D.A. (1999): Nonparametric Statistical Methods. 2nd Ed. Wiley-Interscience, New York, USA.

Huxtable R., Bressler R. (1973): Determination of orthophosphate. Analytical Biochemistry, 54, 604–608.

Karadas F., Grammenidis E., Surai P.F., Acamovic T., Sparks N.H. (2006): Effect of carotenoids from lucerne, marigold and tomato on egg yolk pigmentation and carotenoid composition. British Poultry Science, 47, 561–566.

Kotrbáček V., Doucha J., Offenbartl F. (2004): Use of *Chlorella* as a carrier of organic-bound iodine in the nutrition of sows. Czech Journal of Animal Science, 49, 28–32.

Koutsos E.A., Clifford A.J., Calvert C.C., Klasing K.C. (2003): Maternal carotenoid status modifies the incorporation of dietary carotenoids into the immune tissues of growing chickens (*Gallus gallus domesticus*). Journal of Nutrition, 133, 1132–1138.

Landrum J.T., Bone R.A., Joa H., Kilburn M.D., Moorel L.L., Sprague K.E. (1997): A one year study of the macular pigment: the effect of 140 days of a lutein supplement. Experimental Eye Research, 65, 57–62.

Leeson S., Caston L. (2004): Enrichment of eggs with lutein. Poultry Science, 83, 1709–1720.

Leeson S., Caston L., Namkung H. (2007): Effect of dietary lutein and flax on performance, egg composition and liver status of laying hens. Canadian Journal of Animal Science, 87, 365–372.

Lipstein B., Hurwitz S., Bornstein S. (1980): The nutritional value of algae for poultry. Dried *Chlorella* in layer diets. British Poultry Science, 21, 23–27.

Pizzei H., Bedecarrats G.Y. (2007): Study of the effect of dietary lutein on reproductive performances in chickens. Journal of Poultry Science, 44, 409–415.

Resnikoff S., Pascolini D., Etya´ale D., Kocur J., Pararajasegaram R., Pokharel G.P., Mariotti S.P. (2004): Global data on visual impairment in the year 2002. Bulletin of the World Health Organization, 82, 844–851.

Ribaya-Mercado J.D., Blumberg J.B. (2004): Lutein and zeaxanthin and their potential roles in disease prevention. Journal of American College of Nutrition, 23, 567–587.

Shi X.M., Chen F., Yuan J.P., Chen H. (1997): Heterotrophic production of lutein by selected *Chlorella* strains. Journal of Applied Phycology, 9, 445–450.

Skřivan M., Šimáně J., Dlouhá G., Doucha J. (2006): Effect of dietary sodium selenite, Se-enriched yeast and Se-enriched *Chlorella* on egg Se concentration, physical parameters of eggs and laying hen production. Czech Journal of Animal Science, 51, 163–167.

Steenfeldt S., Kjaer J.B., Engberg R.M. (2007): Effect of feeding silages or carrots as supplements to laying hens on production performance, nutrient digestibility, gut structure, gut microflora and feather pecking behaviour. British Poultry Science, 48, 454–468.

Sun N., Wang Y., Li Y.T., Huang J.C., Chen F. (2008): Sugar-based growth, astaxanthin accumulation and caroteno-

genic transcription of heterotrophic *Chlorella zofingiensis* (Chlorophyta). Process Biochemistry, 43, 1288–1292.

Surai P.F., Sparks N.H.C. (2000): Carotenoids and embryo development. International Hatchery Practice, 15, 17–19.

Svoboda M., Kotrbáček V., Ficek R., Drábek J. (2009): Effect of organic selenium from Se-enriched alga (*Chlorella spp.)* on selenium transfer from sows to their progeny. Acta Veterinaria Brno, 78, 373–377.

VanHeukelem L., Thomas C.S. (2001): Computer assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. Journal of Chromatography A, 910, 31–49.

Vishwanathan R., Gooddrow-Kotyla E.F., Wooten B.R., Wilson T.A., Nicolosi R.J. (2009): Consumption of 2 and 4 eggs yolks/d for 5 wk increases of macular pigment concentrations in older adults with low macular pigment taking cholesterol-lowering statins. American Journal of Clinical Nutrition, 16, 1272–1279.

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