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SPECIAL ISSUE: *Insects on the poultry, swine and rabbit menu*

Feeding value of black soldier fly larvae compared to soybean in methionine- and lysine-deficient laying hen diets

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RESEARCH ARTICLE

Abstract

To increase the sustainability of egg production, alternatives to soybean in poultry nutrition are intensively searched for. Black soldier fly larvae (BSFL) could have a great potential, but the comparative protein value to soybean is not well known. The main objective of this study was to facilitate this comparison by using experimental diets clearly limited in calculated supply of sulphurous amino acids and lysine. Fifty laying hens (Lohmann Brown Classic), aged 40 weeks, were fed one of five diets for 7 weeks (n=10). Two diets were based on soybean cake and oil (SS, SS-) as protein and energy sources, and three diets contained partially defatted BSFL meal and fat from two different origins (AA-, AB-, BB-). Different from SS, all other diets were designed to be deficient in methionine and lysine in relation to requirements by >20%. The realised supply with total sulphurous amino acids and lysine was indeed superior with SS even though this diet was analysed to be more deficient in methionine than the BSFL-based diets. Despite the calculated deficiency in limiting amino acids, laying performance of the hens of all groups was similar and ranged between 93 and 97%. Similarly, egg mass, daily feed intake and feed efficiency were not influenced by the BSFL-based diets. The yolks of group BB- were more intensely coloured compared to the others indicating a difference between BSFL origins. Yolks of SS-, but not of the BSFL-based diets, had lower contents of dry matter and ether extract than those of SS. Including BSFL into the diet did not influence the odour of the eggs tested in scrambled form. The results show that soybean-based feeds for laying hens may be completely replaced by BSFL-based feeds and suggest that the recommendations for amino acid supply of laying hens might need revision.

Keywords: *Hermetia illucens,* soybean, layer diet, larval meal, odour

1. Introduction

With the increasing demand for animal products, global egg production has been steadily expanding and amounted to about 82 million tons in 2019 (FAO, 2021). Therefore, the demand for feed protein is high, which is currently mainly covered by soybean. However, the cultivation of this crop requires substantial land resources and is often associated with deforestation (Taherzadeh and Caro, 2019). In addition to ecosystem destabilisation, the cultivation and transport to consuming countries comes with higher greenhouse gas emissions from carbon release and expenditure of fossil fuels (Pendrill *et al.*, 2019). This knowledge triggered an intensive search for soybean alternatives. Preferable are those which can be produced with low resource input and low need for arable land thus associated with low food-feed competition. Insects as feeds offer a promising solution in this respect, as they are not well accepted as food by most people, especially in Europe (Hartmann and Siegrist, 2017), and have certain sustainability benefits, such as low land demand, possibility of use of residue streams as feed substrates within circular economy concepts, and

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beneficial nutrient composition (Smetana *et al.*, 2019; Van Huis, 2020). In addition to the mealworm (*Tenebrio molitor*), various cricket species (*Gryllidae*), the house fly (*Musca domestica*) and other insects, that are also suitable as feed (Van Huis, 2020), were evaluated. Among them, from the list of insects recently approved by EU for feeding to poultry (Regulation (EU) 2021/1372, Annex IV to EC No 999/2001 (EC, 2021)), the black soldier fly larvae (*Hermetia illucens*; BSFL), showed particularly advantageous characteristics as a substitute for soybean in hens' nutrition (Bejaei and Cheng, 2020; Heuel *et al.*, 2021a; Mwaniki *et al.*, 2020; Patterson *et al.*, 2021). The BSFL are rich in protein and have a beneficial amino acid composition for poultry nutrition (Spranghers *et al.*, 2017). The larvae are a natural source of nutrients for poultry (Bovera *et al.*, 2016), reproduce fast and efficiently convert almost any organic material to biomass (Oonincx *et al.*, 2015) which is characterised by protein of high quality. It has already been shown that rearing larvae on low-value side streams or high-impact waste streams can be more sustainable than using conventional protein sources (Smetana *et al.*, 2019). Results on the feeding value of BSFL compared to soybean are contrasting so far, with either higher (Marono *et al.*, 2017) or lower (Mwaniki *et al.*, 2020) value found for BSFL. To further clarify the feeding value of BSFL compared to soybean-based feeds, we had carried out a layer experiment with diets not supplemented with pure amino acids and with a calculated small deficiency of methionine (Met) and total sulphurous amino acids (S-AA; i.e. Met + cysteine (Cys)) as the first limiting amino acids (Heuel *et al.*, 2021a). However, this did not trigger any differences to soybean and between the BSFL sources in the hens' performance, which left the question about the comparative protein value open. Other potential side-effects may also be important for the implementation of BSFL-feeding to hens in farm practice. The rearing substrates, depending on the type and processing, and the BSFL as such may have a strong and variable odour (Diener *et al.*, 2011; Rana *et al.*, 2015), but the effects of BSFL on the sensory impression of the eggs from laying hens are widely unknown. Although taste, texture, and appearance of boiled eggs were improved, no change in odour was found by Al-Qazzaz *et al.* (2016) when adding either 1 or 5% of BSFL to the diet, a level probably too low to cause off-odour. Bejaei and Cheng (2020) reported no changes in sensory texture, taste and odour of boiled eggs when replacing half or all soybean meal by full-fat dried BSFL (10 and 18% in the diet), but at the same time various other feed ingredients were changed in that study.

The present study aimed to evaluate the differences between soybean protein and BSFL protein by provoking a pronounced deficiency of limiting amino acids that provides lysine (Lys) at a borderline level compared to the calculated requirements of high-performance layer hybrids in their first third of the laying period. The following hypotheses were tested: (1) The feeding value of BSFL is superior to that of soybean, especially in the situation of a deficiency of limited amino acids. (2) The level of superiority depends on the source of BSFL. (3) The use of BSFL meal and fat causes an unfavourable odour of the eggs.

2. Materials and methods

Birds and housing system

The experiment was conducted at the research cooperation AgroVet-Strickhof (Lindau, Switzerland) and was approved by the Cantonal Veterinary Office of Zurich, Switzerland (licence number ZH221/17). For this purpose, 50 Lohmann Brown Classic layers (Burgmer Geflügelzucht, Weinfelden, Switzerland) at 40 weeks of age were individually housed in enriched enclosures (each 80 × 80 × 80 cm). These contained a meshed floor, a nest, a perch and a scratching box filled with sawdust. Feed and water were provided *ad libitum* with one trough and two nipple drinkers each. The room climate was kept constantly at 20 °C and at about 45% humidity. Throughout the 7-week experiment, the health status of the hens was monitored daily. No hen showed signs of illness or died during the experiment. However, in the beginning of week 3 the water system failed on one side of the bird house (for half of the hens of each treatment). Water was provided by an additional trough instead. No bird was harmed during this period. Still feed intake declined to some extent which is why no intake and performance related data from all hens from week 3 were used for statistical analysis.

Diet composition, experimental design, and sampling

The hens were allocated to one of five experimental diets according to a randomised design (n=10 hens per diet). The same hens had been used in the previous experiment (Heuel *et al.*, 2021a). It was ensured that no hen received a diet similar to that type already fed in the preceding experiment. The five diets differed in their main protein and energy sources (Table 1). There were two control diets (SS, SS-) based on soybean cake and soybean oil and three experimental diets (AA-, AB-, BB-) based on different combinations of partially defatted protein meal and pure fat from two different BSFL origins (A and B). The difference between the positive control (SS) and the negative control (SS-) was intended to be the supply with Met and Lys, being deficient in SS- but not in SS. Accordingly, diet SS should meet the breeder's specifications for requirements of hybrids of the used type and age, specified as 0.44% Met and 0.88% Lys/kg diet assuming an average daily feed intake (ADFI) of 110 g (Lohmann Tierzucht, 2020). Diet SS was composed of the same ingredients and proportions as the diet SS used before in Heuel *et al.* (2021a,b). However, new batches except for the BSFL materials and soy components were used. All BSFL-containing diets (AA-, AB-, BB-) were designed to be similarly deficient in Met and Lys as diet

Table 1. Dietary composition of the control and the four protein reduced diets (% of dry matter).

Diet[1]	SS	SS-	AA-	AB-	BB-
Soybean cake	15.0	15.0	-	-	-
Soybean oil	3.0	3.0	-	-	-
Defatted larval meal A	-	-	15.0	15.0	
Defatted larval meal B	-	-	-	-	15.0
Larval fat A	-	-	2.0	-	-
Larval fat B	-	-	-	2.0	-
Wheat	30.0	16.2	15.8	15.8	15.8
Maize	18.0	34.2	34.1	34.1	37.1
Wheat boll meal	3.16	3.00	2.49	2.49	4.49
Broken rice	2.00	7.00	10.5	10.5	8.50
Wheat bran	8.45	8.49	7.00	7.00	6.00
Sunflower cake	7.28	-	-	-	-
Calcium carbonate	2.7	2.7	2.7	2.7	2.7
Limestone grit	7.0	7.0	7.0	7.0	7.0
Dicalcium phosphate	1.0	1.0	1.0	1.0	1.0
Sodium bicarbonate	0.33	0.33	0.33	0.33	0.33
Sodium chloride	0.20	0.20	0.20	0.20	0.20
Choline chloride	0.08	0.08	0.08	0.08	0.08
Vitamin and trace element premix[2]	0.20	0.20	0.20	0.20	0.20
Celite[3]	1.6	1.6	1.6	1.6	1.6

[1] SS = control, soybean cake and soybean oil; SS- = negative control, soybean cake and soybean oil; AA- = larval meal A and larval fat A; AB- = larval meal A and larval fat B; BB- = larval meal B rich in larval fat B.
[2] Contained per kg: Ca, 86.5 g; P, 0.2 g; Mg, 25 g; Cu, 5 g; Mn, 30 g; J, 400 mg; Zn, 25 g; Fe, 25 g; Se, 100 mg; vitamin A, 5,000,000 IE; vitamin D_3, 1,250,000 IU; vitamin E, 15 g; vitamin K, 1.5 g; vitamin B_1, 1 g; biotin, 250 mg; folic acid, 750 mg; niacin, 20 g; pantothenic acid, 8.2 g.
[3] No. 545, acid-washed diatomaceous earth (Schneider Dämmtechnik, Winterthur, Switzerland).

SS-. Diet AB- served to evaluate whether any effects are based on the larval protein meal (BB- vs AA- and AB-) or the larval fat (AA- vs AB- and BB-) or both. Larval meal and fat A were purchased from a commercial BSFL producer (InnovaFeed, Paris, France) with the larvae being reared on >80% wheat bran and solubles from wheat distillation according the producer. Harvesting of the BSFL took place before they became prepupae. The BSFL for meal and fat B were produced in an experimental unit (FiBL, Frick, Switzerland) and grown on 40% fruit and vegetable raw waste and 30% each of brewer's grains and pasta production waste. Harvesting of batch B took place when ≥50% of the larvae reached the prepupal stage. A more detailed description of the production and processing of the two BSFL batches can be found in Heuel *et al.* (2021a). Due to a high residual fat content of BSFL meal B (Table 2), no additional supplementation of fat B was necessary in diet BB-. Except for the BSFL meals and fats and the soybean oil, all components used were certified organic and obtained from local companies. No synthetic amino acids and no yolk colour pigments were added. Celite (1.6% of dry matter (DM)) was added as an indigestible marker to be able to determine metabolisability according to Vucić-Vranješ *et al.* (1994).

Diets were produced on the research station in accordance with the current Swiss regulations for feed production (SR 916.307; https://www.fedlex.admin.ch/eli/cc/2011/772/de). At first the individual dry feedstuffs were mixed in a 100 kg single-shaft feed mixer (Gericke, Zurich, Switzerland). Afterwards, either the soybean oil or the liquefied BSFL fat was added and mixed again (total mixing time approx. 25 min). The feed was then stored in bags at +4 °C. During the experiment, ADFI and body weight (BW) were determined weekly and individually. The eggs per hen were collected and weighed daily. From feeding week 4 on six eggs per hen were collected to determine egg quality. Following the determination of the external quality, yolks, and albumen of four of these eggs per hen were frozen at -20 °C, lyophilised (Beta 1-16 Christ, Osterode am Harz, Germany) and subsequently homogenised with a kitchen mortar (Haldenwanger, Berlin, Germany). In week 7, the hens' excreta were collected daily for 5 days, weighed and frozen (-20 °C) immediately after collection, lyophilised and ground to 0.75 mm (centrifugal mill ZM 1, Retsch, Haan, Germany). Feed samples were taken once before the start of the experiment and once in week 2. Samples of the soybean cake and the two BSFL meals were taken once before the diets were mixed. All feeds were ground to 0.5 mm (same

Table 2. Analysed nutrient contents of the soybean cake, the larval meals A and B and the complete experimental diets (% in dry matter (DM) unless stated otherwise).[1]

Item	Soybean cake	Larval meal[2]		Diet[3]				
		A	B	SS	SS-	AA-	AB-	BB-
DM (% in original substance)	92.9	95.2	93.7	90.4	90.2	90.3	90.3	90.0
Organic matter	87.1	86.7	88.4	76.9	75.8	74.4	74.5	75.2
Nitrogen	7.08	9.67	7.99	2.61	2.36	2.59	2.64	2.48
Ether extract	9.03	13.3	29.9	7.65	6.76	5.60	5.53	6.79
Chitin	-	7.44	6.95	-	-	1.06	1.10	0.93
Phosphorus	0.71	1.21	0.68	0.73	0.65	0.69	0.67	0.63
Calcium	0.20	1.64	0.95	4.02	4.26	5.35	5.18	4.68
Magnesium	0.05	0.06	0.06	0.15	0.14	0.14	0.14	0.13
Chloride	na[4]	0.13	0.14	0.21	0.21	0.24	0.28	0.27
Sodium	na	0.34	0.41	0.19	0.19	0.17	0.22	0.19
Amino acids								
Methionine	0.56	0.95	0.79	0.27	0.25	0.30	0.31	0.29
Methionine + cysteine	1.09	1.37	1.11	0.56	0.52	0.52	0.53	0.51
Lysine	2.62	3.21	2.36	0.77	0.71	0.76	0.79	0.63
Amino acids (% of total amino acids)								
Alanine	0.43	0.74	0.82	0.47	0.51	0.67	0.68	0.68
Arginine	0.75	0.52	0.50	0.69	0.68	0.56	0.57	0.57
Asparagine/aspartic acid	1.13	1.01	0.97	0.91	0.91	0.87	0.88	0.82
Cysteine	0.12	0.08	0.08	0.19	0.19	0.15	0.14	0.16
Glutamine/glutamic acid	1.83	1.19	1.17	2.14	2.02	1.65	1.63	1.75
Glycine	0.42	0.60	0.92	0.47	0.46	0.54	0.54	0.53
Histidine	0.26	0.33	0.30	0.27	0.28	0.30	0.30	0.28
Isoleucine	0.46	0.49	0.50	0.43	0.43	0.43	0.42	0.42
Leucine	0.76	0.74	0.74	0.78	0.84	0.81	0.81	0.82
Lysine	0.61	0.61	0.56	0.50	0.50	0.51	0.51	0.47
Methionine	0.13	0.18	0.19	0.17	0.18	0.20	0.20	0.21
Phenylalanine	0.52	0.45	0.44	0.50	0.51	0.46	0.47	0.47
Proline	0.51	0.61	0.68	0.66	0.65	0.70	0.69	0.75
Serine	0.50	0.45	0.46	0.48	0.48	0.46	0.45	0.45
Threonine	0.39	0.43	0.44	0.37	0.37	0.40	0.40	0.39
Tryptophan	0.13	0.17	0.17	0.14	0.14	0.15	0.15	0.15
Tyrosine	0.34	0.72	0.69	0.33	0.35	0.54	0.53	0.47
Valine	0.48	0.68	0.70	0.51	0.51	0.62	0.62	0.60
Gross energy (MJ/kg dry matter)	21.0	22.5	25.4	17.2	16.8	16.4	16.5	16.9

[1] Nutrient composition of the soybean cake and the larval meals according to Heuel *et al.* (2021a).
[2] Insect meal A was produced on wheat bran and dried wheat distillery solubles; insect meal B was produced on 40% fruit and vegetables raw waste (with seasonal variations); 30% brewers' grain; 30% pasta production waste.
[3] SS = control, soybean cake and soybean oil; SS- = negative control, soybean cake and soybean oil; AA- = larval meal A and larval fat A; AB- = larval meal A and larval fat B; BB- = larval meal B rich in larval fat B.
[4] Not analysed.

mill as for excreta) before being analysed. For the sensory evaluation, 45 eggs each from groups AA-, AB- and BB- and 90 eggs from group SS (no SS- as only the effect of insect feeding was targeted) were collected in weeks 4 and 5 and graded after being stored for 4 days at 4 °C.

Laboratory analyses

All analyses were carried out in duplicate. Feed items, dried yolks and albumens as well as excreta samples were analysed for their proximate composition according to standard procedures (AOAC International, 1997) and methods described in detail in Heuel *et al.* (2021a).

DM and total ash were measured with a thermo gravimetric device (TGS 701, Leco Corporation, St. Josephs, MI, USA; AOAC index no. 942.05). Organic matter was calculated as the difference between the two variables. A C/N-analyser (TruMac CN, Leco Corporation; AOAC index no. 968.06) was used to determine the N content. The crude protein content of the lyophilised egg yolks and albumens was calculated as 6.25 × N. Ether extract (EE) in feeds and yolks was determined using a Soxhlet extraction system (B-811, Büchi, Flawil, Switzerland; AOAC index no. 963.15). The amino acid contents of the diets were analysed using HPLC (Alliance 2690; Waters Corporation, Milford, MA, USA) adjusted for amino acid analysis as outlined in Gangnat *et al.* (2020). Gross energy (GE) was measured by incineration in a bomb calorimeter (Calorimeter System C7000 and Cooling System C7002, IKA-Werke, Staufen, Germany). Chitin in the BSFL protein meals and diets was determined according to Black and Schwartz (1950). Shell breaking strength, shell thickness, yolk colour, yolk and albumen heights as well as the ratios of the inner egg composition were assessed and Haugh units (Haugh, 1937) were calculated as described by Heuel *et al.* (2021a).

Sensory evaluation

For the sensory evaluation, scrambled egg samples were prepared by combining the complete egg content from either three (either from group AA-, AB- or BB-) or six eggs (group SS) from different hens, respectively, laid 4 days earlier. These egg materials were fried separately per diet group in a household pan for 5 min and cooled down to a temperature of 38 to 40 °C. Scrambled egg batches were then divided into 4-5 portions (groups AA-, AB- and BB-) and 8-10 portions (group SS), respectively, and filled into sealable disposable cups (Pacovis, Stetten, Switzerland). All samples were kept at 38 to 40 °C until sensory testing. As BSFL-fed poultry was not yet allowed to be consumed by humans at the time of this evaluation, only the odour of the scrambled egg samples was assessed. As sensory test method an R-index analysis was chosen. The R-index is a probability value for discriminating two samples. An R-index of 100 indicates perfect discrimination, while a value of 50 means that the two samples are distinguished just by chance. Each test series consisted of a reference sample (from SS eggs) and four coded test samples, one of which was again the reference sample. For each of the coded samples the panellist had to decide whether it differed in odour from the reference sample. In addition, a sureness judgement had to be given to each of the decisions. The panel evaluated five test series in total. All egg samples were evaluated by a trained sensory panel (n=12-13) at Agroscope Liebefeld, Switzerland. No explicit egg related panel training was conducted since the test set up did not ask for any product specific odour attributes. All samples were coded with three-digit random numbers and presentation order of the samples followed a William Latin Square design. Tests were conducted at room temperature under day light conditions.

Calculations and statistical analyses

The coefficients of metabolisability of N and GE were determined as outlined by Vucić-Vranješ *et al.* (1994), considering the intake and excretion of acid-insoluble ash as an indicator and as described in detail in Heuel *et al.* (2021a). Measured data were combined to one value per hen, feed item or diet and subjected to analysis of variance using a linear mixed effect model (procedure MIXED of SAS version 9.4, SAS Institute, Cary, NC, USA), including the Tukey-Kramer correction for multiple comparisons. Diet was considered as fixed effect, the individual hen or egg data as experimental unit. Results are expressed as least squares means and standard error of the mean. Effects were assumed to be significant at $P<0.05$. The sensory data were collected and statistically analysed with the software FIZZ (version 2,51 Biosystèmes, Couternon, France). Critical R-indices (two-sided, 5% significance level) were taken from Bi and O'Mahony (2007).

3. Results

Nutrient composition of the main protein sources and the complete diets

The N content as analysed in BSFL meal B was the lowest, followed by soybean cake and BSFL meal A (Table 2). The analysed contents of the limiting amino acids and their proportion of total amino acids differed among the main protein sources. The BSFL meals contained more Met than the soybean cake, with meal A having the highest proportion of Met. The proportion of S-AA was also highest in BSFL meal A, whereby BSFL meal B and the soybean cake barely differed. Concerning the content of Lys, the order from high to low was BSFL meal A, soybean cake and BSFL meal B. In the complete diets, the BSFL-based diets had higher levels of Met than the control diets (SS and SS-) following the differences among the main protein sources, whereas the content of S-AA did not differ from diet SS-. Diet AB- contained most Lys and diet BB- least Lys, with the other diets showing values in between. The EE content in DM of the defatted BSFL meal A was 16.6% lower than that of BSFL meal B and 4.3% higher than that of the soybean cake. The high EE content of BSFL meal B was also reflected in the diet BB- which was richer in EE than the other BSFL-based diets, but equal to SS- and lower than that of SS. Correspondingly, the gross energy content of diet SS was also the highest but showed only a difference of 0.8 MJ/kg DM to diet AA-, which had the lowest gross energy content. With about 7% of DM, the chitin content of the two BSFL meals was comparable. BSFL meal A was richer in P, Ca and Mg compared to meal B and the soybean cake. The two insect meals contained similar amounts of Na and Cl.

Performance

The ADFI was not affected by diet, but groups significantly differed in the daily amounts of Met and Lys consumed (Table 3). Accordingly, the Met intake of the SS and SS- hens was lower by 41 (P=0.003) and 57 mg/day (P<0.001) than those fed AA-, respectively, with the other groups being intermediate. The BB- hens had the lowest (P<0.001) Lys intake, with a consumption being lower by 100 to 190 mg/day compared to the other groups. In all diets, the realised intake of Met and total S-AA was clearly below the requirements assumed for laying hens at this performance stage. For Met this was also true for the positive control diet (SS), which had been designed to meet the actual demand, but the deficiency in S-AA was clearly lower in SS than in the other diets. There was no deficiency in calculated Lys supply with diets SS, AA- and AB-. Compared to the other diets, BB- resulted in the poorest (P<0.001) supply of Lys. The N utilisation was higher (P=0.044) by 5% in the SS- hens compared to the AA- hens. The N metabolisability of group SS- was higher (P=0.016) than that of group BB-, with the other groups ranging in between. The energy metabolisability was higher (P<0.05) in groups AA- and AB- than in group SS. The measured dietary contents of metabolisable energy did not differ significantly between groups.

Internal and external egg quality as well as sensory odour perception

Proportions of albumen, yolk and shell of the total egg, shell breaking strength, shell thickness as well as albumen composition and the Haugh units did not significantly differ between the diet groups (Table 4). The BB- hens had a lower (P=0.019) yolk height than the SS hens. In addition, the yolks of the SS, AA- and AB- hens had an EE content higher (P<0.001) by around 3% compared to the SS- hens. The diet containing BSFL meal B (BB-) intensified yolk colouration (red and yellow colour space) especially when compared to diet SS (P<0.001), whereas the yolks of groups SS-, AA- and AB- were intermediate in colour intensity. With one exception (test series 3), the calculated R-index values of the sensory test of the odour of the scrambled eggs were not significant. Accordingly, the odour perception did not allow discriminating between the BSFL-based diets (AA-, AB- and BB-) and diet SS (Table 5).

Table 3. Effect of the insect feeding over 40 d on performance (n=10 per treatment).[1,2]

	SS	SS-	AA-	AB-	BB-	SEM	*P*-value[3]
Daily intake							
Total (g as fed)	121	119	121	117	115	4.1	ns
Methionine (mg)	290[bc]	274[c]	331[a]	328[ab]	299[abc]	9.6	***
Methionine + cysteine (mg)	617[a]	559[ab]	577[ab]	562[ab]	529[b]	18.4	*
Lysine (mg)	846[a]	761[a]	847[a]	831[a]	656[b]	25.2	***
Supply over requirements[4] (%)							
Methionine	-26.7[b]	-29.5[b]	-16.5[a]	-17.8[a]	-21.9[ab]	1.96	***
Methionine + cysteine	-10.9[a]	-17.9[ab]	-17.1[ab]	-19.7[b]	-21.1[b]	2.09	*
Lysine	6.3[a]	-2.7[a]	6.0[a]	3.5[a]	-14.9[b]	2.52	***
Bodyweight (kg)	1.99	1.92	2.01	2.02	2.03	0.081	ns
Laying performance (%)	93.5	93.0	97.3	93.5	95.0	2.15	ns
Egg weight (g)	65.9	63.6	65.4	65.9	63.2	1.12	ns
Egg mass (g/day)	61.5	59.3	63.6	61.6	60.1	1.69	ns
Feed efficiency (g feed/g egg)	1.97	1.99	1.90	1.91	1.91	0.044	ns
Nitrogen utilisation[5] (%)	42.4[ab]	45.6[a]	40.6[b]	42.1[ab]	42.9[ab]	1.23	ns
Metabolisability[6] (%)							
Nitrogen	46.6[ab]	49.2[a]	47.2[ab]	42.6[ab]	41.4[b]	1.67	**
Energy	77.2[b]	78.6[ab]	80.2[a]	80.1[a]	79.5[ab]	0.67	*
Metabolisable energy (MJ/kg feed dry matter)	13.3	13.2	13.2	13.2	13.4	0.11	ns

[1] Least-square means within a row with no common superscript are differ significantly different (P<0.05).
[2] SS = positive control, soybean cake and soybean oil; SS- = negative control, soybean cake and soybean oil; AA- = larval meal A and larval fat A; AB- = larval meal A and larval fat B; BB- = larval meal B rich in larval fat B; SEM = standard error of the mean.
[3] ns = not significant; Significant diet effects are indicated as *P<0.05, **P<0.01, ***P<0.001.
[4] The requirements were calculated based on performance and recommendations by the National Research Council (1994) and were related to the actual intake of the hens.
[5] Nitrogen excretion via the egg in relation to nitrogen intake.
[6] Calculated as outlined by Vucić-Vranješ *et al.* (1994) for indicator techniques.

Table 4. Egg quality of the hens receiving the experimental diets (n=10 per treatment).[1,2]

	SS	SS-	AA-	AB-	BB-	SEM	*P*-value[3]
Egg composition (g/kg)							
Shell	106	106	111	104	105	2.3	ns
Albumen	634	628	617	630	641	6.7	ns
Yolk	260	266	272	266	253	5.5	ns
Shell breaking strength (N)	48.3	49.0	55.5	49.3	47.6	2.28	ns
Shell thickness (mm)	0.43	0.42	0.43	0.42	0.40	0.008	ns
Albumen composition (g/kg wet weight)							
Dry matter	118	117	116	118	117	1.8	ns
Total ash	6.96	6.69	6.92	6.79	7.32	0.21	ns
Crude protein	93.3	93.6	91.9	93.8	93.3	1.56	ns
Haugh units	86.0	85.4	79.5	84.4	86.7	2.23	ns
Yolk height (mm)	17.9[a]	17.5[ab]	17.3[ab]	17.6[ab]	16.7[b]	0.28	*
Yolk composition (g/kg wet weight)							
Dry matter	507[a]	496[b]	505[ab]	503[ab]	499[ab]	2.4	**
Total ash	22.2	21.6	21.3	20.9	21.2	0.56	ns
Crude protein	163	162	159	158	158	1.4	*
Ether extract	262[a]	253[b]	266[a]	265[a]	261[ab]	2.0	***
Yolk colour[4]							
Lightness (L*)	67.4	67.1	65.5	65.4	65.8	0.64	ns
Red-green axis (a*)	-7.09[c]	-6.13[b]	-5.66[b]	-5.91[b]	-4.72[a]	0.17	***
Yellow-blue axis (b*)	39.5[c]	47.0[ab]	44.6[b]	46.2[ab]	48.5[a]	0.91	***

[1] Least-square means within a row with no common superscript are differ significantly different (P<0.05).
[2] SS = positive control, soybean cake and soybean oil; SS- = negative control, soybean cake and soybean oil; AA- = larval meal A and larval fat A; AB- = larval meal A and larval fat B; BB- = larval meal B rich in larval fat B; SEM = standard error of the mean.
[3] ns = not significant; significant diet effect differences are indicated as *P<0.05, **P<0.01, ***P<0.001.
[4] L* ranges from black (0) to white (100), a* from red (+) to green (–) and b* from yellow (+) to blue (–).

Table 5. Calculated R-indices[1] for the sensory evaluation of the odour of the eggs from three experimental feeds compared to the eggs from the control feed SS (n=13 panellists for series 1, 2 and 3; n=12 panellists for series 4 and 5).

Test series	Session	Diet[2,3]		
		AA-	AB-	BB-
1	1	66.3	61.8	65.7
2	1	55.9	48.2	61.8
3	2	65.7	64.2	75.4*
4	3	61.1	47.2	63.9
5	3	62.2	63.9	51.4

[1] 100 indicate perfect discrimination, 50 indicates that samples are distinguished by chance.
[2] AA- = larval meal A and larval fat A; AB- = larval meal A and larval fat B; BB- = larval meal B rich in larval fat B.
[3] Critical R-index values for a significance level of 5% (two-sided) indicated by * (Bi and O'Mahony, 2007): n=13: 20.24, n=12: 20.93.

4. Discussion

In laying hens, Met and Lys are considered as the first and second limiting amino acids (Toride, 2004). A Met deficiency can be counteracted by extra Cys, as this S-AA can be converted to Met in metabolism. In the present experiment, the realised supply with these amino acids turned out to be somewhat different from that planned. This happened as a result of variations in nutrient composition, intake and performance. Especially the low Met supply with the control diets was unexpected, but the higher Cys supply made the positive control SS still superior in supply with S-AA. Also, different from expectation, the Lys supply appeared to be sufficiently high not only in SS but also in AA- and AB-. The latter shows superiority of BSFL meal A over B. However, overall, the limiting amino acids in the diets were far more deficient than in the previous experiment (Heuel *et al.*, 2021a). Therefore, and by considering the severe calculated deficiency in the order of about 20% of requirements, it was unexpected that again no clear effects on performance of the deficient diets compared to SS were found and this at a still very high laying performance (eggs and egg mass per day). Hens tend to compensate for a lack of nutrients by increasing feed intake (Van Krimpen *et al.*, 2007). Therefore, lack of performance impairment when feeding a deficient diet should be accompanied by a higher ADFI, but this did not happen either. It seems that no indirect compensation for protein deficiency with a higher ADFI was taking place as the energy density of the diet was obviously sufficiently high. Another form of compensation could consist in an increased digestive or metabolic use efficiency. Marono *et al.* (2017) found an improvement in feed efficiency when Lohmann Brown Classic hens were fed BSFL-based diets for 21 weeks. Although this did not occur with statistical significance in the current study, the BSFL-based groups still tended to have a numerically more favourable feed efficiency. However, the defatted BSFL meals seem to even have had a slightly inferior apparent N metabolisability than the soybean cake as it had been also recorded previously (Heuel *et al.,* 2021a). Furthermore, it has been observed that feeding BSFL can lead to morphological changes in the gut, which can impair digestibility and absorption of nutrients (Dabbou *et al.*, 2018). Such an impairment of digestibility could be due to the chitin contained in the insect-based diets. Chitin is assumed to adversely affect digestion for instance by binding proteins and amino acids (Longvah *et al.*, 2011). Accordingly, Bovera *et al.* (2018) observed decline in ileal protein digestibility in layers when substituting half of soybean meal by BSFL meal and explained this by the chitin supplied with the insect-based diet. Cutrignelli *et al.* (2018) found a reduced enzymatic activity in the ileum in the small intestine and an altered production of volatile fatty acids in the caecum when feeding a BSFL-based diet to layers instead of a soybean-based diet which depressed nutrient digestibility. On the other hand, the chitin contained in BSFL may help improve the gut milieu of laying hens by promoting beneficial bacteria producing short chain fatty acids (Borelli *et al.*, 2017). Since we did not assess these traits, it is unclear if chitin might explain the lower N metabolisability found with BSFL material B. A final possible explanation for the lack of effects on performance would be that the recommendations are even more overestimating requirements of Lohmann Brown Classic hens than speculated earlier (Heuel *et al.*, 2021a). Applegate and Angel (2014) have already shown that the recommendations, e.g. of the National Research Council (NRC, 1994) are probably outdated and need to be adapted to the current genotypes of the commercial hybrids. This has yet to be done. Accordingly, a daily supply of 450 mg Met and 858 mg Lys was considered by Applegate *et al.* (2009) to be sufficient to meet the requirements of modern hybrids. For the Lohmann Brown Classic hens used in the present study, the requirements seem to be even lower as the actual supply especially with Met was much lower than these thresholds in the present experiment. It could be, though, that requirements are slightly higher under commercial conditions with group floor housing and access to outside areas. The lack of clear effects on performance made it impossible to demonstrate the presence or absence of BSFL origin differences in the present study.

Another important aspect of the utility of BSFL as feed for laying hens is its influence on egg quality. Regarding the egg weight, certain standards have to be met, which should ensure that as many eggs as possible can be marketed in the best paid category. No feeding-related influences were found in the present study, and the average egg weights of all groups fit into the category of large eggs (63 to 73 g) according to the EU regulation for egg marketing (EC, 2008). Different from this, Mwaniki *et al.* (2020) found a decrease in egg weight by 1 g/hen/day and in egg mass by up to 2 g/day with increasing amounts of BSFL meal (either 10 or 15%) in the diet compared to a control diet with 18% soybean meal. Similarly, Marono *et al.* (2017) reported egg weight and egg mass being lower by 2 g/hen/day and 3 g/day, respectively, in hens fed a diet containing 17% BSFL compared to those fed a maize-soybean meal-based diet. In the present study, shell thickness and stability were also not significantly influenced by integrating BSFL into the diets. Mwaniki *et al.* (2020) reported a numerically higher shell breaking strength when feeding a diet with 15% BSFL meal. The authors explained this by the concomitantly smaller eggs in this group, which have a more favourable egg surface to egg volume ratio. Different from that, Secci *et al.* (2020) found larger eggs as well as lower shell percentage and shell thickness when replacing half of the soybean meal with partially defatted BSFL meal in the diet. Apart from the same argument used by Mwanki *et al.* (2020), the authors presumed that hens can mobilise a limited amount of calcium for shell formation. Considering these controversial findings concerning the influence of BSFL meal on egg quality, further research is needed for the development of feeding recommendations (Heuel *et al.*, 2021a; Marono *et al.*, 2017; Secci *et al.*, 2020).

Another important point for assessing whether BSFL are suitable as feed is their influence on the internal egg quality and composition. Compositional changes of the egg content determine technological properties, nutritional value and sensory perception, which are all decisive for the purchase decision of industry and consumers. Technological properties are affected by the nutritional composition of yolk and albumen and the structure of these two egg fractions. The latter were considered in the present study via yolk height (a variable related to yolk membrane stability) and Haugh units (an indicator of albumen foaming ability). Indeed, there were some diet effects in some of these variables. Yolk height was low with diets SS- and BB-, but no reason for that is apparent. The fat (EE) content of the yolk was increased with diets AA- and AB- compared to SS- which was opposite to the fat content of the corresponding diets. One explanation for this observation could be that diets AA- and AB- had the highest energy metabolisability and that extra energy is typically deposited as fat in the yolk (Grashorn, 2016). Still, the supply with metabolizable energy was not clearly different among diets. In the previous study (Heuel *et al.*, 2021a), a diet based on BSFL meal B was superior in yolk fat content to that with meal A, which is contrary to the present results. In the investigation of Secci *et al.* (2018), BSFL feeding numerically lowered the total lipid content of the yolk by 15 g/kg compared to the yolks from a soybean-based diet. This all points towards influencing factors other than only the exchange of soybean by BSFL.

Sensory perception includes yolk colour quality and absence of off-flavours. Consumers often equate a darker and more intensely coloured yolk with free-range or organic farming, even though it is mainly the feeding that is of influence in this respect (Beardsworth and Hernandez, 2004). Other studies have already shown that, depending on the origin of the larvae, BSFL-based feeding can have a significant impact on yolk colouration (Mwaniki *et al.*, 2020; Secci *et al.*, 2018). In the present study, the BSFL-meal B, but not BSFL fat B, intensified yolk colouration like also found by Heuel *et al.* (2021a). This was likely the results of an enrichment of various colour active compounds in the BSFL produced on correspondingly different feeding substrates. An influence of the proportion of maize, rich in carotenoids, which was low in diet SS, can be excluded as only meal B had a colouring effect. The effect of the type of feeding substrate for the BSFL might be responsible for these findings as they probably were for the sensory analyses conducted by Bejaei and Cheng (2020) where hard-boiled eggs from a control group (soybean-based diet) were rated as more colour-intensive than those from the group fed a diet with 15% BSFL-based feed. However, in their study the intensive colouration coincided with a 20% higher proportion of maize in the soybean-based diet. In addition, Bejaei and Cheng (2020) could not confirm the sensory finding with corresponding measured egg colour differences between groups. It still has to be shown whether the colour intensification noted in yolks of diet BB- is large enough to be apparent in diets containing common levels of carotenoids which had been deliberately omitted in the present study. Consumers are susceptible to off-flavours, as shown, for example, where feeding a diet rich in flaxseeds affected the smell and taste of eggs (Hayat *et al.*, 2010). It could therefore be assumed that a distinct feed substrate composition and the generally unpleasant odour of BSFL might even have more adverse effects. However, except for one deviating test series, the results of the sensory evaluation indicated that none of the tested BSFL feeds changed egg odour in a way that it could be differentiated from the eggs produced without BSFL. This excludes a general effect of feeding substrate which differed clearly between BSFL batches A and B but also a general adverse effect of BSFL feeding. This is consistent with the study of Al-Qazzaz *et al.* (2016) using eggs from laying hens and Dalle Zotte *et al.* (2019) testing eggs from quails. In both studies, 10 to 15% of BSFL material was added to the diet. Al-Qazzaz *et al.* (2016) were even able to show that feeding diets based on BSFL may positively influence sensory palatability and texture of the eggs, traits which could not be sensorily investigated in the present study due to the legal reasons outlined above. One aspect, not specifically looked at in the present study but in the previous experiment (Heuel *et al.*, 2021b), is the degree to which the large amounts of lauric, myristic and palmitic acid present in the BSFL fat are accumulating in the egg lipids. These fatty acids are considered unfavourable for human health (Calder, 2015). When larvae material is used as full-fat meal or, as in the present study, as a combination of meal and fat, the intake with these fatty acids is high. Even when feeding only the meals this might be an issue as these may contain large amounts of residual fats (in the present study especially BSFL fat B). However, according to Heuel *et al.* (2021b) the transfer from BSFL-based diets to eggs is favourably low.

5. Conclusions

The results of the present study showed that the complete replacement of soybean in the diets of laying hens with BSFL meals and fats is possible. In addition, there are no significant impairments concerning performance and egg quality, even when the recommendations for the limiting amino acids are not met. The lack of performance differences did not allow to prove or disprove hypothesis 1 of a superiority of BSFL over soybean. Also, no clear difference in performance was found between origins of BSFL (disproving hypothesis 2), and differences between origins in egg quality were small as well. Of particular importance is the finding that the inclusion of BSFL did not adversely affect the odour of the processed eggs disproving hypothesis 3. It would be important to assess other sensory parameters in further studies.

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Conflict of interest

There are no conflicts of interest to declare by the authors.

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