



Low crude protein diets supplemented with free amino acids in laying hens

Effects on performance, egg quality, N-efficiency, N-excretion, economics and diet carbon footprint

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Low crude protein diets supplemented with free amino acids in laying hens. Effects on performance, egg quality, N-efficiency, N-excretion, economics and diet carbon footprint

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Samenvatting NL

Het verstrekken van voeders aan leghennen met een verlaagd eiwitgehalte aangevuld met vrije aminozuren om in de aminozuurbehoefte van leghennen te voorzien kan bijdragen aan het verminderen van het gebruik van sojaschroot van Zuid Amerikaanse herkomst. In dit rapport worden de resultaten beschreven van een leghennen experiment waarin de effecten van het verstrekken van voeders met een 14, 26 of 41 gram per kilogram verlaagd ruw eiwitgehalte ten opzichte van een controlerantsoen met een eiwitgehalte 161 g/kg, maar met een vergelijkbaar berekend verteerbaar aminozuurgehalte op de productieresultaten, ei-kwaliteit, N-excretie van leghennen van 39 – 51 weken leeftijd.

Summary UK

Providing laying hens diets with a reduced crude protein (CP) content that are supplemented with free amino acids (AA) to cover the AA requirement might be helpful in reducing the soybean meal content of Latin American origin, and thus in reducing the amount of non-EU protein in the diet. This report describes the results of an experiment in which the effects of low CP diets, with a partial replacement of soybean meal by free AA, on laying performance, egg quality, and N-excretion were evaluated in laying hens from 39 – 51 weeks of age.

This report can be downloaded for free at <https://doi.org/10.18174/557184> or at www.wur.nl/livestock-research (under Wageningen Livestock Research publications).



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Public Wageningen Livestock Research Report 1343

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Foreword

Feed4Foodure (F4F) is a public-private partnership between the Dutch Ministry of Agriculture, Nature and Food Quality and a consortium of various stakeholders within the animal production chain and Wageningen Livestock Research. Feed4Foodure aims to contribute to sustainable and healthy livestock farming in the Netherlands, simultaneously strengthening its competitive position in the global market. The F4F program line “MVV5”, aims to reduce the soybean meal content of Latin American origin, and thus reducing the amount of non-EU protein in the diet of pigs and poultry.

The current report describes the results of an experiment with laying hens in which the effects of low CP diets, with a partial replacement of soybean meal by free AA, on laying performance, egg quality, N-excretion, feed margin/economics and diet carbon footprint were evaluated.

The experiment was performed at the layer facility of De Heus in Vietnam. The study was designed with input from scientists of Wageningen Livestock Research and representatives of VDN in the F4F-consortium. The authors thank all members of the project team for their worthwhile input, and the team of the layer facility in Vietnam for performing the experiment.

Jan van Harn, Arya Rezaei Far, Marinus van Krimpen, J. Phuc and Cecile Veiga.

Note: Unfortunately, our dear colleague Marinus passed away suddenly and shortly after completing the work protocol, just before the start of the experiment. His critical input in this project is highly appreciated.

Summary

An experiment was conducted with 2880 Bovans Brown layer hens from 39 to 51 weeks of age to evaluate the effects of reducing the crude protein content of iso-energetic diets supplemented with essential amino acids (AA) in free form on laying performance, egg quality, N-excretion, feed margin/economics and carbon footprint.

The experiment was carried at the experimental layer facility of De Heus Vietnam (Vinh Long, Vietnam), and consisted of four dietary treatments differing in crude protein content. Two diets were produced after analysing the major raw ingredients. Diet A with 16.1% CP and diet D with 12.0% CP. Diet A and D were formulated to meet or exceed the requirements for all commercially available essential AA (Lys, M+C, Thr, Trp, Ile, Arg and Val) and were iso-caloric. Two other treatments were produced by mixing diet A and D in ratios of 65:35 (diet B 14.7% CP) and 35:65 (Diet C 13.5% CP). There were six replicates for each treatment. Each replicate consisted of a row of 20 cages with initially 6 birds in each cage. Laying performance, egg quality characteristics and manure composition (dry matter and nitrogen concentration) were assessed over the experimental period.

Reducing the crude protein content of the diet from 16.1% to 14.7% resulted in a higher feed intake and laying rate. Further reduction of the crude protein content to 13.5% and 12.0% reduced the feed intake and laying rate. Stepwise reduction of the dietary crude protein content across the treatments, reduced the protein intake, amino acids intake, and egg weight, while FCR increased. Laying hens fed the 12.0% CP diet had a significantly higher laying rate of 2nd-grade eggs compared to the other treatments. There was a negative linear relationship between the crude protein content of the diets and the egg weight, and albumen content of eggs. The calculated carbon footprint of the diets linearly increased with the decline of the CP content. The dry matter and nitrogen content of the manure increased when decreasing the crude protein content of the diets, while there was no effect on the ammonium-N content of excreta.

From this study can be concluded that:

- Reduction of the crude protein content of diet from 16.1% to 14.7%, with similar content of digestible EAA, resulted in a higher laying rate, lower egg weight, and fewer Jumbo eggs. Further reduction of crude protein content to 13.5% and 12.0% impaired the laying rate, egg weight, egg mass, and feed efficiency.
- Reducing the crude protein content of the diet from 16.1% to 14.7% did not affect the egg mass, but because of the higher feed intake, the FCR increased.
- Reduction in crude protein content of the diets to 13.7% and 12.0% CP resulted in impaired laying performance.
- The FCR increased with the decreased crude protein contents of the diets.
- The crude protein content of the diets did not affect the mortality rate.
- The crude protein intake decreased linearly with the reduced crude protein content of the diets.
- Egg weight decreased linearly with decreasing crude protein contents of the diets.
- Feeding diets with 12.0% CP increased the laying rate (%) of second-grade eggs. No differences were observed in the laying rate of the second-grade eggs between the 16.1, 14.7 and 13.5% diets.
- Albumen in eggs decreased with the decreased crude protein content of the diets.
- Decreasing dietary protein content with similar digestible content of essential amino acids increases the carbon footprint of the diets.
- The calculated feed price increases with a decrease in the crude protein content.
- The calculated economic feed margin is strongly influenced by the availability and price of the protein-rich raw materials and the price of free amino acids. Under the current Dutch market conditions, the feed margins per 100 placed hens of the low-crude protein diets decreased by €12.82, €56.58 and €70.08 respectively for the 14.7, 13.5 and 12.0% CP-diets.
- The dry matter content of the manure increased linearly with decreasing crude protein content of the diets.
- Total N-content of the manure decreased linearly with decreasing crude protein content of the diets.

In general, we conclude that lowering the crude protein content of laying hens diets from 16.1% to 14.7%, by reducing the soybean meal inclusion level and supplementing free essential amino acids , resulted in similar laying performance in laying hens aged between 39 and 51 weeks. Further lowering of the dietary crude protein to 13.5% and 12.0% deteriorate performance results.

1 Introduction

The growing world population and the expected increase in prosperity imply that the market demand for animal protein will continue to grow (FAO, 2009; FAO, 2017). It is expected that the demand for poultry meat and eggs will increase rapidly worldwide. As a result, the demand for vegetable proteins, e.g. soybean meal, for use in animal feed will increase (Alexandratos and Bruinsma, 2012). In Europe, the rate of self-sufficiency for soybean meal is only 5% (EU, 2017). This strong dependency for this important protein source in poultry diets and the low self-sufficiency makes the EU livestock sector vulnerable to price volatility and trade distortions, causing feed price to rise, thereby, increasing farmers' production costs and reducing the sectors' profitability (Euractiv, 2011). Besides, there is concern about the deforestation of tropical rainforest to fulfil the need for arable land for soybean cultivation (WNF, 2011; Van Gelder and Kuepper, 2012).

As a consequence of the transition of natural ecosystems into agriculture, the rate of biodiversity loss (proportion of extinct species) increases, whereas the current status has already more than ten times exceeded the proposed boundary (Rockström et al., 2009). Moreover, large scale soybean cultivation may increase water and soil pollution, and drive small farmers and the native population out of business (WNF, 2011). Among other reasons, this is driving the urge to find alternatives for imported soybean meal in poultry diets. Besides that, the emission of nutrients such as nitrogen and phosphorus via the poultry manure contributes to the environmental impact of the poultry production (Kumari et al., 2016).

A lower inclusion of soybean protein in poultry diets is one way to reduce the use and import of soybeans and soybean meal. This can be done by partial replacing soybean products with other protein commodities such as peas, rapeseed/rapeseed meal, sunflower meal, fish meal, corn gluten meal, and/or potato protein or in the near future novel proteins such as insect protein. Soybean meal has a balanced amino acids pattern that largely meets the amino acid requirements of poultry, and a complete replacement of soybean meal by the aforementioned protein-rich feedstuffs, with a less balanced pattern of amino acids, will increase the use of free amino acids in poultry diets. Partial replacement of soybean meal by free amino acids seems, in view of the economic aspects, to be a more obvious way forward when reducing the use of soybean meal. Reducing the inclusion of soybean meal in amino acids supplemented diets in poultry is in fact in line with the efforts to reduce nitrogen excretion by reducing the crude protein content of diets and optimizing the ideal protein concept.

However, the provision of reduced-crude protein diets may impair growth or egg performance when (semi-) essential amino acids become limiting. To successfully reduce crude protein levels in poultry diets while maintaining performance results, it is essential to keep the supply and the balance of amino acids in line with the bird's requirement. As a result, free amino acids should be adequately supplied to the diet.

A part of the dietary nitrogen intake is not retained by the animal but excreted into the environment. Lowering the crude protein content of the diet could therefore be a tool to reduce the nitrogen excretion and ammonia emission from poultry houses (Roberts et al., 2007). Moreover, low crude protein levels in poultry diets reduce the risk of digestive problems and necrotic enteritis (Palliyeguru et al., 2010). Besides the fact that providing low protein diets to poultry reduces the use of soybean meal, it could also be an efficient tool to improve intestinal health (less digestive disorders and improved health status), animal welfare and to reduce environmental emissions (less N-excretion and ammonia emission) (Qaisrani et al., 2015; Apajalahti and Vienola, 2016).

Within the Feed4Foodure project, several experiments have been performed to assess the effects of low protein diets on broiler growth performance, however, the available knowledge on the effects of low protein diets on the production performance of the laying hens is still limited. The objective of this study was to evaluate the effects of gradual reduction of dietary crude protein from 16.1% to 12.0% in amino acids supplemented diets by a partial replacement of soybean meal and sunflower seed meal on laying performance, egg quality, N-efficiency and N-excretion in the laying hens from 39 to 52 weeks of age. In addition, the effects of the diets on feed costs and the carbon footprint were evaluated.

2 Material and Methods

2.1 Start date and end date of the experiment

Start adaptation period:	02 December 2019 (week 37 of age)
End adaptation period:	15 December 2019 (week 39 of age)
Start experimental period:	16 December 2019
End experimental period:	22 March 2020 (week 52 of age)

2.2 Experimental animals

The experiment was carried out with approximately 2880 Bovans Brown laying hens. These hens were housed in eight 3-tier battery cages. In total there were 24 rows, and each row includes 20 cages (5-6 hens/cage). The row was the experimental unit. The experiment started at the age of 37 weeks, with two weeks of adaptation, and completed at the age of 52 weeks.

2.3 Experimental design

The experiment was carried out at the experimental layer facility of De Heus Vietnam (Vinh Long, Vietnam). This mechanical ventilated house was equipped with a pad cooling system and a climate computer (Hotraco).

A completely randomized block design was used in this experiment which consisted of four treatments (A to D) and four blocks (1 to 4). The experimental factor was the dietary crude protein content; a control diet (CP 16.1%) and three other treatments with stepwise reduction of crude protein content (CP 14.7%, 13.5%, 12.0%) by excluding soybean meal from the formulation and decreasing the inclusion level of sunflower meal. By including amino acids, i.e. lysine, methionine, threonine, tryptophan, valine, isoleucine, and arginine in free form, diets were balanced for the digestible concentrations of amino acids.

There were eight 3-tier battery cages in the layer facility. Battery cages formed 4 blocks, with each block consisting of two rows of the 3-tier battery cages, making in total 8 rows (Figure 1). Therefore, there were in total 24 rows each containing 20 cages. A row with 20 cages was considered an experimental unit. There were 5-6 animals per cage. Therefore, each experimental unit included approximately 120 laying hens. Treatments were replicated six times. The distribution of the experimental units in the layer farm is illustrated in Figure 2.

Table 1 *Experimental design in summary.*

Item	Number
Treatments	4
Replicates per treatment	6
Number of experimental units (one row)	24
Number of cages in each experimental unit	20
Number of laying hens in each cage	6
Total number of laying hens per treatment	720

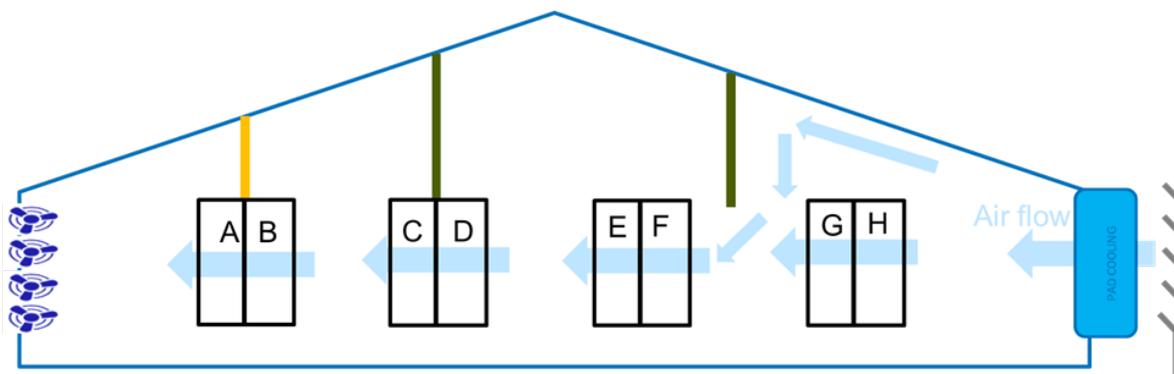


Figure 1 The layer facility was set up in four blocks, with each block consisting of two rows of the 3-tier battery cages, making in total 8 rows (block 1= rows A and B, block 2 = rows C and D, block 3= rows E and F and block 4 = rows G and H). Each row consisted of 3 tiers, which counted up to 24 rows in total. One row is an experimental unit.

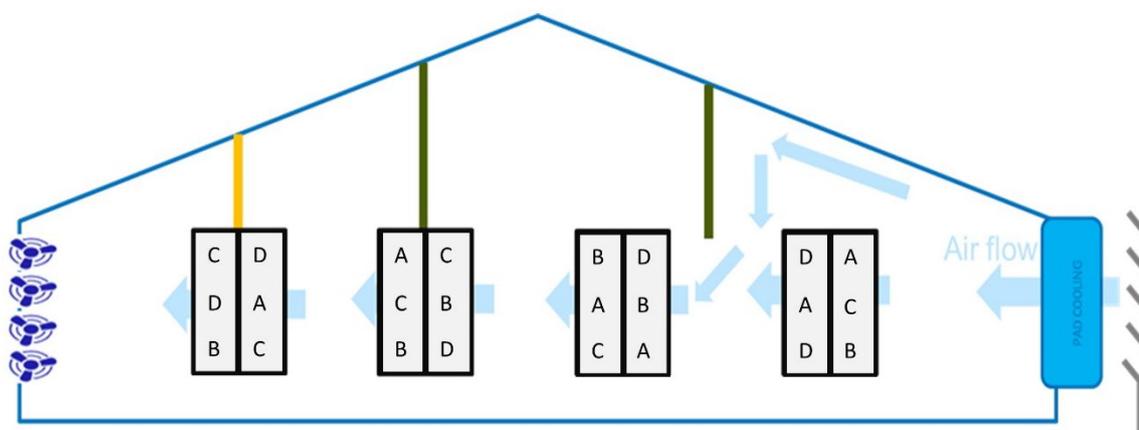


Figure 2 The distribution of treatments (A: CP 16.1%; B: CP 14.7%; C: 13.5%; D: 12.0%) in the experimental units (row) in the layer farm.

2.4 Experimental diets

Prior to formulation, the main raw ingredients were analysed by Near-infrared spectroscopy (NIR) to update the ingredient composition matrix in Bestmix. These ingredients included corn, wheat, wheat middling pellets, soybean meal, sunflower meal which were analysed for macronutrients and essential amino acids. After updating Bestmix, RP075 as the control diet (Trt A; CP 16.1%) and RP078 as the experimental diet with the lowest CP content (Trt D: CP 12%) were formulated.

Table 2 Experimental treatments. Iso-energetic diets with reduced contents of crude protein being supplemented by available free amino acids ¹.

Treatment	Code	Description
A	RP075	Control, CP 16.1%
B	RP076	Control - 1.4% CP; diet with 14.7% CP
C	RP077	Control - 2.6% CP; diet with 13.5% CP
D	RP078	Control - 4.0% CP; diet with 12.0% CP

¹ Lysine, methionine, threonine, valine, arginine, isoleucine, and tryptophan.

Reduction in crude protein in Trt D (12.0% CP) was realized by removing the soybean meal and reducing the inclusion level of sunflower meal. The resulting reduction in essential amino acids was compensated by adding free amino acids, i.e. lysine, methionine, threonine, tryptophan, valine, isoleucine and arginine. The other two dietary treatments (Trt B: RP076 and Trt C: RP077) were

obtained by mixing the control diet (RP075) and the reduced crude protein diet (RP078) in different ratios (Table 3).

Table 3 Composing the experimental diets from the low and high protein diet.

Treatment	Code	Mixing		Expected CP %
		RP075	RP078	
A	RP075	100%	-	16.1
B	RP076	65%	35%	14.7
C	RP077	35%	65%	13.5
D	RP078	-	100%	12.0

Due to the length of the trial, the experimental diets were produced in two batches on different production dates and with different macro raw materials. All these ingredients were assessed by NIR prior to feed formulation.

- Diets for the first 7 weeks were produced in mid-November 2019 (Appendix 1)
- Diets for the last 8 weeks were produced begin January 2020 (Appendix 2)

In total, four iso-energetic experimental diets with the same digestible lysine content (0.69%) were produced to meet or exceed the requirements for commercially available essential AA (Lys, M+C, Thr, Trp, Ile, Arg and Val), metabolizable energy (2800 kcal/kg ME Lay), calcium, digestible phosphorus and minerals recommended by CVB (2018) for laying hens. All diets were produced and pelleted (4.0 mm) on the commercial line and crumbled afterwards at the Dong Nai factory, Vietnam.

2.5 Animal and housing

The experiment started with a total number of approximately 2880 Bovans Brown laying hens at the age of 37 weeks. The laying house was equipped with battery cages and a pad cooling system on one side and the exhausting fans on the other side. The pad cooling and exhaust fans were controlled by a Hotraco climate computer to maintain the indoor climate settings. The indoor target temperature was set at 25°C. The sum of temperature and relative humidity, as a parameter for the prevailing climate conditions in the facility, was allowed to be a maximum of 115. On the occasion that this level was reached ventilation rate was increased gradually. The pad cooling was set on when maximum ventilation alone could not maintain the target temperature. Curtains were hung vertically from the roof to the top of the tier battery cages to maximize air speed through the cages. After fully closing the curtains, the airflow was between 1.1 to 1.6 m/s.

The experiment started with an adaptation period of two weeks, followed by an experimental period of 13 weeks. The age of the hens at the end of the experimental period was 52 weeks. During the adaptation period and the experimental period, the experimental diets were provided. The housing, management, feeding and husbandry conditions were standard and representative for commercial layer operations with battery cages. Water was provided *ad libitum* and feed was distributed three times a day: 9-11h, 13-14h, and 16-17h manually via feeding trolleys above the cages. The feed gift provided was based on the number of hens per row. The lighting program was the same during the entire experimental period; 16 hours light (from 4h to 20h) and 8 hours dark per 24h. Light intensity was the highest in the lower tier (46 Lux), while it was slightly less in the middle and upper tiers (37 and 46 Lux, respectively). The variation in light density was taken into account in the statistical analysis and randomization.

Visual observation of the birds was done twice per day to check animal health. Infectious Bronchitis and Newcastle Disease vaccines were given every six weeks via the drinking water.



Figure 3 *Top left: Laying hen facility Vinh Long, Vietnam; Top right: one cage hosting initially 6 laying hens; Bottom left and right: 3-tiers battery cages. Each tier including 20 cages formed a row (an experimental unit).*

2.6 Measurements

2.6.1 Diet

After pelleting, and just before bagging, each diet was sampled. These samples were pooled to form a composite, representative sample of each diet. All diets (2 batches of the four dietary treatments) were analysed on contents of dry matter, crude protein, crude fat, crude fibre, ash, starch, sodium, chlorine, manganese, magnesium, copper, zinc, and total amino acids content. All proximate analyses were performed by wet chemical analyses and NIR. The amino acid analyses were performed by Eurofins, Ho Chi Minh City, Vietnam (first batch of diets) and Evonik, Singapore (second batch of diets) (EC No 152/2009). In addition, dry sieve analysis and pellet durability index were assessed.

2.6.2 Animal performance parameters

The body weight (BW) of birds was assessed every two weeks by measuring the mean body weight of hens in four marked cages per experimental unit. Feed intake (FI) was measured for each experimental unit, weekly. Regarding the production performance, laying rate (%), egg weight (g), mortality (%), egg mass (EM= egg weight in g × laying rate in %) and feed conversion ratio (FCR= feed intake/egg mass) were recorded in each experimental unit on a weekly basis. The amount of 2nd-grade eggs was recorded visually every day. The 2nd-grade eggs were considered unsellable eggs, i.e. eggs with shell defects, abnormal size (tiny or double yolked), eggs with (blood)stains, and broken eggs.

2.6.3 Egg quality

Characteristics of the eggs produced in a day were measured individually, every two weeks (between 38 to 46 wks) and then in a weekly manner (from 46 to 52 wks). These characteristics included egg weight, breaking strength of the eggshell (BS; Futura device), egg composition (g; the amount of yolk, albumen and eggshell), Haugh unit and thickness of the shell in three points (μm ; top, equator, bottom). The percentage of eggs in each experimental unit with BS <25 N was calculated based on the BS values.

Fifteen eggs from each row (= experimental unit) were selected with a weight of +/- 0.4 g of the mean egg weight of the row and the egg component and Hauge unit were measured. The eggshell thickness of three eggs per row was measured on 3 points (top, equator, bottom) by the means of a micrometre with 0.001 mm accuracy (Helios-Preisser, DE).

Table 4 The measurement of individual egg characteristics during the experimental period (39 - 52 weeks).

Week	Breaking Strength ¹	Egg weight	Egg composition ²	Haugh Unit	Albumen height	Shell thickness ³
40	x	x	x			
42	x	x	x			
44						
46	x	x		x	x	x
47	x	x		x	x	
48	x	x	x	x	x	
49	x	x				x
50	x	x	x	x	x	
51		x		x	x	x
52	x		x	x	x	

¹ BS (N) also calculate the percentage of eggs with BS <25N

² Including the analysed weight of yolk and dry eggshell and calculated weight of albumen in gram to also calculate relative weight of yolk, eggshell and albumen.

³ Shell thickness was measured in top, equator and bottom of eggs (μm)

2.6.4 Manure composition

Representative samples from each experimental unit were taken to assess the dry matter, total nitrogen and $\text{NH}_3\text{-N}$ content (UPScience, VN) as the manure quality indicators, in weeks 40, 41, 45 and 51.

2.7 Statistics

Raw data were analysed for statistical outliers. An outlier was defined as an observation which is deviating more than 2.5 standard deviations from the mean. If the deviation of observation was more than 2.5 times the standard deviation from the mean and there was a plausible reason for this deviation, the observation was excluded from the dataset. The experimental data were analysed using GenStat statistical software (GenStat™ Release 19.1) on Windows 7.

The P-value of the treatment effect and the LSD (least significant difference (P=0.05)) were provided per response parameter. Treatment effects with a P-value <0.05 were considered to be statistically significant.

Response parameters were analysed using ANOVA (analysis of variance) according to the following model:

$$Y_{ijk} = \mu + \text{Age}_i + \text{Treatment}_j + \text{Week/Position}_k + e_{ijk}$$

In which:

Y	= dependent variable,
M	= overall mean
Age	= (fixed effect): age of the layers in weeks
Treatment	= (fixed effect): treatment (crude protein) effect, n=4
Week/Location	=(random term): Week and Position (Height+Row) with height within position being the block
e	= residual error

3 Results

3.1 General

Due to a defect of one of the temperature sensors in the barn, the results obtained in week 5 (41 weeks of age) of the experiment had to be excluded from the statistical analysis. The barn was equipped with four temperature sensors. The sensor located close to the pad cooling was defect and overestimated the actual temperature. As a result, the pad cooling system worked excessively to reach the target temperature and the air temperature in the cages close to the pad cooling system decreased, resulting in increased feed intake in these cages in week 5. Due to this, the results obtained at week 5 of the experiment had to be excluded from the statistical analysis.

In week 15 of the experiment (52 weeks of age), an error was made in the recording of the supply of the diets. As a result, the obtained performance data in this period were not reliable and the performance results obtained in week 15 of the experiment had to be excluded from the statistical analysis.

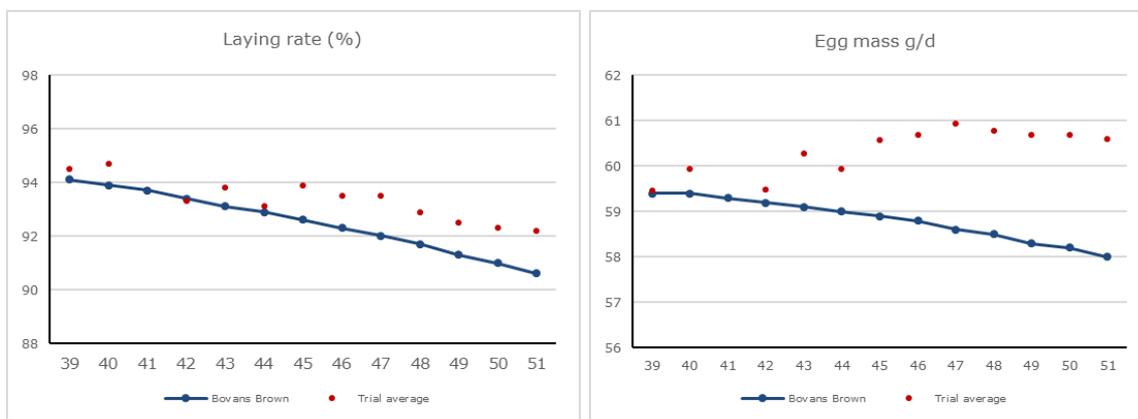


Figure 4 Average laying rate (left) and egg mass (right) of birds in the present experiment compared with Bovans Brown laying performance objectives for the cage-housed hens (Hendrix Genetics, 2021).

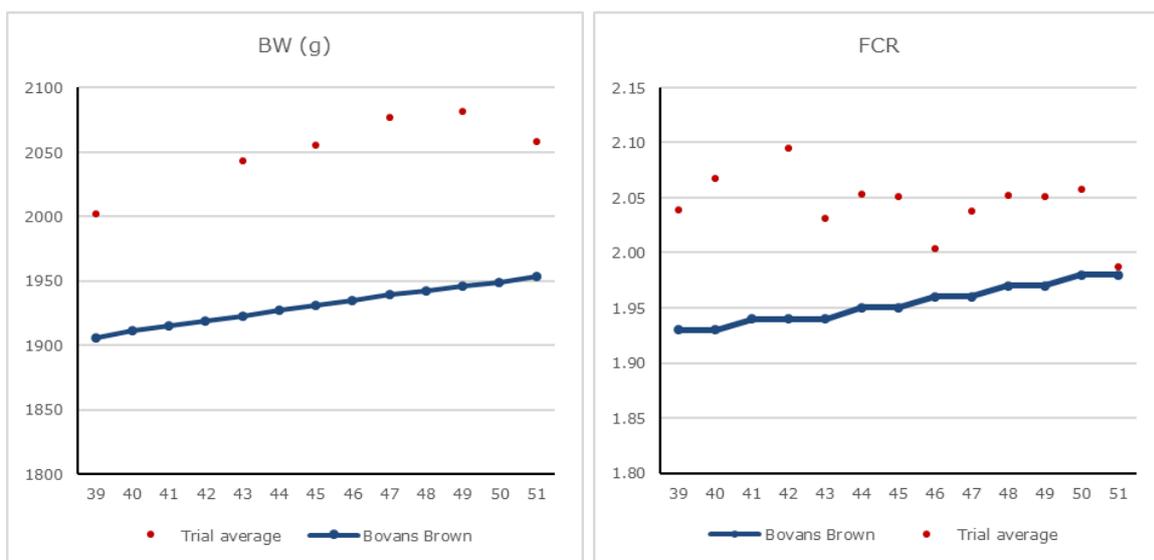


Figure 5 Average body weight (left) and feed conversion ratio (right) of birds in the present experiment compared with Bovans Brown laying performance objectives for cage-housed hens (Hendrix Genetics, 2021).

Figures 4 and 5 illustrate the average laying performance in this experiment in comparison to the performance objectives of Bovans Brown (Hendrix Genetics, 2021). In general, the average performance of the treatments in this trial was higher than the performance objective of Bovans Browns in cages (ISA, 2021). Despite the fluctuations over the first eight weeks of the experiment (39-44 wks), the laying performance showed the same trend as the standard of Bovans Brown. The bodyweight of the laying hens was from the beginning of the experiment higher than the Bovans Brown performance objectives. The higher average laying rate and egg weight in this experiment resulted in a greater egg mass (g/day) than the objectives of Bovans Brown over the experimental period. The average feed intake of each week was higher than the objectives of Bovans Brown and resulted in a higher feed conversion ratio.

The cumulative mortality between weeks 39 to 51 was 4.0%, which is higher than normal in this facility (0.9%) and also higher than the expected cumulative mortality of Bovans Brown, kept in cages under (sub) tropical circumstances. However, the crude protein content of the diet did not affect the cumulative mortality of birds during the study (see 3.3.1).

3.2 Diets

In Table 5, the calculated and analysed nutrient contents of the experimental diets are given. Regarding the crude protein content, the variations between the two batches of each treatment and deviations from the calculated content were small (from -1 to 4% relative difference). In general, the analysed nutrient content of crude protein and crude fat was in line with the calculated content.

Deviations in total phosphorus content between calculated and analysed contents were observed among treatments (between -2 to 13%). Average P-content in diets A, B and D was slightly higher than calculated (5 - 6%), whereas the P-content diet C was on average 5% lower. In particular, the analysed total phosphorus content of the second batch of diet D was 0.6 g/kg higher than the calculated value (5.4 vs 4.8 g/kg), which resulted on average (both batches) in a 6% higher total phosphorus content in the diet (5.1 vs 4.8 g/kg).

Table 5 Analysed and calculated¹ nutrient contents (g/kg) in the experimental diets.

		Moisture	Crude protein	Crude fat	Crude fibre	Crude ash	Calcium	Phosphorus
		g/kg	g/kg	g/kg	g/kg	g/kg	g/kg	g/kg
A	Batch 1	97	164	27	38	120	35	4.9
	Batch 2	100	160	26	41	114	35	5.1
	Average	99 (98)	162 (161)	26 (25)	39 (44)	117 (121)	35 (36)	5.0 (4.7)
B	Batch 1	100	151	27	34	126	37	4.9
	Batch 2	97	146	26	39	127	42	4.9
	Average	98 (99)	148 (147)	26 (26)	37 (42)	126 (121)	39 (36)	4.9 (4.7)
C	Batch 1	101	134	27	34	110	32	4.5
	Batch 2	101	138	28	31	113	36	4.6
	Average	101 (100)	136 (135)	27 (26)	33 (40)	112 (120)	34 (36)	4.6 (4.8)
D	Batch 1	97	125	29	36	108	31	4.7
	Batch 2	104	125	30	30	106	34	5.4
	Average	100 (100)	125 (120)	29 (26)	33 (38)	107 (120)	32 (36)	5.1 (4.8)

¹ Calculated values are given between brackets

The calcium content of treatment B was the highest and treatment D the lowest across the treatments. The average calcium content of treatment B was 8% higher and treatment D was 11% lower than the calculated values. The trend in calcium content was in line with ash content. In a similar way, the ash content in the experimental diet of treatment B was 4% higher and in treatment D 11% higher than the calculated values. Crude ash in all diets was lower than calculated (on average 14%). Simultaneous deviations in the total phosphorus and calcium content varied the Ca to P ratio

from the calculated value (7.5-7.6) among the treatments. In the case of dietary treatment D, the ratio was between 6.3 and 6.6.

Table 6 illustrates the analysed content of the total amino acids in the experimental diets as analysed by Eurofins (first batch) and Evonik (second batch) (EC No 152/2009). In general, the deviations in analysed amino acids concentrations from the calculated values were considered relatively small and acceptable and were in line with the experimental set-up, except for diet C. All analysed major limiting/essential amino acids of this diet were lower than calculated. The deviation (relative difference) between the analysed and calculated values for Lys, M+C, Thr, Val, Arg and Ile was -4, -10, -8, -4, -6 and -7%, respectively.

Table 6 Analysed content of amino acids (g/kg) in the two batches of the experimental diets.

		Lysine	Methionine	M+C	Threonine	Valine	Arginine	Isoleucine
		g/kg	g/kg	g/kg	g/kg	g/kg	g/kg	g/kg
A	Batch 1	8.0	4.0	7.0	6.3	7.4	10.3	6.2
	Batch 2	7.8	3.6	6.4	5.6	7.4	9.7	6.4
	Average	7.9 (7.9)	3.8 (3.9)	6.7 (6.9)	6.0 (5.8)	7.4 (7.4)	10.0 (10.3)	6.3 (6.2)
B	Batch 1	8.3	4.1	6.7	6.1	7.6	9.8	6.2
	Batch 2	8.0	3.8	6.3	5.4	7.1	9.2	6.1
	Average	8.2 (7.8)	4.0 (4.1)	6.5 (6.9)	5.8 (5.7)	7.4 (7.2)	9.5 (9.6)	6.2 (6.1)
C	Batch 1	7.7	3.9	6.4	5.4	6.9	8.6	5.7
	Batch 2	7.1	3.4	5.7	4.9	6.5	8.2	5.6
	Average	7.4 (7.7)	3.7 (4.3)	6.1 (6.8)	5.2 (5.6)	6.7 (7.0)	8.4 (8.9)	5.7 (6.1)
D	Batch 1	7.8	4.6	6.9	5.5	7.0	8.2	5.7
	Batch 2	7.4	4.0	6.0	5.0	6.5	8.0	5.6
	Average	7.6 (7.6)	4.3 (4.5)	6.4 (6.7)	5.3 (5.5)	6.8 (6.8)	8.1 (8.2)	5.7 (6.0)

¹ Calculated values are given between brackets

3.3 Laying performance

3.3.1 General

Table 7 provides an overview of the laying performance results from week 39 to 51. It shows that the crude protein content of the diet affected the laying performance.

The laying hens fed the control diet (Trt A) had the highest average egg weight between week 39 to 51 of age in this experiment. However, the feed intake (g/day) and the laying rate (%) were lower in comparison to the laying hens fed a 14.7% CP diet (Trt B). In other words, reducing the crude protein content of the diet to 14.7% (Trt B) did not affect the egg mass, but the feed conversion ratio increased. The laying hens fed the diets with the crude protein content of 13.5% (Trt C) and 12.5% (Trt D) showed a further decline in feed intake (g/day), body weight (g), laying rate (%), egg weight (g), egg mass (g/day) and FCR in comparison to the control treatment (Trt A; 16.1% CP) and Treatment B (14.7% CP). A gradual reduction in egg weight was observed among the treatments in line with the reduction in CP content (Figure 6).

In comparison to the control diet (Trt A; 16.1% CP), a reduction of the crude protein up to 13.5% (Trt C) did not affect the percentage of the second-grade eggs. The crude protein content of the diets did not affect the number of broken eggs produced. However, further reduction of the crude protein content to 12.0% (Trt D) was associated with an increase of 2nd-grade eggs (1.4%), in particular, the numbers of eggs with a shell defect and tiny size were significantly higher in treatment D than the rest of the treatments. According to Table 7, reducing the crude protein content of the diet to 14.7% or lower was associated with a drop in the production of large-sized double-yolked (jumbo) eggs which were the highest (0.19%) in the control treatment. The laying hens fed the diet with 14.7% CP tended to have the lowest number of eggs with blood spots (0.09%) in comparison to the other treatments.

Table 7 Effects of the crude protein content of the diet on the performance of laying hens from 39 to 51 weeks of age.

Treatment		A	B	C	D	LSD	P-Value
CP (Calc)		16.1%	14.7%	13.5%	12.0%		
CP (Ana)		16.2%	14.8%	13.6%	12.5%		
Bodyweight	(gram)	2070 ^b	2064 ^b	2043 ^a	2034 ^a	19.5	<0.001
Feed intake	(g/day)	123.5 ^b	125.0 ^c	122.6 ^a	122.0 ^a	0.88	<0.001
Crude protein intake	(g/day)	20.0 ^d	18.5 ^c	16.7 ^b	15.2 ^a	0.15	<0.001
FCR		2.005 ^a	2.031 ^b	2.067 ^c	2.073 ^c	0.0138	<0.001
Cum. Mortality 39-51wks	(%)	4.3%	4.6%	3.2%	3.9%	3.48%	0.800
Laying rate	(%)	93.5 ^b	94.8 ^c	92.5 ^a	92.7 ^a	0.60	<0.001
Second grade-eggs	(%)	1.18 ^a	1.08 ^a	1.15 ^a	1.42 ^b	0.180	0.002
Broken eggs	(%)	0.41	0.42	0.38	0.37	0.086	0.625
Jumbo eggs	(%)	0.19 ^b	0.07 ^a	0.06 ^a	0.05 ^a	0.059	<0.001
Tiny eggs	(%)	0.03 ^a	0.04 ^a	0.04 ^a	0.08 ^b	0.037	0.036
Bloody eggs	(%)	0.15 ^{xy}	0.09 ^x	0.20 ^y	0.20 ^y	0.094	0.095
Shell defect	(%)	0.40 ^a	0.46 ^a	0.47 ^a	0.72 ^b	0.125	<0.001
Egg weight	(g)	65.9 ^d	65.1 ^c	64.3 ^b	63.3 ^a	0.18	<0.001
Egg mass	(g/day)	61.6 ^c	61.7 ^c	59.3 ^b	58.7 ^a	0.41	<0.001

As was expected, reducing the crude protein content of the diets was associated with a decline in crude protein intake of laying hens. In other words, by the stepwise reduction of the crude protein content from 16.1% to 12.5%, nitrogen intake reduced gradually from 3.2 to 2.4 g/day. Figure 6 demonstrates the relationship between the crude protein intake and laying performance in laying hens.

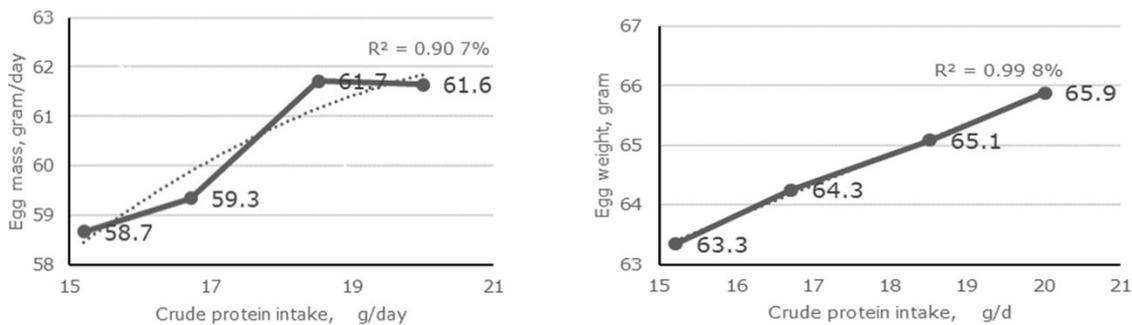


Figure 6 The relationship between the crude protein intake (g/day) and egg mass (left graph) and egg weight (right graph), among the four treatments of this experiment.

3.3.2 Nutrient intake of birds

Table 8 illustrates the average intake of crude protein and total amino acids among the treatments. The crude protein and amino acids intake were significantly different among the treatments. Crude protein intake declined gradually by the stepwise reduction in crude protein content of the dietary treatments. In general, the intake of essential and non-essential amino acids showed a stepwise reduction from Trt A (16.1% CP) to Trt D (12.0% CP). The exceptions were lysine and methionine as the laying hens fed the diet with 14.7% CP (Trt B) had the highest lysine intake among the treatments and the intake of Met+Cys was lower in Trt C than Trt D. In the case of total valine and isoleucine, the difference between the intake of laying hens in treatment C and D was not significant. In spite of the significant effects of reducing dietary crude protein on the intake of amino acids, the magnitude of this reduction was different among essential and non-essential amino acids. In comparison to the control treatment (Trt A; CP 16.1%), the laying hens in Trt C and D had an average lower intake of essential amino acids which was 6% for lysine, 8% for Met+Cys, 11% for valine and isoleucine, 14% for

threonine, and 18% for arginine. However, the intake of non-essential amino acids in Trt C and D was an average between 19 to 44% lower than the intake of the laying hens in Trt A and B.

Table 8 The average intake of crude protein (g/day) and total amino acids (mg/day) based on the feed intake values and the analysed content of nitrogen (wet chemical) and amino acids (EC No 152/2009). in the dietary treatments over the experimental period.

Treatment	A		B		C		D		LSD	P-Value
Calculated CP (%)	16.1		14.7		13.5		12.0			
Crude Protein (g/day)	20.0	^d	18.5	^c	16.7	^b	15.2	^a	1.5	<0.001
Lys	971	^c	1011	^d	902	^a	917	^b	7.7	<0.001
Met	469	^b	487	^c	440	^a	513	^d	3.8	<0.001
Thr	729	^d	712	^c	625	^b	633	^a	6.1	<0.001
Trp	ND		ND		ND		ND			
Val	918	^c	910	^b	818	^a	817	^a	7.8	<0.001
Arg	1225	^d	1180	^c	1025	^b	983	^a	9.1	<0.001
Iso	777	^c	766	^b	692	^a	687	^a	6.0	<0.001
Phe	917	^d	823	^c	707	^b	577	^a	6.2	<0.001
His	487	^d	407	^c	386	^b	325	^a	5.8	<0.001
Leu	1500	^d	1359	^c	1199	^b	1024	^a	10.8	<0.001
Cys	354	^d	320	^c	295	^b	265	^a	2.3	<0.001
M+C	823		807		735		778			
Asp	1661	^d	1407	^c	1134	^b	724	^a	27.5	<0.001
Ser	929	^d	807	^c	697	^b	573	^a	7.6	<0.001
Glu	4167	^d	3744	^c	3361	^b	2843	^a	26.2	<0.001
Pro	1305	^d	1191	^c	1112	^b	1013	^a	9.0	<0.001
Gly	899	^d	820	^c	700	^b	601	^a	6.7	<0.001
Ala	903	^d	840	^c	802	^b	658	^a	11.2	<0.001

In addition to the crude protein and major limiting amino acids, calcium and phosphorus are the other nutrients with potential effects on the response of laying hens in the case of laying performance and egg quality. Figure 7 illustrates the intake of calcium and total phosphorus among the treatments. The intake of calcium was significantly different among the treatments. Laying hens of treatment B had the highest calcium intake, whereas the hens of treatment D had the lowest calcium intake. Calcium intake of the hens of Trt B differed significantly from Trt D, the Ca-intake of Trt A and Trt C were in-between and did not differ from either Trt B or Trt D. The intake of phosphorus was significantly lower in Trt C (CP 13.5%) compared to the other treatments.

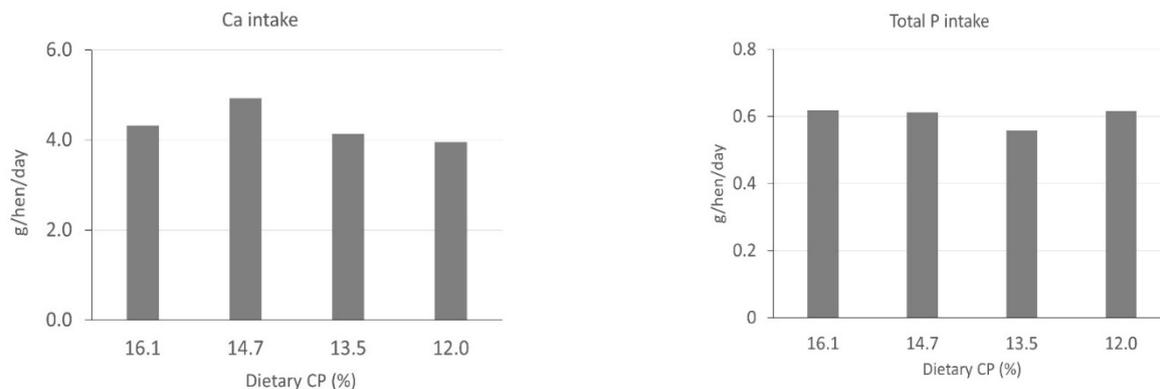


Figure 7 The average intake of calcium and total phosphorus (in g/day) based on the average feed intake values (g/day) and the analysed content of calcium and total phosphorus (g/kg) in the dietary treatments over the experimental period.

3.4 Egg quality

In Table 9, the values of egg characteristics and eggshell quality indicators are given.

Table 9 The effect of the crude protein content of the dietary treatments on egg characteristics and eggshell quality indicators.

Treatment		A	B	C	D	LSD	P-Value
Calculated CP (%)		16.1	14.7	13.5	12.0		
Egg Characteristics							
Egg weight	g	66.25 ^d	65.37 ^c	64.49 ^b	63.66 ^a	0.23	<0.001
Egg weight of analysed egg	g	66.28 ^d	65.42 ^c	64.56 ^b	63.71 ^a	0.23	<0.001
Yolk in egg	g	17.43	17.42	17.31	17.29	0.24	0.529
Albumen in egg	g	42.21 ^d	41.45 ^c	40.83 ^b	40.04 ^a	0.398	<0.001
Shell in egg	g	6.54	6.44	6.32	6.39	0.192	0.143
Yolk in egg	% EW	26.4 ^a	26.7 ^{ab}	26.9 ^{bc}	27.1 ^c	0.37	<0.001
Albumen in egg	% EW	63.8 ^b	63.5 ^b	63.3 ^b	62.8 ^a	0.46	0.001
Shell in egg	% EW	9.9	9.9	9.8	10.0	0.30	0.513
Haugh Units	-	81.3	82.0	82.9	81.1	2.12	0.336
Shell thickness							
Top	mm	0.557	0.558	0.570	0.557	0.0379	0.881
Equator	mm	0.500	0.490	0.501	0.497	0.0225	0.767
Bottom	mm	0.542	0.527	0.538	0.515	0.0280	0.218
Eggshell quality							
Breaking strength	N	41.2 ^c	40.3 ^a	40.8 ^b	40.6 ^{ab}	0.38	<0.001
Eggs with shell strength < 25N	%	2.51 ^a	3.41 ^b	2.39 ^a	2.82 ^{ab}	0.064	0.009

The crude protein content of the diets affected the egg weight, the relative content of albumen and yolk, and the eggshell quality. Reduction in CP content of the diets was associated with a gradual reduction in egg weight and absolute and relative content of albumen. There were no significant differences in Haugh units among the treatments. In the case of eggshells, the crude protein content of the diets did not affect shell content and thickness. Further investigations of eggshell quality characteristics showed that the breaking strength reduced as the laying hens fed the reduced-cp diets (14.7 – 12.0% CP). The laying hens fed the diet with 14.7% CP (Trt B) produced a higher percentage of the eggs with shell breaking strength lower than 25 N.

3.5 Excreta quality

Table 10 illustrates the results of the excreta composition assessment. Gradual reduction of the CP content in diets was associated with a stepwise reduction in nitrogen content of manure. According to Figure 8, the reduction of diet CP from 16.1% to 14.7%, was associated with a 7.5% reduction in nitrogen intake (from 3.20 to 2.96 g/day) and a 12.5% reduction in nitrogen excretion. It is worth mentioning that the egg mass was similar among these two treatments (see 3.3.1). The effect of the crude protein content of the diet on the moisture content of manure was only significant when CP was reduced to 12.0% (Trt D). In other words, the rise in DM content of manure from 25.9% (Trt A) to 26.8% (Trt C) was not significant. In addition, the crude protein content of the diet had no significant effect on NH₃-N % in the protein content of the manure.

The DM content of the manure of Trt D was significantly higher than the manure of the other three treatments. The dry matter content of the manure did not differ between treatments A, B and C.

Table 10 The effect of the crude protein content of the diets on dry matter, nitrogen content and NH₃-N in excreta at weeks 45 and 51.

Treatment		A	B	C	D	LSD	P-Value
Calculated CP (%)		16.1	14.7	13.5	12.0		
Dry matter	g/kg	259	^a 261	^a 268	^a 281	^b 13.9	0.008
Total nitrogen	g/kg	16	^c 14	^b _c 13	^a _b 12	^a 1.9	0.001
NH ₃ -N	(%/Total protein)	10.2	9.0	10.6	10.3	1.93	0.349

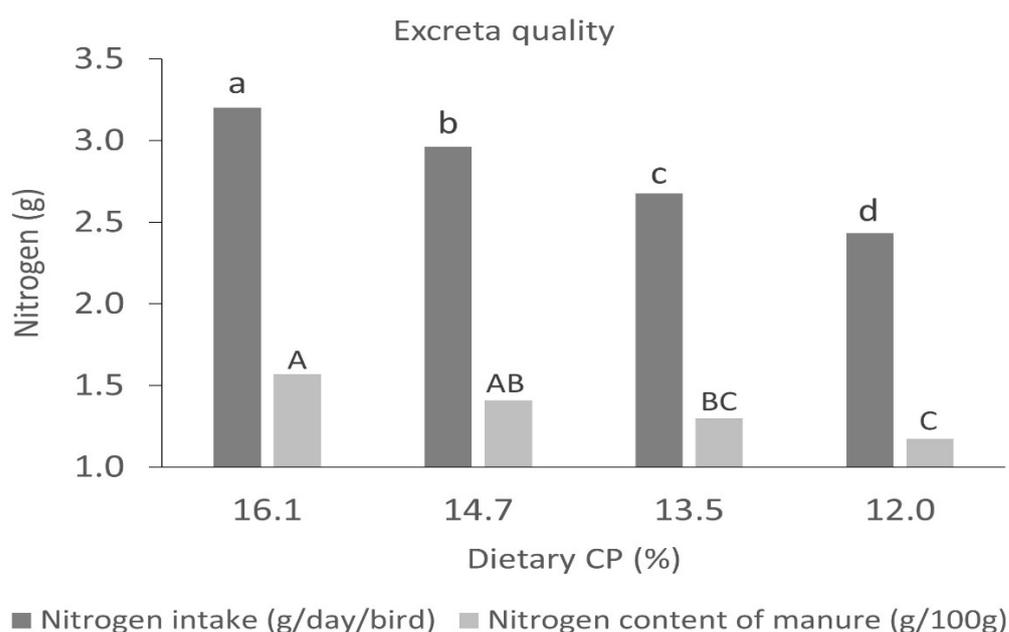


Figure 8 The relationship between the average nitrogen intake (g/day) over the experimental period (39 to 51 weeks) and the average nitrogen content of excreta (g/100g) at weeks 45 and 51 among the treatments.

3.6 Carbon footprint of the diets and economic feed margin as affected by the dietary treatments

In Figure 9 the effect of the crude protein content of the diet on the carbon footprint of the diets is given. In Table 11A and 11B, the calculated economic revenues on the farm (feed margins) as affected by the experimental treatments are provided. In appendix 1 and 2 the feed composition of both feed batches is given. In Appendix 4 the contribution of the individual feedstuffs used in the experimental diets on the carbon footprint (CFP) and the calculated CFP per batch of each diet is

given. Data were generated from the FeedPrint database (Vellinga et al., 2013). The CFPAN tool and database FeedPrint calculates the carbon footprint of feed raw materials during their complete life cycle. This ranges from crop production, via the processing of crops and animal products, compound feed production to utilization by the animal, including transport and storage between all steps of the production chain. For feed prices, the prices of the feed ingredients from January 2021 were taken. From this figure, it can be concluded that a decrease in the crude protein content of layer diets increased the carbon footprint, excluding effects of potential changes in land use (CFP excl. LUC). In the range 16.1 – 12.0% CP, each percent lower crude protein leads to an increase of CFP with 40 points.

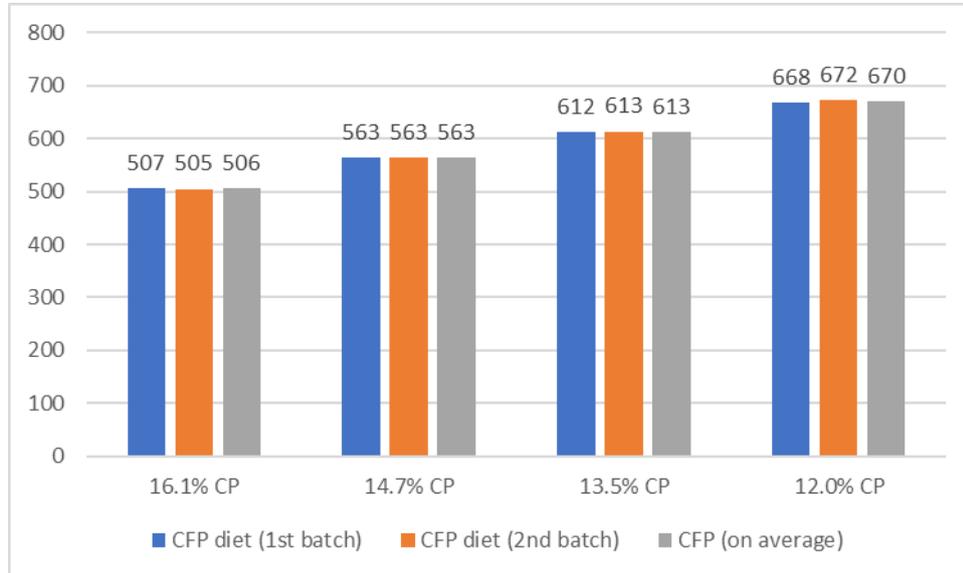


Figure 9 Calculated carbon footprint (excl. LUC, in g CO₂-eq/kg diet) of the experimental diets.

In Vietnam, the feed price largely depends on the availability and price of the protein sources and the price of free amino acids. At the time of the experiment, sunflower seed meal was sufficiently available in Vietnam and the calculated feed price difference between the 16.1% CP control diet and the 12.0% CP diet was only 3.8%, with the value being higher for the low-protein diet. However, in the current Vietnamese market, sunflower seed meal is not available and soybean meal would become the main protein supplier in the diet, making the 12.0% CP diet 8.9% more expensive than the control diet. In the Netherlands a wide range of protein-rich raw materials is available, so that the price of low-protein diets is less dependent on the availability of protein sources, but mainly dependent on the price of the free amino acids. Based on the current Dutch raw material prices and prices of the free amino acids, the diet price of the 12.0% CP diet is 9.5 percent higher than the control diet with 16.1% CP. Because a wide range of protein sources is available in the Netherlands and the feed price is therefore less dependent on the availability of protein sources, it was decided to base the feed margin calculations on the current Dutch market situation (Table 11A). Assuming that the prices of the free amino acids will decrease in the near future, we also calculated feed margins taking into account a feed price difference between the control diet and the 12.0% CP diet of 3.8% (Table 11B).

In the current situation laying hens fed the control diet showed the highest feed margin, whereas the hens fed the 12.0% CP diet showed the lowest feed margin. The feed margin of the hens receiving diets with 14.7% CP was €12.82 per 100 hens placed lower than the control group. The difference in feed margin between Trt C (CP = 13.5%) and Trt D (CP = 12.0%) with the control group was -€ 56.58 and -€70.08 per 100 placed hens, respectively. In the situation that the difference in feed price between the control diet and the 12.0% CP-diet is only 3.8%, the feed margin of the laying hens fed the 14.7% CP was slightly higher than the control group (€125.97 vs. €125.72, Table 11B). The feed margin of the hens receiving diets with 13.5% and 12.0% CP was €30.74 and €31.67 per 100 hens placed lower than the control group.

Table 11A Feed margin (in € per 100 hens placed) at farm level per treatment per flock – current situation.

	A	B	C	D
CP content diet	16.1%	14.7%	13.5%	12.0%
Revenues				
Eggs	1258.60	1275.03	1244.03	1246.20
Meat	35.66	35.44	35.60	35.18
<i>Total revenues (A)</i>	1294.26	1310.47	1279.63	1281.38
Costs hens and feed				
Hens	440.32	440.32	440.32	440.32
Feed 17 – 20 weeks	46.41	46.41	46.41	46.41
Feed > 20 weeks	681.81	710.84	723.76	739.02
<i>Total costs (B)</i>	1168.54	1197.57	1210.49	1225.75
Feed Margin (A – B)	125.72	112.90	69.14	55.64

Prices based on KWIN 2020/2021. Egg price: €6.20/100 eggs. Meat price: €0.18/kg. Feed prices: Diet A: €26.00/100kg; Diet B: €26.82/100kg; Diet C: €27.65/100kg; Diet D: €28.47/100kg.

Table 11B Feed margin (in € per 100 hens placed) at farm level per treatment per flock – future scenario.

	A	B	C	D
CP content diet	16.1%	14.7%	13.5%	12.0%
Revenues				
Eggs	1258.60	1275.03	1244.03	1246.20
Meat	35.66	35.44	35.60	35.18
<i>Total revenues (A)</i>	1294.26	1310.47	1279.63	1281.38
Costs hens and feed				
Hens	440.32	440.32	440.32	440.32
Feed 17 – 20 weeks	46.41	46.41	46.41	46.41
Feed > 20 weeks	681.81	697.77	697.92	700.60
<i>Total costs (B)</i>	1168.54	1184.51	1184.65	1187.33
Feed Margin (A – B)	125.72	125.97	94.98	94.05

Prices based on KWIN 2020/2021. Egg price: €6.20/100 eggs. Meat price: €0.18/kg. Feed prices: Diet A: €26.00/100kg; Diet B: €26.33/100kg; Diet C: €26.66/100kg; Diet D: €26.99/100kg.

4 Discussion

In this study, laying hens between 39 to 51 weeks of age fed iso-energetic diets with a reduced content of crude protein (16.1% vs 14.7%, 13.5%, and 12.0%) supplemented with commercially available free essential amino acids up to CVB recommendations (CVB, 2018). This study aimed to evaluate the effect of low protein diets on laying performance, egg quality, N-efficiency, N-excretion, farm economics/feed margin and carbon footprint. The experiment was performed at the experimental layer facility in a tropical climate in Vietnam and the laying hens were kept in battery cages in a mechanical ventilated house. The results showed that lowering the crude protein content of the diets from 16.1% to 14.7%, by partial replacement of soybean meal and sunflower seed meal, resulted in similar egg mass in laying hens between 39 to 51 weeks of age. Further lowering of the crude protein content deteriorated the production performance. The laying hens fed the 13.5% or 12.0% CP diets had a significantly lower laying rate, egg weight, and feed efficiency compared with the hens fed the control diet (16.1% CP) and the hens fed the 14.7% CP diet.

Reducing the crude protein content of diets in laying hens has been the aim of several studies and the increasing availability of free amino acids such as Met, Lys, Thr, Trp, Val, Ile and Arg for diet supplementation facilitates the implementation of the concept. The aims are, on the one hand, to reduce nitrogen excretion and to improve N-efficiency, and on the other hand to reduce the dependency on imported soybean meal from countries outside Europe, mainly Latin America. However, studies on the subject in laying hens show in part contrasting results, as further outlined below.

Reducing the dietary crude protein from 18% to 15% had no effects on egg mass and laying rate in laying hens between 21 to 72 weeks (Rao et al., 2011). Mousavi et al. (2013) also observed a similar laying rate in laying hens fed a reduced CP diet (15.5% vs 18.5% CP) between 25 to 33 weeks of age. Keshavarz & Austic (2004) found that hens fed a low crude protein diet (13% CP) supplemented with free essential amino acids had a comparable laying performance with the control diet with 16–16.5 % CP. Recently, Parenteau et al. (2020) reported that CP in laying hen diets between the age of 28 – 46 weeks can be reduced by 2% units (from 16 to 14%) as long as it is fortified with synthetic AA (Met, Lys, Thr, Trp and Ile). These studies concluded that supplying the low protein diets with free amino acids met the requirements of the laying hens to have the same laying performance as the control diets.

Stepwise reduction of the dietary crude protein from 18% to 16% in laying hens between 21 to 34 weeks of age affected the average egg weight. Laying hens fed the 16 to 16.5% CP diet had 2-3% lower average egg weight while the changes in feed intake, feed efficiency, and laying rate were not significant (Ji et al., 2014). In contrast, the results of several studies indicated negative effects of low protein diets on laying performance (Azzam et al., 2017a; Wang et al., 2020). In the present study, the reduction of the crude protein content of the diet up to 14.7% did not affect egg mass, but a further reduction to 13.5% and 12.0% reduced feed intake, body weight, laying rate, and egg mass, despite supplementation of essential AA.

Several factors cause this inconsistency which could be related to the characteristics of the experimental diets (e.g. basal diet composition, feed ingredients, metabolisable energy content), laying hens (e.g. strain and age), environmental conditions (climate, temperature, and humidity) or interacting factors, for example, the feed intake which is influenced by the animal-related (e.g. age) and diet-related (e.g. ME content and concentrations of other nutrients) factors, and environmental conditions such as temperature or the housing system.

One critical factor is the extent to which crude protein was reduced or free amino acids were supplemented to the experimental diets. For example, reducing the crude protein content of the diet to 9.2% impaired the laying performance in comparison to the laying hens fed a 16.5% CP diet, between 28 to 32 weeks of age (Wang et al., 2020). However, the low protein diets in the above-mentioned studies had a crude protein content of 15.5% (Mousavi et al., 2013) or 15% (Rao et al., 2011). Besides that, in the study of Mousavi et al (2013), the levels of digestible lysine (0.87% vs 0.69%), threonine (0.62% vs 0.48%), arginine (0.93% vs 0.75%), and isoleucine (0.60% vs 0.55%) were higher than the levels in the diets in our study. The reason for the higher contents of the essential amino acids could be the younger age of the laying hens (25 to 33 weeks) when hens are

highly productive and related amino acid requirements are high, compared with the age of the laying hens over the experimental period in our study (39 to 51 week).

Another reason for inconsistencies in the results is the difference in the intake of other nutrients. For example, laying hens fed a diet with 250 kcal/kg higher metabolizable energy had a higher body weight and a higher laying performance in battery cages in a tropical region, regardless of the diet crude protein content (15% to 18% CP) (Rao et al., 2011). In our study, feed intake was lower in the laying hens fed 13.5% and 12.0% CP diets which reduced the intake of other nutrients, as well. The inconsistencies among the studies resulted in contrasting conclusions on the effectiveness of free amino acids supplementation in low crude protein diets to prevent the drop in laying performance

In the present experiment, the feed intake was lower in laying hens fed the diets with 13.5% or 12.0% CP compared with the diets with 14.7 or 16.1% CP. In contrast, feed intake increased (101.9 vs 104.1 g/day) as laying hens fed a low crude protein diet (15.5% vs 18.5% CP) with the same content of the essential amino acids (Mousavi et al., 2013). Considering the similar content of digestible amino acids among diets, the higher feed intake of the laying hens fed the low crude protein diet led to a higher intake of essential amino acids. The laying rate (%) was similar regardless of the crude protein content of the diets, however, the egg weight, body weight and feed efficiency decreased (Mousavi et al., 2013). A similar feed intake when laying hens fed a 14% CP diet instead of a 16% CP diet prevented a drop in egg weight (Bunchasak & Silapasorn, 2005). In contrast, Shim et al (2013) suggested that the drop in feed intake was the reason for the deteriorated laying performance in laying hens fed a low crude protein diet (4 % CP lower than control) supplemented with EAA. In the current study, reducing the crude protein content of diets to 13.5% and 12.0% was associated with a drop in feed intake and an impaired laying performance.

The drop in feed intake in chickens fed a low protein diet has been observed in other studies (Viana, 2017; Shim et al., 2013; Wang et al., 2020) and several reasons have been suggested. Shim et al. (2013) suggested that the laying hens reduced the feed intake in response to a drop in the dietary CP content because the limited availability of protein had impaired the egg size and laying rate which reduced the energy requirement of the laying hens. In contrast, the laying hens increased the feed intake in response to a higher level of dietary CP to provide the required energy to synthesise protein for egg formation. It indicates that the supplemented amino acids in the reduced protein diets did not meet the requirements of the laying hens for egg production, therefore, the feed intake did not increase.

Viana (2017) discussed four reasons for the drop in feed intake when laying hens fed a reduced protein diet (CP 17% vs 13%) supplemented with EAA. Firstly, the lower heat increment in reduced-CP diets could increase the net energy value which affects the feed intake. Secondly, the more rapid intestinal absorption of free amino acids could lead to amino acid imbalances in the postabsorptive metabolism of amino acids. Thirdly, the high level of free amino acids could affect appetite regulation. Lastly, it was emphasised that the balance between the nitrogen being supplied by EAA and NEAA could affect performance in laying hens and it is critical to find an ideal balance. In other words, the lower content of NEAA in reduced-CP diets increases the ratio of EAA to NEAA, in particular in the diets that are supplemented with free EAA. Therefore, the amount of dietary nitrogen could become limiting and EAA would be used as a nitrogen source rather than for their specific functions. In the present study, the content of NEAAs reduced gradually by the stepwise reduction in crude protein content led to an imbalance in the content of NEAA and the rise in the ratio of EAA to NEAA in the low crude protein diets.

Differences in the kinetics of intestinal absorption of free and protein-bound amino acids have been recently suggested in broilers (Selle & Liu, 2018), however, to the best of our knowledge, the potential differences in the absorption rate, utilisation in enterocytes, and post-absorptive metabolism between free and protein-bound amino acids have not been studied yet in laying hens.

Regarding the effects of amino acids on satiety stimulation in laying hens, it has been shown that supplementing a reduced-CP diet (CP 13%) with 79.2 g/kg glutamic acid reduced the feed intake (93.4 vs 90.7 g/kg) in laying hens between 28 and 44 weeks of age, while supplementing 37.6 g/kg glutamic acid in a 15% CP diet did not affect feed intake in comparison to diets with the same level of CP (Viana, 2017). The author considered the anorectic effects of glutamic acid as a neurotransmitter in chickens to cause a drop in feed intake (Viana, 2017). It is not the case in the present study, as the content of glutamic acid, as well as other non-essential amino acids, gradually declined by reducing the crude protein content of the diets. Further understanding the pathways by which the non-essential amino acids affect satiety in laying hens and the importance of NEAA concentration in diets, could facilitate the application of low crude protein diets in laying hens.

According to a study reviewed the outcome of research in amino acids requirements of laying hens being published between 1994 and 2019, supplying low protein diets with a lysine content of over 8.0 g/kg might reduce feed intake (Macelline et al., 2021). Wang et al (2018) emphasized the regulatory role of amino acids on the appetite centres in CNS and argued that a high level of lysine might suppress the appetite in chickens by the means of L-pipecolic acid, an important metabolite of lysine in the brain. In the current study, the 14.7% CP diet had the highest lysine content (8.2 g/kg). The laying hens fed this diet had the highest feed (125 g/day) and lysine (1011 mg/day) intake across the treatments. In contrast, the lower content of lysine (7.4-7.6 g/kg) in diets with 12.0% and 13.5% CP was associated with a drop in feed intake and laying performance. It might indicate that a rise in lysine content even more than 8.0 g/kg might prevent the drop in laying performance in the case of low CP diets. In our study, the imbalance of amino acids due to the large reduction of the non-essential amino acids in 13.5% and 12.0% CP diets (Figure 11) along with the rise in free essential amino acids could have impaired the feed intake of the hens.

Bunchasak & Silapasorn (2005) suggested the imbalance in amino acids content of the low crude protein diets, in particular methionine content, as the reason for the drop in feed intake in laying hens. It was shown that the feed intake in laying hens consumed either 14% or 16% CP diets were similar in the case that diets contained 3.8 g/kg methionine (Bunchasak & Silapasorn, 2005). The results of studies in laying hens stated a linear positive relationship between total sulfur-containing amino acids (TSAA) content of the diets and feed intake (Carvalho et al., 2018). In our study, the intake of TSAA was 5 to 10% higher in the laying hens fed 16.1% and 14.7% CP diets which were associated with a higher feed intake in comparison to the low crude protein diet treatments. Besides that, TSAA content was higher (6.4 vs 6.1 g/kg) in the 12.0% CP diet (Trt D) than the 13.5% CP diet (Trt C), while the feed intake of the laying hens between these two dietary treatments were similar. It suggests that increasing the content of TSAA might prevent a further drop in feed intake in very low CP diets.

In general, there are two approaches in diet formulation in studies on the response of laying hens to low protein diets. Firstly, the ratio of amino acids to crude protein remained constant while the crude protein content reduced gradually (Rao et al., 2011; Ji et al., 2014; Torki et al., 2017). Secondly, the content of amino acids remained constant while crude protein content reduced gradually (Mousavi et al., 2013). In the present study, the diets were formulated to have similar concentrations of digestible, first limiting essential amino acids. However, the analysed contents of total amino acids indicated slight differences between the analysed and calculated concentrations, somewhat varying between treatments (Table 6). Deviations from the expected content of amino acids along with the significant differences in feed intake among the treatments, affected the intake of digestible amino acids by the laying hens. In general, the intake of amino acids in laying hens fed 13.5% and 12.0% CP diets was lower than the dietary treatments with 14.7% and 16.1% CP (Table 8). Reduction in the crude protein or essential amino acids intake in addition to the imbalance of amino acids led to a decreased laying performance (Mousavi et al., 2013).

In fact, there was a linear relationship between the crude protein intake and the bodyweight of the laying hens in the current experiment. The laying hens fed the 13.5% or 12.0% CP diets had on average 16.7 or 15.2 g/day crude protein intake and these hens had a lower body weight and laying rate compared to the hens fed diets with 14.7% or 16.1% CP which had a crude protein intake of 18.5 or 20 g/day, respectively. The drop in body weight, egg weight, and absolute and relative content of albumen in eggs might indicate that protein synthesis was limited. Besides essential amino acids, the intake of non-essential amino acids was lower in these treatments. Considering the existence of potential differences in the absorption and post-absorptive metabolism between free and protein-bound amino acids, high content of free EAA in reduced-CP diets might cause an imbalance in the post-absorptive profile of amino acids (Selle & Liu, 2018). The existence of this phenomenon in laying hens requires further study to reach an ideal amino acids balance.

Moreover, the low intake of NEAAs could exacerbate the limiting effects of EAA in reduced-CP diets by enhancing the conversion of EAAs which impair protein synthesis (Novak et al., 2006; Rao et al., 2011; Wang et al., 2020). In a study on the interaction of lysine content and low crude protein diets on laying performance of laying hens between 21 to 72 weeks of age, Rao et al. (2011) showed the limiting effect of low NEAAs on the performance response of laying hens fed the low protein diet. In other words, when dietary protein decreases to a relatively low level, a detrimental influence on laying performance may appear. The requirement for the nonessential amino acids was suggested to be one of the underlying reasons for the decreased laying performance at low protein diets (Han et al., 1992), however, requirement values for NEAA are not yet available for laying hens.

In the present experiment, the intake of amino acids reduced gradually by the stepwise reduction in the crude protein content of the diets. Therefore, laying hens fed the 13.5% and 12.0% CP diets had

the lowest intake of essential as well as non-essential amino acids. Methionine is the first limiting amino acid in poultry feed (Carvalho et al., 2018). Increasing the methionine content (4.4 vs 3.8 g/kg) of a low protein diet (14% CP) prevented the drop in feed intake while the intake of crude protein and other amino acids was still lower than the control diet (16% CP) (Bunchasak & Silapasorn, 2005). The higher methionine intake (440 vs 373 mg/day) in laying hens between the age of 24 to 44 weeks improved the egg weight to the same level as the control diet while the egg mass and feed efficiency were still deteriorated (Bunchasak & Silapasorn, 2005). Kakhki et al. (2016) suggested that 710 mg/d TSAA is required for optimum egg production and 641 mg/d for optimum egg weight in laying hens (Hy-line W36) between the age of 32 to 44 weeks. In our study, TSAA intake of 779 mg/d did not prevent the drop in laying performance when laying hens fed a 12.0% CP diet.

Another example of the lower EAA intake in our study is threonine, the third limiting amino acid (Fernandez et al., 1994). Azzam et al (2017) found that supplying free amino acids to a low protein diet (14.1% vs 16.1%) prevented the drop in laying performance in Lohman Brown laying hens between 29 to 40 weeks of age. However, a reduction in threonine intake from 562 to 467 mg/day in laying hens fed the low protein diets, impaired the laying performance. Azzam et al (2017) stated that a digestible threonine content of 5.7 or 6.6 g/kg, corresponding to 652 or 757 mg daily threonine intake, ensured the highest laying performance. In our experiment, the digestible threonine content was higher in diets with CP content of 16.1 and 14.7% than the diets with the crude protein content of 13.5 and 12% (5.2 vs 4.8 g/kg) which affected the threonine intake. The laying hens with a threonine intake of 712-729 mg/day (Trt A & B) had a higher laying performance in comparison to the laying hens with a threonine intake of 625-633 mg/day (Trt C & D). Therefore, the result of our experiment related to threonine was in line with the observations in the literature.

In the present study, the laying hens fed the 13.5% and 12.0% CP diets (Trt C & D) had a lower intake of essential and non-essential amino acids in comparison to the laying hens fed the 14.7% and 16.1% CP diets. However, it is disputable to suggest that the digestible EAA intake met the requirement of the laying hens in Trt C & D or caused marginal deficiencies and imbalance of EAA. The intake of NEAA in the laying hens in Trt C & D was remarkably lower than the laying hens in Trt A & B, however, the daily requirement of the NEAA has not been studied yet. Moreover, the lack of nitrogen to synthesise NEAA could impair protein synthesis and lead to metabolic degradation of EAA, as well. This possibility along with the potential differences in the intestinal absorption of free and protein-bound EAA could impair the timely availability of all required amino acids for maximum protein synthesis in organs and tissues.

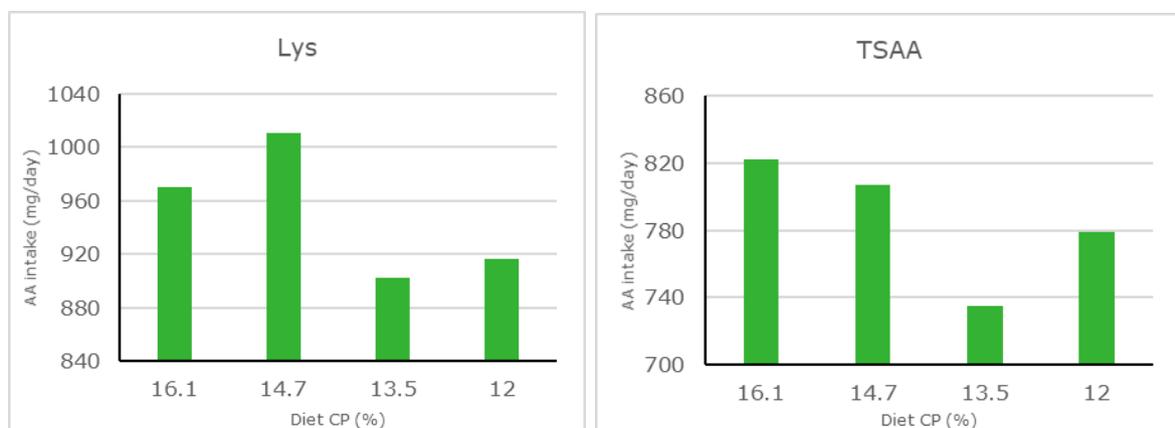


Figure 10 Average intake (mg/day) of lysine and total sulphur-containing amino acids (TSAA) based on the average feed intake (g/day) and the analysed content of amino acids (g/kg) in each dietary treatment, between week 39 to 51. Note: recommended daily intake of total lysine and TSAA in laying hens up to the age of 55 weeks is 980 mg/day and 860 mg/day, respectively (Hendrix Genetics, 2020).

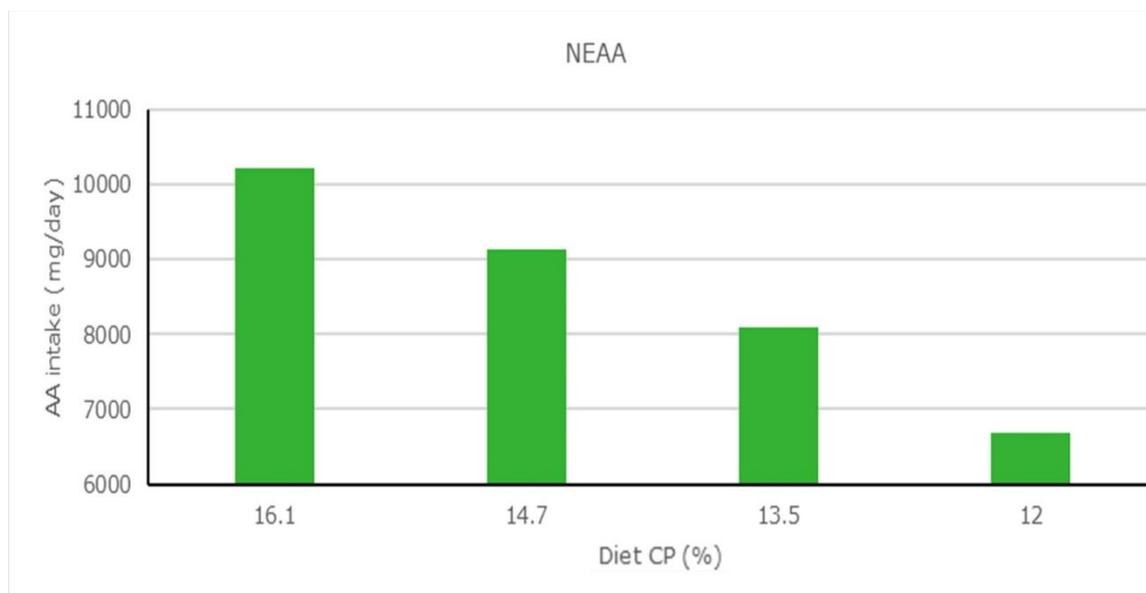


Figure 11 Average intake (mg/day) of non-essential amino acids (NEAA) based on the average feed intake (g/day) and the analysed content of amino acids in each dietary treatment, between weeks 39 to 51.

In the present experiment egg weight decreased linearly (65.9 vs 63.3 g) with the stepwise reduction of crude protein content in diets (Table 9 and Figure 12). Stepwise reduction of diet CP from 18.5% to 15.5% did not affect laying rate (%) in laying hens between 25 to 33 weeks of age, however, the egg weight reduced gradually (57.1 vs 55.9 g) (Mousavi et al., 2013). In spite of supplementing free amino acids to the low crude protein diet, egg weight reduced in the laying hens and the lower intake of crude protein was suggested as the reason for the drop in egg weight (Mousavi et al., 2013; Novak et al., 2006).

While it is generally accepted that the content of NEAA is adequate in the laying hen diets, in the low CP diets the dietary nitrogen content to synthesise NEAA could become limiting. In fact, NEAA such as glycine and glutamine are critical sources of nitrogen. The limited intake of NEAA in the case of low protein diets impaired the protein synthesis in the laying hens which might be reflected as a drop in body weight or albumen synthesis for egg production. In our study, the drop in egg weight was associated with the reduction in the absolute content of albumen (42.2 to 40.0 g/egg) in eggs. In laying hens fed the 12.0% CP diet, the relative content of albumen (%/egg) was significantly lower than the eggs from other treatments. In laying hens fed low protein diets supplemented with free amino acids, the intake of crude protein and non-essential amino acids declined which could impair protein synthesis in the body. Several researchers (Mousavi et al., 2013 and Torki et al., 2017) suggested that in laying hens fed low protein diets with similar content of major essential amino acids, the reduction in protein synthesis could be the cause of the drop in egg weight and body weight. In our study, body weight, egg weight, albumen content of the eggs, and in the extreme case (Trt D) the relative content of albumen in eggs declined in response to the reduced intake of crude protein and amino acids across the treatments.

Regarding the limiting role of low NEAA content in reduced-CP diets in laying hens, Viana (2017) showed that supplying glutamic acid (38 g/kg) to a reduced-CP diet (15% vs 17% CP) in laying hens between 28 and 44 weeks of age increased egg weight and improved FCR to the same values as the laying hens fed the control diet. In other words, in laying hens fed a reduced CP diet (15% vs 17% CP), the egg weight (62.8 vs 65.0 g), egg mass (53.8 vs 55.9 g), and feed conversion ratio (1.76 vs 1.70) were impaired, however, supplementing 38 g/kg glutamic acid to the 15% CP diet increased the egg weight (64 g), egg mass (55.3 g), and improved the FCR (1.71) to the similar level as the laying hens in the 17% CP dietary treatment. The rise in performance by including glutamic acid in the reduced-CP diet indicated that the glutamic acid level was limiting. Another explanation is supplementing glutamic acid in the reduced-cp diet could provide enough nitrogen for protein synthesis. In fact, the researchers at Texas A&M University suggested that the requirements of laying hens for NEAA could be higher than the content in the conventional diets. Therefore, the content and ratio of the non-essential amino acids in laying hen diets should be considered in the Ideal Protein concept, even in conventional diets (He et al., 2021).

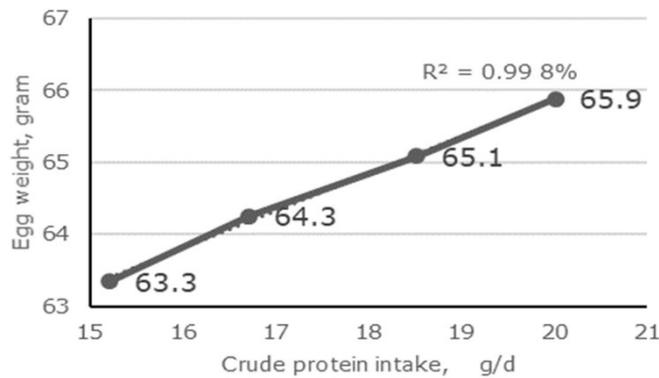


Figure 12 Correlation between crude protein intake and egg weight of laying hens from 39 – 51 weeks of age.

Haugh unit of eggs is an important indicator of egg quality parameter related to egg protein content and egg freshness. In the current study, the crude protein content of diets did not affect the Haugh unit, while the relative content of albumen (%/egg weight) was reduced in laying hens consumed the 12% CP diet. This finding is in line with the outcome of several studies (Alagawany & El-hindawy, 2020; Azzam et al., 2017b). A drop in Haugh unit (P-value 0.044; 77 vs 84) was observed in laying hens fed a low protein diet (CP 13% vs 16%) in hot weather at 42 to 55 weeks of age (Torki et al., 2017). Surprisingly, the reduction in egg weight was not significant, but egg mass reduced from 58% to 52% in comparison to the control group. Feeding the low protein diet was associated with a significant decline in the intake of crude protein (14.4 vs 17.8 g/day), valine (792 vs 948 mg/day), and arginine (955 vs 1123 mg/day) (Torki et al., 2017). It was suggested that the low intake of valine affected the albumen synthesis in laying hens. In our study, the lowest intake of crude protein (15.2 g/day), valine (817 mg/day) and arginine (983 mg/day) was observed in the laying hens fed the 12.0% CP diet which was still higher than the value in the study of Torki et al. (2017).

In the present study, the breaking strength of the eggs was lower when laying hens fed the reduced CP diets (14.7 to 12.0% CP) in comparison to the control diet (16.1% CP), while the effect on shell content and shell thickness were not significant. The drop in specific gravity of eggs due to consuming a reduced-cp diet (4% unit lower CP) has been observed which could be related to the alterations in the protein matrix of the eggshell and the proteinous eggshell membranes (reviewed by Shim et al., 2013; Scheideler et al., 2005). The interaction between the organic and the mineral components of the eggshell determine the eggshell strength. Assessing the ultrastructural changes in the eggshell in the case of low protein diets in the laying hens could provide further explanation about the reason for the drop in the breaking strength.

Moisture content is a major determinant of ammonia production in excreta. The nitrogen content of manure includes N in uric acid, bacterial protein, urea, ammonia, and endogenous nitrogen. In our study, manure moisture and nitrogen content declined gradually by reducing the crude protein content of diets (Table 10). As expected, the stepwise reduction in the crude protein content of the diets reduced the nitrogen intake and subsequently the nitrogen excretion (Figure 8), but the ammonium-N of the manure was not affected (Table 10). Figures 13 and 14 show a strong relationship between crude protein intake and the manure dry matter and nitrogen content, respectively. However, it did not affect the ammonia content of manure. Like our study, Roberts et al. (2006) observed a reduction in nitrogen excretion but not the ammonia excretion in laying hens fed a low protein diet. The authors suggested that the imbalance in amino acids intake due to a low protein diet could impair protein metabolism which resulted in an impaired laying performance (Roberts et al., 2006). In a recent study, reducing the crude protein content of the diet (17.2 vs 13.5 % CP) in laying hens between weeks 20 to 39 was associated with a drop in excreta moisture and nitrogen (Dao et al., 2021).

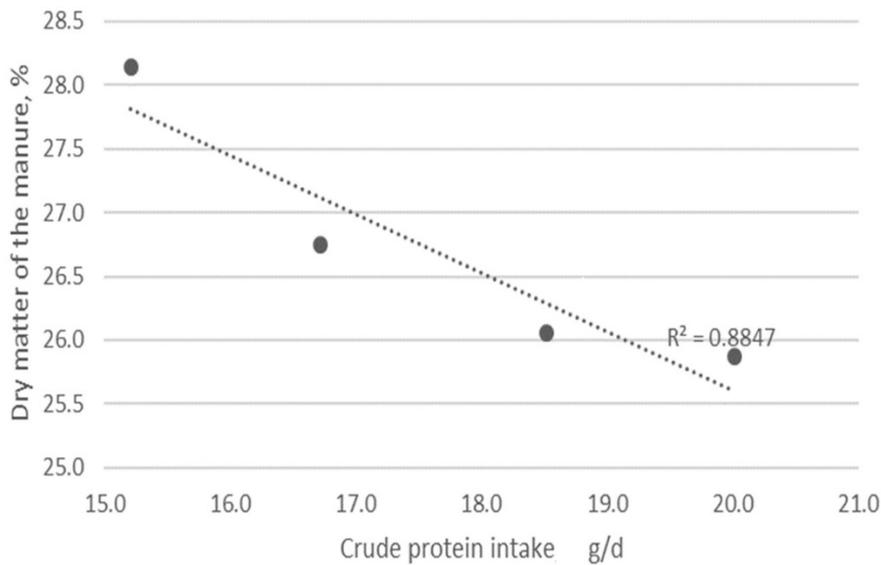


Figure 13 Correlation between the crude protein intake on the dry matter content of the manure of laying hens from 39 – 51 weeks of age.

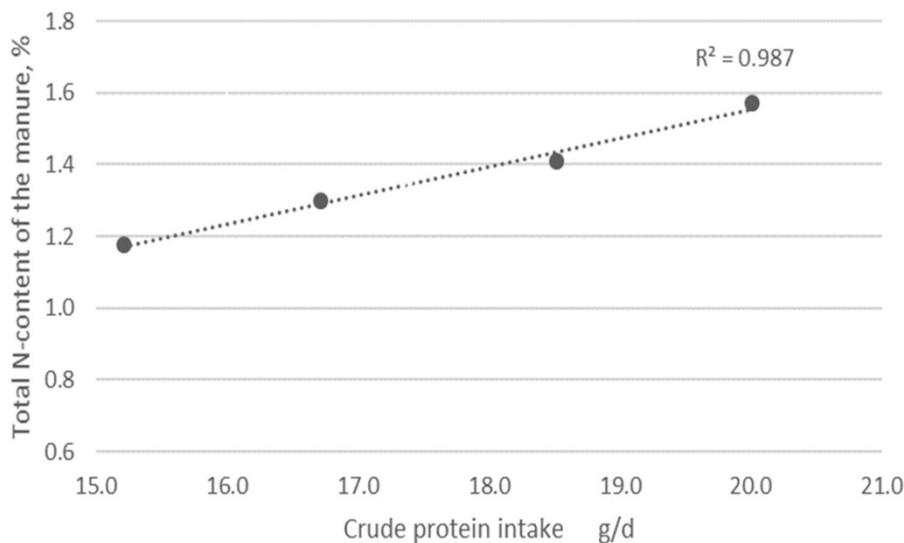


Figure 14 Correlation between the crude protein intake and the total nitrogen content of the manure of laying hens from 39 – 51 weeks of age.

Despite the lower SBM content in the low protein diets, the carbon footprint (CFP) was not lower compared to the control diet. The use of free amino acids is the main reason for the higher CFP. For example the CFP (excl. LUC) for free L-lysine, DL-methionine, L-tryptophan, L-valine, L-arginine, L-isoleucine, L-threonine is 6437, 3049, 12865, 6351, 6351, 7197 and 6437 g CO₂-eq/kg, respectively (FeedPrint database). Marinussen and Kool (2010) describe the environmental impact of the production of free amino acids L-lysine, DL-methionine and L-threonine. For the production of L-threonine, high quantities of sulphate compounds are required, which significantly contribute to the acidification of the environment and explain the high CFP value of L-threonine.

The current experiment was performed in a trial facility in Vietnam and in battery cages. There is evidence indicating the existence of interaction between the dietary crude protein content and the production system on the production responses of the laying hens. It seems that the housing system affects the protein requirements of the laying hens or their response to the balanced protein diets (Eits et al., 2005). For example, the laying hens in cages reduced the feed intake in response to a decline in the dietary crude protein content from 17.5 to 13%, while the feed intake increased in laying hen in the floor pens (Eits et al., 2005). Viana et al. (2017) stated differences in the responses of the laying

in cages and floor pens to the crude protein content of diets between the age of 30 to 45 weeks. The interaction between the crude protein content of diets (14 to 18% CP) and the production system was significant in the internal quality of the eggs, i.e. albumen height (mm) and albumen relative weight (%). In addition, the laying performance and egg quality of laying hens fed a similar diet were significantly different in various housing systems (Englmaierová et al., 2014). Therefore, the outcome of studies in a conventional cage system might not be representative for hens kept in aviary systems as common practice in the EU. Considering the limited amount of knowledge on the effects of low protein diets in laying hens, it is suggested to also perform research on low-protein diets in laying hens in facilities representative for production systems and climate conditions of the laying sector in the EU.

5 Conclusion

From this study, feeding laying hens between 39 – 51 week of age diets with 16.1, 14.7, 13.5 or 12.0% crude protein content but similar digestible amino acids contents of the first limiting and free available amino acids, and thereby reducing gradually the soybean meal content from approx. 10% to 0%, it can be concluded that:

- Reduction of the crude protein content of diet from 16.1% to 14.7% with similar content of digestible EAA resulted in a higher laying rate, lower egg weight and fewer Jumbo eggs. Further reduction of crude protein content to 13.5% and 12.0% impaired the laying rate, egg weight, egg mass, and feed efficiency
- Reducing the crude protein content of the diet from 16.1% to 14.7% did not affect the egg mass, but because of the higher feed intake, the FCR increased.
- Reduction in crude protein content of the diets to 13.7% and 12.0% CP resulted in impaired laying performance.
- The FCR increased with the decreased crude protein contents of the diets.
- The crude protein content of the diets did not affect the mortality rate.
- The crude protein intake decreased linearly with the reduced crude protein content of the diets.
- Egg weight decreased linearly with decreasing crude protein contents of the diets.
- Feeding diets with 12.0% CP resulted in a higher % second-grade eggs. No differences were observed in % second-grade eggs between the 16.1, 14.7 and 13.5% diets.
- Albumen in eggs decreased with the decreased crude protein content of the diets.
- Decreasing dietary protein content with similar digestible content of essential amino acids increases the carbon footprint of the diets.
- The calculated feed price increases with a decrease in the crude protein content.
- The calculated economic feed margin is strongly influenced by the availability and price of the protein-rich raw materials and the price of free amino acids. Under the current Dutch market conditions, the feed margins per 100 placed hens of the low-crude protein diets decreased by €12.82, €56.58 and €70.08 respectively for the 14.7, 13.5 and 12.0% CP-diets.
- Dry matter content of the manure increased linearly with decreasing crude protein content of the diets.
- Total N-content of the manure decreased linearly with decreasing crude protein content of the diets.

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Appendix 1 Experimental diets 1st batch

Treatment Recipe code		37 to 46 weeks			
		A RP075.00	B RP076.00	C RP077.00	D RP078.00
RAW MATERIALS					
CORN *	%	33.65			41.15
WHEAT *	%	33.19			27.52
WHEAT MIDLINGS MEAL *	%				13.49
SBM 48.5-50%CP *	%	9.55			
SFM 35-38%CP *	%	12.50			3.81
SOYBEAN OIL CRUDE *	%	0.69			0.25
LIMESTONE 0.4-0.8MM MEDIUM	%	8.68			8.74
DICALCIUM PHOSPHATE DIHYDRATE	%	0.47			0.55
SALT VACUUM	%	0.23			0.24
NA BICARBONATE	%	0.27			0.27
XYLANASE	%	0.03			0.03
PHYTASE	%	0.03			0.03
L-LYSINE SULPHATE 55%	%	0.28			0.76
DL-METHIONINE 99%	%	0.12			0.27
L-THREONINE 99% POWDER	%	0.01			0.21
L-TRYPTOPHANE 98%	%				0.06
L-VALINE 20% LOW CA PX	%				1.09
ISOLEUCINE 24.77%	%				1.00
L-ARGININE 98%	%				0.23
CHOLIN CHLORID 60%	%	0.04			0.04
DH400 LAYERpx0.20%VN	%	0.20			0.20
CANTHAXANTHIN 1% PX	%	0.04			0.04
Biofill yellow	%	0.03			0.03
Diet A	%		65.00	35.00	
Diet D	%		35.00	65.00	
Total	%	100.00	100.00	100.00	100.00
Calculated nutrients					
	Unit				
Crude protein	%	16.1	14.7	13.5	12.0
Crude fat B (EEh)	%	3.3	3.3	3.3	3.3
Crude fat Xtr (EE)	%	2.6	2.6	2.6	2.6
Crude fibre	%	4.3	4.1	4.0	3.8
Ash	%	12.2	12.1	12.0	11.9
Moisture	%	9.9	10.0	10.0	10.1
Starch Amyloglucosidase	%	40.7	42.2	43.5	45.1
ME poultry enz	Kcal/kg	2764	2764	2765	2765
ME layer enz	Kcal/kg	2800	2800	2801	2801
ME poultry (WPSA)	MJ/kg	11.1	11.1	11.1	11.1
LYS - Lysin	%	0.79	0.78	0.77	0.76
afd LYS po	%	0.69	0.69	0.69	0.69
afd MET po	%	0.36	0.38	0.40	0.42
afd CYS po	%	0.25	0.23	0.21	0.19
afd M+C po	%	0.61	0.61	0.61	0.61
afd THR po	%	0.48	0.48	0.48	0.48
afd TRP po	%	0.16	0.16	0.16	0.16
afd ILE po	%	0.55	0.55	0.55	0.55
afd ARG po	%	0.92	0.86	0.81	0.75
afd VAL po	%	0.64	0.63	0.62	0.61
afd LEU po	%	1.08	0.96	0.85	0.73
afd GLU po	%	3.1	2.74	2.45	2.07
afd PHE po	%	0.68	0.58	0.5	0.41
afd TYR po	%	0.44	0.38	0.33	0.27
afd HIS po	%	0.36	0.31	0.27	0.23
afd ALA po	%	0.61	0.55	0.49	0.44
afd ASP po	%	1.16	0.95	0.75	0.56
afd PRO po	%	0.93	0.86	0.81	0.71
afd GLY po	%	0.59	0.52	0.44	0.37
afd SER po	%	0.66	0.57	0.5	0.41
afd G+S po	%	1.25	1.09	0.94	0.78
Starch Ewers	%	42	43	45	46
Sugar	%	2.7	2.6	2.4	2.3
C16:0+C18:0	%	0.4	0.4	0.4	0.4
C18:2 - Linoleic	%	1.5	1.5	1.5	1.5
Calcium	%	3.70	3.70	3.70	3.70
Phosphor tot	%	0.46	0.47	0.47	0.48
dP poultry	%	0.30	0.30	0.30	0.30
Sodium	%	0.18	0.18	0.18	0.18
Chlorine	%	0.20	0.20	0.20	0.20
Potassium	%	0.63	0.58	0.55	0.50
dEB	meq	182	171	161	150
Choline (-OH) tot	mg/kg	1184	1081	993	891
Vit D3 eq sum add	IU/kg	3023	3023	3023	3023
Vit A add (3a672a)	IU/kg	10076	10076	10076	10076
Vit D3 add (3a671)	IU/kg	3023	3023	3023	3023
Vit E add (3a700)	mg/kg	30	30	30	30
Xanthophyll sum	mg/kg	12.7	12.6	12.6	12.5
Canthaxanthin (2a161g)	mg/kg	4.0	4.0	4.0	4.0
Phytase equivalents	sFU/kg	300	300	300	300

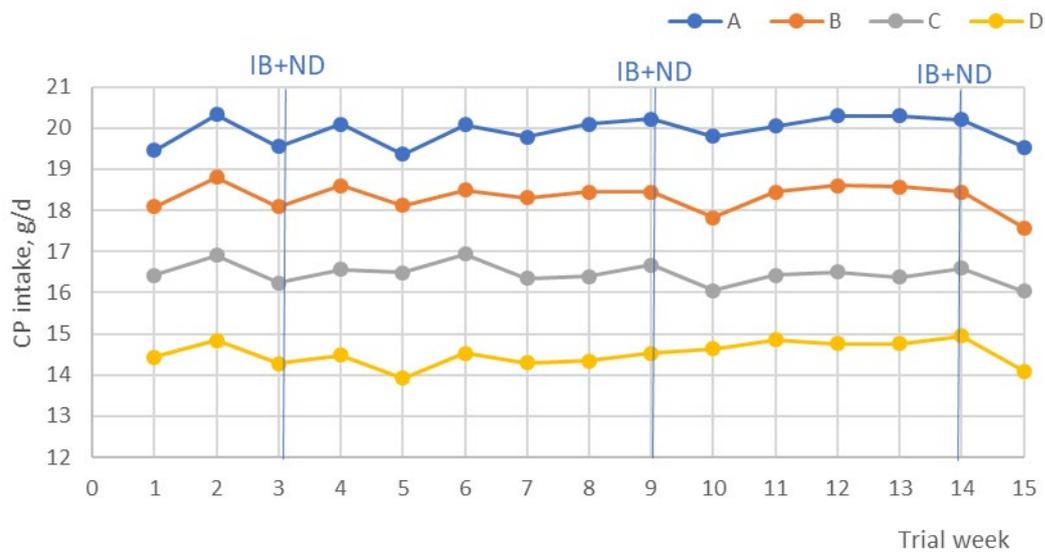
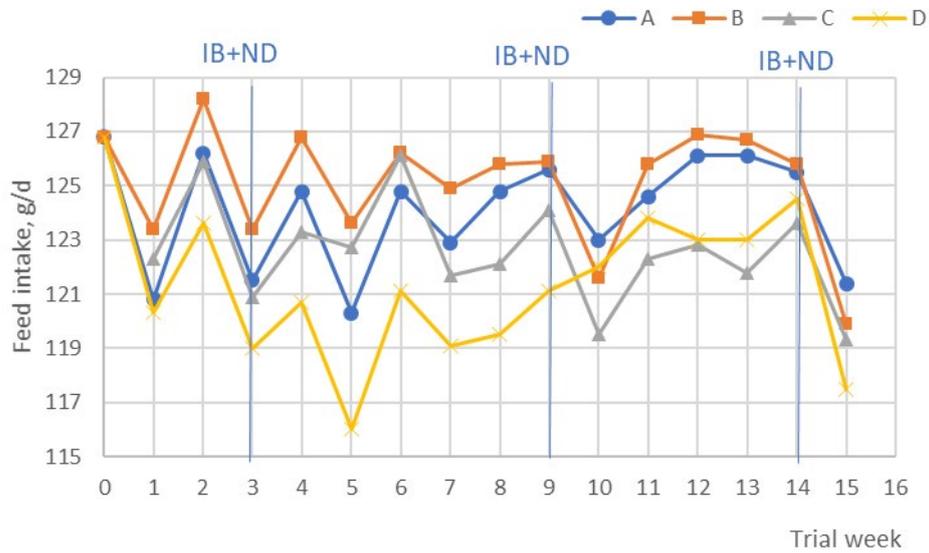
* analysed on AMINONir

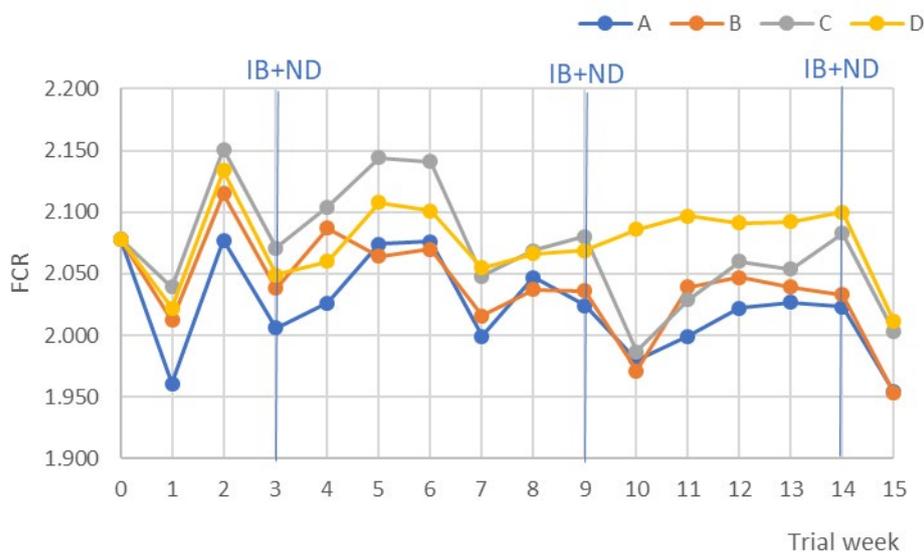
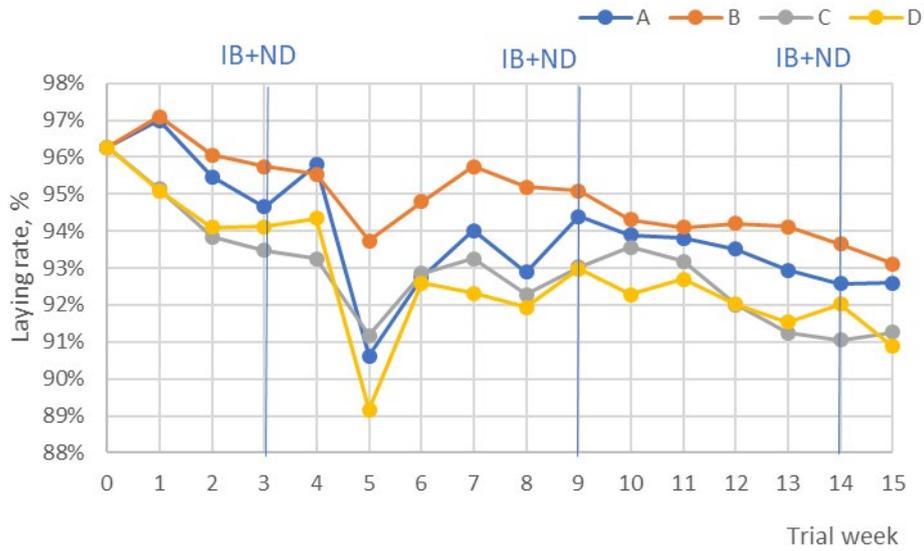
Appendix 2 Experimental diets 2nd batch

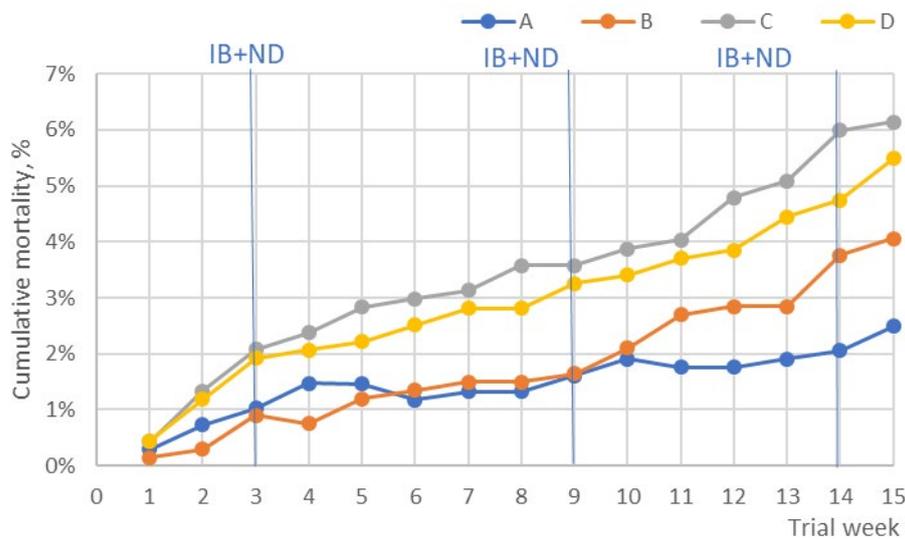
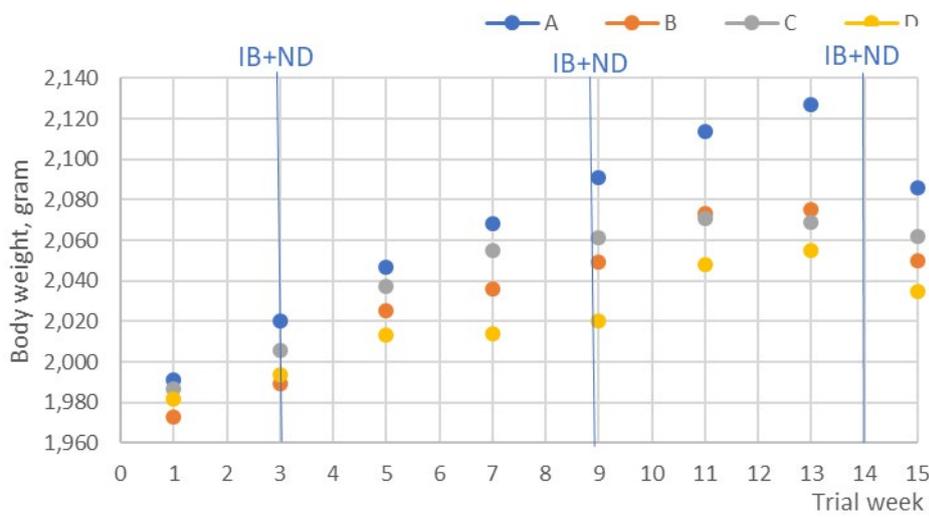
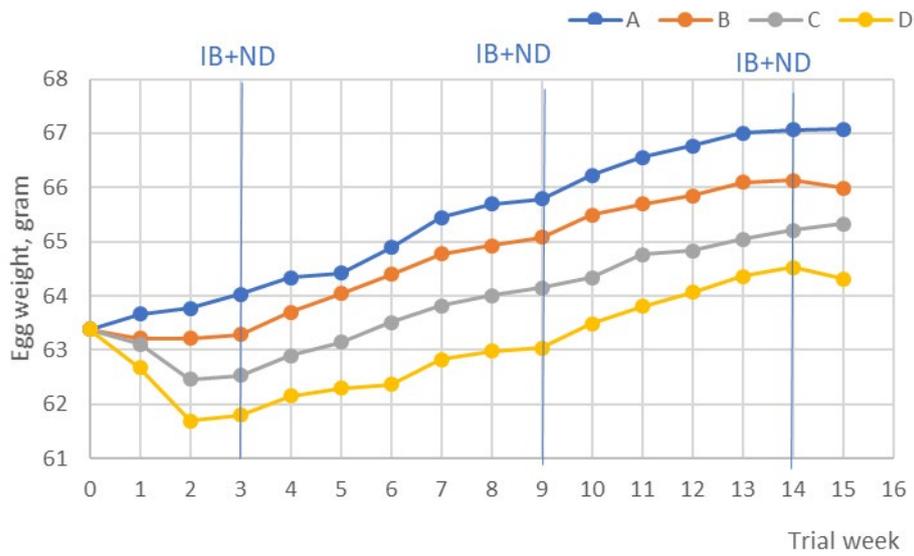
		46 to 52 weeks			
		A	B	C	D
		RP075	RP076	RP077	RP078
Raw materials					
CORN*	%	30.56			40.35
WHEAT*	%	36.76			27.93
WHEAT MIDDLINGS PELLET*	%				15.56
SBM 48.5-50%CP*	%	11.06			
SFM 30-35%CP*	%	10.97			2.03
SOYBEAN OIL CRUDE*	%	0.53			0.25
LIMESTONE COARSE	%	8.47			8.74
DICALCIUM PHOSPHATE DIHYDRATE	%	0.40			0.54
SALT VACUUM	%	0.23			0.23
NA BICARBONATE	%	0.27			0.28
XYLANASE	%	0.03			0.03
PHYTASE	%	0.03			0.03
L-LYSINE SULPHATE 55%	%	0.25			0.77
DL-METHIONINE 99%	%	0.12			0.27
L-THREONINE 99% POWDER	%	0.02			0.22
L-TRYPTOPHANE 98%	%				0.06
L-VALINE 20% LOW CA PX	%				1.13
ISOLEUCINE 24.77%	%				1.04
L-ARGININE 98%	%				0.25
CHOLIN CHLORID 60%	%	0.04			0.04
DH400 LAYERpx0.20%VN	%	0.20			0.20
CANTHAXANTHIN 1% PX	%	0.04			0.04
Biofill yellow	%	0.04			0.03
F4F trial control diet A	%		65.00	35.00	
F4F trial control diet D	%		35.00	65.00	
Total	%	100.00	100.0	100.0	100.00
Calculated nutrients					
Crude protein	%	16.1	14.7	13.5	12.0
Crude fat B (EEh)	%	3.1	3.2	3.2	3.2
Crude fat Xtr (EE)	%	2.4	2.5	2.5	2.6
Crude fibre	%	4.5	4.3	4.0	3.8
Ash	%	12.0	12.0	12.0	12.0
Moisture	%	9.7	9.8	9.9	10.0
Starch Amyloglucosidase	%	40.8	42.2	43.5	44.9
ME poultry enz	Kcal/kg	2757	2759	2761	2763
ME layer enz	Kcal/kg	2791	2793	2796	2798
ME poultry (WPSA)	MJ/kg	11.1	11.1	11.1	11.1
LYS - Lysin	%	0.79	0.78	0.77	0.76
afd LYS po	%	0.69	0.69	0.69	0.69
afd MET po	%	0.36	0.38	0.40	0.42
afd CYS po	%	0.25	0.23	0.21	0.19
afd M+C po	%	0.61	0.61	0.61	0.61
afd THR po	%	0.48	0.48	0.48	0.48
afd TRP po	%	0.16	0.16	0.16	0.16
afd ILE po	%	0.55	0.55	0.55	0.55
afd ARG po	%	0.90	0.85	0.81	0.75
afd VAL po	%	0.64	0.63	0.62	0.61
afd LEU po	%	1.09	0.96	0.85	0.73
afd GLU po	%	3.17	2.79	2.47	2.08
afd PHE po	%	0.68	0.58	0.5	0.4
afd TYR po	%	0.45	0.39	0.33	0.27
afd HIS po	%	0.36	0.31	0.28	0.23
afd ALA po	%	0.61	0.55	0.49	0.43
afd ASP po	%	1.16	0.94	0.75	0.53
afd PRO po	%	0.97	0.89	0.82	0.73
afd GLY po	%	0.58	0.5	0.44	0.36
afd SER po	%	0.67	0.58	0.5	0.41
afd G+S po	%	1.25	1.08	0.94	0.77
Starch Ewers	%	42	44	45	46
Sugar	%	2.9	2.7	2.5	2.3
C16:0+C18:0	%	0.4	0.4	0.4	0.4
C18:2 - Linoleic	%	1.4	1.4	1.5	1.5
Calcium	%	3.4	3.5	3.5	3.5
Phosphor tot	%	0.48	0.48	0.48	0.48
dP poultry	%	0.30	0.30	0.30	0.30
Sodium	%	0.18	0.18	0.18	0.18
Chlorine	%	0.20	0.20	0.20	0.20
Potassium	%	0.69	0.63	0.57	0.51
dEB	meq	198	182	169	153
Choline (-OH) tot	mg/kg	1181	1072	979	870
Vit D3 eq sum add	IU/kg	3023	3023	3023	3023
Vit A add (3a672a)	IU/kg	10076	10076	10076	10076
Vit D3 add (3a671)	IU/kg	3023	3023	3023	3023
Vit E add (3a700)	mg/kg	30	30	30	30
Xanthophyll sum	mg/kg	13	13	13	13
Canthaxanthin (2a161g)	mg/kg	4	4	4	4
Phytase equivalents	SFU/kg	300	300	300	300

* analysed on AMINONir

Appendix 3 Weekly performance parameters







Appendix 4 Carbon footprint diets

Batch 1	A	B	C	D	CFP (excl. LUC)	CFP (excl. LUC)			
	(16.1% CP) RP075	(14.7% CP) RP076	(13.5% CP) RP077	(12.0% CP) RP078		RP075	RP076	RP077	RP078
CORN	33.65	36.27	38.52	41.15	489	165	177	188	201
WHEAT	33.19	31.21	29.51	27.52	439	146	137	130	121
WHEAT MIDLINGS	0.00	4.72	8.77	13.49	270	0	13	24	36
SBM 48.5-50%CP	9.55	6.20	3.34	0.00	602	57	37	20	0
SFM 35-38%CP	12.50	9.46	6.85	3.81	404	51	38	28	15
SOY BEAN OIL	0.69	0.53	0.40	0.25	1630	11	9	7	4
LIMESTONES	8.68	8.70	8.72	8.74	519	45	45	45	45
DICALCIUMPHOSPHATE	0.47	0.50	0.52	0.55	575	3	3	3	3
SALT	0.23	0.24	0.24	0.24	180	0	0	0	0
NA BICARBONATE	0.27	0.27	0.27	0.27	490	1	1	1	1
XYLANASE enzyme	0.03	0.03	0.03	0.03	1174	0	0	0	0
AXTRA PHY 10000 TPT	0.03	0.03	0.03	0.03	1894	0	0	0	0
L-LYSINE SULPHATE 55%	0.28	0.45	0.59	0.76	6437	18	29	38	49
DL-METHIONINE 99%	0.12	0.17	0.21	0.27	3050	4	5	7	8
L-THREONINE 99%	0.01	0.08	0.14	0.21	6437	1	5	9	13
L-TRYPTOPHANE 98%	0.00	0.02	0.04	0.06	12865	0	3	5	8
L-VALINE 20%	0.00	0.38	0.71	1.09	6351	0	24	45	69
test ingredient 1 Isoleucine	0.00	0.35	0.65	1.00	7197	0	25	47	72
test ingredient 2 L-ARGININE 98%	0.00	0.08	0.15	0.23	6351	0	5	10	15
CHOLIN CHLORID 60%	0.04	0.04	0.04	0.04	4159	2	2	2	2
LAYERpx0.20%VN	0.20	0.20	0.20	0.20	1174	2	2	2	2
CANTHAXANTHIN 1% PX	0.04	0.04	0.04	0.04	1174	0	0	0	0
Yellow colorant	0.03	0.03	0.03	0.03	1174	0	0	0	0
						507	563	612	668

Batch 2	A	B	C	D	CFP (excl. LUC)	CFP (excl. LUC)			
	(16.1% CP) RP075	(14.7% CP) RP076	(13.5% CP) RP077	(12.0% CP) RP078		RP075	RP076	RP077	RP078
CORN	30.56	33.98	36.92	40.35	489	149	166	181	197
WHEAT	36.76	33.67	31.02	27.93	439	161	148	136	123
WHEAT MIDLINGS	0.00	5.44	10.11	15.56	270	0	15	27	42
SBM 48.5-50%CP	11.06	7.19	3.87	0.00	602	67	43	23	0
SFM 35-38%CP	10.97	7.84	5.16	2.03	404	44	32	21	8
SOY BEAN OIL	0.53	0.43	0.35	0.25	1630	9	7	6	4
LIMESTONES	8.47	8.56	8.64	8.74	519	44	44	45	45
DICALCIUMPHOSPHATE	0.40	0.45	0.49	0.54	575	2	3	3	3
SALT	0.23	0.23	0.23	0.23	180	0	0	0	0
NA BICARBONATE	0.27	0.27	0.28	0.28	490	1	1	1	1
XYLANASE enzyme	0.03	0.03	0.03	0.03	1174	0	0	0	0
AXTRA PHY 10000 TPT	0.03	0.03	0.03	0.03	1894	0	0	0	0
L-LYSINE SULPHATE 55%	0.25	0.43	0.59	0.77	6437	16	28	38	49
DL-METHIONINE 99%	0.12	0.17	0.22	0.27	3050	4	5	7	8
L-THREONINE 99%	0.02	0.09	0.15	0.22	6437	1	6	9	14
L-TRYPTOPHANE 98%	0.00	0.02	0.04	0.06	12865	0	3	5	8
L-VALINE 20%	0.00	0.39	0.73	1.13	6351	0	25	47	72
test ingredient 1 Isoleucine	0.00	0.37	0.68	1.04	7197	0	26	49	75
test ingredient 2 L-ARGININE 98%	0.00	0.09	0.17	0.25	6351	0	6	10	16
CHOLIN CHLORID 60%	0.04	0.04	0.04	0.04	4159	2	2	2	2
LAYERpx0.20%VN	0.20	0.20	0.20	0.20	1174	2	2	2	2
CANTHAXANTHIN 1% PX	0.04	0.04	0.04	0.04	1174	0	0	0	0
Yellow colorant	0.04	0.03	0.03	0.03	1174	0	0	0	0
						505	563	613	672

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



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