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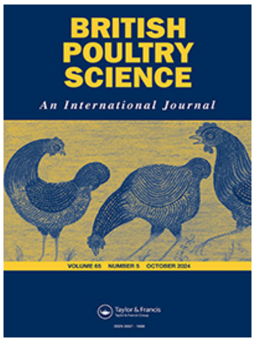

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Dietary stinging nettle (*Urtica dioica*) improves carotenoids content in laying hen egg yolk

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ABSTRACT

1. This study assessed the addition of dried stinging nettle (SN) leaves at 0%, 2.5% and 5.0% in feed on egg production, egg quality, chemical composition and antioxidant content in eggs from laying hens.
2. Seventy-two Hy-Line Brown laying hens, housed in 36 enriched layer cages, were used in the study from 43 to 47 weeks of age. Feeding dry SN leaves did not affect ($p > 0.05$) egg production variables.
3. Dietary SN inclusion linearly increased ($p < 0.001$) carotenoid content more than six-fold, in addition to yellowness and redness of the yolks at the maximum inclusion. Providing eggs from hens fed carotenoid enriched diets, *e.g.* SN, may be used to increase carotenoids in human diets.

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Introduction

The use of carotenoids in poultry production to increase the pigmentation of egg yolk and meat is common practice (Kljak, Carović-Stanko, et al. 2021a; Ortiz et al. 2021). Changes in pigmentation of yolks, ranging from yellow to intense orange, and meat, from white to yellow, can be influenced by the dietary concentration of xanthophylls and their bioavailability (Kljak, Duvnjak, et al. 2021b). However, the awareness of the role of xanthophylls in human health, and in particular, the roles of lutein and zeaxanthin in the prevention of certain eye disorders, *e.g.* age-related macular degeneration (AMD), has increased in recent years (Wilson et al. 2021). Although, there is no recommended daily allowance (RDA) for carotenoids, a consumption of 5–10 mg/day lutein/zeaxanthin can reduce the risk of AMD (Wilson et al. 2021).

Eggs are an essential part of the human diet and provide available amino acids, fatty acids, vitamins and minerals. Annual world consumption is about 150 eggs *per capita*, and the EU consumption is on average 217 eggs *per capita* (Gautron et al. 2022). Egg composition can be manipulated to substantially increase concentrations of omega-3 fatty acids, vitamins E, D and folic acids, selenium, iodine and carotenoids (Surai 2002). Surai (2002) suggested that, by producing antioxidant-enriched eggs, it is possible to improve diets for various categories of consumers, helping to prevent AMD and other diseases. It has been reported that the total xanthophyll content in the yolk in eggs from hens fed yellow or orange maize-based diets or free-range hens may exceed 20 µg/g egg yolk (Ortiz et al. 2021). Feeding 20 g/kg of dry stevia leaf almost doubled the total carotenoid content in egg yolk to 17 µg/g, compared to 9 µg/g from hens fed an unsupplemented control diet (V. R. Pirgozliev et al. 2022). Thus, consumption of 15 g dried yolk from enriched eggs could meet the RDA for humans.

Besides carotenoids, vitamin E (tocopherols) is another antioxidant of interest in human health. Reports have shown that vitamin E is relatively easy to transfer from the diet to the egg yolk (Surai 2002). When an egg is enriched with both vitamin E and lutein, lipids within the egg yolk could help antioxidant absorption. The amount and profile of yolk lipids provides an ideal environment for antioxidant absorption by the human intestine (Surai 2002). Like other carotenoids, the bioavailability of lutein increases when co-consumed with fat (Unlu et al. 2005). In addition, feed source and matrix can influence bioavailability, *e.g.* lutein from lutein-enriched eggs is more bioavailable than lutein from supplements or spinach (Chung et al. 2004).

The stinging nettle (*Urtica dioica* L.; SN) is an herbaceous plant that originates from the colder regions of Northern Europe and Asia, but today is spread all over the world (Grigorova et al. 2022; Loetscher et al. 2013). Throughout history, the use of SN as a herbal remedy and nutritious addition to the diet is well known. An important group of nutrients present in SN leaf are carotenoids, including lutein and its isomers, β-carotene and its isomers as the major groups (Guil-Guerrero et al. 2003). The leaf of certain SN may contain a substantial amount of tocopherols (Loetscher et al. 2013). Feeding SN to laying hens at levels of up to 25 g/kg led to an increase in yolk yellowness (Loetscher et al. 2013; Mierlita 2022), although differences between different SN batches have been reported (Loetscher et al. 2013). Nettle batches affected tocopherol content in the yolk, whereby some result in greater tocopherol levels compared to others (Loetscher et al. 2013). To ensure adequate antioxidant supply, this variability may be reduced by feeding greater levels of SN.

The aims of this study were to (1) establish whether graded levels of SN in hen feed can increase the subsequent concentration of antioxidants, including carotenoids and

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tocopherols in the eggs, and (2) examine the effects on egg production and eggshell quality variables. It was hypothesised that feeding SN to laying hens will increase the antioxidant content in eggs without having an impact on egg production variables.

Materials and methods

Ethical statement

The animal study protocol was approved (0150–202102-STAFF) by the Research Ethics Committee of Harper Adams University (UK). The experiment has been designed to comply with the ARRIVE 2.0 guidelines (Percie du Sert et al. 2020).

Experimental design

Seventy-two, 43-week-old Hy-Line Brown laying hens (Cyril Bason, Craven Arms, Shropshire, SY7 9NG, UK) were randomly allocated in pairs to 36 enriched layer cages. All cages were enriched with a nest box, perch and sand bath. Water and feed (meal form) were provided *ad libitum*. The rearing conditions followed industry recommendations (www.hyline.com).

Diet formulation

Before the start of the experiment, birds were fed a diet formulated to meet the nutrient requirement of the hens (Hy-Line International, Studley, UK), containing 11.56 MJ/kg AME and 172 g/kg crude protein (Table 1).

Table 1. Formulation of laying hen experimental basal diet.

Ingredients (%)	Basal diet
Barley	10.00
Wheat	54.00
Soya meal	17.50
Full fat soya	5.00
L Lysine	0.05
DL Methionine	0.15
Soya oil	2.00
Limestone	10.00
Monocalcium Phosphate	0.80
Salt	0.25
Sodium bicarbonate	0.15
Layer Vit-Min Premix[a]	0.10
Calculated provisions	
AME (MJ/kg)	11.56
Crude protein (g/kg)	172.0
Crude fat (g/kg)	43.0
Digestible Lysine (g/kg)	8.26
Digestible Methionine + Cysteine (g/kg)	5.98
Digestible Threonine (g/kg)	6.01
Digestible Tryptophan (g/kg)	2.04
Ca (g/kg)	41.7
Available P (g/kg)	31.0
Analysed values	
Ca (g/kg)	43.2
Total P (g/kg)	5.0

The vitamin and mineral premix contained vitamins and trace elements to meet the requirements specified by the breeder. The premix provided (units/kg diet) the following: [a]Premix (per kg feed): vitamin A (retinyl acetate) 10.000 IE; vitamin D3 (cholecalciferol) 2.000 IE; vitamin E (DL-α-tocopherol) 25 mg; vitamin K3 (menadione) 1,5 mg; vitamin B1 (thiamin) 1,0 mg; vitamin B2 (riboflavin) 3,5 mg; vitamin B6 (pyridoxine-HCl) 1,0 mg; vitamin B12 (cyanocobalamin) 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.

The experiment began when the birds were 43 weeks old. The same batch of dried and milled SN leaves were obtained locally (Target Feeds, Whitchurch SY13 3LT, UK). The basal diet was used as a control (C). The treatment diets were made by adding 2.5 kg SN to 97.5 kg of the C diet (2.5% SN) or 5 kg SN to 95 kg of the C diet (5% SN). The diets were fed to assess the transfer of antioxidants (carotenoids and vitamin E) from hens into the yolk. The experimental diets were fed in a meal form for 4 weeks, between 43 and 47 weeks of age and did not contain any coccidiostat, antimicrobial growth promoters or other similar additives. All experimental diets were formulated to meet the nutritional requirements of laying hens (Hy-Line International, Studley, UK).

Hen performance, egg production and determination of egg quality

The hens were individually weighed at the beginning of the study at 43 weeks of age. Feed intake (FI) for each cage was recorded for the entire 4-week study period. Egg numbers, weights, feed conversion ratio (FCR) and percentage egg production were measured according to previous reports (Pirgozliev et al. 2023; Whiting et al. 2019). Egg and shell quality analyses were performed on a total of 36 eggs which had been collected, one from each cage, on the last day of the experiment, when hens were 47 weeks old. All egg analyses were performed after 1 day of storage at 15°C as previously described (Pirgozliev et al. 2022; Whiting et al. 2019).

Analysis of experimental diets and eggs

At the end of the study, an additional egg was collected from each cage. The shell was removed, the yolk was separated from the albumen and was freeze dried, ground and analysed for carotenoids and tocopherols content (Karadas et al. 2014; Kljak, Carović-Stanko, et al. 2021a, 2021b). Dry matter (DM) of feed and collected excreta samples was determined by drying of samples in a forced draft oven at 105°C to a constant weight (AOAC (2000); method 934.01). Crude protein (6.25 × N) in samples was determined by the combustion method (AOAC (2000); method 990.03) using a LECO FP-528 N (Leco Corp., St. Joseph, MI, U.S.A.). Oil (as ether extract) was extracted with diethyl ether by the ether extraction method (AOAC (2000); method 945.16) using a Soxtec system (Foss Ltd., Warrington, UK). Dietary Ca and P were determined as described in previous reports (Oso et al. 2017; V. Pirgozliev et al. 2011). The egg yolk colour was determined by DSM YolkFanTM (YF) and with Chroma Meter CR-400 from Konica Minolta (Sunderland, UK) to determine luminance and chromaticity scores using CIELAB scoring. For colour analysis, L* refers to lightness, a* refers to redness and b* refers to yellowness (Whiting et al. 2019).

Statistical analysis

Experimental data were analysed using Genstat (23rd edition) statistical software package (IACR Rothamstead, Hertfordshire, UK) by one-way ANOVA, followed by orthogonal polynomials contrasts to test for a linear response (L) and deviations (D) from linearity. All tests were considered significant at $p < 0.05$. Data are expressed as means and their pooled standard errors of means (SEM).

Results

Hen performance, egg production and quality

All birds completed the study in good health. The study began when the birds were 43 weeks old with an average body weight of 2.058 kg (SD ± 0.186 kg). Compared to the basal diet, supplementing with SN had a much higher concentration of carotenoids, primarily lutein (Table 2). There was almost double the amount of total vitamin E in the SN compared to the basal diet.

There were no statistically significant differences ($p > 0.05$) in feed intake, daily egg mass and FCR for egg production due to the experimental diets (Table 3). Although there was no significant ($p > 0.05$) difference in egg production, there was a linear trend (L = 0.055) for reduced production with increasing inclusion of SN. The effect of SN on both internal and external egg quality variables showed no significant difference among the three diets in egg weight, shell weight or thickness, deformities, Haugh units (HU), albumen height or yolk pH ($p > 0.05$). However, albumen pH tended ($p = 0.081$) to decrease in a linear fashion (L = 0.031) with increasing SN.

Table 2. Carotenoids, vitamin E, crude protein, crude fat, neutral detergent fibre and dry matter contents of stinging nettle and the laying hen basal diet*.

Determined values (µg/g)	Stinging nettle	Basal diet
Lutein (µg/g)	111.87	0.192
Zeaxanthin (µg/g)	11.47	0.019
β-cryptoxanthin (µg/g)	0.65	0.036
β-carotene (µg/g)	6.12	0.052
Total carotenoids (µg/g)	130.11	0.259
α-tocopherol (µg/g)	10.69	1.995
γ-tocopherol (µg/g)	8.36	6.845
δ-tocopherol (µg/g)	1.64	2.556
Vitamin E (µg/g)	20.69	11.397
Crude protein (g/kg)	226.0	172.0
Crude fat (g/kg)	24.0	39.5
Neutral detergent fibre (g/kg)	351.0	83.0
Dry matter (g/kg)	905.6	903.0

*Analyses were performed in technical duplicates.

Supplementing with SN increased yolk colour intensity ($p < 0.001$) measured using the DSM YolkFanTM (YF) scale, compared to eggs from the control fed birds, but there was no linear relationship (D = 0.001) between the levels of SN. Dietary inclusion of SN reduced lightness (L*; $p < 0.001$; L < 0.001) and increased redness (a*; $p < 0.001$; L < 0.001); however, yellowness (b*; $p < 0.001$; L < 0.001; D < 0.001) of the yolk plateaued at 2.5% SN inclusion.

Effect of SN on carotenoids and vitamin E content on yolk

The impact of dietary SN on carotenoids and vitamin E content in egg yolk is presented in Table 4. Feeding SN increased ($p < 0.001$) yolk lutein, zeaxanthin, β - carotene, peak β - carotenoids and total carotenoids linearly ($p < 0.001$). Only β - cryptoxanthin was not affected ($p > 0.05$). There was no impact ($p > 0.05$) of dietary SN on vitamin E in yolks compared to eggs from hens fed the control. Only the δ - tocopherol fraction tended ($p = 0.095$) to increase with SN inclusion in the diets.

Discussion

This study investigated the impact of graded levels of dietary SN on yolk pigmentation, carotenoid and tocopherol contents when fed to Hy-Line Brown laying hens at peak production. There were some small differences between calculated and determined feed composition, which was probably due to differences in the software database and composition of ingredients. However, these differences were negligible and are unlikely to have had an impact on the study outcome.

Production and quality results were within the expected range for eggs from Hy-Line Brown laying hens at this stage of production. Supplementing with SN had no adverse effect on the overall productive performance of hens, which agreed with some trials (Grigorova et al. 2022; Mierlita 2022; Zhang et al. 2020) and disagreed with others (Hashemi et al. 2018; Tabari et al. 2016). An increase in eggshell thickness from hens receiving SN has been reported (Zhang et al. 2020), but

Table 3. Effect of dietary stinging nettle on hen performance and egg quality variables at study endpoint.

	C	2.5% SN	5.0% SN	SEM	P	L	D
Feed intake (g/bird/day)	119	117	115	2.3	0.546	0.278	0.917
Egg mass (g/bird/day)	56.7	55.3	53.5	1.77	0.443	0.207	0.929
FCR egg production (g:g)	2.094	2.128	2.182	0.0590	0.582	0.307	0.896
Egg production (%)	92.5	86.9	85.4	2.48	0.124	0.055	0.495
Egg weight (g)	62.2	62.1	59.7	1.20	0.258	0.144	0.450
Eggshell thickness (mm)	0.362	0.344	0.348	0.0124	0.579	0.446	0.477
Eggshell weight (g)	6.07	5.88	5.69	0.224	0.504	0.247	0.991
% double yolk	0.0	0.3	0.2	0.18	0.403	0.455	0.264
% soft shell	0.2	0.5	0.4	0.31	0.721	0.643	0.513
% target shell	0.0	0.2	0.2	0.14	0.473	0.276	0.588
Albumen height (mm)	7.08	6.73	6.74	0.247	0.531	0.339	0.560
Haugh unit	83.0	80.6	81.5	1.67	0.605	0.532	0.437
Albumen pH	8.89	8.84	8.71	0.055	0.081	0.031	0.553
Yolk pH	6.18	6.22	6.15	0.047	0.628	0.677	0.389
Yolk colour (DSM YolkFan^{TM})	2.03^{a}	4.50^{b}	5.67^{c}	0.147	<0.001	<0.001	0.001
Yolk colour (Minolta Chroma Meter)							
L*	16.81^{a}	15.89^{b}	15.22^{b}	0.216	<0.001	<0.001	0.631
a*	0.54^{a}	1.46^{b}	2.80^{c}	0.137	<0.001	<0.001	0.227
b*	15.76^{a}	19.93^{b}	19.53^{b}	0.500	<0.001	<0.001	<0.001

C = control diet; SN = stinging nettle; SEM = pooled standard error of means; p = Fisher's probability; L = orthogonal polynomial contrast for linearity; D (deviation) = orthogonal polynomial contrast for deviation from linearity; FCR = feed conversion ratio; L* = lightness; a* = redness; b* = yellowness; Values in a row with different letters differ significantly.

Table 4. The effect of dietary stinging nettle on egg yolk carotenoids and vitamin E concentration (µg/g).

	C	2.5% SN	5.0% SN	SEM	P	L	D
Lutein	4.4[a]	14.0[b]	27.7[c]	1.25	<0.001	<0.001	0.186
Zeaxanthin	0.5[a]	1.6[b]	3.0[c]	0.16	<0.001	<0.001	0.442
β - cryptoxanthin	0.14	0.08	0.14	0.022	0.407	0.955	0.267
β - carotene	0.1[a]	0.5[b]	0.7[c]	0.04	<0.001	<0.001	0.277
All peaks β-carotenoids	5.6[a]	18.2[b]	35.6[c]	1.59	<0.001	<0.001	0.221
Total carotenoids	5.0[a]	16.1[b]	31.5[c]	1.41	<0.001	<0.001	0.222
α-tocopherol	26.1	26.0	27.4	1.17	0.661	0.459	0.602
δ-tocopherol	2.5	2.4	3.0	0.19	0.095	0.087	0.169
γ-tocopherol	18.1	18.7	19.3	0.83	0.643	0.352	0.986
Vitamin E	46.8	47.1	49.6	2.05	0.572	0.339	0.665

C = control diet; SN = stinging nettle; SEM = pooled standard error of means; p = Fisher's probability; L = orthogonal polynomial contrast for linearity; D (deviation) = orthogonal polynomial contrast for deviation from linearity; Values in a row with different letters differ significantly.

this was not supported in the current study or previous reports (Grigorova et al. 2022; Loetscher et al. 2013; Mierlita 2022). Such differences in the literature may have been due to differences in geographical location, rearing conditions, bird production type or breed, or dietary inclusion level of SN (Loetscher et al. 2013; V. Pirgozliev et al. 2014; Zhang et al. 2020; Zurak et al. 2024a, 2024b).

The study compared yolk pigmentation, carotenoid and tocopherol deposition of eggs produced from diets with the same proximate composition, but with different dry SN leaf inclusion. Yolk colour was previously found to reflect the dietary SN inclusion (Grigorova et al. 2022; Mierlita 2022; Zhang et al. 2020). Dietary carotenoids are usually associated with yolk colouration (Kljak, Carović-Stanko, et al. 2021a, 2021b; Zurak, Gunjević, et al. 2024a, 2024b) and is important regarding visual impact on consumer perceptions (Johnson et al. 2018; Ortiz et al. 2021). Hens fed diets containing SN produced egg yolks with a higher YF colour, a* (redness) and b* (yellowness) index values and reduced L* compared with those fed C. In addition, eggs produced by birds fed 5% SN had all those yolk colour measures significantly higher than eggs obtained from birds fed 2.5% SN or C. In contrast, feeding 2.5% SN resulted in an increase in yolk b* only (Loetscher et al. 2013), while L* and a* were not significantly affected, which suggested that the origin and batch of the SN may be important. However, feeding coloured maize to layers led to higher YF yolk colour (Moreno et al. 2020; Ortiz et al. 2021) compared to the current results. The high inclusion rate of dietary maize (over 500 g/kg) compared to SN (50 g/kg) may have been the reason, although the SN contained 130.11 µg/g carotenoids compared to 5.7 µg/g and 24.9 µg/g for yellow and orange maize, respectively (Ortiz et al. 2021).

The lack of differences between experimental diets regarding production variables and the linear increase of yolk carotenoids in the current study, which suggested that carotenoid enrichment of eggs may benefit from even higher SN inclusion levels. The optimum inclusion level of SN for both antioxidant content in eggs and production variables remains to be determined. It should be noted that hens avoid eating SN in pasture because of the stinging or burning effect from the fresh plant (Hammershøj et al. 2010); thus, it cannot be used as a suitable enrichment for laying hens in free-range systems but can be fed as a component of the diet.

Both β-carotene and β-cryptoxanthin contain provitamin A activity and are converted to vitamin A, which may explain the reduced deposition in the yolk (Hencken 1992; Kljak, Carović-Stanko, et al. 2021a). The concentration of the carotene portions in yolk was within the range of previous reports on different carotenoid sources (Kljak, Carović-Stanko, et al. 2021a; Loetscher et al. 2013; Ortiz et al. 2021). This further confirmed that a feeding period of 4 weeks was long enough for deposition of dietary pigments into eggs (Hammershøj et al. 2010; Kljak, Duvnjak, et al. 2021b; V. R. Pirgozliev et al. 2022).

Lutein and zeaxanthin are present in the retina of the human eye (Ranard et al. 2017) and a high concentration of these carotenoids in blood serum is an indicator of protection of the retina from light-induced damage (Landrum et al. 2013). However, lutein and zeaxanthin cannot be synthesised *de novo* and must be provided through dietary consumption (Ranard et al. 2017). Data from 2016 (Shanahan 2017) showed that in the EU, the cost of managing the consequences of AMD in adults over 50 years of age was estimated to be €89.46 billion per year. In 2020, 196 million people worldwide had AMD and this number is expected to rise to 288 million by 2040 (Wong et al. 2014). This suggests that the cost of managing the consequences of AMD in the EU in 2040 may well exceed €200 billion annually. Thus, an increase of daily lutein/zeaxanthin intake to help prevent AMD and other associated diseases is important. The overall carotenoid content in the dry yolk at the end of the study for hens fed C, 2.5% SN and 5.0% SN diets were 5.0, 16.1 and 31.5 µg/g, respectively. This suggested that an egg a day from hens fed SN could increase daily carotenoid consumption and potentially prevent the prevalence of AMD in an older human population. However, it must be recognised that only one batch of SN was evaluated in the present study over a 4-week feeding period, therefore variation in the antioxidant composition of other batches or longer feeding periods may enrich the yolk to different levels.

Although the SN used in the present study contained almost twice the amount of vitamin E compared to the control diet, the relatively low SN inclusion and lower feed intake may explain the lack of further enrichment of the yolk with vitamin E. However, the diets were supplemented with 25 mg/kg vitamin E, which contributed to the similarity in egg yolks vitamin E content in the current study.

In another broiler study, feeding stevia for 2 weeks improved hepatic antioxidant status (V. Pirgozliev et al. 2021), suggesting potential health benefits for the birds. It may be speculated that this increased total carotenoid content, in the egg yolk and the liver of laying hens, may reduce oxidative stress.

Conclusions

Hens fed diets containing either 2.5 or 5% SN increased carotenoid levels, a* and b* of the yolks compared to the control diet. Feeding diets containing 5% SN resulted in higher carotenoid content in egg yolks compared to the control diet and the diet containing 2.5% SN. When comparing published data with the current results, the concentration of carotenoids in the egg yolks from hens fed SN was similar or more than that recorded in free-range reared hens or those fed maize or marigold. Dietary SN did not affect other egg production variables in this study. Thus, it was concluded that laying hens supplemented with SN can produce eggs enriched in lutein and zeaxanthin, potentially helping with the prevention of AMD in the human population.

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Conceptualisation, V.R.P., S.P.R. and K.K.; methodology, V.R.P., I.M.W., M.T. and K.K.; software, V.R.P., I.M.W. and S.C.M.; validation, V.R.P.; formal analysis, V.R.P., I.M.W., M.T. and K.K.; investigation, V.R.P., I.M. W., K.K., S.C.M., S.B.E., A.G.A., and S.P.R.; resources, V.R.P.; data curation, V.R.P. and S.C.M.; writing original draft preparation, V.R.P., C.S.M., I.M.W. and S.P.R.; writing review and editing, A.G.A, M.T., S.C.M., S.B. E. and K.K.; visualisation, V.R.P. and S.C.M.; supervision, V.R.P.; project administration V.R.P., I.M.W. and K.K.; funding acquisition, V.R.P. All authors have read and agreed to the published version of the manuscript.

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