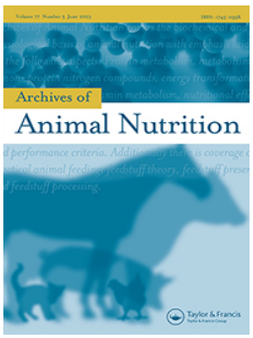

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Genotype-dependent impact of dietary vitamin D_3 on laying hens

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ABSTRACT

Vitamin D_3 has an integral part in calcium and phosphorus homoeostasis, which in turn plays a key role in egg production of hens. The present study aimed to investigate whether an additional vitamin D_3 supplementation improves the laying performance and egg quality of hens according to their genetic potential. For this purpose, four layer lines (low performing: R11 and L68; high performing: WLA and BLA) supplemented either with 300 or 3000 IU vitamin D_3 per kg feed were compared concerning serum 25-hydroxyvitamin D_3 (25-OHD_3), calcium, phosphorus and alkaline phosphatase (ALP), laying performance and egg quality. The higher supplementation of vitamin D_3 increased 25-OHD_3 serum concentrations in all genotypes, except for R11 and WLA hens in week 49, and also elevated vitamin D_3 and 25-OHD_3 content in the egg yolk ($p < 0.05$). In week 29, 3000 IU vitamin D_3 decreased pooled least squares means (LSMeans) of serum calcium concentrations considering all genotypes and increased the ALP concentrations in BLA hens ($p < 0.05$). Considering the whole experimental period daily egg mass of R11 hens was increased by an additional vitamin D_3 supplementation ($p < 0.001$). Regarding all genotypes and the whole experimental period the pooled LSMeans of breaking strength of eggs from hens fed 3000 IU vitamin D_3 were higher than those of hens fed 300 IU ($p = 0.044$). In conclusion, present results give evidence that the higher vitamin D_3 supplementation might have genotype-dependently beneficial effects on calcium and phosphorus homoeostasis of hens, which might improve feed efficiency in the early laying period and promote the persistence of the laying period irrespectively of genotype. The increase of serum 25-OHD_3 by the higher vitamin D supplementation supported the higher transfer of vitamin D in the egg yolk and improved genotype-dependently the breaking strength of the eggshell.

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Laying hens; performance; egg quality; vitamin D_3; calcium; phosphorus; alkaline phosphatase

1. Introduction

Vitamin D_3 can be synthesised in the skin during UV exposure or ingested as vitamin D_2 or D_3 from dietary sources or supplements (Soares et al. 1995; de Matos 2008;

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Proszkowiec-Weglarz and Angel 2013). Birds that are kept indoors rely on a dietary vitamin D supplementation (Edwards et al. 1994; de Matos 2008). In the avian nutrition vitamin D_3, which has a markedly higher biological activity in birds, is the commonly used vitamin D isoform (de Matos 2008; Proszkowiec-Weglarz and Angel 2013). Vitamin D_3 undergo two hydroxylation steps which initially lead to the formation of 25-hydroxyvitamin D_3 (**25-OHD$_3$**) and subsequently to the active form 1,25-dihydroxy vitamin D_3 or 24,25-dihydroxy D_3 (de Matos 2008; Proszkowiec-Weglarz and Angel 2013). The vitamin D hormone plays a key role in the intestinal calcium and phosphorus homoeostasis and utilisation which are crucial for bone and eggshell mineralisation and on the other hand, the activation of vitamin D_3 depends on plasma calcium concentration (de Matos 2008; Adedokun and Adeola 2013; Proszkowiec-Weglarz and Angel 2013). Factors influencing the vitamin D requirements are for example the species, age, breeding status, egg production, growth, fertility or hatchability as well as the calcium and phosphorus levels in the diet (de Matos 2008).

In previous studies, it was shown that an additional vitamin D_3 supplementation could influence the laying performance of hens by increasing the daily egg production or the egg weight (Atencio et al. 2006; Browning and Cowieson 2015). Besides the laying performance of the hens also the egg quality plays an important role. The eggshell quality representing an integral part of the egg quality influences the percentage of broken eggs. Thus, the eggshell quality has a high economic priority and depends on breeds, lines and families of laying hens as well as the age of hens (Ketta and Tumová 2016). Additionally, dietary supplementation with vitamin D_3 or 25-OHD$_3$ also shows beneficial effects on the eggshell quality (Soares et al. 1995).

Given the specific role of vitamin D for calcium homoeostasis and in turn of eggshell stability, we hypothesised, that the current recommendation of 300 IU vitamin D_3/kg diet (GfE 1999) is insufficient for modern laying hybrids which are characterised by a high laying performance. We assume that these hens might benefit from higher vitamin D_3 doses. According to EC No 1831/2003 (2003) the maximum permissible vitamin D_3 content in laying hen feed is 3000 IU vitamin D_3/kg diet. Moreover, hens laying brown-coloured-coloured eggs are characterised by a calcium and bone metabolism which is different from those laying hens that produce white-coloured eggs (Habig and Distel 2013). Thus, it was further hypothesised that phylogenetically divergent layer lines would respond differently to low and high doses of vitamin D_3. The current study investigated the effects of 300 IU vs. 3000 IU vitamin D_3/kg feed in phylogenetically divergent layer lines with marked differences in laying performance. Therefore, the four layer-line model at first reported by Lieboldt et al. (2015) was used to investigate the interactions between genetically determined laying performance and vitamin D_3 supply.

Summarising, the present study aimed to investigate the influences of a vitamin D_3 supplementation of 300 IU vs. a vitamin D_3 supplementation of 3000 IU on laying performance, egg quality and calcium-phosphorus homoeostasis in various genotypes of laying hens differing in performing type and phylogeny according to brown and white layer lines at different ages. It was hypothesised that a higher vitamin D_3 supplementation improves the laying performance and also the eggshell mineralisation in the hens and might reduce the genotype dependent differences.

2. Materials and methods

The current study is a companion study of an interconnected research project dealing with the effects of phylogeny and performance divergence on the adaptability of layer lines (Jansen et al. 2020). The present study was performed at the experimental station of the Institute of Farm Animal Genetics, Friedrich Loeffler Institute (FLI), Federal Research Institute of Animal Health in Neustadt, Germany. The housing conditions of the animals and experimental procedures described in this study were in accordance with the guidelines of the German animal protection law and were approved by the Lower Saxony State Office for Consumer Protection and Food Safety, LAVES, Germany (registration number: AZ 33.19 -42502-04-15/1988).

2.1. Experimental diets and design

Laying hens of four genotypes (72 hens per genotype; 36 hens per feeding group) were supplemented either with 300 IU vitamin D_3 (c, recommendation according to GfE (1999)) or +, 3000 IU (the maximum permissible vitamin D_3 content according to EC No 1831/2003 (2003)) from week 18 of life (Supplemental Table S1). The four genotypes included two low performing genotypes (L68 and R11 hens) and two high performing genotypes (BLA and WLA hens) differing in their phylogeny (brown layer lines (L68 and BLA) vs. white layer lines (R11 and WLA)). The basal diets were formulated according to the requirements of the different performance periods and the recommendations of the National Research Council (NRC 1994) and the Society of Nutrition Physiology (GfE 1999) (Table 1). Feed and water were available *ad libitum* by feeding troughs and nipple drinkers. The hens were equipped with win-tags and reared in groups in conventional floor-range pens until starting the laying trial. Within this period the light and temperature program followed usual specifications of rearing layer chickens. During rearing birds were vaccinated against Marek´s and Newcastle Disease and Infectious Bronchitis.

2.1.1. Laying trial

After rearing at week 17, birds were placed in random order into a layer facility with a three-floor cage system including single cages (50 cm × 46 cm × 43 cm) containing a perch, a nipple drinker and a feeding trough. Cages contained no litter material. The birds were housed at a temperature of 18°C without artificial ultraviolet B light exposure. From week 17, the daily lighting period was increased by half an hour per week up to a constant illumination period of 14 h from week 23 on. The feed intake and the number of laid eggs were recorded weekly. In 4-week intervals the eggshell weight including inner egg membrane, eggshell thickness and strength were determined.

2.1.2. Balance trial

To study the apparent nutrient retention the laying hen feed was blended with Sipernat® (Evonik Industries, Essen, Germany) as indigestible marker. In week 29 and 49, the excrements of 8 animals per group were collected over three consecutive days and pooled per animal. The collected excrements were stored at −20°C until further analyses. On the last sampling day of both balance periods and at the end of the experiment (week 69) blood

Table 1. Composition of diets.

Item	Laying hen diet	
Vitamin D supplementation group*	c	+
Ingredients [%]		
Corn	20.0	
Soybean toasted	10.63	
Soybean meal, toasted	8.00	
High protein soybean meal, toasted	5.00	
Wheat	39.8	
Lucerne pellets	2.44	
Soybean oil	2.00	
Calcium phosphate	2.16	
Calcium carbonate	7.53	
Sodium chloride	0.29	
DL-methionine	0.15	
Premix[†]	1.00	
Sipernat[§]	1.00	
Supplement		
Vitamin D_3 [IU][‡]	300	3000
Calculated nutrients		
ME [MJ · kg^{-1}]	11.6	
Crude protein [g · kg^{-1}]	170	
Lysine [g · kg^{-1}]	8.80	
Methionine + Cysteine [g · kg^{-1}]	7.50	
Threonine [g ·kg^{-1}]	6.60	
Tryptophan [g · kg^{-1}]	2.00	
Calcium [g · kg^{-1}]	39.0	
Phosphorus [g · kg^{-1}]	7.50	

*c, control group, 300 IU vitamin D_3; +, maximum permissible vitamin D_3 supplementation according to EC No 1831/2003, 3000 IU vitamin D_3.
[†]Vitamin- mineral premix delivers per kg feed, starter, pullet and laying hen diet: 32, 40 and 40 mg iron, 12, 15 and 10 mg copper, 80, 80 and 80 mg zinc, 100, 80 and 100 mg manganese, 0.40, 0.32 and 0.25 mg selenium, 1.6, 1.6 and 1.2 mg iodine, 12,000, 10,000 and 10,000 IU vitamin A, 40, 25 and 20 mg tocopherol, 4.5, 3.0 and 4.0 mg menadione, 2.5, 2.5 and 2.5 mg thiamine, 8.0, 5.0 and 7.0 mg riboflavin, 6.0, 4.0 and 4.0 mg pyridoxine, 32, 18.5 and 20 μg cobalamin, 45, 30 and 40 mg nicotinic acid, 15, 9.0 and 10 mg pantothenic acid, 1.2, 0.8 and 0.6 mg folic acid, 50, 0.0 and 25 μg biotin, 550, 300 and 400 mg choline chloride acid.
[‡]Sipernat® 22 Hydrophilic Silcia (Evonik Industries, Essen, Germany).
[§]Vitamin D_3, product code: SLDF01 (NHU Europe GmbH, Bardowick, Germany).

samples of the observed hens were taken from *V. ulnaris s. basilica* and collected in conventional serum tubes.

During the sampling period in week 29 also the laid eggs from the 8 birds (corresponding to birds from which excrements were collected) were collected and the oviposition time was noted (time interval 1: before or at 7:30 am; 2: between 7.30 am and 12:00 pm, 3: after 12:00 pm). The weights of egg yolk, egg white, and eggshell including inner shell membrane were determined. The thickness and breaking strength of the eggshell were measured. The yolk colour was determined according to a Roche-fan (1-white to 15-intense orange). Thereafter, the yolk was frozen at −20°C until further analyses.

2.2. Analyses of feed and excreta

Feed and excreta samples were analysed according to methods of the Association of German Agricultural Analysis and Research Centres (VDLUFA). DM of feed and excreta

were analysed by methods of Naumann and Bassler (2006). Nitrogen was determined by the combustion-method according to Dumas (Naumann and Bassler 2006). Methods of VDLUFA 8.1 and 8.2 were used to analyse crude ash and HCL- insoluble ash, respectively. The mineral concentrations of calcium and phosphorus were analysed with optical emission spectrometry according to method number 10.8.2.

2.3. Vitamin D, calcium, phosphorus and alkaline phosphatase (ALP) analyses in blood

The determination of 25-OHD_3 in serum was conducted by high performance liquid chromatography with diode array detection (HPLC-DAD; Shimadzu, Kyoto, Japan). First, a solid phase extraction (Phree^{TM} Phospholipid Removal 1 ml tube, Phenomenex, Aschaffenburg, Germany) with acetonitrile/methanol was performed. After that, the combined extracts were evaporated at 40°C under nitrogen-flow, the dried residues were dissolved in methanol/distilled water (40/60) and filtered (syringe filters, 13 mm, 0.45 μm, PVDF, amchro GmbH) before HPLC injection. The HPLC parameters were as follows: oven temperature: 40°C, auto sampler temperature: 4°C, column: Synergi 4 μ Hydro-RP 80 A; 4 μm; 250 × 3.0 mm, gradient elution with acetonitrile (mobile phase A) und methanol/distilled water (40/60) (mobile phase B), DAD at 265 nm.

Serum concentrations of total calcium and inorganic phosphate were determined spectrophotometrically (Unicam UV/Vis Spectrometer UV4; Unicam, Kassel, Germany) using commercial kits of Greiner Diagnostic GmbH (Bahlingen, Germany). ALP activity in serum was measured by an automatic clinical chemistry analyser (Indiko plus, Thermo Fisher Scientific Oy, Vantaa, Finland).

2.4. Vitamin D analyses in egg yolk

Freeze-dried egg yolk samples were analysed for their vitamin D_3 and 25-OHD_3 content by high-performance liquid chromatography tandem-mass spectrometry (HPLC-MS /MS) as described by Kühn et al. (2019). In brief, samples were spiked with deuterated internal standards. After saponification under alkaline conditions, samples were extracted with *n*-hexane, washed with ultrapure water and subjected to normal phase HPLC (1100 Series, Agilent Technologies, Waldbronn, Germany) for further purification. Based on the specific retention times, vitamin D_3 and 25-OHD_3 were collected in separate fractions. Both fractions were derivatised with 4-phenyl-1,2,4-triazoline-3,5-dione and injected into the HPLC-MS/MS (1200 Series, Agilent Technologies; QTRAP 5500, Sciex, Darmstadt, Germany). Ionisation was achieved by positive electrospray. Data were recorded in the multiple-reaction monitoring mode with the following ion transitions: vitamin D_3: 560 > 298, deuterated vitamin D_3: 563 > 301, 25-OHD_3: 576 > 298, deuterated 25-OHD_3: 582 > 298. In case of 25-OHD_3 concentrations in egg yolk below the quantification limit (<16 ng/g DM), a concentration of half of the quantification limit was used for statistical calculations.

2.5. Calculations and statistical analyses

The beginning of the laying period and therefore the laying maturity were defined as the time point reaching a laying intensity of at least 50% per week for two consecutive weeks.

In contrast, the end of the laying period was defined as the time point when the laying intensity dropped below 50% per week for at least three consecutive weeks. If the laying intensity did not drop below the reference point before the end of the trial (week 69), an end of the laying period in week 70 was supposed. Five birds did never reach a laying intensity of at least 50% because of premature exclusion from the trial. They were not considered for the analyses of parameters in the laying period.

The nutrient retention was calculated by the following equation:

$$Apparent\ nutrient\ retention\ [\%] = 100 - \left(\left(\frac{HCl\ indigestible\ ash\ in\ feed\ \left[\frac{g}{kg\ DM}\right]}{HCl\ indigestible\ ash\ in\ excreta\ \left[\frac{g}{kg\ DM}\right]} \right) \times \left(\frac{Analyzed\ nutrient\ content\ in\ excreta\ \left[\frac{g}{kg\ DM}\right]}{Analyzed\ nutrient\ content\ in\ feed\ \left[\frac{g}{kg\ DM}\right]} \right) \times 100 \right)$$

The calcium and phosphorus intake was calculated according to analysed calcium and phosphorus concentrations in feed related to the real feed intake of each bird.

The feed conversion ratio is defined as the quotient between the daily feed intake (g) and the daily egg mass (g). The eggshell density was calculated by dividing the eggshell mass (g) by the eggshell thickness (mm).

The SAS Enterprise Guide 8.1 for Windows (SAS Institute Inc., Cary, NC) was used to evaluate the present results. The MIXED procedure was used to analyse the apparent nutrient retention, blood traits, traits of laying performance and eggshell traits measured during the laying trail. The model included the fixed effects "week of life", "genotype" (L68; R11; BLA; WLA) and "vitamin D supplementation" (300 IU vitamin D_3 vs. 3000 IU vitamin D_3). Repeated measurements of the birds were considered using the REPEATED statement. In case of blood traits, the SLICE statement of the MIXED procedure was used to conduct partitioned analyses for the LSMeans for interactions. In case of egg quality parameters and vitamin D content in egg yolk determined during the balance trail in week 29 as well as the analyses of the laying period or of the ELP ratio, the model of the MIXED procedure included the fixed effects "genotype" and "vitamin D supplementation". To analyse the effects of oviposition time on eggshell thickness or strength the time interval (1–4) of oviposition was also considered as a fixed factor. Least squares means (LSMeans) and their standard errors were calculated for each fixed effect, and all pairwise differences in LSMeans were tested with the Tukey-Kramer procedure. Effects and differences were defined as significant if $p < 0.05$. The SAS Enterprise Guide 6.1 was also used to estimate Pearson correlation coefficient; r. Correlations were assessed as significant at $p < 0.05$.

3. Results

Twenty-eight birds (L68c = 4; L68+ = 4; R22c = 2; R11+ = 2; BLAc = 3; BLA+ = 5; WLAc = 1; WLA+ = 7) died or were excluded from the experiment before the end of the trial because of clinical signs.

3.1. Feed analyses and apparent nutrient retention

The calcium content in laying hen feed was unintentionally higher in the diet containing 300 IU vitamin D_3 (50.41 g/kg DM) compared to the diet containing

Table 2. Apparent nutrient retention [%] depending on vitamin D supplementation in different genotypes (LSMeans, n = 8).

Week of life	Genotype	Vitamin D supplementation [IU]	Organic matter	Crude ash	Nitrogen	Phosphorus	Calcium
29	L68	300	88.0	74.2	72.1^{b}	65.5^{ab}	85.8
	R11	300	88.1	71.7	75.7^{ab}	63.7^{ab}	86.1
	BLA	300	87.1	74.5	75.5^{ab}	58.1^{b}	82.5
	WLA	300	88.7	77.3	77.4^{a}	69.2^{a}	86.8
	L68	3000	87.5	69.5^{B}	71.9^{B}	63.9	78.9
	R11	3000	88.3	68.9^{B}	76.2^{AB}	65.9	80.2
	BLA	3000	87.9	76.0^{A}	78.8^{A}	64.4	84.1
	WLA	3000	89.5	78.2^{A}	79.8^{A}	71.4	82.1
49	L68	300	89.3^{b}	71.3^{b}	72.0	83.9	77.7^{b}
	R11	300	89.9^{ab}	76.3^{ab}	73.9	84.1	82.8^{ab}
	BLA	300	90.6^{ab}	79.3^{a}	78.1	85.1	87.0^{a}
	WLA	300	91.6^{a}	81.5^{a}	80.1	87.2	89.3^{a}
	L68	3000	89.9	75.6	73.1	67.8***	82.7
	R11	3000	90.4	79.3	76.5	67.9***	88.0
	BLA	3000	90.6	79.6	79.5	68.3***	88.8
	WLA	3000	90.6	81.7	79.1	73.3***	90.1
SE†			0.42	1.28	1.36	1.61	1.67
p-Values							
Week			<0.001	<0.001	<0.001	<0.001	<0.001
Genotype			<0.001	<0.001	0.292	<0.001	0.004
Vitamin D			0.407	0.614	0.115	<0.001	0.635
Week x Genotype			0.130	0.012	0.471	0.087	0.010
Genotype x Vitamin D			0.828	0.938	0.818	0.432	0.484
Week x Vitamin D			0.489	0.010	0.685	<0.001	<0.001
Week x Genotype x Vitamin D			0.073	0.009	0.246	0.258	0.054

†SE, standard error; $^{ab;\ AB}$different superscripts mark significant differences between genotypes in the control group (lowercase letters) and the supplemented group (upper case letters) at $p < 0.05$; ***asterisks mark significant differences between groups differently supplemented with vitamin D_3 considering similar genotypes ($p < 0.001$).

3000 IU vitamin D_3 (44.12 g/kg DM). In contrast, phosphorus content was slightly higher in the diet containing 3000 IU vitamin D_3 (8.52 g/kg DM) compared to the diet containing lower amounts of vitamin D_3 (7.96 g/kg DM). Therefore, the calcium: phosphorus ratio was 6.35 for the 300 IU vitamin D_3 and 5.18 for the 3000 IU vitamin D_3.

In general, pooled LSMeans of organic matter, crude ash, nitrogen, phosphorus and calcium retention were lower at week 29 compared to week 49 of life ($p < 0.001$) (Table 2). The nitrogen retention was lower in L68c hens compared to WLAc hens in week 29 of life. Crude ash and nitrogen retention were lower in L68+ and R11+ hens compared to BLA+ and WLA+ in week 29 of life ($p < 0.05$) except for nitrogen retention of R11+ hens. The phosphorus retention was lower in BLAc hens compared to WLAc hens in week 29 of life ($p < 0.05$). In week 49 of life the organic matter, crude ash and calcium retention was lower in L68c hens compared to the high performing hens supplemented with 300 IU vitamin D_3 except for the organic matter of BLAc hens. In week 49, a 3000 IU vitamin D_3 supplementation decreased the phosphorus retention in all genotypes ($p < 0.001$).

3.2. Concentrations of 25-OHD$_3$, calcium and phosphorus in serum

The 25-OHD_3 concentrations in serum of control groups did not differ between genotypes ($p > 0.05$) (Figure 1(a)). The higher supplementation of vitamin D_3 increased 25-OHD_3 serum concentrations in all genotypes and at all sampling time points ($p < 0.001$),

except for white layer lines (R11; WLA) in week 49 showing only a numerical increase ($p > 0.05$). In week 49 and 69, the 25-OHD_3 concentrations in serum were higher in BLA+ compared to WLA+ hens ($p < 0.01$). In week 69, the 25-OHD_3 serum concentrations were also higher in the BLA+ group compared to the R11+ group ($p = 0.002$).

Serum concentrations of calcium and phosphorus were affected in an age dependent manner ($p < 0.05$), showing highest values in week 29 and lowest in week 49 (Figure 1(b,c)). In week 29, pooled LSMeans of serum calcium concentrations were lower in the group fed 3000 IU vitamin D_3 compared to pooled LSMeans of birds supplemented with 300 IU ($p = 0.045$). There were no differences between the layer lines at similar time points.

A significant feed-by-genotype-by-week interaction was detected in case of the serum activity of ALP ($p = 0.049$) (Figure 1(d)). In WLAc and BLAc hens the ALP activity was increased in week 29 compared to week 49 and 69 ($p < 0.001$). Furthermore, in week 29 WLAc hens showed higher serum ALP activity compared to R11c and L68c hens ($p < 0.05$). Supplementation of 3000 IU vitamin D_3 decreased the ALP activity in BLA hens compared to hens fed 300 IU in week 29 ($p = 0.018$). In week 49, WLA+ hens showed higher ALP activity compared to L68+ hens ($p = 0.015$).

There was a negative correlation between phosphorus retention and serum 25-OHD_3 concentrations ($r = -0.651$; $p < 0.001$).

3.3. Vitamin D_3 and 25-OHD_3 concentrations in egg yolk

The vitamin D_3 and 25-OHD_3 content in egg yolk was similar in all control groups but tended to be slightly higher in the BLAc group compared to the WLAc group ($p = 0.058$) (Figure 2(a,b)). The higher vitamin D_3 supplementation increased the vitamin D_3 and 25-OHD_3 content in the egg yolk of all genotypes ($p < 0.05$). The BLA+ group showed significantly higher vitamin D_3 content in egg yolk compared to the other genotypes with similar vitamin D supplementation ($p < 0.05$). The 25-OHD_3 content in egg yolk was also higher in the BLA+ group compared to the R11+ and the WLA+ group ($p < 0.05$).

3.4. Performance

While the laying maturity was reached earlier in the high performing genotypes compared to the low performing genotypes ($p < 0.001$) showing also a difference between latter ones ($p < 0.001$) (Figure 3(a)), the end of the laying period was similar between all genotypes. In turn, the laying period of high performing genotypes lasted longer than that of low performing birds ($p < 0.01$) (Figure 3(b)). The vitamin D_3 supplementation had no influence on the beginning, end or duration of the laying period ($p > 0.05$). There was a significant week-by-genotype-by-vitamin D_3-interaction in case of egg production rate ($p < 0.05$) (Figure 3(c)). In all genotypes the egg production rate increased until week 28. While the production rate remained on a high level in BLA and WLA hens irrespectively of the vitamin D supplementation the egg production rate of low performing hens seemed to decrease steadily after week 32 except for R11 hens receiving 3000 IU vitamin D_3. These hens show no difference in the egg production rate compared to BLA+ hens until week 64 of life.

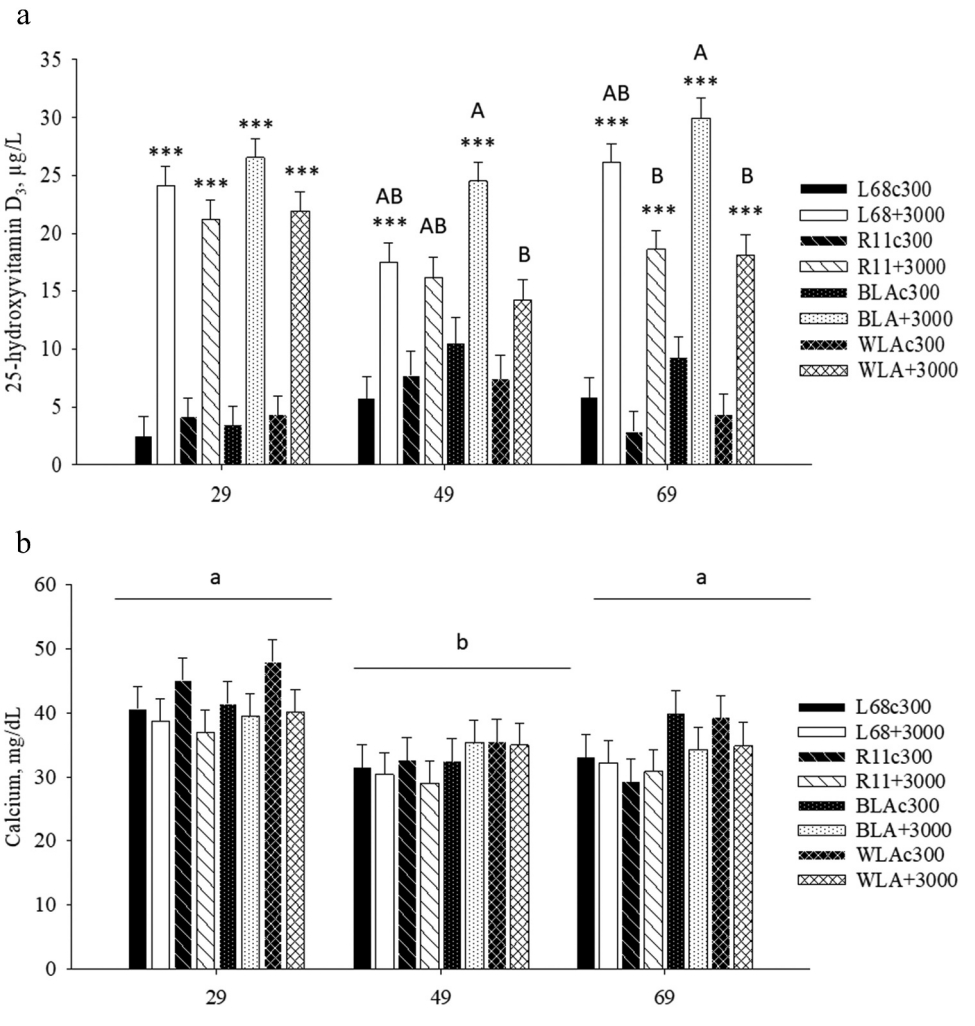

Figure 1. Serum parameters depending on vitamin D3 supplementation (c, 300 IU; +, 3000 IU) in different laying hen genotypes. (a) 25-hydroxyvintamin D_3 ***Asterisks mark significant differences between feeding groups of similar genotypes ($p < 0.001$). a-c; A-C different superscripts mark significant differences between genotypes in the control group (lowercase letters) and the supplemented group (upper case letters) at $p < 0.05$. $p_{week} = 0.205$; $p_{genotype} < 0.001$; $p_{vitamin\ D} < 0.001$; $p_{week\ x\ genotype} = 0.007$; $p_{genotype\ x\ vitamin\ D} < 0.001$; $p_{week\ x\ vitamin\ D} = 0.026$; $p_{week\ x\ genotype\ x\ vitamin\ D} = 0.995$; (a) calcium ^{ab} different superscripts mark significant differences between weeks ($p < 0.05$). $p_{week} < 0.001$; $p_{genotype} = 0.148$; $p_{vitamin\ D} = 0.119$; $p_{week\ x\ genotype} = 0.799$; $p_{genotype\ x\ vitamin\ D} = 0.910$; $p_{week\ x\ vitamin\ D} = 0.376$; $p_{week\ x\ genotype\ x\ vitamin\ D} = 0.699$; (c) phosphorus, $p_{week} = 0.035$; $p_{genotype} = 0.763$; $p_{vitamin\ D} = 0.743$; $p_{week\ x\ genotype} = 0.589$; $p_{genotype\ x\ vitamin\ D} = 0.917$; $p_{week\ x\ vitamin\ D} = 0.228$; $p_{week\ x\ genotype\ x\ vitamin\ D} = 0.674$; (d) alkaline phosphatase, ***Asterisks mark significant differences between feeding groups of similar genotypes ($p < 0.001$). a-c; A-C different superscripts mark significant differences between genotypes in the control group (lowercase letters) and the supplemented group (upper case letters) at $p < 0.05$. $P_{week} < 0.001$; $p_{genotype} < 0.001$; $p_{vitamin\ D} = 0.910$; $p_{week\ x\ genotype} = 0.004$; $p_{genotype\ x\ vitamin\ D} = 0.151$; $p_{week\ x\ vitamin\ D} < 0.001$; $p_{week\ x\ genotype\ x\ vitamin\ D} = 0.049$.

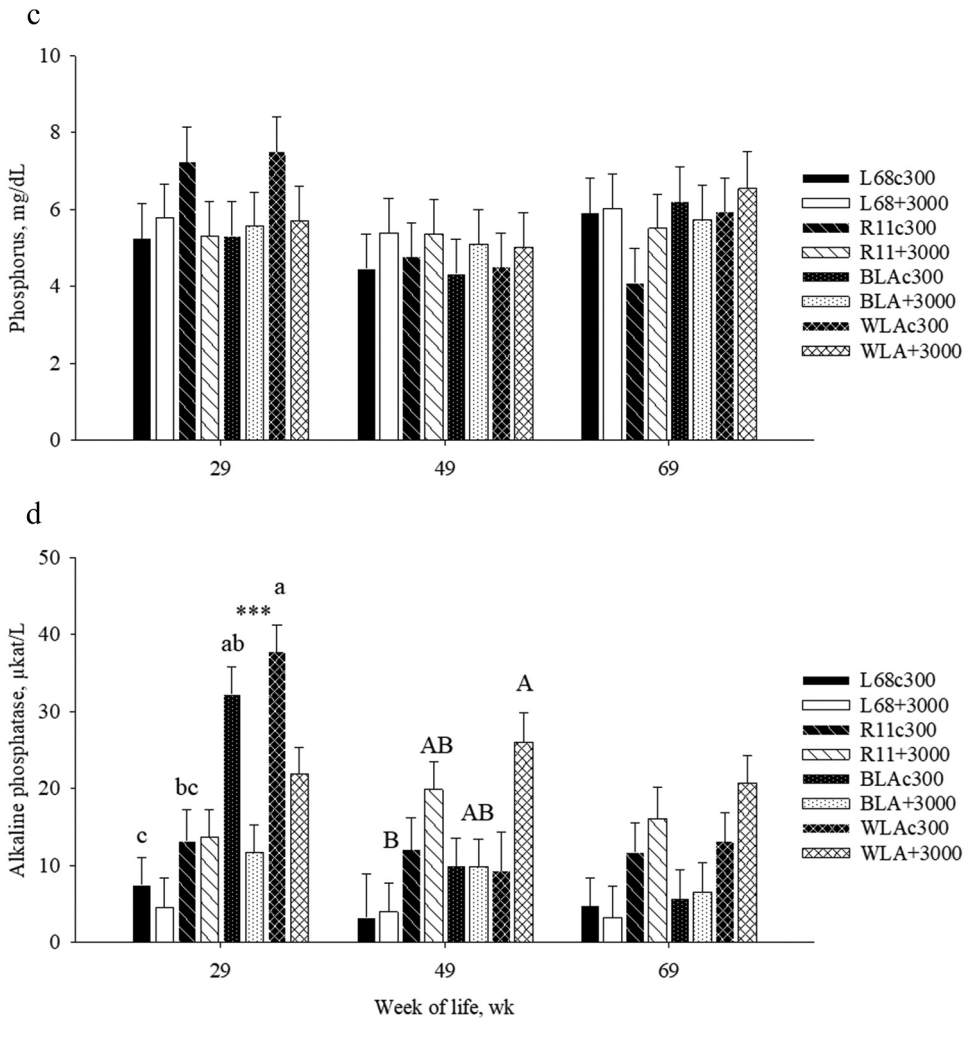

Figure 1. (Continued).

The feed intake increased from the beginning of the trial in all genotypes and lastly reached a plateau in R11 hens at week 28 of life (Figure 4(a)). The feed intake of BLA hens increased markedly until week 24 of life ($p < 0.05$) and was higher compared to all other birds from week 22 until week 44 of life ($p < 0.05$). Thereafter, the feed intake of the different genotypes aligned except for the feed intake of R11 hens. A markedly increase of the feed intake in R11 hens was observed at first from week 20 to 22 of life ($p < 0.05$). From week 18 until week 26 of life these hens consumed less feed compared to the other genotypes. Thereafter, the feed intake became closer to the feed intake of L68 and WLA hens but remained significantly lower compared to BLA hens until the end of the trial ($p < 0.05$).

Considering the whole experimental period the pooled LSMeans of calcium intake of all genotypes fed 3000 IU vitamin D_3 were lower compared to the 300 IU vitamin D_3 supplemented hens ($p < 0.05$) (Figure 5(a)). The phosphorus intake was affected in

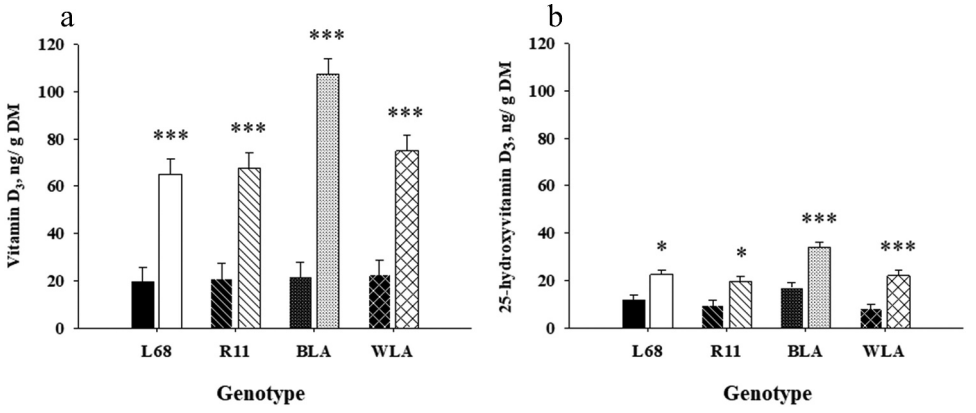

Figure 2. Vitamin D_3 (a) and 25-hydroxyvitamin D_3 (b) in egg yolk collected in week 29 of life depending on vitamin D3 supplementation (■ - c, 300 IU; □ - +, 3000 IU) in different laying hen genotypes. DM, dry matter. * Asterisks mark significant differences between feeding groups of similar genotypes (*$p < 0.05$; ***$p < 0.001$). (a) $p_{genotype} = 0.005$; $p_{vitamin\ D} < 0.001$; $p_{genotype\ x\ vitamin\ D} = 0.009$; (b) $p_{genotype} < 0.001$; $p_{vitamin\ D} < 0.001$; $p_{genotype\ x\ vitamin\ D} = 0.334$.

an opposite manner ($p < 0.05$) except for WLA hens, which showed no significant difference between differently supplemented hens (Figure 5(b)). In week 48, the phosphorus intake was higher in L68c hens compared to L68+ hens ($p = 0.029$). The daily calcium intake and the phosphorus retention tended to correlate with each other at week 49 ($r = 0.242$; $p = 0.056$). Furthermore, a negative correlation between the daily phosphorus intake and the phosphorus retention was detected ($r = -0.364$; $p = 0.003$).

Egg weight increased over time in all genotypes except for the egg mass of R11 hens, which seemed to decrease from week 22 to week 24 of life (Figure 4(b)). However, this effect was mainly based on the low number of laid eggs and was not significant ($p > 0.05$). Until week 40, the eggs of low performing hens were lighter compared to high performing hens ($p < 0.05$). Thereafter, the egg weight of L68 hens became closer to the egg weight of the high performing hens but the egg weight of R11 hens remained significantly lower compared to the high performing hens ($p < 0.05$). From week 54 of life, the egg weight of R11 hens was even lower compared to L68 hens ($p < 0.05$). From week 32 to week 50, the eggs of WLA hens were lighter than eggs of BLA hens ($p < 0.05$).

Initially, in all genotypes a marked increase of the daily egg mass was observed, however the high performing hens reached a plateau faster compared to low performing hens (Figure 4(c)). During the whole observation period, low performing animals showed a significantly lower daily egg mass compared to high performing hens ($p < 0.05$). Furthermore, L68 hens reached faster the plateau than R11 hens. In contrast to high performing hens the daily egg mass began to decrease from week 40 on resulting in a significantly lower daily egg mass in week 68 of life than determined in week 40 ($p < 0.001$). Considering the whole experimental period the daily egg mass of R11 hens was significantly increased by an additional vitamin D_3 supplementation (pooled LSMeans: R11c = 30.7 ± 0.4 g/d; R11+ = 33.5 ± 0.4 g/d; $p < 0.001$) and tended to be decreased in BLA hens (BLAc = 53.1 ± 0.4; BLA+ = 51.6 ± 0.04; $p = 0.064$).

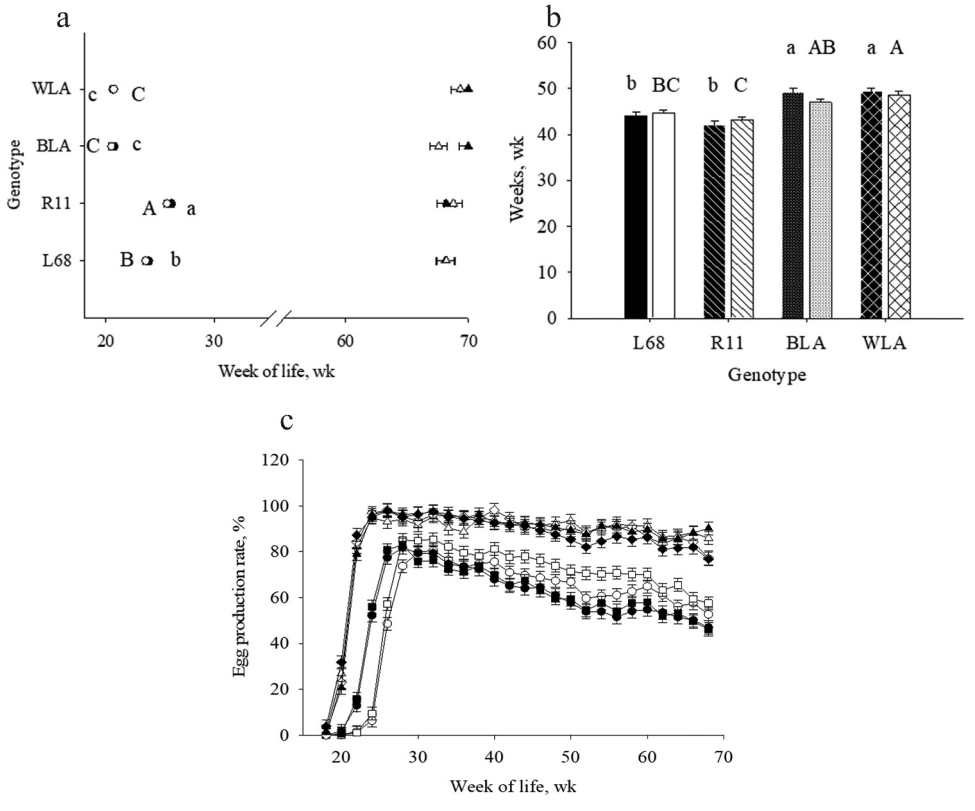

Figure 3. Laying period and egg production rate depending on vitamin D_3 supplementation in different genotypes (LSMeans ± SE, $n = 36$), (a) Laying maturity depending on genotype and vitamin D_3 supplementation (black–300 IU; white–3000 IU), (●, ○ - laying intensity of at least 50%); $p_{genotype} < 0.001$; $p_{vitamin\ D} = 0.144$; $p_{genotype\ x\ vitamin\ D} = 0.677$ and end of laying period (▲, Δ - laying intensity < 50% for at least three consecutive weeks after reaching the laying maturation); $p_{genotype} = 0.193$; $p_{vitamin\ D} = 0.243$; $p_{genotype\ x\ vitamin\ D} = 0.181$. $^{a\text{-}c;\ A\text{-}C}$ Different superscripts mark significant differences between genotypes in the control group (lowercase letters) and the additionally supplemented group (upper case letters) at $p < 0.05$. (b) Duration of the laying period depending on genotype and vitamin D supplementation (black–300 IU; white–3000 IU); $p_{genotype} < 0.001$; $p_{vitamin\ D} = 0.547$; $p_{genotype\ x\ vitamin\ D} = 0.135$. $^{a\text{-}c;\ A\text{-}C}$ Different superscripts mark significant differences between genotypes in the control group (lowercase letters) and the additionally supplemented group (upper case letters) at $p < 0.05$. (c) egg production rate depending on different genotypes and vitamin D supplementation (● - L68c (c, 300 IU); ■ - L68+ (+, 3000 IU); ○ - R11c; □ - R11+; ▲ - BLAc; ♦ - BLA+; Δ - WLAc; ◊ - WLA+); $p_{week} < 0.001$; $p_{genotype} < 0.001$; $p_{vitamin\ D} = 0.454$; $p_{week\ x\ genotype} < 0.001$; $p_{genotype\ x\ vitamin\ D} = 0.068$; $p_{week\ x\ vitamin\ D} = 0.045$; $p_{week\ x\ genotype\ x\ vitamin\ D} = 0.026$.

The feed conversion ratio decreased initially in all hens and became closer from week 28 on until the ratios began to differ once again between genotypes (Figure 4(d)). From week 42 on, the feed conversion ratio slightly increased in L68 hens, which in turn resulted in a higher ratio compared to the high performing hens ($p < 0.05$). In week 54 of life, also the R11 hens showed a higher feed conversion ratio compared to the high performing hens and at week 62 and 64 compared to WLA hens ($p < 0.05$). While the 3000 IU vitamin D_3 supplementation increased the feed conversion ratio in L68 hens at week 20 of life (L68c = 7.9 ± 0.6; L68+ = 17.1 ± 1.0 g/g; $p < 0.001$), in BLA hens the feed

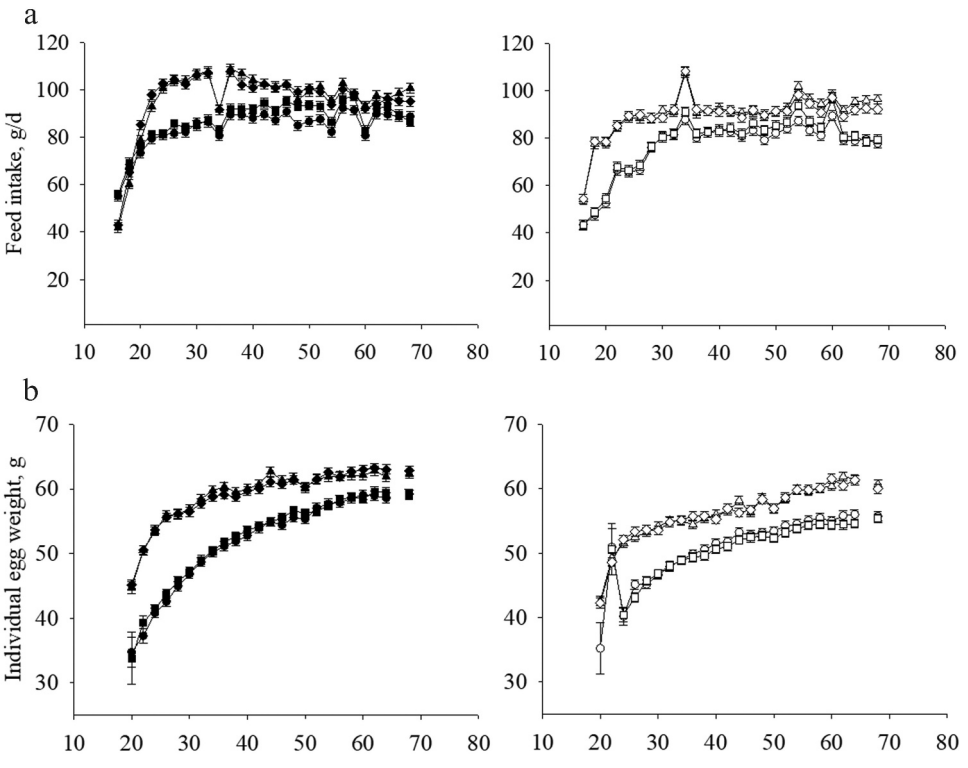

Figure 4. Feed intake (a), individual egg weight (b), daily egg mass (c) and feed conversion ratio (d) depending on different genotypes and vitamin D supplementation (● - L68c (c, 300 IU); ■ - L68+ (+, 3000 IU); ○ - R11c; □ - R11+; ▲ - BLAc; ◆ - BLA+; Δ - WLAc; ◊ - WLA+) (LSMeans ± SE, n = 72). (a) $p_{week} < 0.001$; $p_{genotype} < 0.001$; $p_{vitamin\ D} = 0.483$; $p_{week\ x\ genotype} < 0.001$; $p_{genotype\ x\ vitamin\ D} = 0.645$; $p_{week\ x\ vitamin\ D} < 0.001$; $p_{week\ x\ genotype\ x\ vitamin\ D} = 0.519$. (b) $p_{week} < 0.001$; $p_{genotype} < 0.001$; $p_{vitamin\ D} = 0.910$; $p_{week\ x\ genotype} = 0.004$; $p_{genotype\ x\ vitamin\ D} = 0.151$; $p_{week\ x\ vitamin\ D} < 0.001$; $p_{week\ x\ genotype\ x\ vitamin\ D} = 0.049$. (c) $p_{week} < 0.001$; $p_{genotype} < 0.001$; $p_{vitamin\ D} = 0.483$; $p_{week\ x\ genotype} < 0.001$; $p_{genotype\ x\ vitamin\ D} = 0.645$; $p_{week\ x\ vitamin\ D} < 0.001$; $p_{week\ x\ genotype\ x\ vitamin\ D} = 0.519$. (d) $p_{week} < 0.001$; $p_{genotype} < 0.001$; $p_{vitamin\ D} = 0.374$; $p_{week\ x\ genotype} < 0.001$; $p_{genotype\ x\ vitamin\ D} = 0.002$; $p_{week\ x\ vitamin\ D} < 0.001$; $p_{week\ x\ genotype\ x\ vitamin\ D} < 0.001$.

conversion ratio was decreased by the higher vitamin D_3 supplementation (BLAc = 5.7 ± 3.9; BLA+ = 3.9 ± 0.2; $p < 0.001$). In week 24 of life, R11+ showed a higher feed conversion ratio compared to R11c hens (R11c = 5.0 ± 0.4; R11+ = 7.2 ± 0.3; $p = 0.031$). No further differences were observed between hens with and without additional vitamin D supplementation at specific time points but L68+ hens showed a significantly higher feed conversion ratio compared to L68c hens considering the whole experimental period (L68c = 3.2 ± 0.1; L68+ = 3.6 ± 0.1; $p = 0.008$).

3.5. Egg quality laying trial

Initially, the eggshell weight increased slightly. At each observed time point the eggshells were lighter in low performing hens compared to high performing hens irrespective of the vitamin D supplementation ($p < 0.05$) (Figure 6(a)).

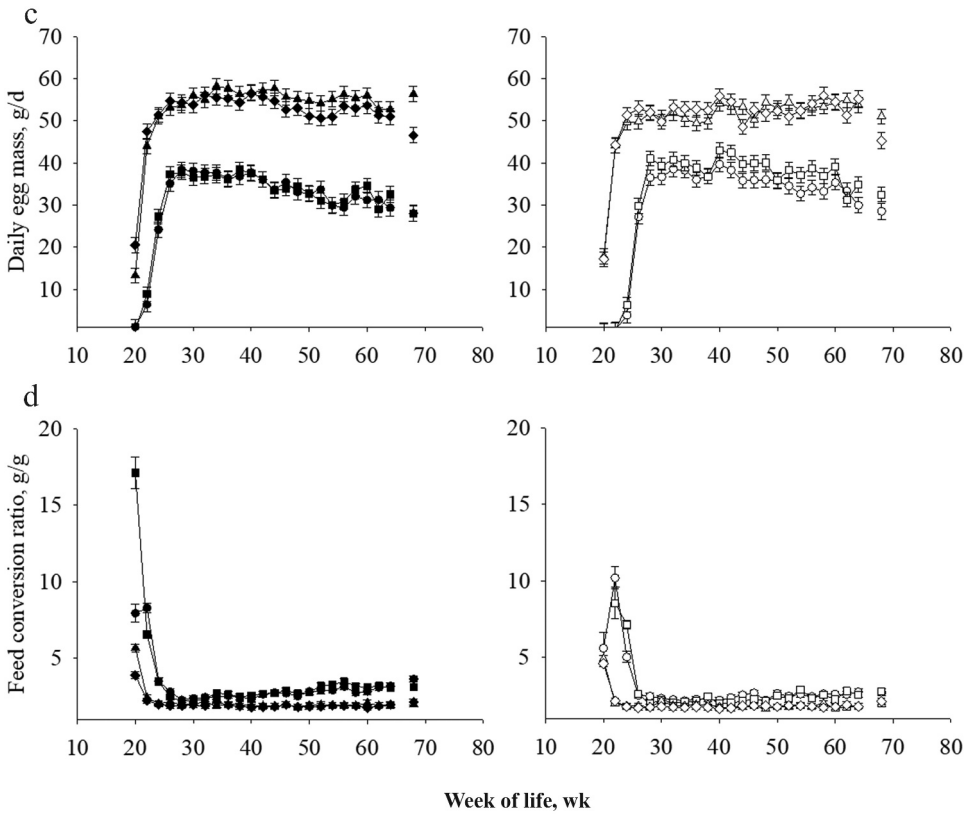

Figure 4. (Continued).

A significant week-by-genotype-by-vitamin D interaction was observed in case of eggshell thickness ($p = 0.015$) (Figure 6(b)). While the eggshell thickness in WLA hens was higher compared to the low performing hens from the beginning of the experiment ($p < 0.05$) except for WLA+ hens in week 52, the eggshell thickness of BLA hens increased at first from week 44 of life. The eggshell thickness of L68c remained on a low level during the whole experimental period. In contrast, the thickness in R11c and R11+ hens increased slightly from week 36 until week 52 ($p < 0.05$).

The breaking strength of eggshell decreased over the experimental period especially in WLA hens which initially showed significantly higher breaking strengths compared to the other hens ($p < 0.05$) and became closer to them over the experimental period (Figure 6(c)). Pooled LSMeans of the breaking strength of eggs were higher in genotypes that received 3000 IU vitamin D_3 supplementation in week 36 of life ($p = 0.020$). Furthermore, considering all genotypes and the whole experimental period the pooled LSMeans of breaking strength of hens fed 3000 vitamin D_3 were higher compared to hens with lower vitamin D supplementation ($p = 0.044$).

The eggshell density increased from week 28 until week 44 in all hens ($p < 0.001$) (Figure 6(d)). At this period the eggshell density was higher in BLAc and BLA+ hens compared to the low performing hens ($p < 0.05$) except for the eggshell density in WLA+ hens in week 44. WLA+ hens showed higher eggshell density compared to low

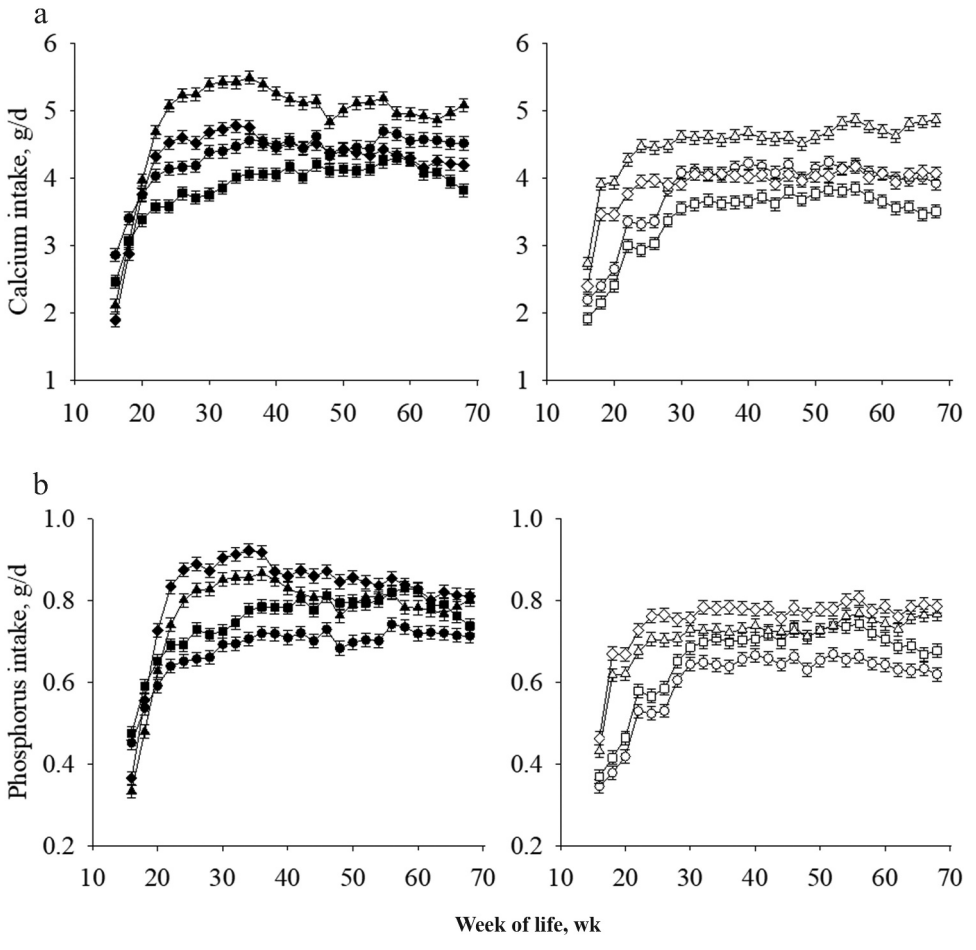

Figure 5. Calcium (a) and phosphorus (b) intake depending on different genotypes and vitamin D supplementation (● - L68c (c, 300 IU); ■ - L68+ (+, 3000 IU); ○ - R11c; □ - R11+; ▲ - BLAc; ◆ - BLA+; Δ - WLAc; ◊ - WLA+) (LSMeans ± SE, $n = 36$). (a) $p_{week} < 0.001$; $p_{genotype} < 0.001$; $p_{vitamin\ D} < 0.001$; $p_{week\ x\ genotype} < 0.001$; $p_{genotype\ x\ vitamin\ D} = 0.265$; $p_{week\ x\ vitamin\ D} < 0.001$; $p_{week\ x\ genotype\ x\ vitamin\ D} = 0.352$. (B) $p_{week} < 0.001$; $p_{genotype} < 0.001$; $p_{vitamin\ D} < 0.001$; $p_{week\ x\ genotype} < 0.001$; $p_{genotype\ x\ vitamin\ D} = 0.766$; $p_{week\ x\ vitamin\ D} < 0.001$; $p_{week\ x\ genotype\ x\ vitamin\ D} = 0.431$.

performing hens in week 60 ($p < 0.05$). Additionally, BLA+ hens showed higher eggshell density compared to R11+ hens in this week ($p < 0.05$).

There was a positive correlation between the breaking strength and the weight ($r = 0.251$; $p < 0.001$) as well as the thickness ($r = 0.414$; $p < 0.001$) of the eggshell.

3.6. Egg quality balance trial

During balance trial in week 29, there was no effect of oviposition time on eggshell thickness or eggshell breaking strength ($p > 0.05$) (Supplemental Table 2).

The proportions of eggshell, egg white and egg yolk differed between the genotypes ($p < 0.05$) but not between the two feeding groups but the higher vitamin

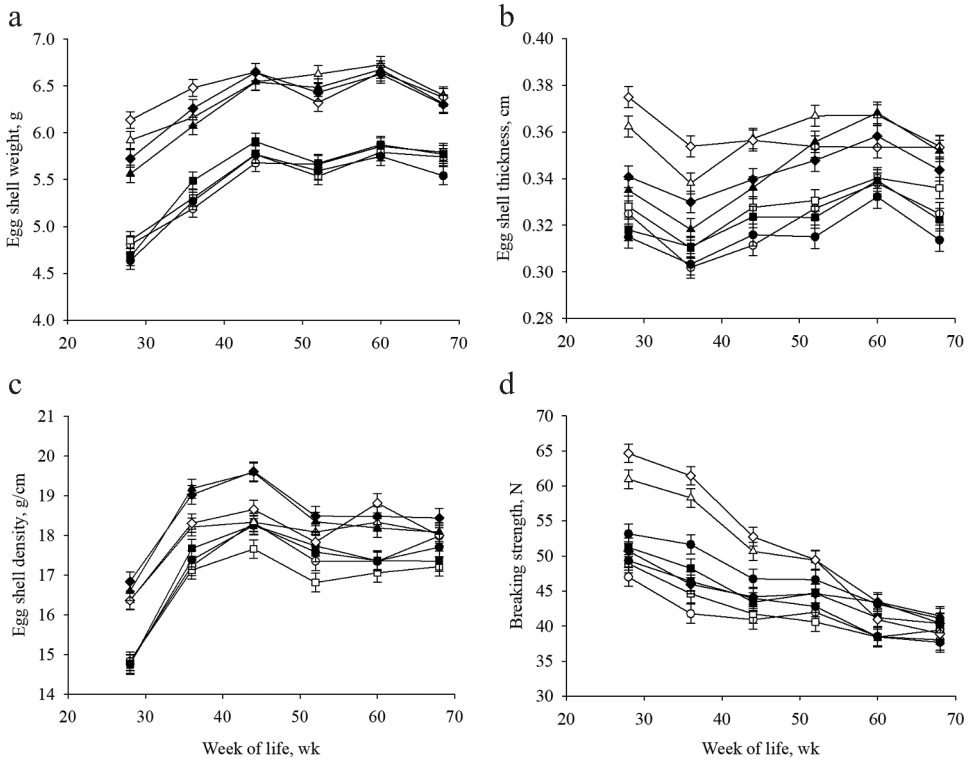

Figure 6. Eggshell weight (a), eggshell thickness (c), eggshell density (c) and breaking strength of eggshell (d) depending on different genotypes and vitamin D supplementation (● - L68c (c, 300 IU); ■ - L68+ (+, 3000 IU); ○ - R11c; □ - R11+; ▲ - BLAc; ◆ - BLA+; Δ - WLAc; ◊ - WLA+) (LSMeans ± SE, n = 36). (a) $p_{week} < 0.001$; $p_{genotype} < 0.001$; $p_{vitamin\ D} = 0.258$; $p_{week\ x\ genotype} < 0.001$; $p_{genotype\ x\ vitamin\ D} = 0.818$; $p_{week\ x\ vitamin\ D} < 0.001$; $p_{week\ x\ genotype\ x\ vitamin\ D} = 0.293$. (b) $p_{week} < 0.001$; $p_{genotype} < 0.001$; $p_{vitamin\ D} = 0.147$; $p_{week\ x\ genotype} < 0.001$; $p_{genotype\ x\ vitamin\ D} = 0.448$; $p_{week\ x\ vitamin\ D} < 0.001$; $p_{week\ x\ genotype\ x\ vitamin\ D} = 0.015$. (c) $p_{week} < 0.001$; $p_{genotype} < 0.001$; $p_{vitamin\ D} = 0.496$; $p_{week\ x\ genotype} < 0.001$; $p_{genotype\ x\ vitamin\ D} = 0.022$; $p_{week\ x\ vitamin\ D} = 0.658$; $p_{week\ x\ genotype\ x\ vitamin\ D} = 0.936$. (d) $p_{week} < 0.001$; $p_{genotype} < 0.001$; $p_{vitamin\ D} = 0.044$; $p_{week\ x\ genotype} < 0.001$; $p_{genotype\ x\ vitamin\ D} = 0.842$; $p_{week\ x\ vitamin\ D} = 0.009$; $p_{week\ x\ genotype\ x\ vitamin\ D} = 0.230$.

D supplementation increased the eggshell weight ($p = 0.044$) (Table 3). The pooled LSMeans of eggshell proportion were significantly increased in R11 and WLA hens compared to BLA hens ($p < 0.05$). The pooled LSMeans of eggshell thickness were lower in R11 and BLA hens compared to L68 and WLA hens ($p < 0.05$). The pooled LSMeans of the breaking strength in WLA hens were higher compared to R11 and BLA hens and tended to be higher compared to L68 hens ($p = 0.058$). In week 29 of life, the breaking strength of eggshell tended to be increased by a vitamin D_3 supplementation of 3000 IU ($p = 0.052$).

A positive correlation between the eggshell proportion and the serum calcium (r = 0.365, $p = 0.003$) and phosphorus concentrations ($r = 0.356$, $p = 0.004$) were detected.

The pooled LSMeans of egg white proportion were significantly higher and LSMeans of egg yolk proportions were lower in BLA hens compared to the other genotypes ($p <$ 0.001). The colour of egg yolk was more intensive in L68 hens compared to the other

Table 3. Egg quality traits depending on vitamin D supplementation in 29 week old hens of different genotypes (LSMeans, n = 8).

			Egg shell				Egg white		Egg yolk		
Genotype	Vitamin D supplementation*	Total egg weight [g]	Weight[g]	Percentage of total egg [%]	Thickness [cm]	Breaking strength [N]	Weight[g]	Percentage of total egg [%]	Weight[g]	Percentage of total egg [%]	Color
L68	300	45.8^{a}	5.6^{c}	12.1	0.36^{ab}	40.2	26.7^{b}	58.3^{c}	13.5^{ab}	29.5^{a}	13.3
R11	300	45.1^{a}	5.7^{bc}	12.6	0.31^{c}	36.4	26.5^{b}	58.9^{c}	12.9^{b}	28.4^{ab}	12.9
BLA	300	56.4^{b}	6.4^{ab}	11.3	0.32^{bc}	37.8	36.6^{a}	65.0^{a}	13.4^{ab}	23.7^{b}	13.0
WLA	300	54.0^{b}	6.9^{a}	12.8	0.37^{a}	44.6	31.9^{b}	58.9^{b}	15.2^{a}	28.3^{ab}	13.0
L68	3000	46.3^{A}	5.9^{C}	12.7	0.36^{A}	42.3^{AB}	27.6^{B}	59.5^{C}	12.9^{B}	27.8^{A}	13.6^{A}
R11	3000	46.5^{A}	5.9^{BC}	12.7	0.31^{B}	37.3^{B}	28.0^{AB}	60.1^{BC}	12.7^{B}	27.3^{AB}	12.9^{AB}
BLA	3000	59.1^{B}	7.1^{A}	12.1	0.33^{AB}	42.7^{AB}	37.8^{A}	63.7^{A}	14.3^{AB}	24.3^{B}	12.8^{B}
WLA	3000	54.3^{B}	6.9^{B}	12.7	0.36^{A}	50.8^{A}	32.5^{B}	59.9^{B}	14.9^{A}	27.4^{AB}	12.8^{B}
SE†		1.4	0.22	0.35	0.010	2.5	1.06	0.78	0.44	0.72	0.19
p-Values											
Genotype		<0.001	<0.001	0.010	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	0.008
Vitamin D		0.211	0.044	0.181	1.000	0.052	0.273	0.367	0.779	0.117	0.817
Genotype x Vitamin D		0.817	0.438	0.601	0.707	0.697	0.996	0.338	0.363	0.457	0.314

*c, vitamin D_3 supplementation 300 IU; +, vitamin D_3 supplementation 3000 IU; †SE, standard error; $^{a\text{-}c;\ A\text{-}C}$different superscripts mark significant differences between genotypes in the control group (lowercase letters) and the supplemented group (upper case letters) at $p < 0.05$.

genotypes ($p < 0.05$). No effect of the vitamin D supplementation on the yolk colour was observed ($p > 0.05$).

4. Discussion

Given the specific role of vitamin D for calcium homoeostasis of laying hens with egg(shell) output as the major calcium sink, it was hypothesised that the nutritional-physiological requirement of 300 IU vitamin D_3 as recommended by GfE (1999) does not fit to the requirements of modern laying hybrids compared to hybrids with a genetically lower laying performance potential. The present study was performed to investigate whether an additional vitamin D_3 supplementation might have beneficial effects on the laying performance and egg quality of hens and therefore might support their genetic performing potential.

BLA and WLA hens belonging to the high performing layer lines reached their laying maturity earlier compared to L68 and R11 hens belonging to the low performing layer lines. This aspect resulted in a longer duration of laying period. The laying period did not appear to be affected by the vitamin D_3 supplementation. However, it has to be emphasised, that only approximately 23% of all birds reached the actual end (laying intensity dropped below 50% per week for at least three consecutive weeks) of the laying period before the 70th week of life (end of the experimental trial). It cannot be ruled out that different completions and therefore different durations of the laying period between birds might become significant if all animals were observed until the actual end of the laying period.

Vitamin D_3 is involved in the transcellular and paracellular intestinal absorption of calcium (Adedokun and Adeola 2013). Adhikari et al. (2020) showed beneficial effects of high dietary vitamin D_3 concentrations on calcium digestibility. In the present study, no effect of the vitamin D_3 supplementation on the calcium retention was found; however, Adhikari et al. (2020) used concentrations which were double and three times higher than those used in the present study. The slight differences in calcium and phosphorus content between the laying-hen diets resulted in a lower calcium: phosphorus ratio and a lower calcium but a higher phosphorus intake over the whole laying period in hens consuming the diet containing 3000 IU vitamin D_3. In studies of Kebreab et al. (2009) it was shown, that increased dietary calcium concentrations decreased the calcium retention in the body and egg, whereas phosphorus retentions increased. Especially in week 49, the lower calcium and higher phosphorus intake might be the explanation for the lower phosphorus retention in hens consuming the 3000 IU vitamin D_3 diet. This assumption was supported by the correlations between the intake of both major elements and the phosphorus retention in this week. Rodehutscord et al. (2002) traced a reduced phosphorus utilisation and net absorption in consequence of a higher calcium supplementation to a reduced phytate hydrolysis. Adedokun and Adeola (2013) explained that high dietary calcium increased the pH of the gastrointestinal content, which adversely affects the phytase efficacy. Consequently, the effects of the vitamin D_3 supplementation on phosphorus retention might have been influenced by the diet formulation and were not only direct vitamin D_3 effects, because vitamin D_3 is normally to a high extent involved in the activation of genes, which are responsible for the phosphorus transport in the intestine (Hattenhauer et al. 1999; Adedokun and Adeola 2013). It cannot be fully

explained why this effect was not detected in week 29 of the present trial; however it is assumed that this might be based on the changes in the laying performance. Nevertheless, a lower phosphorus retention contributes to a higher environmental pollution which might also be considered with regard to the selection of the layer line. Especially at the beginning of the laying period in week 29, a higher phosphorus retention was shown in WLA hens compared to BLA hens which might be based on the differences in the calcium and phosphorus metabolism between the layer lines. Also, Adedokun and Adeola (2013) emphasised the influences of age, sex, productions and genetics on the metabolism of these minerals. A compensatory effect of vitamin D supplementation on these impact factors cannot be supported by the present results.

In the present study, it was clearly demonstrated that a higher vitamin D_3 supplementation leads to the 25-OHD_3 enrichment in serum of all used layer lines. Already Soares et al. (1995) reported correlations between the dietary vitamin D intake and the 25-OHD_3 concentrations in blood. In week 49, especially in white layer lines the 25-OHD_3 enrichment in serum was smaller than in the other analysed weeks. The 25-OHD_3 is the major vitamin D_3 form in the circulation (Soares et al. 1995; de Matos 2008). A decrease of this form might indicate that vitamin D_3 is converted to a higher extent to the most metabolically active form 1, 25-dihydroxy vitamin D_3 or to 24, 25-dihydroxy vitamin D_3. Both were; however, not measured in the present study. An up-regulation of the 1, 25-dihydroxy vitamin D_3 would be supported for example in case of hypocalcaemia but in case of high calcium and phosphorus concentrations, the major hydroxylation product is 24, 25-hydroxy vitamin D_3 (de Matos 2008; Proszkowiec-Weglarz and Angel 2013). Indeed it might be possible that lower calcium intake of hens fed 3000 IU vitamin D_3 provoked hypocalcaemia. Hypocalcaemia is also associated with an increased urinary phosphorus excretion, which is important for the maintenance of the blood phosphorus balance (Soares et al. 1995). Therefore, hypocalcaemia might also be a further explanation for the lower phosphorus retention in white layer lines, but not in brown layer lines which showed no reduced enrichment of 25-OHD_3 serum concentrations after the 3000 IU vitamin D_3 supplementation. The correlations between the phosphorus retention and the blood 25-OHD_3 concentrations in week 49 supported possible interrelations between both parameters. Presented calcium plasma levels were higher compared to reference values (3.3–5.9 mmol/l ≈ 13.2–23.6 mg/dl) for domestic fowls (*Gallus gallus*) described by Moritz (2014). Therefore, there were no indications of hypocalcaemia. Nevertheless, it is possible that hens suffered from calcium deficiency. As summarised by de Matos (2008) a high or low calcium: phosphorus ratio increases the requirements for vitamin D_3 in birds. According to the present results of the feed analyses the calcium: phosphorus ratio was lower in the 3000 IU vitamin D_3 group compared to the group with lower vitamin D_3 content. This might also have evoked a higher vitamin D_3 requirement in these birds especially in the middle of the laying period being characterised by a high laying performance and as indicated by a high eggshell density and weight also by a high eggshell mineralisation.

Hypovitaminosis of vitamin D_3 can also provoke signs of a calcium deficiency in birds (de Matos 2008). In the present trial; however, there were no adverse effects on calcium retention in week 49, which would have been expected in this case. Nevertheless, the blood 25-OHD_3 concentrations of control groups of the present study were below reference ranges of domestic chicken reported by de Matos (2008). Especially, in week

29 the vitamin D values of all control hens were extremely low while the calcium levels were increased. Elevated activity of ALP might indicate a calcium deficiency in this period especially in WLAc and BLAc hens. de Matos (2008) described that in case of calcium deficiencies, generally ALP is elevated and calcium concentrations are normal or elevated based on the higher activity of the parathyroid hormone. In week 29, the vitamin D_3 supplementation seemed to decrease the ALP activity in the high performing layer lines although the difference was not significant in WLA hens. In studies of Attia et al. (2020) no effect of vitamin D_3 supplementation on ALP values in serum were observed in brown egg layers which were supplemented with different calcium levels. However, these authors chosen lower values of vitamin D_3 concentrations and birds were older than birds used in the present study. ALP activity of 60–72-week old hens of Attia et al. (2020) equalled ALP activity of 69-old brown layers from the present study but were markedly lower than ALP activity of 29-old high performing hens receiving 300 IU vitamin D_3. As a consequence, this could mean that especially in high performing layer lines an additional vitamin D supplementation might support the calcium metabolism in an early stage of the laying period. This might be also an explanation for the decrease of the feed conversion ratio in this period in BLA+ hens. The temporary increase of the feed conversion ratio of L68+ and R11+ hens is mainly based on the fact, that only a low number of hens reached the laying maturity at this time point and therefore showed no constant oviposition as well as a low and volatile daily egg mass production at this period. The fact that considering the whole experimental period L68+ hens exhibited a higher feed conversion ratio compared to L68c hens attracted more attention. A concrete explanation is not possible presently. However, the nutrient retention of L68 hens was lower compared to the other genotypes especially in week 49 which might support the effects on the feed conversion ratio.

Considering the whole experimental period R11+ hens exhibited a higher daily egg mass production compared to R11c hens despite similar individual egg weight. This indicates an improved constant production or higher number of produced eggs laid from R11+ compared to R11c hens, which was also shown by the constant egg production rate also in the later laying phase. Atencio et al. (2006) showed also a stabilisation of the daily egg production by a higher vitamin D_3 supplementation in broiler breeders especially at a later stage of the laying period. The decrease of the daily egg mass production in BLA+ compared to BLAc might be based on the numerically lower egg weight. It is suggested, that because of their higher calcium and vitamin D requirements the deficiency of calcium in the 3000 IU vitamin D_3 diet lead to the lower performance of BLA+ hens. In contrast, R11 hens are in general low performing hens, so that the lower calcium intake might be balanced by the higher vitamin D_3 supplementation in R11+ hens.

The enrichment of 25-OHD_3 in serum was also reflected in the egg yolk. Both the total vitamin D_3 and 25-OHD_3 increased with 3000 IU vitamin D_3 supplementation. The amount of the enrichment in egg yolk was similar to the vitamin D enrichment in indoor housed hens which were exposed at least 3 h with artificial ultraviolet B light (Kühn et al. 2019). BLA+ hens reached also amounts of total vitamin D_3 and 25-OHD_3 in egg yolk which equalled the amounts in egg yolk of hens which could choose freely between inside and outside pens (Kühn et al. 2014). As described by de Matos (2008) also in the present study vitamin D_3 showed a higher affinity to be deposited into the egg yolk than 25-OHD_3. Although the difference was not

significant the numerically higher serum 25-OHD_3 in BLA+ hens might be the explanation for the higher vitamin D_3 and 25-OHD_3 concentrations in egg yolk of these hens. Unfortunately, only in week 29 the vitamin D concentrations in egg yolk were determined. It would have been interesting to know, if the lower serum 25-OHD_3 concentrations were also reflected in the egg yolk in week 49. The higher deposition of vitamin D_3 with increasing dietary vitamin D_3 levels was also shown in studies of Yao et al. (2013).

From an economic point of view, the eggshell quality including eggshell weight, percentage, thickness, strength and density is extremely important because of its crucial role in egg production and hatchability (Ketta and Tumová 2016). Internal factors affecting eggshell quality described in the literature (Ketta and Tumová 2016) such as age and genotype were also in the present study prominent impact factors on eggshell quality. An increased eggshell weight is associated with increased egg size related to an increased shell surface area (Ketta and Tumová 2016). These relationships are also the explanation for the differences in the egg shellweight between high and low performing hens. Especially with regard to the eggshell strength WLA hens seemed to be superior compared to the other genotypes at the beginning of the observations which seemed to be mainly related to the shell thickness indicated by the positive correlation between both parameters. This relationship was already demonstrated in studies of Tumova et al. (2014). At no time point the other genotypes reached the breaking strength of WLA eggs measured in week 28 or 36. Corresponding to Pavlík et al. (2009) eggshell breaking strength decreased in an age dependent manner. They discussed relationships between the blood calcium levels and the eggshell breaking strength as well as the eggshell thickness but found no relations on blood phosphorus levels (Pavlík et al. 2009). In the present study a positive correlation between the eggshell proportion and serum calcium and phosphorus concentrations was detected but no correlation between eggshell strength and calcium or phosphorus concentrations in serum in week 29 (data not shown). Considering all genotypes and the whole experimental period the pooled LSMeans of breaking strength of hens fed 3000 IU vitamin D_3 were higher compared to hens with lower vitamin D supplementation. Although they did not concretely determine the breaking strength of the eggshell, also Atencio et al. (2006) reported a softer eggshell of eggs of broiler breeders receiving low amounts of vitamin D_3.

5. Conclusion

The higher vitamin D_3 concentrations in feed used in the present study might have beneficial effects on the calcium and phosphorus homoeostasis in the early laying period of high performing layer lines. In turn, the feed efficiency can be improved. Additionally, the higher vitamin D_3 supplementation seemed to lead to an improved constant production of eggs in R11 hens. In general, positive effects appeared to be influenced to a high extend by the genotype and laying performance of birds. However, further studies need to guarantee a balanced calcium-phosphorus content in the feed. The increase of serum 25-OHD_3 by the higher vitamin D_3 supplementation supported the higher transfer of vitamin D in the egg yolk and improved genotype-dependently the breaking strength of the eggshell. Therefore, the present study gives evidence that there are economic

advantages and nutritional-physiological benefits for hens and consumers of eggs with a higher vitamin D_3 supplementation in laying hens especially at the beginning of the laying period.

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References

Adedokun SA, Adeola O. 2013. Calcium and phosphorus digestibility: metabolic limits. J Appl Poult Res. 22:600–608.

Adhikari R, White D, House JD, Kim WK. 2020. Effects of additional dosage of vitamin D3, vitamin D2, and 25-hydroxyvitamin D3 on calcium and phosphorus utilization, egg quality and bone mineralization in laying hens. Poult Sci. 99:364–373.

Atencio A, Edwards HM, Pesti GM, Ware OG. 2006. The vitamin D3 requirement of broiler breeders. Poult Sci. 85:674–692.

Attia YA, Al-Harthi MA, Abo el-Maaty HM. 2020. Calcium and cholicalciferol levels in late-phase laying hens: effects on productive traits, egg quality, blood biochemistry, and immune responses. Front Vet Sci. 7:Article 389.

Browning LC, Cowieson AJ. 2015. Interactive effects of vitamin D-3 and strontium on performance, nutrient retention and bone mineral composition in laying hens. J Sci Food Agr. 95:1080–1087.

de Matos R. 2008. Calcium metabolism in birds. Vet Clin North Am Exot Anim Pract. 11:59–82.

EC No 1831/2003. 2003. Regulation (EC) No. 1831/2003 of the European parliament and of the council on additives for use in animal nutrition.

Edwards HM, Elliot MA, Sooncharernying S, Britton WM. 1994. Quantitative requirement for cholecalciferol in the absence of ultraviolet-light. Poult Sci. 73:288–294.

GfE, Ausschuss für Bedarfsnormen der Gesellschaft für Ernährungsphysiologie. 1999. Empfehlungen zur Energie- und Nährstoffversorgung der Legehennen und Masthühner (Broiler). Frankfurt am Main: DLG-Verlag.

Habig C, Distel O. 2013. Evaluation of bone strength, keel bone status, plumage condition and egg quality of two layer lines kept in small group housing systems. Br Poult Sci. 54:413–424.

Hattenhauer O, Traebert M, Murer H, Biber J. 1999. Regulation of small intestinal Na-P-i type IIb cotransporter by dietary phosphate intake. Am J Physiol-Gastr L. 277:G756–762.
Jansen S, Baulain U, Habig C, Weigend A, Halle I, Scholz AM, Simianer H, Sharifi AR, Weigend S. 2020. Relationship between bone stability and egg production in genetically divergent chicken layer lines. Animals. 10:850.
Kebreab E, France J, Kwakkel RP, Leeson S, Kuhi HD, Dijkstra J. 2009. Development and evaluation of a dynamic model of calcium and phosphorus flows in layers. Poult Sci. 88:680–689.
Ketta M, Tumová E. 2016. Eggshell structure, measurements, and quality-affecting factors in laying hens: a review. Czech J Anim Sci. 61:299–309.
Kühn J, Schutkowski A, Kluge H, Hirche F, Stangl GI. 2014. Free-range farming: a natural alternative to produce vitamin D- enriched eggs. Nutr. 30:481–484.
Kühn J, Wassermann C, Ebschke S, Schutkowski A, Thamm K, Wensch-Dorendorf M, von Borell E, Stangl GI. 2019. Feasibility of artificial light regimes to increase the vitamin D content in indoor-laid eggs. Poult Sci. 98:5177–5187.
Lieboldt M-A, Halle I, Frahm J, Schrader L, Weigend S, Preisinger R, Dänicke S. 2015. Effects of long-term graded L-arginine supply on growth development, egg laying and egg quality in four genetically diverse purebred layer lines. J Poult Sci. 53:8–21.
Moritz A. 2014. Klinische Labordiagnostik in der Tiermedizin. Stuttgart, Germany: Schattauer Verlag.
Naumann C, Bassler R. 2006. Die chemische Untersuchung von Futtermitteln. Darmstadt, Germany: VDLUFA Verlag.
[NRC] National Research Council. 1994. Nutrient requirements of poultry. 9th revised ed. Washington (DC): National Academic Press.
Pavlík A, Lichovníkova M, Jelínek P. 2009. Blood plasma mineral profile and qualitative indicators of the eggshell in laying hens in different housing systems. Acta Veterinaria Brno. 78:419–429.
Proszkowiec-Weglarz M, Angel R. 2013. Calcium and phosphorus metabolism in broilers: effect of homeostatic mechanism on calcium and phosphorus digestibility. J Appl Poult Res. 22:609–627.
Rodehutscord M, Sanver F, Timmler R. 2002. Comparative study on the effect of variable phosphorus intake at two different calcium levels on P excretion and P flow at the terminal ileum of laying hens. Archiv für Tierernaehrung. 56:189–198.
Soares JH, Kerr JM, Gray RW. 1995. 25-hydroxycholecalciferol in poultry nutrition. Poult Sci. 74:1919–1934.
Tumova E, Gous RM, Tyler N. 2014. Effect of hen age, environmental temperature, and oviposition time on egg shell quality and egg shell and serum mineral contents in laying and broiler breeder hens. Czech J Anim Sci. 59:435–443.
Yao LX, Wang T, Persia M, Horst RL, Higgins M. 2013. Effects of vitamin D3-enriched diet on egg yolk vitamin D3 content and yolk quality. J Food Sci. 78:C178–C183.