Effects of dietary alfalfa protein concentrate supplementation on performance, egg quality, and fatty acid composition of raw, freeze-dried, and hard-boiled eggs from Polbar laying hens

E. R. Grela,[*] S. Knaga,[†,1] A. Winiarska-Mieczan,[*] and G. Zięba[†]

[*]*Institute of Animal Nutrition and Bromatology, University of Life Sciences, Lublin 20-950, Poland; and* [†]*Institute of Biological Bases of Animal Production, University of Life Sciences, Lublin 20-950, Poland*

ABSTRACT The aim of this study was to determine the effect of addition of alfalfa protein concentrate (**APC**) at the dose of 15 g or 30 g per 1 kg of complete feed ration for the native Polbar breed on selected production traits and the fatty acid profile in the yolk of raw, hard-boiled or freeze-dried eggs. Laying hens were assigned randomly to 3 experimental groups, each comprising 30 birds. The control group received a standard diet without the APC addition and the experimental groups received APC, which partially replaced postextraction soybean meal. Egg laying performance, feed intake, and feed conversion ratio were controlled throughout the experiment. At 33 wk of age, 45 eggs were randomly selected for assessment of the quality of the egg content and eggshell, and 30 eggs were taken for each of the cooking and freeze-drying treatments. The fatty acid composition was determined in the yolks of hard-boiled, freeze-dried, and raw eggs. There was no effect of the APC addition on the laying performance, feed intake and feed conversion ratio, and a majority of egg quality traits. Hens fed with the APC laid eggs with a darker colored eggshell and yolk and a slightly lower breaking strength. The yolks in the eggs from hens receiving the APC addition were characterized by distinctly higher content of polyunsaturated fatty acids (**PUFA**). The group fed with a higher dose of APC produced eggs with a substantially lower level of saturated fatty acids (**SFA**). Boiling resulted in an increase in the SFA content and a decline in the level of PUFAs and carotenoids. Freeze-drying led to an increase in the total SFA content and a decrease in the level of n-3 PUFA. The APC addition to feed can replace the genetically modified soybean meal without reducing the values of production traits and egg quality and with a beneficial effect on the yolk color and fatty acid profile.

Key words: alfalfa, laying hen, hard-boiled, freeze-dried, fatty acid

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INTRODUCTION

Owing to the recent global increase in animal production combined with the stabilization of soybean production, a steady increase in the price of postextraction soybean meal, which is the basic protein source in poultry nutrition, can be observed (Bianchi and Szpak, 2017). The growth in the production costs related to the increase in the feed prices, the evident trend in the EU countries toward the use of genetically nonmodified components, and the constantly growing demand for organic products from poultry fed with native plant fodder have created a need to search for alternative sources of protein, such as alfalfa (*Medicago sativa* L.). Besides its high protein content, alfalfa is rich in vitamins and minerals, and its xanthophyll pigments, β-carotene, and flavonoids ensure its high antioxidant activity (Xie et al., 2008). Alfalfa is also a rich source of polyunsaturated fatty acids, in particular, those from the n-3 PUFA, which exert a positive effect on human health when consumed (Kremmyda et al., 2011). Through an appropriately composed diet for laying hens, the quantitative composition of fatty acids contained in egg yolks can be manipulated to achieve, for example, eggs rich in the n-3 PUFA (Beynen, 2004). This is particularly important when the consumption of PUFA is reduced, for example, due to limited availability of n-3-rich food products (fish meal). This problem can be mitigated by providing the organism with essential nutrients via consumption of cheaper and more readily available poultry products (meat, eggs).

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[1]Corresponding author: sebastian.knaga@up.lublin.pl

Hence, dried alfalfa material seems to be an excellent ingredient in poultry feed. However, owing to its high fiber content, the use of the plant as the main protein source is highly limited. This problem can be solved by application of alfalfa protein concentrate (**APC**) containing only 0.59% of crude fiber in dry matter. In addition, APC is characterized by high content of protein (approx. 50–55%), crude fat (10.2%), xanthophylls, and valuable bioactive compounds, such as saponins, flavonoids, vitamins, and minerals (Grela and Pietrzak, 2014). Previous investigations have demonstrated the suitability of APC in the nutrition of swine (Grela et al., 2008; Pietrzak and Grela, 2015, 2016), broiler chickens (Kwiatkowska et al., 2017), laying hens (Laudadio et al., 2014), turkeys (Krauze and Grela, 2010; Grela et al., 2014), lambs (Ognik et al., 2012; Szymanowska et al., 2017), and fish (Rechulicz et al., 2014). The results of these studies indicated improvement of production performance (higher daily gains, better feed conversion ratio [**FCR**]) and meat quality. Studies on laying hens nutrition showed improvement of antioxidant indices and yolk color (Grela et al., 2014).

At present, consumers pay increasing attention to the welfare of farm animals, hence they prefer products from animals reared in the so-called alternative farming systems (litter, free-range, or organic farming). These systems require laying hens with an adequate behavioral profile, which is very often typical of native breeds of chickens adapted to caged, free-range, and organic farming and producing eggs with exceptional sensory properties. The Polish autosexing Polbar chicken breed used in this study exhibits such traits (Gryzińska and Niespodziewański, 2009).

Most eggs are consumed after processing (boiling, baking, frying) and storage in different temperature conditions even for a long time (Ruiz-Rodriguez et al., 2008). Therefore, the aim of the present study was to determine the effect of addition of alfalfa (*M. sativa* L.) protein concentrate at a dose of 15 g or 30 g per 1 kg of a complete feed ration for the native Polbar breed on selected production traits, the fatty acid profile, and the content of carotenoids in the yolk of raw, hard-boiled, or, freeze-dried eggs.

MATERIAL AND METHODS

Birds and Diets

The study involved eggs laid by Polbar laying hens (**Pb**). The birds were reared in a cage system (5 birds/cage) in a building equipped with modern technological devices. The birds on the farm were tagged with barcode wing bands for identification. Data were read, collected, and recorded using barcode readers connected to computers and portable terminals. Throughout the production period (from the 19th to 55th wk of age), the birds were fed ad libitum with the standard full-feed mixture for laying hens – DJ (control group) or mixtures containing different levels of APC (experimental groups). They had constant access to drinking water (Table 1). The birds were randomly assigned to 3 groups, each comprising 30 individuals with 5 birds in each subgroup (6 replications). The subgroup was regarded as an experimental unit. Group C receiving the standard feed mixture without the experimental addition was the control (Table 1).

Layers from groups E-1 and E-2 were administered 15 g (E-1) and 30 g (E-2) of APC, respectively, as a partial replacement of soybean meal (Table 1). The APC concentrate was produced by a cooperative plant from Planschez, Marne Department, which is a part of the France Luzerne concern. The content of nutrients and biologically active APC was specified by Pietrzak and Grela (2015). The animal care and experimental procedures were performed in accordance with the Polish and European regulations. The mean body weight of hens at the beginning of the experiment was 1,520 ± 15 g.

Assessment of Production Performance and Egg Traits

Throughout the production period, the feed intake (**FI**) and FCR were controlled in the subgroups (5 birds in each).

At week 33 of age (egg-laying peak), 45 eggs were collected randomly from each group over 2 D and subjected to a qualitative assessment the next day. The measurements of the egg weight; Haugh units; the color and weight of the yolk; and the color, thickness, and density of the eggshell were performed using a

Table 1. Composition of ingredients and nutrients in the diet of the experimental hens (air-dry basis).

Item	Treatment[1]		
Ingredients (g kg^{-1})	C	E-1	E-2
Yellow corn	442.4	442.4	442.4
Triticale	200.0	200.0	200.0
Soybean meal (44% CP)	250.8	235.8	220.8
Vegetable oil	11.5	11.5	11.5
Salt	3.0	3.0	3.0
Dicalcium phosphate	10.7	10.7	10.7
Mineral-vitamin premix[2]	3.0	3.0	3.0
Limestone	78.0	78.0	78.0
DL-methionine	0.6	0.6	0.6
Alfalfa protein concentrate (APC)	0.0	15.0	30.0
Chemical analysis (g kg^{-1})			
Dry matter	901.3	901.7	901.8
Crude protein	170.2	170.2	170.2
Crude fiber	40.8	40.5	40.4
Ether extract	30.5	30.6	30.7
Crude ash	95.9	96.1	96.1
Calcium	33.1	33.2	33.2
Total phosphorus	64.5	64.6	64.7
Calculated of nutrients in 1 kg:			
Metabolisable energy, MJ	11.29	11.29	11.29
Available phosphorus, g	5.44	5.45	5.46
Methionine	3.48	3.49	3.49
Lysine	9.05	9.07	9.08

[1]C = control group; E-1 = group fed with the diet supplemented with 1.5% of APC; E-2 = group fed with the diet supplemented with 3.0% of APC.

[2]Supplied per kg of diet: vitamin A 12,000 IU, vitamin D_3 3,000 IU, vitamin E 40 IU, vitamin K_3 3 mg, vitamin B_1 2 mg, vitamin B_2 6 mg, vitamin B_6 5 mg, vitamin B_{12} 0.02 mg, niacin 45 mg, biotin 0.075 mg, folic acid 2 mg, pantothenic acid 12 mg, choline 300 mg, manganese 100 mg, zinc 100 mg, iron 30 mg, copper 10 mg, iodine 1 mg, selenium 0.2 mg, cobalt 0.1 mg.

semiautomatic egg quality assessment system (TSS-Technical Services and Supplies, York, UK) consisting of a microprocessor (EQM+), 2 electronic scales (NavigatorXT model AV8101CM from Ohaus Corporation, Pine Brook, NJ, and KERN 440–49 from KERN & Sohn, GmBH, Balingen, Germany), a colorimeter for measurement of the yolk color in a 15-point Roche scale (QCC), a reflectometer for measurement of the eggshell color, and a micrometer screw (QCT). The reflectometer uses light reflection for determination of the color (the percentage of light reflected). The lower the value, the darker the color of the eggshell. The eggshell strength was determined using an Instron Testing Machine model Mini 55 (Instron Ltd., High Wycombe, England).

Egg-Processing Procedures

To determine the fatty acid composition as well as the β-carotene and lutein content in the raw, hard-boiled, and freeze-dried yolks, 30 eggs were randomly collected from each group at 33 wk of age and divided into 3 equal parts. The first part of the eggs was boiled in a water bath at 100°C for 12 min (Parpinello et al., 2006). Before boiling, the eggs were kept at room temperature for 30 min. After boiling, the eggs were cooled in running water for 5 min. Egg yolks that reached room temperature were collected and minced. Another 10 eggs were broken, the yolks were separated from the albumens, and the albumen remnants adhering to the vitelline membrane were removed by rolling the yolk on the filter paper. The yolks were then homogenized and freeze-dried. The same procedure, but without freeze-drying, was used for preparation of raw yolks.

Fatty Acid Analysis

The fat content in the yolks of raw, freeze-dried, and hard-boiled eggs was determined by homogenization in a chloroform:methanol solution (2:1, v/v) using a procedure proposed by Folch et al. (1957). Fatty acid methyl esters (**FAMEs**) were determined on a Varian CP 3800 gas chromatograph (Varian, Inc., Palo Alto, CA) equipped with an FID detector and a CP-WAX 52CB capillary column. The determinations were carried out in the following conditions: 100-m length; inner diameter of 0.25 mm; gas carrier, helium; hydrogen flow rate, 30 mL/min; air flow rate, 300 mL/min; helium flow rate, 1.4 mL/min; initial column temperature, 120°C gradually increasing by $20°C/min^{-1}$ to the final temperature of 210°C; determination time, 157 min; injector temperature, 160°C; detector temperature, 160°C; other gases, hydrogen and air. The volume of the injected sample was 1 μL. FAMEs were identified by comparison of retention times with known standards, a 37 Component FAME Mix (Supelco, Bellefonte, PA). The results of the percentage content of fatty acids in the sample were obtained using Star GC Workstation version 6.30 (Varian Inc., Walnut Creek, CA).

The quality indices of egg fat, that is, the atherogenic index (**AI**) and the thrombogenic index (**TI**), were calculated as well using a formula developed by Ulbricht and Southgate (1991):

$$\mathrm{AI} = [(4 \times C14:0) + C16:0] / [n-6\ \mathrm{PUFA} + n-3\ \mathrm{PUFA} + \mathrm{MUFA}];$$

$$\mathrm{TI} = [C14:0 + C16:0 + C18:0] / [(0.5 \times \mathrm{MUFA}) + (0.5 \times n-6\ \mathrm{PUFA}) + (3 \times n-3\ \mathrm{PUFA}) + n-3 / n-6\ \mathrm{PUFA}].$$

The ratio of hypocholesterolemic to hypercholesterolemic acids (**h**/**H**) was calculated according to the equation proposed by Fernández et al. (2007).

$$h / H = (C18:1 + C18:2 + C18:3 + C20:4 + C20:5 + C22:4 + C22:5 + C22:6) / (C14:0 + C16:0).$$

β-Carotene and Lutein Content

The β-carotene and lutein contents in the yolk samples were determined by high-pressure liquid chromatography (**HPLC**) after saponification and extraction into heptane according to the study by Grela et al. (1999). Finally, the yolk (about 0.3 g) was suspended in a mixture of 24.0 mL 96% v/v ethanol, 9.0 mL methanol, 10.0 mL 10% w/v ascorbic acid in water, and 7.0 mL KOH-water (1:1 w/v). The mixture was saponified for 30 min at 70°C in the dark and cooled in cold water. Two milliliters of the saponified mixture were diluted with 0.50 mL of distilled water, after which carotenoids were quantitatively extracted with 2 portions of 5.0 mL of heptane. The HPLC equipment consisted of a Perkin Elmer LC pump series 410, a Perkin Elmer Advanced LC

Sample Processor ISS 200, and a Perkin Elmer LC 240 fluorescence detector (Perkin-Elmer, Überlingen, Germany). Carotenoids were separated on a 4.6 × 250-mm Supelcosil LC-NH2-5 µm HPLC column (Supelco, Bellefonte, PA). The mobile phase consisted of heptane modified with 2-propanol (50 mL/L) and triethylamine (1.0 mL/L). The eluate was monitored at 450 nm by using a Perkin Elmer LC95 UV/VIS Spectrophotometer Detector.

Statistical Analysis

Based on explanatory analyses, the models took into account factors that significantly affected the studied traits. The statistical calculations of production and egg quality traits were performed by a one-way analysis of variance taking into account the effect of the feeding group (the pen was the experimental unit).

The fatty acid profile was analyzed with a 2-way analysis with interaction in general linear models (proc GLM) in the SAS 9.4 software (SAS Institute, Cary, NC). The model included diet and egg processing as the main factors. The research hypotheses (comparisons of the effect of the experimental factors) were verified with predefined orthogonal contrasts using the Bonferroni correction.

RESULTS

Production Traits and Egg Quality

The addition of APC to the feed ration did not cause a decrease in the laying performance or an increase in the FI and FCR parameters (Table 2). The values of most of the analyzed eggshell quality were similar in all the feeding groups. Eggs laid by the group E-2 hens were characterized by a slightly lower eggshell breaking strength (Table 3). The mean difference between the control group, which exhibited the highest value of this parameter, and group E-2 was only 2.25 N. There was a highly significant effect of the protein concentrate administration in the feed on the color of the eggshell and yolk. The eggshell in the eggs laid by hens receiving the APC-supplemented feed was darker, and a significantly darker color was noted in the yolk of these eggs (Table 3). The mean difference in the yolk color between the control group and the APC-supplemented groups was 2.0 points in the Roche scale.

Table 2. Production performance in laying hens fed with the DJ mixture (standard full-feed mixture for laying hens) with addition of the alfalfa protein concentrate (**APC**).

Trait	Feeding groups[1]			SEM
	C	E-1	E-2	
Egg production (%)	76.9	76.8	75.9	0.18
FI (g/layer/D)	121.3	120.4	123.5	0.34
FCR (g feed/g egg)	1.97	1.96	1.93	0.15

Abbreviations: FCR, feed conversion ratio; FI, feed intake.
[1]C = control group; E-1 = group fed a diet with addition of 1.5% of APC; E-2 = group fed a diet with addition of 3.0% of APC.

Table 3. Traits of eggs from hens receiving the DJ mixture (standard full-feed mixture for laying hens) with addition of the protein concentrate (**APC**).

Trait	Feeding groups[1]			SEM
	C	E-1	E-2	
Egg weight (g)	60.5	58.8	59.7	0.71
Egg mass (g/layer/D)	47.4	47.1	48.5	1.45
Eggshell color (%)	51.50[a]	54.25[b]	55.15[b]	0.41
Egg specific gravity (g/cm^3)	1.11	1.10	1.10	0.08
Haugh units	71.6	71.3	73.4	1.86
Breaking strength (N)	42.15[a]	41.25[a,b]	39.90[b]	0.29
Eggshell thickness (µm)	329.0	326.0	327.0	1.85
Eggshell density (g/cm^3)	99.0	97.5	97.5	0.25
Eggshell weight (g)	7.05	6.85	6.95	0.02
Yolk weight (g)	15.4	15.3	15.9	0.07
Yolk color (pts)	6.55[a]	8.55[b]	8.96[b]	0.16

Different superscript lowercase letters denote statistically significant differences at $P \leq 0.05$ (means with the same letter are not significantly different).
[1]C = control group; E-1 = group fed a diet with addition of 1.5% of APC; E-2 = group fed a diet with addition of 3.0% of APC.

Fatty Acid Profile—APC

The addition of the APC to the feed, especially at the dose of 3.0%, contributed to a significant increase in the total concentration of PUFA in the egg yolks both from the PUFA n-3 (linolenic acid [**ALA-C18:3 n-3**] and docosahexaenoic acid [**DHA-C22:6 n-3**]) and from the PUFA n-6 (in linoleic acid **LA-C18:2 n-6**) in comparison with the control group (Table 4). Consequently, the n3/n6 ratio increased, and the n6/n3 ratio decreased in both experimental groups. The yolk from eggs laid by the E-2 hens exhibited a change in the content of some monounsaturated fatty acids (**MUFA**), that is, an increase in the content of palmitoleic (**C16:1**) and margaroleic (**C17:1**) acids and a reduced level of oleic acid (**C18:1 n-9**). In group E-1, there was a decline in the content of palmitoleic, vaccenic (**C18:1 n-7**), and gondolic (**C20:1n-9**) acids in the yolks. However, these changes did not have an influence on the total MUFA content in the yolks from the experimental groups. With the exception of myristic acid (**C14:0**), there were no significant differences in the concentration of saturated fatty acids between the experimental groups and the control group; however, group E-2 was characterized by significantly lower total SFA content than the control. The addition of APC resulted in a significant increase in the h/H ratio and a decrease in AI (both groups) and TI (only group E-2).

Fatty Acid Profile—Egg Processing

In comparison with raw yolks, the hard-boiled yolks exhibited higher content of palmitic (**C16:0**) and stearic (**C18:0**) acids and, consequently, significantly higher total SFA content. Boiling resulted in a decline in the content of palmitoleic and vaccenic acids; however, it did not affect the total MUFA content in the yolks. The thermal treatment of the eggs resulted in an increase in the content of arachidonic acids (**C20:4 n-6**) and DHA and a simultaneous decrease in the content of LA and docosapentaenoic acid (**DPA-C22:5 n-3**). Consequently,

Table 4. Crude fat and fatty acid composition (%) in yolk fat of hens.

	Processed egg			Feeding groups[1]			
Fatty acid	Raw	Boiled	Lyophilized	C	E-1	E-2	SEM
C12:0	0.02	0.01	0.01	0.01	0.01	0.01	0.02
C14:0	0.30	0.32	0.32	0.34^{b}	0.29^{a}	$0.31^{a,b}$	0.06
C16:0	23.67^{a}	24.52^{b}	24.25^{b}	24.40	24.00	24.04	0.01
C16:1 n-7	0.89	0.90	0.85	0.88	0.93	0.84	0.004
C16:1 n-9	3.10^{b}	2.66^{a}	2.94^{b}	2.91^{b}	2.64^{a}	3.15^{c}	0.07
C17:0	0.14	0.19	0.17	0.15	0.17	0.18	0.26
C17:1	0.13	0.12	0.13	0.12^{a}	0.12^{a}	0.14^{b}	0.04
C18:0	6.71^{a}	7.98^{c}	7.14^{b}	$7.30^{a,b}$	7.48^{a}	7.05^{b}	0.23
C18:1 n-9	42.46	42.43	42.23	42.52^{a}	43.27^{a}	41.32^{b}	0.02
C18:1 n-7	2.21^{b}	2.03^{a}	2.18^{b}	2.18^{b}	2.02^{a}	2.22^{b}	0.001
C18:2 n-6	16.99^{b}	15.31^{a}	16.86^{b}	16.00^{a}	15.84^{a}	17.32^{b}	0.01
C18:3 n-3	0.70	0.66	0.68	0.52^{a}	0.69^{b}	0.83^{c}	0.005
C20:0	0.03	0.02	0.02	0.03	0.02	0.02	0.02
C20:1n-9	0.20^{b}	0.20^{b}	0.16^{a}	0.20^{b}	0.16^{a}	0.20^{b}	0.01
C20:2 n-6	0.15	0.14	0.14	0.14	0.14	0.15	0.02
C20:4 n-6	1.41^{b}	1.58^{c}	1.31^{a}	1.49^{b}	$1.43^{a,b}$	1.37^{a}	0.14
C22:5n-3	0.17^{c}	0.05^{b}	0.04^{a}	0.09	0.08	0.10	0.27
C22:6 n-3	0.35^{a}	0.59^{b}	0.30^{a}	0.38^{a}	0.37^{a}	0.50^{b}	0.24
SFA	30.72^{a}	32.86^{c}	31.75^{b}	32.09^{b}	$31.79^{a,b}$	31.44^{a}	0.03
MUFA	48.98	48.34	48.48	$48.81^{a,b}$	49.14^{a}	47.85^{b}	0.23
PUFA	19.86^{b}	18.32^{a}	19.25^{b}	18.62^{a}	18.55^{a}	20.26^{b}	0.002
PUFA n-3	1.23^{b}	1.30^{b}	1.02^{a}	0.99^{a}	1.14^{b}	1.42^{c}	0.003
PUFA n-6	18.63^{b}	17.02^{a}	18.23^{b}	17.63^{a}	17.41^{a}	18.84^{b}	0.01
n-3/n-6	0.07^{b}	0.08^{c}	0.06^{a}	0.06^{a}	0.07^{b}	0.08^{c}	0.02
n-6/n-3	15.15^{a}	13.57^{a}	17.87^{b}	18.18^{c}	15.83^{b}	13.56^{a}	0.45
AI	0.36^{a}	0.39^{c}	0.38^{b}	0.37^{b}	0.36^{a}	0.36^{a}	0.02
TI	0.82^{a}	0.90^{c}	0.87^{b}	0.88^{b}	$0.86^{a,b}$	0.83^{a}	0.06
h/H	2.69^{c}	2.53^{a}	2.59^{b}	2.56^{a}	2.62^{b}	2.62^{b}	0.01
Lutein (mg/kg wet mass)	6.87^{b}	5.16^{a}	6.52^{b}	1.23^{a}	7.14^{b}	12.23^{c}	0.24
Lutein (mg/kg dry mass)	15.25^{b}	11.46^{a}	14.47^{b}	2.73^{a}	15.85^{b}	27.15^{c}	0.52
β-carotene (mg/kg wet mass)	0.13^{b}	0.10^{a}	0.11^{b}	0.04^{a}	0.16^{b}	0.19^{b}	0.03
β-carotene (mg/kg dry mass)	0.29^{c}	0.22^{a}	0.24^{b}	0.09^{a}	0.36^{b}	0.42^{b}	0.07
Crude fat	31.92	31.88	31.85	31.89	31.83	31.93	0.37

Different superscript lowercase letters denote statistically significant differences at $P \leq 0.05$ (means with the same letter are not significantly different).

Abbreviations: AI, atherogenic index; h/H, hypocholesterolemic-to-hypercholesterolemic acids ratio; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TI, thrombogenic index.

[1]C = control group; E-1 = group fed the diet supplemented with 1.5% of APC; E-2 = group fed the diet supplemented with 3.0% of APC.

boiling resulted in a decline in the total PUFA content, particularly that of PUFA n-6. The n-3/n-6 ratio changed as well. In comparison with the raw yolks, the hard-boiled yolks exhibited higher values of AI and TI and a lower h/H acid ratio (Table 4).

Yolk freeze-drying increased the total SFA content mainly through the increase in the content of palmitic and stearic acids. In turn, there was a decrease in the quantity of arachidonic acid and a 4-fold decline in the content of DPA acid, which resulted in a drop in the amount of PUFA n-3 and, consequently, an increase in the n-6/n-3 ratio. The yolk freeze-drying process did not change the total MUFA and PUFA content. In comparison with raw yolks, the freeze-dried material was characterized by a lower h/H acid ratio and higher values of the AI, TI, and n-6/n-3 ratio.

In comparison with freeze-dried yolks, the hard-boiled egg yolks were characterized by a higher concentration of SFA (mainly stearic acid) and a lower total content of PUFA n-6 (reduced LA amounts). The content of long-chain n-3 PUFAs (DPA and DHA) and, hence, the n3/n6 ratio were higher as well. Higher values of the AI and TI indices and a reduced h/H value were determined in the hard-boiled yolks, compared with the freeze-dried eggs. There was no effect of the boiling and freeze-drying processes on the total content of MUFA, despite the changes in the content of some fatty acids (palmitoleic, vaccenic, and gondolic acids).

Carotenoids Content Depending on Feeding Group and Egg Processing

The addition of 1.5% of APC to the feed (group E-1) caused an almost 6-fold increase in the lutein content, in comparison with the control group. The 2-fold increase in the APC dose in the feed (from 1.5 to 3%) resulted in a 1.7-fold increase in the lutein content in the egg yolks from E-2 group chickens compared with group E-1 (Table 4). The eggs of chickens receiving 1.5% of APC (group E-1) were characterized by over 4-fold higher content of β-carotene than the eggs from the control birds (Table 4). The 2-fold increase in the APC dose in the feed (from 1.5 to 3.0%) resulted in an increase in the β-carotene content in the egg yolks of chickens receiving the higher amount of the supplement (group E-2). However, the differences between the 2 groups were not statistically significant.

The freeze-drying process did not exert a significant effect on the content of lutein and β-carotene in the yolks, compared with the control group, that is, the raw yolks. In turn, cooking induced a significant decrease in both the lutein and β-carotene contents in egg yolks subjected to this process, compared with the raw and freeze-dried yolks (Table 4).

DISCUSSION

Production Traits

Phytobiotics, including alfalfa products, may contribute to achievement of varied production effects. Xie et al. (2008) underlined the unfavorable sensory values of APC, which may have a negative effect on feed intake in laying hens and thus on the other production traits. The present study did not reveal any impact of APC on the mean feed intake during the production period. Other authors investigating the effect of administration of various forms and doses of alfalfa on the production traits in turkeys, hens, and Japanese quails did not report changes in the feed intake between experimental groups and controls (Güçlu et al., 2004; Czech et al., 2012; Laudadio et al., 2014) or described only a slight decrease in the intake (Mourão et al., 2006; Krauze and Grela, 2010; Olgun and Yıldız, 2014; Wüstholz et al., 2017). The present study did not show an effect of the APC supplementation of hen diets on FCR. This was confirmed by results reported by other authors (Güçlu et al., 2004; Laudadio et al., 2014; Olgun and Yıldız, 2014). In a study conducted by Czech et al. (2012), significant improvement of the FCR value was noted in meat female turkeys receiving feed supplemented with 1.5% of APC. Mourão et al. (2006) observed an increased FCR value in hens in the initial rearing period in comparison with the control group. The results of the present investigations did not reveal a negative impact of the APC administration on the laying performance trait. This is in line with results reported by other researchers in investigations of supplementation of Japanese quail diets with various forms of alfalfa (protein concentrate, dried, meal with reduced raw fiber content, and crushed, extruded, or granulated silage) supplied in a dose range from 1.0 to 15% (Güçlu et al., 2004; Grela et al., 2014; Laudadio et al., 2014; Olgun and Yıldız, 2014; Wüstholz et al., 2017). A clear decrease in the laying performance in the initial period of production was only observed in investigations conducted by Mourão et al. (2006), where a dose of 15.1% of alfalfa meal was administered.

Egg Quality Traits

The present study did not reveal a negative effect of the APC addition on the weight egg, egg mass (g/layer/D), egg specific gravity, Haugh units, eggshell weight, thickness, and density, and yolk weight. Analogous investigations were carried out by Grela et al. (2014), who supplemented 1.5 and 3.0% of APC to the feed for high-performance ISA Brown hens. The authors did not report a significant effect of the nutritional supplement on the egg weight, specific gravity, yolk weight, albumen height, albumen quality, eggshell thickness and density, and the incidence of meat and blood spots. Administration of various forms and amounts of alfalfa in poultry nutrition did not induce changes in egg quality traits in Japanese quails and hens studied by other authors (Güçlu et al., 2004; Laudadio et al., 2014; Olgun and Yıldız, 2014; Wüstholz et al., 2017). Only Mourão et al. (2006) have demonstrated that partial replacement of soybean meal by 15.1% of alfalfa meal can significantly reduce the egg weight and egg mass (g/D). As shown in the present study, the addition of 3.0% of APC caused a slight decline in the eggshell breaking strength, with a simultaneous beneficial effect on eggshell color. This has not been demonstrated by other authors (Grela et al., 2014; Laudadio et al., 2014; Olgun and Yıldız, 2014). Only Güçlu et al. (2004) reported a beneficial effect of supplementation of feed with 6 and 9% of alfalfa meal on the mean eggshell thickness in the Japanese quail.

One of the most important criteria applied by consumers in their choice of table eggs is the yolk color. Their preferences differ depending on the country or geographical region. Nevertheless, they usually choose eggs with a yolk color between 11 and 12 points in the La Roche scale (Nys and Sauveur, 2004). In the present study, the addition of 1.5 and 3.0% of APC to the feed resulted in a clearly more intensive yolk color. This is in line with other reports demonstrating a beneficial effect of administration of various forms of alfalfa on the yolk color (Mourão et al., 2006; Grela et al., 2014; Laudadio et al., 2014). The beneficial effect of APC on the yolk color presented in this study may be associated with the high content of β-carotene in the concentrate, and this pigment is responsible for the more intense color of yolk. Laudadio et al. (2014) demonstrated that laying hens receiving 15% of low-fiber alfalfa meal in the diet were characterized by a higher concentration of β-carotene in both blood serum and egg yolk, which was also reflected in the darker yolk color, in comparison with eggs from control birds. Similar conclusions were reported in earlier studies conducted by Karadas et al. (2006) on Japanese quails fed with diet supplemented with 2% concentrate of alfalfa (PX). The researchers demonstrated that the addition of PX to the feed caused an over 10-fold increase in the content of lutein and β-carotene in the egg yolks of the experimental birds compared with the control. As shown by Carrasco et al. (2016) in laying hens kept in the organic rearing system and fed chopped, extruded, and pelleted alfalfa silage, the deposition of carotenoids in egg yolks may be dependent on the amount and availability of dietary lipids. Similarly, there was a significantly higher concentration of β-carotene and lutein in the yolks of birds fed with the APC-containing feed in the present study. However, the increase in the content of both these pigments was not directly proportional to the dose of the APC in the diet. The addition of 3% APC did not cause such a significant increase in their concentrations, especially β-carotene, as in the case of the 1.5% APC dose in

relation to the control group. A similar relationship was noted in the case of the yolk color between groups E-1 and E-2 and the control.

Fatty Acid Composition—APC Dose

In the present study, a significant decrease in the total SFA content was observed only in the egg yolks from the E-2 hens. This may be related to the fact that this group was characterized by the lowest stearic acid content and lower but statistically insignificant content of palmitic acid in comparison with the control group. Palmitic acid accounts for approximately 24% of all fatty acids contained in the egg yolk; hence, even statistically insignificant differences in its level between groups can determine the total SFA content in the yolk. In analogous research conducted on high-performance layer hybrids, Grela et al. (2014) did not observe a significant effect of feeding the birds with APC-supplemented diet on the SFA content in the egg yolk. The same findings were reported in a study on hens kept in a free-range system with access to alfalfa-sown pastures (Karsten et al., 2010). In turn, the yolks of eggs laid by laying hens kept in a free-range system and fed various forms of young alfalfa silage exhibited a significantly lower level of all individual saturated fatty acids and substantially lower total SFA content than the control group (Carrasco et al., 2016).

Oleic acid in all feeding groups represented from 41.32 to 42.52% of all fatty acids in the yolk, whereas the total MUFA amount was in the range of 47.84 to 49.14%. The lowest mean content of this acid was recorded in the yolks of eggs laid by the E-2 hens. Oleic acid accounts for only 5.2% of all fatty acids contained in APC (Grela and Pietrzak, 2014). Therefore, it is possible that an increase in the APC content in the feed will be accompanied by a decrease in the oleic acid level in yolks. The yolks of eggs laid by the E-2 hens were characterized by significantly higher content of palmitoleic and margaroleic acids than that in the other groups. However, the variable amount of MUFAs did not have an impact on the total amount of MUFA in both experimental groups compared with the control. Yet, the groups differed significantly between each other. In their investigations of the effect of APC on commercial laying hens, Grela et al. (2014) showed the lowest MUFA content in the yolks of hens fed diets supplemented with 3.0% of APC, but the differences between the groups were not significant. A reduced level of oleic acid in yolks from eggs laid by hens kept on alfalfa-sown pastures in comparison with hens receiving conventional feed was demonstrated by Karsten et al. (2010) as well. The investigations conducted by Carrasco et al. (2016) showed a significantly higher concentration of MUFA in egg yolks in all groups of hens receiving various forms of alfalfa silage than that in the control. In the study, the total amount of MUFA in yolks was determined by the content of oleic acid, which was significantly higher in the yolks from groups fed with alfalfa silage. However, the authors did not specify the fatty acid profile of the silage.

In the present study, there was a positive effect of APC, especially at the dose of 3%, on the PUFA content in the egg yolks. The addition of APC contributed to a significant increase in the content of PUFA in the yolks, both n-3 PUFA and n-6 PUFA, a higher n-3/n-6 ratio, and a lower n-6/n-3 ratio. In group E-2, the content of all polyunsaturated fatty acids increased, in particular that of LA and ALA, with the exception of arachidonic acid. The increase in LA and ALA in the groups receiving the APC-supplemented feed seems logical because APC contains 18.8 and 41.7% of these acids, respectively, which accounts for almost 60% of all fatty acids (Grela and Pietrzak, 2014). It may seem surprising that at the high ALA level in the concentrate, the amount of this acid in the egg yolks increased only by 0.31%, compared with the control group, especially given the report by Poureslami et al. (2012) in which the ALA content in yolk was highly positively correlated with the ALA amount in the diet. The increase in the content of LA in the yolks in group E-2 may have induced the decline in the content of some MUFAs, as their level is determined by their availability in the diet and is negatively correlated with the amount of LA (Poureslami et al., 2012). The n-3 and n-6 PUFAs are not synthesized by mammalian cells and must be supplied to the organism with nutrition. Long-chain fatty acids (EPA, DPA, DHA) are especially important for human health, as they serve a number of important functions in the human organism and their appropriate levels prevent the development of many civilization diseases (Kremmyda et al., 2011). These acids can be synthesized in the organism from LA and ALA; however, as demonstrated by kinetic analysis, the total conversion of ALA can only amount to 0.2% in the case of EPA, 0.13% for DPA, and 0.05% for DHA (Pawlosky et al., 2001). Hence, they must be supplied with food. Therefore, the significant increase in the content of long-chain fatty acids, especially DHA, in the egg yolks in group E-2 is worth noting. These results are in agreement with the reports provided by Karsten et al. (2010). The authors found that, in comparison with caged hens fed with conventional fodder, the eggs from the hens kept on alfalfa-sown pastures had higher LA content, 5-fold higher ALA content, and a significantly higher level of long-chain fatty acids: EPA (2-fold higher level), DPA (2.7-fold higher level), and DHA (2.2-fold higher level). The total content of n-3 acids was 3-fold higher than that in yolks from eggs laid by the control hens. This relationship was not confirmed by other authors who did not detect a positive effect of alfalfa silage or protein concentrate on the total content of PUFA in egg yolks (Grela et al., 2014; Carrasco et al., 2016). In a study conducted by Grela et al. (2014), there was an increase in the n-6/n-3 ratio in the yolks from both APC-supplemented groups, whereas this parameter declined highly significantly in the yolks from hens receiving alfalfa silage in the study conducted by Carrasco et al. (2016). Egg yolks from hens fed with alfalfa silage exhibited 2.4-fold higher amounts of n-3 PUFA than those in the control group and a lower level

of n-6 PUFA (Carrasco et al., 2016). Similarly, in a study conducted by Karsten et al. (2010), the ratio of n-6/n-3 acids was 12.05 in the control group and 4.44 in the alfalfa-supplemented group.

The results of the present study indicate that the 1.5% APC addition contributed to a significant increase in the h/H ratio and a decline in the AI index. The 3.0% APC dose resulted in a reduction of the AI and TI indices and the h/H ratio. The AI values were comparable to those reported by Grela et al. (2014) and slightly lower than those presented by Attia et al. (2015). The TI was lower than that reported by Grela et al. (2014) but higher than or comparable to that reported by Attia et al. (2015) in investigations on consumer eggs available in stores. Analogous studies carried out on high-performance laying eggs did not show an effect of APC of the aforementioned parameters, although yolks from eggs laid by hens receiving 3.0% of APC were characterized by the lowest values of these indices (Grela et al., 2014).

Fatty Acid Profile and Carotenoid Content—Egg Processing

The present study demonstrated that the egg boiling process contributed to an increase in the SFA content and the n-3/n-6 ratio as well as a decrease in the PUFA level (in particular n-6 PUFA) in the yolks. The yolks of hard-boiled eggs exhibited higher values of the atherogenic and thrombogenic indices and a lower h/H ratio. The quantity of long-chain fatty acids changed, that is, the DHA level increased and DPA declined. While analyzing the effect of boiling on the fatty acid content in egg yolks enriched with n-3 acids, other authors did not observe any obvious changes in the content of SFA and MUFA (Cortinas et al., 2003; Ren et al., 2013; Douny et al., 2015). Only investigations conducted by Botsoglou et al. (2012) on eggs enriched with n-3 acids demonstrated that boiling induced an increase in the SFA content in yolks, although there was no clear effect of the thermal treatment on the content of the individual saturated fatty acids. These authors also showed an increase in the content of oleic acid in the yolks and, consequently, the total level of MUFA. In the present study, the egg boiling treatment contributed to an increase in the content of arachidonic acid and DHA and to a simultaneous decline in the content of LA and DPA. Most studies carried out to date have indicated an adverse effect of cooking on the content of individual polyunsaturated fatty acids in egg yolk (Murcia et al. 1999; Cortinas et al., 2003; Botsoglou et al., 2012). The investigations conducted by Botsoglou et al. (2012) and Ren et al. (2013) demonstrated only an increase in the LA content in boiled yolks compared with raw yolks. The present study showed a decline in the total content of PUFA and n-6 PUFA and an increase in the n-3/n-6 PUFA ratio in the thermally treated yolks. Similar conclusions were formulated by Cortinas et al. (2003). The results reported by other authors are ambiguous. Botsoglou et al. (2012) observed a decrease in the content of n-3 PUFA and PUFA and an increase in the level of n-6 PUFA and the n-6/n-3 ratio in cooked yolks, whereas results reported by other authors did not show any effect of cooking on the aforementioned parameters (Ren et al., 2013; Douny et al., 2015).

The present study showed that lyophilization of yolks contributed to an increase in the total SFA content and a decline in the level of n-3 PUFA; however, no changes in the total PUFA content was noted. The MUFA level was unchanged as well. The available literature does not provide information about the effect of freeze-drying on the fatty acid profile in the egg yolks. Dehydration and low temperature can affect the conformation and stability of egg proteins and the fatty acid profile. Wenzl et al. (2010) reported an increase in the content of carotenoids in freeze-dried yolks. The researchers found that the freeze-drying process might have led to denaturation of proteins associated with carotenoid pigments and contributed to their higher availability in freeze-dried eggs. Dehydration in the freeze-drying process can also result in destabilization of the low-density lipoprotein structure (Thierau et al., 2014) and affect the conformation of ovalbumin (Kitabatake et al., 1989). This is not confirmed by the results of this study, as there was no increase in the concentration of carotenoids in freeze-dried yolks compared with raw yolks.

The present study showed that the process of cooking eggs caused a significant decrease in the content of lutein in the yolk. Schlatterer and Breithaupt (2006) reported a 19% decrease in the lutein content in boiled egg yolks, compared with raw egg yolk, while Nimalaratne et al. (2012) observed a 18% decrease in the lutein content in boiled egg yolk. Similar conclusions were formulated by Perry et al. (2009), who determined the content of this pigment in fresh and cooked eggs. In turn, contrasting findings were presented by Surai et al. (2000) in their investigations of eggs enriched with lutein, selenium, vitamin E, and DHA (designer eggs). They found no significant effect of cooking on the content of carotenoids in the egg yolks. This may have been related to the presence of a large amount of antioxidants in the analyzed eggs, which significantly reduce the oxidation of carotenoids (Zaheer, 2017).

CONCLUSIONS

The addition of 3% of APC to the feed for Polbar hens partially replacing soybean meal resulted in enhanced intensity of the yolk and eggshell color and an increased proportion of PUFA and carotenoid content. There was no negative effect of the APC supplementation on the other egg quality and egg production traits, FI and FCR. The supplementation led to a decline in eggshell strength, reduction of the SFA content, a decrease in the PUFA n-6/n-3 ratio, and lower AI and TI indices.

Boiling and freeze-drying induced changes in the fatty acid profile. The yolk of the hard-boiled eggs was characterized by increased content of SFA, a lower level of n-6 PUFA and carotenoids, and an increase in the AI and TI indices. The freeze-drying process contributed to

reduction of the n-3 PUFA proportion and an increase in the n-6/n-3 PUFA ratio.

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