Effects of riboflavin supplementation level on health, performance, and fertility of organic broiler parent stock and their chicks

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ABSTRACT

Data from breeder hens and chicks are particularly critical for the development of vitamin B2 recommendations for organic poultry. To test safe thresholds of supplementation, 100 breeder hens (Hubbard JA 57 K) and ten cockerels were allocated to ten housing groups, with each receiving supplementation of either 4.0 (treatment **CON**) or 2.5 mg (treatment **RED**) riboflavin per kg feed. After 15 weeks of experimental feeding, 256 eggs were incubated. From the hatched chicks (Hubbard S757), 192 were allocated to four treatments based on their parents' treatment (CON- or RED-) and on their own supplementation of either 4.0 (-CON) or 2.5 mg (-RED) riboflavin per kg feed. Two groups of each combination (CON-CON, CON-RED, RED-CON, RED-RED), each with 24 chicks of both sexes, were fattened for nine weeks and slaughtered. Analysis of total riboflavin (sum of native concentrations and supplements) in the parent's feeds revealed an average, over the 15 weeks, of 7.8 and 5.8 mg per kg feed for CON and RED, respectively. Body weight, plumage integrity, gait appearance, footpad, claw and keel bone integrity, lesion scores, laying performance, and egg mass were found to be of similarly high levels for all hens without any treatment effects. Performance traits of the hens in both treatments reached the specifications of the producer for this genotype. Yolk and albumen riboflavin concentrations were not affected although yolk colour in the RED treatment group became darker. Fertility was not affected, and hatchability was 100 per cent. Total riboflavin concentrations in the broiler diets were on average, over 9 weeks, 8.2 and 6.1 mg/kg for CON and RED, respectively. In chicks, RED treatment of their parents led to significant depressions of feed intake and growth. The RED treatment of the chicks themselves also impaired growth. Growth rates of the CON-CON treatment were in line with the specifications of the producer for this genotype. Plumage scores, footpad integrity and walking appearance of the broilers, and dressing percentage of the carcasses were not affected by treatment. The RED treatment of chicks caused lower spleen and heart weights, while pancreas and liver weights, and liver riboflavin concentrations were not affected. In conclusion, supplementation of 4.0 mg/kg to organic diets did not evoke any health or performance problems for breeder hens or broiler chicks, while a supplementation level of 2.5 mg per kg feed led to reduced weight gains, although without any other riboflavin deficiency symptoms.

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Implications

In order to assess the impact of low riboflavin supplements to breeders and their offspring, we applied two different supplementation levels (4.0 and 2.5 mg/kg feed) to slow-growing broiler parent hens and subsequently to the broiler chicks hatched from their eggs. We found that 4.0 mg riboflavin supplementation was sufficient for the animals at all stages to prevent deficiency symptoms or performance depressions, while 2.5 mg/kg feed, supplemented to either parent hens or chicks, impaired growth during broiler fattening. These data are relevant for the development of riboflavin supplementation requirements in organic poultry production.

Introduction

Riboflavin (Vitamin B2) is essential for birds and is therefore recommended as an additive substance to all agricultural poultry feeds (National Research Council, 1994; Fefana, 2014). The situation for organic production is not fundamentally different because yields and growth rates of the animals in organic systems also greatly exceed the natural metabolic turnovers in wild chicken or ducks. However, organic production systems aim to minimise inputs of synthetic or isolated substances to livestock feed due to the aspiration of naturalness of production.

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A further reason for minimising inputs of riboflavin to organic poultry diets is economic. Riboflavin, like all B-vitamins, is produced by microbiological fermentation (Rychen et al., 2018; Lambertz et al., 2020), which is genetically modified organisms (**GMO**)-based in most industrial productions. However, GMO are prohibited in organic feed (European Union, 2018) so separate production lines for organic, GMO-free riboflavin are needed (Lambertz et al., 2020). These production lines are on lower scales and the yeast strains used may be less efficient, which makes the currently available GMO-free riboflavin in Europe significantly more expensive than the conventional products.

However, the basis for deciding the minimum input must always be that the supply of riboflavin is sufficient to maintain animal health, welfare, and productivity. No specific recommendations for vitamin supply to any livestock species exist for organic agriculture so far (Leiber et al., 2021). Genotypes with somewhat lower performance levels, as well as differences in feed formulations, give reason to assume specific, and potentially lower, requirements for vitamin supply of organic poultry compared to conventional. Furthermore, also for conventional requirement definitions, the scientific background appears to be rather outdated (National Research Council, 1994; Leeson, 2007). Against this background, riboflavin requirements for poultry in organic conditions are currently being re-evaluated (for broilers: Lambertz et al., 2020 and 2021; for laying hens: Leiber et al., 2021).

Chick production is an essential part of poultry production systems; so particular attention has to be paid to breeder hens. Riboflavin deficiency is likely to be reflected in negative effects on energy metabolism (Tang et al., 2019) and antioxidant capacity in the egg (Zhang et al., 2020). For example, insufficient supply (6.5 mg riboflavin per kg feed) of riboflavin to breeder hens was found to lead to increased embryonic mortality and impaired hatchability of chicks under tropical conditions (Arijeniwa et al., 1996) with similar effects found in ducks (Tang et al., 2019; Zhang et al., 2020). It has furthermore been shown that vitamin B2 supplementation of layers' diets with less than 3.0 mg per kg feed leads to decreased riboflavin concentrations in egg yolk of chickens (Leiber et al., 2021) and ducks (Zhang et al., 2020). A reasonable conclusion is that this is the pathway to impaired embryonic development and low hatching rates (Tang et al., 2019).

The present study aims to assess the influence of parent hen supplementation with riboflavin on egg fertility and hatchability and on the subsequent development of the chicks. Although lower than the critical level reported by Arijeniwa et al. (1996), experiments (Lambertz et al., 2021; Leiber et al., 2021), presumed intrinsic contents of the feed components (Witten and Aulrich, 2019), and long-term practice in Swiss organic poultry production indicated a supplementation level of 4 mg per kg feed should be adequate for a sufficient riboflavin concentration in the egg. Furthermore, and in contrast to other European countries, Swiss organic regulations prescribe a maximum supplementation of 4 mg per kg feed since more than 10 years (Bio Suisse, 2021). To explore the effects of even lower riboflavin supplementation, we compared the 4 mg/kg level with a supplementation of 2.5 mg per kg feed, with the hypothesis that the lower supplementation level would lead to lower riboflavin concentration in the eggs and to impaired fertility, hatchability, and subsequent growth. Furthermore, we hypothesised that the supply of parent animals with riboflavin would interact with the subsequent supply of the chicks on growth and health parameters.

Material and methods

Two consecutive feeding experiments were carried out; both in accordance with the Swiss law on animal welfare and the EU directive 2010/63/EU for animal experiments (European Union, 2010).

Experiment A compared different riboflavin supplementations in parent stock (Hubbard JA 57(K) hens and cockerels), which were purchased from a local breeding company. Experiment B compared different levels of riboflavin supplementations in their offspring (Hubbard S757 chicks).

Experiment A – parent stock

Housing and feeding

In this first experiment, a total of 100 hens and 10 cockerels were individually marked with leg rings and randomly divided into two treatment groups: treatment group "**CON**" received a diet supplemented with 4 mg riboflavin per kg total feed; treatment group "**RED**" received a diet added up with 2.5 mg riboflavin per kg total feed. Animals of each treatment group were randomly allocated to five pens (10 hens and one cock per pen). Pens (3 × 2.5 m) were equipped with perches, dust-baths, litter, nests, feeders and drinkers, so were compliant with the Swiss national animal husbandry regulations (Bundesamt für Lebensmittelsicherheit und Veterinärwesen, 2020) and, with the exception of the absence of an outdoor run, with Swiss organic regulations (Bio Suisse, 2021). All animals had *ad libitum* access to water and bales of straw for occupation purposes. Whole cereal grains (a mixture of wheat and corn) were additionally administered at 100 g per pen and day. In a pretrial phase of 6 weeks (life weeks 17–23), all animals received the same commercial organic feed for layers *ad libitum*. All feedstuffs used in this trial were produced by UFA AG (Herzogenbuchsee, Switzerland). The experimental feeding started in life week 23 and ended in life week 38. In the first 2 weeks of the experiment, animals were fed *ad libitum*; but due to excessive BW gains, feed was restricted to 125 g feed per day and animal from trial week three until trial week 15 when the parent trial was terminated. The control treatment (CON) consisted of a commercial organic feed for layers (Table 1) including a supplementation of 4 mg riboflavin per kg total feed. The experimental treatment (RED) consisted of the same feed (same production date and batch), but with the addition of only 2.5 mg riboflavin per kg total feed. Feed components and nutrient compositions, and total feed riboflavin concentrations, which were measured by HPLC, are listed in Table 1.The total riboflavin concentration in the treatments was higher than the supplementation levels due to native riboflavin in the diet components.

Measured traits

To calculate feed intake on pen level, feed residues were weighed daily (Kern GmbH, Balingen, Germany; scale range: 0–15 kg ± 0.01). Laying performance was determined at the pen level by collecting eggs daily. Clinical health and mortality were documented daily at the pen level. Every second week, all animals were individually weighed and clinically examined for health traits. Plumage was assessed at six body regions, along with lesions at the head, integument, and footpads: all with a rating scale in which "4″ signified full integrity, "3" slight damages, "2" serious damages, and "1" full lack of plumage, open wounds, or severely developed footpad abscesses, respectively (Tauson et al., 2005). In addition, keel bone integrity was assessed on a four-step scale, with "4" signifying a fully intact keel bone, through to "1" as a disintegrated fracture. We counted lost claws. Gait was assessed according to the 5-level scale developed by Kestin et al. (1992) in which "0" signifies symptomless walking, "1" signifies slight irregularities and "4" indicates inability to walk or stand.

Once every 2 weeks, all eggs per pen from the last 48 h were examined for total, eggshell, yolk and albumen weights (PM 3000, Mettler, Columbus, Ohio). Yolk colour was evaluated with a Yolk Colour Fan (scale 1–15; DSM Animal Nutrition and Health, Kaiseraugst, Switzerland).

Table 1
Composition, nutrient and riboflavin concentrations in diets for breeder hens and chicks containing different levels of riboflavin supplementation (as fed, at 90% DM).

	Treatment			
	Parent stock feed		Chick fattening feed	
	CON[1]	RED[1]	CON[1]	RED[1]
Product[2]	UFA 607-9	UFA 1607-90	UFA 678-3	UFA 678-4
Composition[3]	Corn, soybean cake, sunflower cake, calcium carbonate, wheat, soft-wheat bran, alfalfa, corn gluten, potato protein, soya oil, monocalcium phosphate, sodium bicarbonate, paprika powder, sodium chloride, vitamins		Corn, soybean cake, wheat, sunflower cake, soybeans, corn gluten, minerals, rapeseed cake, soya oil, vitamins	
CP [g/kg][3]	185	185	230	230
Ether extract g/kg[3]	60	60	85	85
Crude fibre [g/kg][3]	60	60	45	45
Crude ash [g/kg][3]	135	135	55	55
Metabolisable energy [MJ/kg][3]	11.2	11.2	12.8	12.8
Lysine [g/kg][3]	8.2	8.2	10.7	10.7
Methionine [g/kg][3]	3.7	3.7	4.0	4.0
Calcium [g/kg][3]	39	39	8.5	8.5
Phosphor [g/kg][3]	5.3	5.3	5.8	5.8
Sodium [g/kg][3]	1.7	1.7	1.5	1.5
Riboflavin [mg/kg][4] start of trial[5]	8.1	5.9	7.80	5.67
Riboflavin [mg/kg][4] mid of trial[6]	7.6	6.0	8.53	6.47
Riboflavin [mg/kg][4] end of trial[7]	7.8	5.6	8.17	6.11

[1] Diets supplemented with either 4 mg riboflavin / kg feed (CON) or 2.5 mg riboflavin / kg feed (RED).
[2] Feedstuff used in this trial was produced by UFA AG, Herzogenbuchsee, Switzerland.
[3] Values as provided by the producer.
[4] Riboflavin concentration as measured by HPLC in the total feed (contains native and added riboflavin) in three samples per diet.
[5] Parent trial: week 0; broiler trial: week 1.
[6] Parent trial: week 8; broiler trial: week 5.
[7] Parent trial: week 15; broiler trial: week 9.

In trial weeks 0, 8, and 15, the riboflavin content of egg yolk in one mixed sample from all eggs from the last 48 h from each pen was measured with HPLC. At the same time points, the riboflavin content of feed was measured in three samples per feed. In week 15, the riboflavin content in albumen was also quantified. Riboflavin analyses were performed by LUFA-ITL GmbH, AGROLAB Group, Kiel, Germany according to DIN EN 14152:2014-08 (European Committee for Standardization, 2014).

Incubating and hatching

During trial week 15, 256 clean and intact hatching eggs (weight: min. 53 g, max. 67 g) were sampled over 3 days, stored in a closed container (13 °C; air humidity 75–85%), and turned over daily. One day before the start of incubation, eggs were slowly warmed-up to 24 °C and disinfected with Röhnfried Desinfektion Pro (Dr. Hesse Tierpharma, Hohenlockstedt, Germany) according to the manufacturer's specifications. Eggs were placed, pen-wise, in an automatic hatchery machine (Heka-Favorit-Olymp 256, HEKA-Brutgeräte, Rietberg, Germany) and were incubated for 21 days according to the manufacturer's specifications (prebrood: temperature 37.8 °C, humidity 53%, turning of eggs every 120 min for 3 min, cooling down every 24 h for 25 min; hatching brood: temperature 37.8 °C, humidity day 19: 63%, day 20: 73%, day 21: 78%). Eggs were weighed and sheared on days 1, 7, 14, and 19 of brood. The fertilisation rate, mortality and other abnormalities were documented. One day after hatching, chicks were vaccinated against Marek's disease and coccidiosis (Nobilis® Rismavac and Paracox® 5, MSD animal health, Lucerne, Switzerland). For the next 4 days, they were kept in tempered chick rearing boxes (in small groups according to the treatment of their parents) and received chicken starter diet (UFA 671 Crumbs). Congenital abnormalities or malformations, difficulties whilst hatching, vitality and mortality were recorded twice daily from hatching until the start of experiment B.

Experiment B – broilers

Housing and feeding

At an age of 6 days, 96 chicks from each of the CON and RED groups were individually marked with leg rings and randomly distributed to two feeding groups: feeding group "CON" received a commercial organic feed for fattening supplemented with 4 mg riboflavin per kg feed; feeding group "RED" received the same feed (same production date and batch) supplemented with 2.5 mg riboflavin per kg feed. This allocation led to four treatment groups with 48 chicks each: chicks from parents from CON, receiving CON feed were designated as "**CON-CON**"; chicks from parents from CON, receiving RED feed were designated as "**CON-RED**"; chicks from parents from RED, receiving CON feed were designated as "**RED-CON**" and chicks from parents from RED, receiving RED feed were designated as "**RED-RED**". Chicks of each treatment group were randomly allocated to two pens (leading to 24 chicks/pen) sized as explained above and equipped for broilers in accordance with the national animal husbandry regulations (Bundesamt für Lebensmittelsicherheit und Veterinärwesen, 2020) and, with the exception of the absence of an outdoor run, with the Swiss organic regulations (Bio Suisse, 2021). Chicks received water, whole cereal grains (a mixture of wheat and corn) and the allocated experimental feed *ad libitum* (Table 1).

Measured traits

To calculate feed intake at the pen level, feed residues were weighed daily (Kern GmbH, Balingen Germany; scale range: 0–15 kg ± 0.01). Clinical health and mortality were documented daily on pen level. Once in 2 weeks, all broilers were individually weighed and clinically examined for health traits according to Tauson et al. (2005) and gait traits according to Kestin et al. (1992) using the same evaluation scales as in Experiment A. After 63 days of experimental feeding, which is the minimum lifespan permitted by the Swiss organic standards (Bio Suisse, 2021), broil-

ers were slaughtered after electrical stunning in a commercial poultry slaughterhouse. Carcass weights (without head, neck, feet, viscera and feathers) and weights of heart, liver, spleen and pancreas were determined, and pathological alterations of inner organs were documented. After slaughter, the riboflavin content of livers were measured in two mixed samples per treatment group with HPLC. At the beginning, middle, and end of the trial, the riboflavin content of three mixed samples of feed was measured as described above.

Statistical analysis

For the parent experiment (A), we analysed three exemplary weeks (baseline-week 0, week 8, and week 15, which was the week in which breeding eggs were sampled) with generalised mixed models, considering 'treatment' and 'week' as fixed factors and 'pen' as a random effect. Since the contribution to variance of the pen effect was always negligibly small (less than 1% of the residual effect), we considered the individual animal as the basic statistical unit, with the exception of feed consumption and laying performance, which were assessed at pen level. For egg fertility rates, we used the pen as the statistical unit to account for the clear effect of the cockerels in the groups.

For the broiler experiment (B), we considered overall averaged feed consumption and daily weight gains per pen, slaughter data per animal, and health scores per animal averaged over the whole treatment time. Data were analysed with generalised mixed models, considering 'parent treatment', 'chick treatment', and their interaction as fixed factors and 'pen' as a random effect. Since the contribution to variance of the pen effect was always negligibly small (less than 1% of the residual effect), we used the individual animal as basic statistical unit for slaughter data and health scores.

We provide the significance levels (P-values), based on Type III F-Test statistics with Kenward-Roger degrees of freedom. Statistical significance was defined at $P < 0.05$. Averages are LSMeans, based on Tukey posthoc tests. We validated model assumptions by visually inspecting residual distribution plots. All statistics were calculated with SPSS (IBM Analytics, Zurich, Switzerland).

Validation

The chosen designs contained two constraints: (i) the application of only two supplementation treatments (CON and RED), and (ii) the fact that experiment B had, if considered on a pen basis, only two replicates. The CON treatment was validated by ensuring that absolutely no deficiency symptoms and no deviations from the performance specifications (Hubbard, 2019; Bio Suisse, 2021) for either hens or broilers occurred. Furthermore, we analysed the contribution of 'pen' to the overall residual effects. Since it

was below 1% in all cases, we considered it valid to analyse the data on basis of the individual animal, wherever possible.

Results

The supplementation of 4.0 mg riboflavin per kg feed resulted in total concentrations of 7.6–8.5 mg/kg in the CON feeds of the parent stock (A) and broiler (B) experiments (Table 1), while the RED feeds, supplemented with 2.5 mg/kg contained total concentrations of 5.6–6.5 mg/kg. The total values include the supplemented plus the native riboflavin, which is intrinsically contained in the feed components. There was some variation between the sampling weeks, but no linear effect of storage time.

Experiment A – parent stock

Laying performance and egg mass increased during the experiment. In week 15, laying performance was above 95% in both treatments. A significant difference in feed consumption between the groups designated to different treatments was found in week zero but there was no observable effect of treatment on feed consumption for the remainder of the experiment. No treatment effect was found for laying performance or weights of eggs and their parts (Table 2). Throughout the entire course of the experiment, animals of treatment RED were slightly heavier than those of CON. With time, yolk colour became darker in treatment RED than in CON. No treatment effect on riboflavin concentrations in yolks and albumen could be observed.

Health scores were generally high, and no cases of clinical relevance occurred in any of the assessed traits (Table 3). Mortality in parent stock was zero. Some decline in plumage integrity was found with time, but with no effect of treatment. Only few and light footpad lesions and almost no motion disorders were observed. The treatment had no observable impact on these traits.

The fertility rate of breeding eggs after 7, 14, and 18 days of breeding was not statistically different between the RED and CON treatments (Table 4). After 18 days, 81% of CON eggs and 92% of RED eggs contained living embryos. The fertility rates were not normally distributed between pens. The hatching rate from eggs that had been fertile at day 18 was 100% in both treatments. The BW of hatched chicks was 39.9 g on average and did not differ between treatments.

Experiment B – broilers

Immediately after hatching, and while still in the breeding apparatus, 18 of 25 chicks in one parent group of the RED treat-

Table 2
Feed consumption, laying performance and egg quality of breeder hens supplemented with riboflavin at either 4.0 or 2.5 mg per kg feed. Data are least-squares means.

	Week 0		Week 8		Week 15			P-values		
	CON[1]	RED[2]	CON	RED	CON	RED	SEM	Treatment	Week	T*W
Feed consumption [g/animal/day, as fed]	115[b]	123[a]	122	120	125	122	0.86	<0.01	<0.01	< 0.05
BW [kg]	2.02	2.09	2.21	2.28	2.32	2.38	0.031	<0.1	<0.001	0.90
Laying performance [eggs/animal/day]	0.83	0.85	0.93	0.94	0.96	0.98	0.009	0.27	<0.001	0.61
Egg weight [g]	48.6	48.9	54.2	54.4	58.0	57.6	0.34	0.47	<0.001	0.56
Eggshell weight [g]	6.69	6.65	7.09	7.03	7.38	7.25	0.062	0.77	<0.001	0.78
Egg yolk weight [g]	12.3	12.6	15.3	15.3	17.3	17.4	0.143	<0.1	<0.001	0.34
Albumen weight [g]	29.6	29.6	31.8	32.1	33.3	33.0	0.283	0.83	<0.001	0.52
Yolk colour score	12.2	12.3	12.7[b]	13.8[a]	12.3[n]	13.6 [m]	0.212	0.30	<0.001	<0.001
Riboflavin concentrations [mg/100 g]										
Yolks	0.664	0.708	0.644	0.652	0.698	0.714	0.009	0.23	<0.05	0.71
Albumen	n.a.	n.a.	n.a.	n.a.	0.390	0.378	0.006	0.36	n.a.	n.a.

n.a. = not applicable. T = Treatment. W = Week.
[1] Control group supplemented with 4.0 mg riboflavin per kg feed (N = 50, nested in 5 pens).
[2] Treatment group supplemented with 2.5 mg riboflavin per kg feed (N = 50, nested in 5 pens).
[a,b,n,m] Least-squares means within one week, carrying different letters are significantly different at $P < 0.05$.

Table 3
Plumage, lesion and health scores of breeder hens supplemented with riboflavin either at 4.0 or 2.5 mg per kg feed. Data are least-squares means.

	Week 0		Week 8		Week 15			P-values		
	CON[1]	RED[2]	CON	RED	CON	RED	SEM	Treatment	Week	T*W
Plumage scores[3]										
Neck	4.0	4.0	4.0	4.0	4.0	4.0	0.00	1.0	1.0	1.0
Back	3.98	4.00	3.69	3.69	3.35	3.33	0.034	0.72	<0.001	0.64
Breast	4.00	4.00	3.96	4.00	3.57	3.73	0.015	<0.1	<0.001	0.38
Tail	3.96	4.00	3.57	3.69	3.33	3.27	0.039	0.84	<0.001	0.72
Wings	3.84	3.92	3.55	3.73	3.20	3.21	0.044	0.21	<0.001	0.95
Belly/Cloaca	4.00	3.98	4.00	4.00	4.00	4.00	0.002	0.95	0.56	0.35
Lesions[3]										
Footpads	4.0	4.0	4.0	4.0	4.0	3.96	0.011	0.50	<0.001	0.79
Abdomen	4.0	4.0	4.0	3.96	4.0	4.0	0.003	0.33	<0.05	<0.05
Head and comb	3.55	3.41	3.65	3.65	3.71	3.69	0.042	0.19	<0.001	0.71
Further scores										
Keel bone integrity[3]	3.96	3.98	3.94	3.98	3.98	3.98	0.037	0.69	<0.1	0.99
Missing claws [no/animal]	0.00	0.00	0.00	0.00	0.00	0.00	0.002	0.31	0.41	0.41
Motion disorders[4]	0.00	0.00	0.04	0.00	0.06	0.00	0.011	0.16	0.38	0.72

T = Treatment. W = Week.
[1] Control group supplemented with 4.0 mg riboflavin per kg feed (N = 50, nested in 5 pens).
[2] Treatment group supplemented with 2.5 mg riboflavin per kg feed (N = 50, nested in 5 pens).
[3] Scores from 4 (no incidence) to 1 (severe disorders).
[4] Score from 0 (regular motion behaviour) to 4 (disability to walk).

Table 4
Fertility and hatching rate of eggs from breeder hens supplemented with riboflavin at either 4.0 or 2.5 mg per kg feed. Data are least-squares means.

	CON[1]	RED[2]	SEM	P-value
Fertility rate day 7[3]	0.834	0.940	0.086	0.41
Fertility rate day 14[3]	0.808	0.926	0.105	0.45
Fertility rate day 18[3]	0.808	0.919	0.104	0.48
Hatching rate[4]	1.0	1.0	n.a.	n.a.
BW after hatch [g]	40.1	39.6	0.29	0.19

n.a. = not applicable.
[1] Control group supplemented with 4.0 mg riboflavin per kg feed (128 eggs collected from 5 pens during 3 days; statistical N = 5 pens).
[2] Treatment group supplemented with 2.5 mg riboflavin per kg feed (128 eggs collected from 5 pens during 3 days; statistical N = 5 pens).
[3] Proportion of eggs with embryos alive relative to eggs initially put into breeding apparatus.
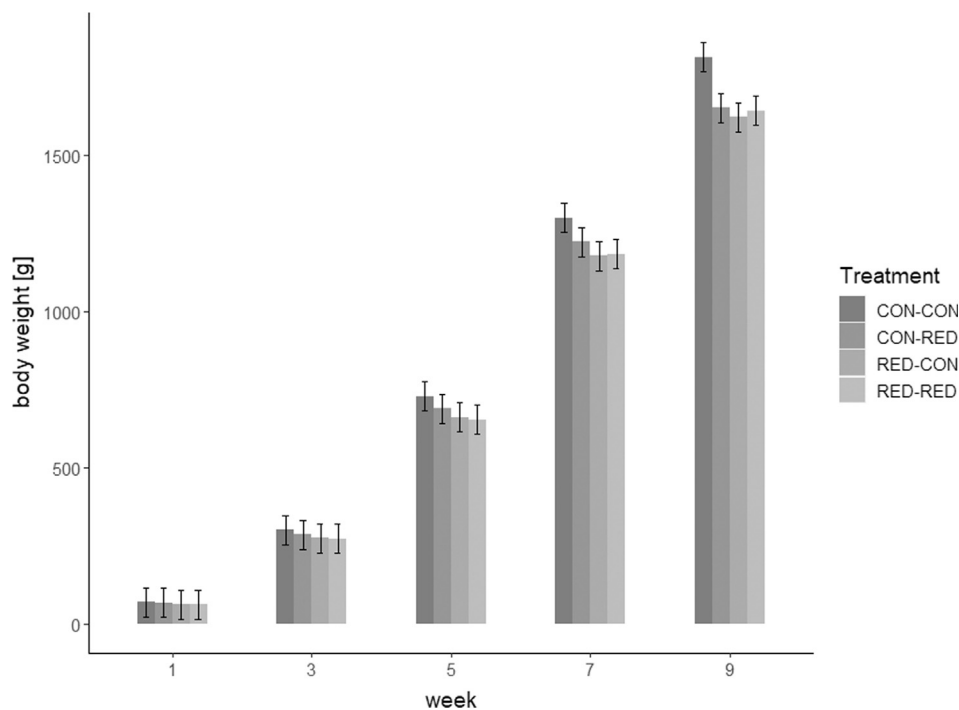[4] Proportion of chicks hatched relative to fertile eggs at day 18.

Fig. 1. Life weight of growing broilers after weeks of life 1, 3, 5, 7, 9 (slaughter). Abbreviations of treatment groups are defined in Table 5.

Table 5
Feed consumption, daily weight gain, slaughter traits, organ weights and health scores of broilers based on different riboflavin supplementation of their parent hens and the broilers themselves. Data are least-squares means.

	Treatments					P-values for treatments		
	CON-CON[1]	CON-RED[2]	RED-CON[3]	RED-RED[4]	SEM	Parent	Chick	P*C
Feed consumption [g/animal/day, as fed]	74.1[a]	68.7[ab]	68.0[ab]	67.0[b]	1.44	<0.05	<0.1	<0.01
Average daily gain [g]	28.8[a]	26.2[b]	25.8[b]	26.1[b]	0.57	<0.01	<0.05	<0.01
BW at slaughter [g]	1 815[a]	1 653[b]	1 623[b]	1 644[b]	35.9	<0.01	<0.05	<0.01
Carcass weight at slaughter [g]	1 276[a]	1 156[b]	1 161[b]	1 151[b]	26.5	<0.05	<0.05	<0.05
Dressing percentage [%]	70.3	69.8	70.8	70.0	0.58	0.42	0.13	0.65
Liver weight [g]	33.9	39.4	32.0	32.2	4.31	0.27	0.50	0.52
Spleen weight [g]	3.18	2.77	2.90	2.66	0.173	0.25	<0.1	0.63
Pancreas weight [g]	3.59	3.53	3.57	3.59	0.161	0.89	0.91	0.81
Heart weight [g]	13.1	11.8	12.6	12.0	0.51	0.77	<0.1	0.43
Riboflavin in liver [mg/100 g]	2.39	2.37	2.43	2.31	0.025	0.87	0.28	0.47
Plumage score[5]	4.00	4.00	4.00	4.00	0.00	1.0	1.0	1.0
Footpad lesion score[5]	3.99	3.99	3.99	3.99	0.009	0.63	0.67	0.99
Motion disorders[6]	0.0	0.0	0.0	0.0	0.00	1.0	1.0	1.0

P = Parent. C = Chick.
[1] Parent supplementation 4.0, chick supplementation 4.0 mg riboflavin per kg feed (N = 48, nested in 2 pens).
[2] Parent supplementation 4.0, chick supplementation 2.5 mg riboflavin per kg feed (N = 48, nested in 2 pens).
[3] Parent supplementation 2.5, chick supplementation 4.0 mg riboflavin per kg feed (N = 48, nested in 2 pens).
[4] Parent supplementation 2.5, chick supplementation 2.5 mg riboflavin per kg feed (N = 48, nested in 2 pens).
[5] Scores from 4 (no incidence) to 1 (severe disorders).
[6] Score from 0 (regular motion behaviour) to 4 (disability to walk).
[a,b] Least-squares means carrying different letters are significantly different at $P < 0.05$.

ment died, although the hatching rate had been 100% and BWs did not differ from the average. During fattening, four chicks of treatment CON-CON, two from RED-CON and one from each other treatment died. Five deaths were caused by ascites syndrome. For three deaths, no clear diagnosis was found, but riboflavin deficiency was definitely excluded in all cases. Not accounting for the deaths in the breeding apparatus, overall mortality of the broilers during nine weeks of fattening was thus 4.1%. Detailed, mortality was 8.3% in CON-CON, 4.2% in RED-CON, and 2.1% in the other treatments, respectively.

From week 3 onwards, a better growth of broilers from CON parents was observable and became significant in week 7 (Fig. 1). Animals in the CON-CON treatment grew better than in CON-RED, leading to a significant advantage of CON-CON over all other treatments regarding BW in week 9 and at slaughter (Fig. 1, Table 5), average daily gain, and carcass weight. The separate effects of parent treatments and chick treatments were all significant for daily weight gain, BW at slaughter, and carcass weight; always in favour of CON over RED. Feed consumption was significantly higher in CON-CON than in RED-RED, but CON-RED and RED-CON were closer to RED-RED than to CON-CON in feed consumption. Parent treatment caused significantly higher feed consumption in CON than in RED, and chick treatment showed a tendency in the same direction.

Dressing percentage, organ weights and riboflavin concentrations in livers were not significantly affected by either treatment but there was a tendency for lower spleen and heart weights in the broiler treatment RED compared to CON. No plumage irregularities and no motion disorders of the chicks were observed, and only few and light footpad lesions were found, with no relationship to treatments.

Discussion

Riboflavin levels in the diets

The aim of this study was to assess whether lower riboflavin supplementation levels than in conventional practice can be applied without harming parent hens or their offspring under organic feeding conditions. The basis we used was the Swiss

organic regulation, which prescribes a maximum supplementation level of 4 mg/kg feed, which is lower than that allowed in the EU (Villamide and Fraga, 1999; Fefana, 2014; Miavit, 2018). However, even with this lower standard supplementation, no deficiency problems are encountered in the Swiss organic poultry industry.

The total measured riboflavin concentrations in the CON and RED feeds of the two experiments indicated that the aspired difference between the treatments was achieved. The differences between supplements and total values indicate native riboflavin concentrations of roughly 3.0–4.5 mg/kg, which is higher than found in a former study of commercial organic chicken feed (Leiber et al., 2021). In contrast to Leiber et al.'s (2021) study, no riboflavin losses occurred during storage.

The supplementation level applied for diets CON (4 mg/kg) was the maximum allowed by the Swiss regulations for organic feed (Schneider and Früh, 2021), and is common practice in the Swiss organic poultry industry. According to experimental data, this level of supplementation matches the requirements for growing broilers (Ruiz and Harms, 1988; Chung and Baker, 1990; Lambertz et al., 2020) and breeder hens (Squires and Naber, 1993). However, recommendations and practice may differ from experimental results. For breeder hens, the 4.0 mg/kg supplementation level is lower than the recommendations of the European Association of Specialty Feed Ingredients and their Mixtures (Fefana, 2014) and of commercial premix producers (Miavit, 2018), and is half of the average reported from a monitoring of breeding hen feeds in Spain (Villamide and Fraga, 1999). For the broilers, the CON supplementation matched the Spanish practice (Villamide and Fraga, 1999) but was only half of Fefana (2014) and Miavit (2018) recommendations.

The 2.5 mg supplementation (RED) was far below common practice (Villamide and Fraga, 1999) and recommendations (Fefana, 2014) for breeder hens. However, based on experimental literature (Squires and Naber, 1993), it was still within the range in which no detrimental effects on hens and eggs are expected to appear. Following a recent study by Leiber et al. (2021), we expected no detrimental effects on hen health or performance and only small effects on egg riboflavin concentrations. For the broilers, the 2.5 mg supplementation was rather low with respect to recommendations and practice, but still above the thresholds

of harmful deficiency effects (Roth-Maier and Kirchgessner, 1997; Roth-Maier and Paulicks, 2002).

Parent stock

After the initial week 0, no effects of treatment on feed consumption or laying performance were found in the hens. Laying performance increased with age to a high level of above 95% in all pens, which was above the specification for this genotype (Hubbard, 2019). No treatment effects on the weights of eggs and their parts were found although egg yolk was affected and was significantly darker from RED animals in weeks 8 and 15. The mechanism behind this phenomenon is not clear. Czarnowska-Kujawska et al. (2021) report lighter yolk colour in eggs with higher folate concentrations but were similarly unable to provide a mechanistic causality.

The lack of diet effects on riboflavin concentrations in yolks and albumen was not unexpected given the total dietary riboflavin levels of approximately 8 and 6 mg/kg in the CON and RED feeds (Leiber et al., 2021). The finding that there were no observable treatment effects on any health or performance, except for the darker yolks for which there is no clear reason or meaning, allows the conclusion that a 2.5 mg/kg riboflavin supplementation on the background of >3 mg/kg native concentration in the feeds does not cause clinical symptoms of deficiency of laying hens. This finding provides further support to the conclusions of Squires and Naber (1993) and Leiber et al. (2021). However, the findings of Zhang et al. (2020), in their study of riboflavin deficient duck breeders, suggest that plasma riboflavin level and antioxidant status of the hens may have reacted more strongly to low riboflavin intake than the clinical indicators used in this study.

Breeding eggs

Combining fertility and hatchability rates to an overall hatchability per egg, the eggs acquired from the parent hen trial showed an average hatching rate of 87%, which was below the specification of 91% for this genotype (Hubbard, 2019). Detailed by treatment, the rates were 81% hatched eggs for CON and 92% for RED. Skew distribution between pens indicates the individual performance of the cockerels in the groups as the reason for variability in the fertility results.

Broilers

Two different accumulations of broiler mortality need discussion, in order to consider any relationship with the riboflavin treatments. First, 72% of the chicks from one parent group (one pen, RED treatment) died within the few hours between hatching and removal from the breeding apparatus. They all hatched completely and BWs were fully within the normal range. The pathological report from the Veterinary Faculty of the University of Zurich for five of the chicks did not reveal any signs of riboflavin deficiency in the histology of neck muscle tissue, brain, heart, proventriculus, liver, lung, kidney, keel bone, yolk sac, marrow, Bursa fabricii, or thymus. Furthermore, no infections were detected. The report concluded a technical issue with the breeding apparatus as the most likely reason for death. Second, a total of eight broilers died during the second half of the fattening period, of which four animals were from the CON-CON treatment group. Again, no symptoms of riboflavin deficiency were detected during pathological analysis. In five cases, ascites syndrome (Kalmar et al., 2013) was identified, while the cause remained unclear in the other cases (one from CON-CON and two from RED-CON).

The clear effects of maternal riboflavin supplementation on feed consumption and growth were not expected following the lack of

differences in yolk and albumen riboflavin concentrations. It is possible that riboflavin deficiency leads to retarded growth (Ruiz and Harms, 1988; Chung and Baker, 1990) and can be explained by poorer expression of proteins responsible for energy metabolism and fatty acid oxidation in the liver of embryos from undersupplied breeders (Tang et al., 2019). It may be possible that such disadvantageous shifts in energy metabolism continue after hatching and impair growth. However, a direct effect of egg riboflavin concentration cannot have been the mechanism in the current study, so such an impact on gene expression must have originated from riboflavin deficiency in the maternal organism. In contrast to previous studies (Squires and Naber, 1993; Arijeniwa et al., 1996), embryo survival and hatching were not affected and the effects occurred only in the growing chicks.

In duck parent stock, the impact on antioxidant status of the egg has been observed at higher supplementation levels than the onset of decrease of riboflavin concentration in the yolk (Zhang et al., 2020). This may serve as another hypothetical explanation for the effects found in the present study: antioxidant status of the egg would have been impaired by parent treatment RED, leading to poorer feed consumption and growth after hatching. Unfortunately, the antioxidant status of the eggs was not analysed in our study and the hypothesis can therefore not be tested. Understanding of the functional causalities requires physiological research, albeit the effect came out clearly: at a total dietary riboflavin concentration of 6 mg/kg for breeder hens, their offspring may show impaired growth. In contrast, the CON-CON treatment group showed growth rates fully within the range of the control groups in other studies under organic conditions using the same genotype Hubbard 757 (Eleroglu et al., 2014; Leiber et al., 2017; Ammer et al., 2017). Furthermore, the growth rates of CON-CON matched the specifications of the producer and of the Swiss organic regulations (Bio Suisse, 2021). We therefore conclude that total dietary riboflavin at 8 mg/kg (CON) for parent hens was sufficient for their broiler offspring to reach their genetic growth potential.

Regarding the direct riboflavin supplementation of broilers, the picture was similar: 2.5 mg/kg supplementation level reduced growth and, by tendency, feed consumption, while supplementation with 4.0 mg/kg resulted in normal growth rates. This result is in line with the findings of Lambertz et al. (2020) for organic rearing of another broiler genotype at different levels of riboflavin supply. The combination of both, maternal and broiler treatments, resulted in identical growth rates whenever either parents or chicks were fed the RED diet. Reduced supplementation to both, parents and chicks, did not have additive effects.

Although clear maternal and direct effects of riboflavin undersupply on intake and growth were found, no impact was evident on plumage scores, footpad integrity, or gait appearance, which would be the most expected symptoms of riboflavin deficiency (Ruiz and Harms, 1988; Shepherd and Fairchild, 2010). In contrast to the studies of Lambertz et al. (2020 and 2021), no effects on organ weights were found, despite a tendency for lower spleen and heart weights within the RED treatment group in the broilers.

General

No signs of riboflavin deficiency occurred with regard to health, performance, or egg quality in parent hens or health, weight gain, and organ weights in broilers, when the supplementation level was 4.0 mg/kg for both parents and chicks. Similarly, no health disorders and none of the common indications of riboflavin deficiency were observed in the groups with the low supplementation rate of 2.5 mg/kg, despite the lower growth rates. We conclude that both supplementation levels, which resulted in total dietary riboflavin of approximately 8 and 6 mg/kg respectively, were within a safe range regarding animal health. However, achieving this total

dietary riboflavin level with the supplementation used in this study requires a native dietary riboflavin content of 3–4 mg/kg, which is usually the case in organic feeds (Lambertz et al., 2020; Witten and Aulrich, 2019) but can also be lower (Leiber et al., 2021). The recommendation for riboflavin supplements to organic breeder hens on the basis of the present study is thus a supplementation of 4.0 mg riboflavin per kg feed to hens as well as to broilers. However, attention should be paid to achieving sufficiently high levels of native riboflavin, which can be supported by lupins, beans and grass-clover silages, but not by cereals (Witten and Aulrich, 2019).

Conclusions

Two comparably low levels of riboflavin supplementation to parent hens and their offspring (broilers) resulted in no clinical symptoms of vitamin B2 deficiency in either hens or chicks. However, lower supplementation levels to parent hens, although not causing effects on laying performance, egg quality, or hatchability, evoked lower growth rates in their offspring. The physiological causality is not clear since a riboflavin deficiency in the eggs was not found. Impacts on gene expression related to energy metabolism and oxidation appear to be a likely cause based on literature references. A supplementation level of 4 mg riboflavin per kg feed for hens and offspring resulted in healthy animals, which fully matched the performance targets of their genotypes. For organic chick production, this supplementation level is therefore recommended. However, it always important to observe broiler growth rates as early signs of deficiency in parent stock or in the chicks themselves.

Ethics approval

The trial was approved by the Cantonal Veterinary Office Aargau (Aarau, Switzerland; animal experiment permission number: 75733). The approval procedure included consideration of the 3R principles.

Data availability statement

The data that support the study findings are available upon request from the corresponding author. Data will be deposited in an official repository at a later time.

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Declaration of interest

None.

Acknowledgments

Financial support statement

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