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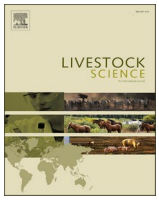

Performance and mineral status of laying hens fed diets with different levels of glyphosate

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HIGHLIGHTS

- Despite intended, the control diet was not entirely free of glyphosate residues.
- No impact of diet glyphosate on blood serum mineral status was observed.
- Glyphosate was present in eggs of hens fed diets with 0.03 mg/kg glyphosate residue.
- There were no clear adverse effects of diet glyphosate on performance.
- Post hoc contrasts showed diet glyphosate $\geq$ 20 mg/kg to reduce rate of lay 0.9%-point.

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ABSTRACT

Concern has been raised about the potential influence of glyphosate on livestock health and performance due to its antimicrobial and mineral-chelating properties. The present study investigated potential effects of feeding diets amended with glyphosate (as a commercial product, Glyphomax HL, or as pure isopropylamine salt, IPA) to laying hens (layers). At the age of 22 weeks, a total of 320 Lohmann LSL-Lite layers started on one of four treatment diets: Control (CON), 20 mg glyphosate/kg as Glyphomax HL (GM_{20}), 20 mg glyphosate/kg as IPA (IPA_{20}) and 200 mg glyphosate/kg as IPA (IPA_{200}). The EU-defined maximum residue level (MRL) of glyphosate for several feed crops is 20 mg/kg. The layers were housed in 32 enriched cages for 27 weeks. Glyphosate levels in the diets (mg/kg feed) were analysed to 0.03 for CON, 19.3 for GM_{20}, 18.5 for IPA_{20} and 191.7 for IPA_{200}. Using preselected contrasts, there were no clear effects of diet glyphosate amendment on average egg weight, egg mass, feed intake, feed conversion ratio and rate of lay, while post hoc contrasts suggested that diet glyphosate concentrations above 20 mg/kg may reduce rate of lay by 0.9%-point. Mineral levels of Mg, Ca, Mn, Fe, Cu and Zn in blood as well as indicators of welfare measured by plumage scoring and by a novel object test were not significantly affected by treatment. Glyphosate levels in blood serum (μg/L) were 0.29 for CON, 22.5 for GM_{20}, 25.0 for IPA_{20} and 128.9 for IPA_{200}. Glyphosate levels in egg yolk and egg white (ng/g) were respectively 0.26 and 0.01 for CON, 20.1 and 0.20 for GM_{20}, 21.4 and 0.25 for IPA_{20} and 223.3 and 3.09 for IPA_{200}. Glyphosate concentrations in blood serum, egg yolk and egg white reflected diet concentrations. The glyphosate degradation product, AMPA, followed the same trend as glyphosate in all analysed compartments. In conclusion, there were no clear adverse effects of glyphosate-amended diets on performance, welfare indicators and mineral status of Lohmann LSL-Lite layers even at a level approximately 10x higher than the MRL for soybeans and other common feed crops (20 mg glyphosate per kg feed). However, post hoc contrasts suggested that diet glyphosate concentrations above 20 mg/kg may reduce rate of lay by 0.9%-point. Moreover, the layers exposed down to 0.03 mg of glyphosate per kg feed produced eggs with residues of this pesticide.

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1. Introduction

The extensive use of glyphosate-based herbicides (GBH) has raised concern about the potential influence of these products on livestock health and performance, due to their antimicrobial properties and ability to form mineral complexes (Sørensen et al., 2021). Besides pre-plant application, glyphosate-based herbicides are used on genetically modified glyphosate-resistant (GR) crops for weed control and on non-GR crops for pre-harvest desiccation. Feed crops exposed to GBH are used for livestock, and data are available regarding glyphosate levels in soybeans and in cereal grains (Xu et al., 2019). However, to our knowledge, there are no studies examining glyphosate residue levels in complete livestock diets, except for the resent work of Foldager et al. (2021), who reported levels between 0.004 and 0.19 mg glyphosate per kg in diets for conventional broiler breeders.

Glyphosate works by inhibiting an enzyme in the shikimate pathway, involved in aromatic amino acid synthesis in plants, fungi, archaea and bacteria. Thus, whereas higher animals do not possess the shikimate pathway, some microorganisms in their gut do. In an *in vitro* study on poultry gut bacteria, Shehata et al. (2013) characterised a range of commensal bacteria (e.g. *Bifidobacterium adolescentis and Lactobacillus* spp.) as being more susceptible to glyphosate than certain pathogenic bacteria (e.g. *Clostridium perfringens, C. botulinum* and members of *Salmonella*). Sørensen et al. (2021) estimated glyphosate levels in individual gut compartments of livestock receiving diets with a glyphosate residue level of 20 mg/kg (the EU-defined maximum residue level (MRL) for several feed crops) and concluded that glyphosate might reach levels in the gut, affecting at least the most glyphosate-sensitive gut bacteria. We are not aware of *in vivo* studies examining glyphosate effects on the gastrointestinal tract of poultry, however in a recent study by Krogh et al. (2021), diet levels of glyphosate, equal to MRL for common feed crops, were not observed to affect faeces score and performance of newly weaned pigs. Nielsen et al. (2018) orally gavaged rats daily for two weeks with glyphosate (0, 2.5 and 25 mg/kg body weight either as glyphosate acid or as a commercial product), resulting in digesta glyphosate levels up to approximately 50 mg per kg colon digesta for the highest dose; no significant effects on gut microbiota composition was reported from this study.

As a chelator, glyphosate forms reversible complexes with cations of metals, such as Cu, Mg, Ca, Mn and Zn (Madsen et al., 1978). By forming complexes, glyphosate potentially makes these cations biologically less available to both gut microbes and the host animal. In an attempt to shed light on a potential connection between mineral status and glyphosate in livestock, dairy cows were analysed for urinary glyphosate and blood Co and Mn (Krüger et al., 2013). All cows contained urinary glyphosate and all cows also had blood concentrations of Co and Mn below the specified minimum reference levels. As Co is a central element of vitamin B12, the low Co level might indicate a deficiency of this vitamin potentially due to glyphosate chelation of Co. However, in the study by Krogh et al. (2021), it was concluded that dietary levels of glyphosate up to 10x the EU-defined MRL for common feed crops, did not affect blood serum mineral status in weaning pigs.

A memorandum on "The feeding of genetically modified glyphosate resistant soy products to livestock" to the Danish Ministry of Food and Agriculture (Sørensen et al., 2014) refer to an egg producer who observed less stress (e.g. not disturbed by loud noises), less feather pecking and higher egg production after changing from GR soybean meal to non-GR soybean meal in the diet.

Based on the referred research and farmer observations, the present study was conducted to investigate potential effects of dietary glyphosate on performance, mineral status and welfare indicators in laying hens (layers), receiving diets amended with 20 mg glyphosate/kg feed (the EU-defined MRL for common feed crops) and 10x this level (200 mg glyphosate/kg feed).

2. Material and methods

2.1. Animals and housing

All experimental procedures involving animals were approved by the Danish Animal Experimentation Council; approval no. 2017-15-0201-01229.

Lohmann LSL-Lite layers (Lohmann Breeders GmbH, Cuxhaven, Germany) 18 weeks of age and with an average body weight of 1.27 kg (SD = 0.021) were acquired from a conventional farm and 11 layers were randomly allocated to each of 32 cages at the poultry facility located at Aarhus University Foulum. The cage was the experimental unit for all variables and cages were arranged in two levels and two double-rows with back of cages facing each other. The cages had a floor area of 0.74 m^2 and were enriched with perches, a 0.15 m^2 nesting box, nipple drinkers, a manure belt underneath and a feed trough out front. On top of the nesting box, peat moss was placed daily to allow the layers to dust bathe. Peat moss was used to ensure glyphosate was not present in the bathing material. The lighting program was gradually increased from 11 to 14 h per day during the pre-treatment period and was 14 h throughout the experimental period. Light was slowly turned on and off to simulate dawn and dusk.

2.2. Treatments and feeding

Each treatment comprised eight replicates and one replicate equalled one cage with layers. The replicates were randomized so that each treatment was evenly represented in rows and levels. The layers had *ad libitum* access to feed and water; feed was supplied 2-3 times per day in order to minimize wastage. In a 4-week pre-treatment period, all layers were fed an organic layer starter diet that, compared to the diets in the treatment period, also included maize products. In the following experimental period, the layers were fed a layer diet that was adjusted according to age when the layers were 30 weeks of age. The age at diet adjustment also defines the border between two experimental periods, i.e. period 1 with layers being 22–29 weeks of age and period 2 with layers being 30–49 weeks of age. The diets were formulated to fulfil the recommendations from Lohmann Breeders GmbH. The number of layers per cage was reduced to ten during the pre-treatment period by removing a randomly selected bird from each cage at 20 weeks of age when individual weights were recorded.

Table 1
Composition of diets used in the experiment for layers being 22-29 (Per. 1) and 30-49 (Per. 2) weeks of age.

Ingredient, %	Per. 1	Per. 2	Nutrients	Per. 1	Per. 2
Wheat	53.00	58.00	Dry matter, %	89.16	88.77
Oats	8.700	7.748	Crude ash, %	12.45	12.33
Soybean meal, toasted	21.50	19.00	Crude fat, %	7.381	5.760
Fishmeal	3.000	3.000	Crude protein, %	18.35	17.76
Rapeseed oil	3.500	2.000	Crude fibre, %	3.594	3.487
Oyster shells	4.200	4.200	Starch, g/kg	350.3	373.7
Chalk, 37% Ca	4.463	4.512	Sugar, g/kg	20.49	18.49
Monocalcium phosphate	0.746	0.662	Calories, kcal/kg	2745	2677
DL-Methionine	0.110	0.110	Lysine, g/kg	9.104	8.585
Salt	0.126	0.122	Methionine, g/kg	3.952	3.882
Sodium bicarbonate	0.240	0.232	Cystine, g/kg	3.170	3.093
Vitamin premix[1]	0.400	0.400	Calcium, g/kg	36.00	36.00
Ronozyme P[2]	0.015	0.015	Phosphorus, g/kg	5.814	5.600

[1] Per kg premix: vitamin A, 2,500,000 IU; vitamin D3, 375,000 IU; 25-hydroxycholecalciferol, 9.37 mg; vitamin E/DL-α-tocopherol, 27,473 IU; vitamin K3, 1,500 mg; vitamin B1, 750 mg; vitamin B2, 3,750 mg; D-pantothenate, 3,750 mg; vitamin B6, 1,500 mg; vitamin B12, 7.5 mg; niacin, 12,500 mg; folic acid, 600 mg; biotin, 75 mg; Fe, 7,500 mg; Zn, 12,500 mg; Mn, 16,250 mg; Cu, 2,500 mg; I, 500 mg; Se, 75 mg.
[2] Phytase product from DSM, Heerlen, Netherlands.

Diet composition is shown in Table 1. The wheat used in the treatment diets was not treated with any GBH before harvest and the oat and soybean cake were of organic origin and, consequently, not treated with any GBH either. These ingredients were used to aim for no residues of glyphosate and the glyphosate degradation product, aminomethylphosphonic acid (AMPA), in the diet and to be able to control the glyphosate level in the treatment diets. The treatment diets were prepared by amending different levels and sources of glyphosate into the basic diet. The commercial GBH Glyphomax HL (Albaugh UK Ltd.; 480 g glyphosate/L, a density of 1.22 kg/L) or an isopropylamine (IPA) salt solution (Monsanto Company, USA: 459 g glyphosate/kg) were prepared for amending by first mixing the glyphosate sources into premixes and then adding these premixes into the basic diet to obtain a uniform distribution of glyphosate in the treatment diets. We are aware that there are many other GBH than Glyphomax HL on the market, however, there was capacity for including only one commercial product at a single concentration in the present study. According to the Danish safety data sheet, Glyphomax HL is with the adjuvants D-glucopyranose, oligomer, decyloctylglycoside, disodium cocoamphodipropionat and methanol but without POEA (polyethoxylated tallow amine). Glyphomax HL was also used in another of a series of experiments regarding potential effects of glyphosate on livestock performance (Krogh et al., 2021). Four treatment diets were formulated: no added glyphosate (CON), 20 mg glyphosate/kg added as Glyphomax HL (GM_{20}), 20 mg glyphosate/kg added as IPA salt (IPA_{20}) and 200 mg glyphosate/kg added as IPA salt (IPA_{200}). The rationale behind inclusion of these treatments were: IPA_{20} to investigate the glyphosate impact at a level corresponding to the maximum residue level (MRL) of glyphosate in common feed crops as set by the European Commission, GM_{20} to study influences attributable to adjuvants in the commercial glyphosate product and IPA_{200} to have a more profound difference from CON. This led to preselection of the following three treatment contrasts: (a) IPA_{20} vs CON (effect of 20 mg/kg of glyphosate), (b) IPA_{200} vs CON (effect of 200 mg/kg of glyphosate) and (c) GM_{20} vs IPA_{20} (effect of magnification with adjuvants in the commercial product). Diets were analyzed for glyphosate before use to ensure that treatments were as planned, see Table 2. The organic diet used in the pre-treatment period contained background level of 0.02 mg/kg glyphosate.

2.3. Recording and sampling

After allocation to the cages upon arrival, all layers were weighed together (per cage) and then individually at 20, 31 and 48 weeks of age. The last recordings were during the week of layer age 49 after which the layers were euthanized by a lethal dose of sodium pentobarbital.

2.3.1. Feed intake and egg production

Once per week the feed troughs were emptied and remains weighed to measure feed intake, which was determined per cage as an average daily feed intake (ADFI) per layer. Eggs were collected from each cage and counted and weighed daily. Additionally, pecked eggs, cracked eggs, eggs dirty from light manure and wind eggs were noted. Wind eggs are eggs laid without a hard shell and were not taken into account when calculating egg production data. Egg production measures and calculations included rate of lay (%) (i.e. percentage laid eggs of total layer days), average egg weight (g), eggs mass (g/layer/day) and % eggs dirty from light manure. All egg production parameters were calculated per cage per week, considering removed layers when determining per layer values (hen-day basis). From feed intake and egg production, average feed conversion ratio (FCR) was calculated as the ratio between ADFI and egg mass (g feed/g egg).

Blood was sampled from three random layers per cage at the end of the study from the vena jugularis in 6 ml silicone-coated (serum cloth activator) BD Vacutainer tubes for trace element analysis (VWR, Søborg, Denmark). Serum was harvested by centrifugation (3000 × g, 10 min, 4 °C) and stored at -20 °C until analysis; serum from each layer was analyzed for glyphosate, glyphosate degradation products and minerals.

2.3.2. Welfare indicators

A novel object test was performed at 29 and 39 weeks of age to measure fearfulness to a novel object. Layers were tested as a whole cage and not individually. A wooden stick painted with multiple colours was used as the novel object. This object was placed in the feed tray so that the object was clearly visible to the layers. The object remained in place for a period of 30 s. The 30 seconds started from when the person conducting the test had been standing still for 10 s in front of the cage. After the 30 s the object was removed, and the layer behaviour for the cage was noted as falling under one of three categories: fearful, unfearful or mixed reaction. Fearful behaviour included crowding in corners and nesting box away from the object and turning to face away from the object. Unfearful behaviour included no crowding and layers evenly spaced out, bodies facing towards or parallel to object, unmoving or moving around and layers watching or approaching object. Behaviours were categorized as having a mixed reaction when some layers displayed fearful behaviour whereas others did not.

After weighing at 48 weeks of age, plumage of individual layers was scored according to Tauson et al. (2005); a score of 4 indicates full feathering and a score of 1 indicates no feathering at all. At start of the

Table 2
Glyphosate and AMPA in diet, serum and egg yolk, and glyphosate in egg white. Layers were fed standard diets amended glyphosate as indicated (treatment). Eggs were collected at 49 weeks of age. Back-transformed EM-means (robust SEM grouped by cage) from log-normal Tobit regressions left censored at LLOQ.

	Glyphosate treatment[1]				
	CON	GM_{20}	IPA_{20}	IPA_{200}	P-value[4]
Diet[2], mg/kg					
Glyphosate	0.03	19.3	18.5	191.7	-
AMPA	0.009	0.096	0.073	0.657	-
Serum, µg/L					-
Glyphosate	$0.29_{(0.110)}$	$22.5_{(1.19)}$	$25.0_{(1.48)}$	$128.9_{(6.19)}$	<0.001 ***,¤¤¤,ns3
AMPA[3]	$<0.01_{(<0.001)}$	$0.20_{(0.032)}$	$0.118_{(0.032)}$	$2.21_{(0.161)}$	<0.001 ***,¤¤¤,ns3
Egg yolk[5], ng/g					
Glyphosate	$0.26_{(0.079)}$	$20.1_{(0.86)}$	$21.4_{(1.09)}$	$223.3_{(8.83)}$	<0.001 ***,¤¤¤,ns3
Egg white, ng/g					
Glyphosate[3]	$0.01_{(0.002)}$	$0.20_{(0.021)}$	$0.25_{(0.029)}$	$3.09_{(0.136)}$	<0.001 ***,¤¤¤,ns3

[1] Glyphosate amended per kg standard diet feed; CON: 0 mg, GM_{20}: 20 mg Glyphomax HL, IPA_{20}: 20 mg isopropylamine (IPA) salt of glyphosate, IPA_{200}: 200 mg IPA salt of glyphosate.

[2] Measured in single composite samples, thus no standard error of mean.

[3] All values in CON were below LLOQ.

[4] ^{ns1} $p_{adj} \geq 0.05$, * $p_{adj} < 0.05$, ** $p_{adj} < 0.01$, *** $p_{adj} < 0.001$ (IPA_{20} vs CON); ^{ns2} $p_{adj} \geq 0.05$, $^{¤}$ $p_{adj} < 0.05$, $^{¤¤}$ $p_{adj} < 0.01$, $^{¤¤¤}$ $p_{adj} < 0.001$ (IPA_{200} vs CON); ^{ns3} $p_{adj} \geq 0.05$, $^{\#}$ $p_{adj} < 0.05$, $^{\#\#}$ $p_{adj} < 0.01$, $^{\#\#\#}$ $p_{adj} < 0.001$ (GM_{20} vs IPA_{20}).

[5] AMPA was below LLOQ in all treatments except for IPA_{200} with a mean of 6.5 ng/g (SD = 1.8).

treatments, the plumage was not scored individually, however all layers were judged to have full feather coverage.

2.3.3. Chemical analyses

Glyphosate and glyphosate degradation products in diets and serum were analysed according to the microLC-MS/MS method described and validated by Nørskov et al. (2019). Whereas the glyphosate degradation product AMPA could be quantified, the degradation products N-acetyl glyphosate and N-acetyl aminomethylphosphonic acid were below the Lower Limits of Quantification (LLOQ). Analyses of glyphosate and AMPA in egg yolk and white were not included in our description of the method (Nørskov et al., 2019) however performed on the same microLC-MS/MS system with the following sample preparation procedure. Egg white and egg yolk were separated and homogenised with a blender for 5–10 s to get a smooth textured liquid. Two gram of egg white and 0.5 g of egg yolk were weighted and added 7.24 and 9.75 mL of 1% formic acid in water, containing internal standards for glyphosate and AMPA with the total volume was 9 and 10 mL, respectively. The samples were mixed for 10–20 min on a vortex mixer and centrifuged for 15 min at 4000 × g at 5 °C. Further, 2 mL supernatant were transferred to a new tube and centrifuged for 10 min at 14,000 × g at 5 °C. In case of egg white, supernatant was further centrifuged through a protein 10 kD cut-off filter (centrifugal filter with modified PES and MWOC, VWR, Germany) for 30 min at 14,000 × g at 30 °C and analysed on microLC-MS/MS. In case of egg yolk, defatting was performed on C18 SPE column and the flow through was collected and centrifuged through the 10 kD cut-off filter for 60 min at 14,000 × g at 30 °C and analysed on microLC-MS/MS. Sample preparation procedure was validated using spiking experiments with five replicates for each of the three spiked concentrations (0.1, 1.0 and 5.0 ng/g for both glyphosate and AMPA) and using egg white and yolk from CON group as spiking matrix. Average recoveries were 101–108% for glyphosate and 98–106% for AMPA; LLOQ were 0.1 ng/g for glyphosate and 1.0 ng/g for AMPA. Quality control samples spiked with low and high concentration of glyphosate were run with each batch and used for calculation of the accuracy. The deviations were within ± 15% of the spiked concentrations, which is the generally accepted range in guidelines for validation of bioanalytical methods.

The reported minerals are based on the following isotopes: ^{57}Fe, ^{66}Zn, ^{63}Cu, ^{55}Mn, ^{43}Ca and ^{24}Mg. Serum was prepared and analysed for minerals by ICP-MS as described by Krogh et al. (2021).

2.4. Statistical analyses

Statistical analyses were carried out using the statistical software R 4.1.2 (R Core Team, 2021) and a significance level of 0.05. In the following, LME will refer to normal linear mixed effects models fitted by use of *lme* and NLME will refer to nonlinear mixed effects models using *nlme*, both from the *nlme* package v. 3.1–153. Main effect of diet treatment was examined by χ^2 likelihood ratio testing on 3 degrees of freedom (df) and the three contrasts as defined in Section 2.2 were tested, generally using *glht* from the *multcomp* package v. 1.4–17. These contrast tests were adjusted for multiple testing by the default single-step method in *glht*. In cases where an interaction between a factor and treatment was included, the number of contrasts tested simultaneously were three times the number of categories of the factor. For NLME, the three contrasts were examined separately for each parameter, i.e. as separate hypotheses with regard to adjustment for multiple testing. *P*-values that are adjusted for multiple testing will be denoted by p_{adj}.

2.4.1. Glyphosate and AMPA in blood serum and eggs

Glyphosate and AMPA in serum, and glyphosate in egg yolk and white were analysed by log-normal Tobit regressions by use of *Surv* and *survreg* from the *survival* package v. 3.2–13. The Tobit regressions had left censoring at LLOQ (0.05 μg/L for serum, 0.1 ng/g for glyphosate in egg yolk and white), glyphosate treatment included as a fixed effect and robust standard errors grouped by cage (cluster option). Results shown as back-transformed estimated marginal means (EM-means) and standard error of means (SEM) using *emmeans* from the *emmeans* package v. 1.7.1–1.

2.4.2. Performance

Average (in cage) layer weight at ages 20, 31 and 48 were analysed by LME with layer age (categorical), treatment and their interaction as fixed effects, and cage as random effect. Interaction and main effect of treatment after removal of interaction (i.e. in the additive model of age and treatment) were tested by χ^2 likelihood ratio tests.

ADFI was modelled by NLME using the following 4-parameters logistic function

$$f_{ADFI}(\widetilde{w}) = \alpha + \frac{(\beta - \alpha)}{1 + e^{(\gamma - \widetilde{w})/\delta}}$$

of layer age (in weeks, w) centred at age 22, i.e. $\widetilde{w} = w - 22$. Here β is the limit as w increase (i.e. the plateau reached a few weeks into period 2) and α is the limit to the left. The parameter γ is the inflection point, i.e. $g(\gamma) = (\alpha + \beta)/2$, and δ is a scale parameter. Note that if γ/δ is relatively large, $f_{ADFI}(0) \sim \alpha$. Cage was included as random effect, allowing each cage to differ from the common parameters of α and β. Corresponding random effects for γ and δ were close to zero and left out to avoid numerical instabilities during estimation. Moreover, a first order autoregressive (AR1) correlation structure was included to handle correlation among observations over weeks from the same cage. The only fixed effect was diet treatment allowing different mean values of the three model parameters α, β and γ for each treatment. Models allowing δ to depend on treatment could not converge. Results are shown as EM-means and SEM for α and β with *p*-values from χ^2 likelihood ratio test on 3 df given for the overall treatment effect on each of these parameters. Moreover, an overall test on 9 df is shown corresponding to omission of treatment dependence simultaneously for α, β and γ. Apart from this, estimates and tests are not shown for γ and δ.

Average egg weight and egg mass were modelled by NLME using the following asymptotic function

$$f_{egg}(\widetilde{w}) = \beta + (\alpha - \beta)e^{-\widetilde{w}e^{-\gamma}}$$

Here β is the limit as w increase (i.e. the plateau reached a few weeks into period 2) and α is the egg mass for $\widetilde{w} = 0$, i.e. at age 22 weeks. Finally, γ is a numeric parameter representing the natural logarithm of the rate constant. We included cage as random effect, allowing each cage to differ from the common parameters of α, β and γ. Moreover, we allowed for heterogeneous variance in treatment and an AR1 correlation structure was included to handle correlation among observations over weeks from the same cage. The only fixed effect was diet treatment allowing different mean values of the three model parameters for each treatment. Results are shown along the lines described for ADFI but only for α and β, except from an overall 9 df test.

In addition, we determined ADFI at age 22, 30 and 48 weeks, i.e. after the first week of period 1, first week of period 2 and last week of period 2, and the egg weight and egg mass at age 30 and 48, e.g. $f_{ADFI}(w = 22)$ and $f_{egg}(w = 30)$. To calculate SEM for each treatment and the three contrasts for these predictions, we applied bootstrapping using 1000 replications with stratification on treatment and age, and with maintenance of cage number. That is, a bootstrap iteration consist of sampling with replacement from the observations within each treatment and age combination a sample of the same size as the original (i.e. 8) and assigning these 8 values to the eight 'original' cages. A simpler bootstrapping procedure would ruin the possibility of running the models with AR1 correlation, as some cages would then have more than one sample for same ages. Wald's z-tests (standard normal) of the contrasts were calculated as the contrast estimate divided by the bootstrapped

SEM, and with p-values adjusted by the (Holm, 1979) method.

The feed conversion ratio was calculated directly from ADFI and egg mass as

$$f_{FCR}(\widetilde{w}) = f_{ADFI}(\widetilde{w}) / f_{egg}(\widetilde{w})$$

Predictions are shown at ages 22, 30 and 48 with SEM and contrast testing via bootstrapping as described above.

For rate of lay as well as percentage of cracked, pecked, wind and dirty eggs in layer ages 22–48 weeks, back-transformed EM-means were obtained from mixed effects logistic regressions using *glmmTMB* from the *glmmTMB* package v. 1.1.2.3 including period, glyphosate treatment and their interaction as fixed effects. Week was included as random effect and an AR1 correlation structure was used to handle correlation among observations over weeks from the same cage. However, due to convergence problems cage was included as a random effect for the analysis of wind eggs, i.e. using a compound symmetry correlation structure within cage.

2.4.3. Welfare indicators

Indicators of animal welfare measured from layers 48 weeks of age. The total sum of the six body scores (i.e., 6–24) was examined by a mixed effects Poisson regression using *glmer* from the *lme4* package v. 1.1–27.1, whereas Poor (total sum $\leq$ 12) and Good (total sum $\geq$ 18) plumage were analysed by mixed effects logistic regressions using *glmer*. Results shown as back-transformed EM-means with diet treatment as fixed effect and cage as random effect.

The novel object test data presented as total number of cages by category were tested separately for the two layer ages by Fisher's exact test. Moreover, the change within cage from age 29 to 39 were investigated as more fearful, unchanged or less fearful and tested by Fisher's exact test.

2.4.4. Mineral status

Serum minerals at 49 weeks of age. Serum was extracted from blood samples of three randomly selected layers from each cage. Results are shown as EM-means from LME with diet treatment group as the fixed effect of interest and cage as random effect. For the micro minerals (Mn, Fe, Cu and Zn), log-transformation was applied to not violate the assumption of normal distribution and for these, EM-means and SEM are shown after back-transformation.

Egg characteristics at the end of study (49 weeks of age). Two eggs from each cage were measured (except from three cages with only 1 egg and one cage without measured eggs). Egg weight, shell weight and shell thickness were analysed by LME with diet treatment as the fixed effect of interest and cage as random effect. Analyses of shell weight and shell thickness were furthermore adjusted for egg weight and included heterogeneous treatment-dependent variances.

3. Results

During the experiment, six layers were removed from the cages and euthanized by cervical dislocation. Two of the six layers were from treatment GM_{20} (day 30 and 32 after commencing the treatments), three were from treatment IPA_{20} (day 84, 141 and 176) and one was from IPA_{200} (134 days after treatment start). The layer euthanized on day 30 had stopped eating and was thin. The layer euthanized on day 32 was thin and was suspected to have an intestinal infection. The layers euthanized on day 84, 134 and 141 had toe or neck injuries. The layer euthanized on day 176 was thin. The number of layers euthanized is accounted for in the data on feed consumption and egg production, i.e. data are per layer per day.

3.1. Glyphosate and AMPA in diets, blood and eggs

The glyphosate levels in each of the three amended treatment diets were close to the planned levels, however, the level in CON was not entirely zero (Table 2). The amount of AMPA was approximately 0.4% of that of glyphosate except for CON where it amounted to 30% of that of glyphosate. As mentioned in Section 2.2, we only selected three treatment contrasts: IPA_{20} vs CON, IPA_{200} vs CON and GM_{20} vs IPA_{20}.

The concentrations of glyphosate blood serum, egg yolk and white increased with increasing concentrations in the diet (Table 2). The same trend was apparent for AMPA in serum, whereas AMPA in egg yolk and white was below LLOQ for all samples except for AMPA in yolk of the IPA_{200} treatment. The glyphosate degradation products N-acetyl glyphosate and N-acetyl aminomethylphosphonic acid were below LLOQ in all samples.

3.2. Performance

Due to the many experimental procedures taking place in the last week of the study (49 weeks of age), including euthanising of all layers over two days, performance data from this week was excluded from the analyses. Layer weight, ADFI, egg weight, egg mass, rate of lay and FCR are presented in Table 3 and the estimated curves from the described models for ADFI, egg weight, egg mass and FCR are illustrated in Fig. 1. Neither average layer weight, ADFI, egg weight, egg mass nor FCR were found to be affected by addition of Glyphomax HL or IPA to the diet. With regard to rate of lay, the only significant result was IPA_{20} being lower than CON in the main effects model ($p_{adj} = 0.021$) and for period 2 in the interaction model (P $\times$ T, $p_{adj} = 0.044$); IPA_{200} was not different from CON though. Post hoc contrasts comparing the three treatment groups with diets amended glyphosate above 20 mg/kg to the CON group indicated a difference for period 2 ($p_{adj} = 0.021$) in the interaction model. The corresponding contrast in the main effects model also showed a difference ($p = 0.007$).

Across all treatments and experimental periods, we found no significant differences in the percentage of cracked, pecked or wind eggs or in the occurrence of eggs dirty from light manure that was monitored as an indicator of intestinal health (Table 4).

3.3. Welfare indicators

In addition to live weight that is mentioned above (Table 3), welfare indicators included plumage score (Table 5) and a novel object test (Table 6). Before the start of experimental treatments, all layers were judged to have full feather coverage. Individual plumage scores were attained from six body parts (tail, breast, cloaca, back, neck, wings) at the same time as weighing the layers at 48 weeks of age. The data were accumulated over the six body parts and in addition presented as probabilities of having good or a poor plumage (Table 5). Generally, the scores were very different among cages regardless of treatment and no differences among the selected contrasts were seen. In Table 6, the novel object test results are presented as a total number of cages within each category: fearful, unfearful or mixed reaction. No differences were found across treatments. In addition, change in reaction within cage from age 29 to age 39 was examined as being worse (more fearful), unchanged or better (less fearful).

3.4. Mineral status

Mineral status as indicated by blood serum concentrations are presented in Table 7. Neither for the micro minerals (Mn, Fe, Cu and Zn) nor the macro minerals (Mg and Ca) did we find effects of treatments. In accordance, we did not find treatment effects for eggshell weight or for shell thickness (Table 8).

4. Discussion

Based on the EU-defined MRL for soybeans and other common feed crops, the dietary treatment level of 20 mg of glyphosate/kg and tenfold

Table 3
Layer weight, rate of lay of layers from 22 to 48 weeks of age, average daily feed intake (ADFI), average egg mass and average feed conversion ratio (FCR). Layers were fed standard diets amended glyphosate as indicated (treatment). EM-means (SEM) from (generalised) linear or non-linear mixed effects models including mainly glyphosate treatment (T) as fixed effect. The models for layer weight and rate of lay also included interaction between week (W) and T, respectively between period (P) and T. See further model details in Statistical analyses section.

	Glyphosate treatment[1]				P-value[5]	
	CON	GM_{20}	IPA_{20}	IPA_{200}	Int.act.	T[6]
					W × T	
Layer weight, kg[7]					0.47 ^{ns1,ns2,ns3}	0.73 ^{ns1,ns2,ns3}
Age 20 weeks	$1.45_{(0.017)}$	$1.45_{(0.015)}$	$1.44_{(0.014)}$	$1.44_{(0.016)}$	^{ns1,ns2,ns3}	
Age 31 weeks	$1.66_{(0.017)}$	$1.66_{(0.015)}$	$1.65_{(0.014)}$	$1.65_{(0.016)}$	^{ns1,ns2,ns3}	
Age 48 weeks	$1.64_{(0.017)}$	$1.64_{(0.015)}$	$1.63_{(0.014)}$	$1.60_{(0.016)}$	^{ns1,ns2,ns3}	
					P × T	
Rate of lay, %[2]					0.92 ^{ns1,ns2,ns3}	0.06 ^{ns2,ns3}
Period 1	$97.9_{(0.41)}$	$97.2_{(0.51)}$	$97.0_{(0.55)}$	$97.1_{(0.52)}$	^{ns2,ns3,}	
Period 2	$98.1_{(0.28)}$	$97.3_{(0.39)}$	$96.9_{(0.44)}$	$97.4_{(0.37)}$		
Average egg weight, g						0.27
Age 22 (α in model)	$55.9_{(0.51)}$	$54.8_{(0.56)}$	$55.3_{(0.48)}$	$55.5_{(0.57)}$	-	0.55 ^{ns1,ns2,ns3}
Age 30 (prediction)[3]	$62.4_{(0.50)}$	$62.8_{(0.24)}$	$61.8_{(0.37)}$	$62.2_{(0.34)}$	-	- ^{ns1,ns2,ns3}
Age 48 (prediction)[3]	$63.1_{(0.48)}$	$63.2_{(0.26)}$	$63.0_{(0.41)}$	$63.1_{(0.51)}$	-	- ^{ns1,ns2,ns3}
Right limit (*β in model*)	$63.1_{(0.38)}$	$63.2_{(0.38)}$	$63.1_{(0.38)}$	$63.1_{(0.40)}$	-	>0.99 ^{ns1,ns2,ns3}
ADFI, g/layer/day						0.55
Left limit (α)	$111.9_{(1.02)}$	$110.2_{(1.01)}$	$110.0_{(1.05)}$	$109.7_{(1.04)}$	-	0.47 ^{ns1,ns2,ns3}
Age 22 (prediction)[3]	$61.2_{(0.44)}$	$61.0_{(0.42)}$	$59.9_{(0.73)}$	$60.7_{(0.30)}$	-	- ^{ns1,ns2,ns3}
Age 30 (prediction)[3]	$61.2_{(0.44)}$	$61.0_{(0.42)}$	$59.9_{(0.73)}$	$60.7_{(0.30)}$	-	- ^{ns1,ns2,ns3}
Age 48 (prediction)[3]	$61.2_{(0.44)}$	$61.0_{(0.42)}$	$59.9_{(0.73)}$	$60.7_{(0.30)}$	-	- ^{ns1,ns2,ns3}
Right limit (β)	$118.3_{(1.40)}$	$116.0_{(1.40)}$	$115.3_{(1.41)}$	$115.9_{(1.41)}$	-	0.47 ^{ns1,ns2,ns3}
Egg mass, g/layer/day						0.46
Age 22 (α in model)	$53.6_{(0.95)}$	$51.3_{(1.01)}$	$50.6_{(1.01)}$	$51.1_{(1.13)}$	-	0.17 ^{ns1,ns2,ns3}
Age 30 (prediction)[3]	$61.2_{(0.44)}$	$61.0_{(0.42)}$	$59.9_{(0.73)}$	$60.7_{(0.30)}$	-	- ^{ns1,ns2,ns3}
Age 48 (prediction)[3]	$61.7_{(0.56)}$	$61.3_{(0.46)}$	$60.3_{(0.75)}$	$61.1_{(0.70)}$	-	- ^{ns1,ns2,ns3}
Right limit (*β in model*)	$61.7_{(0.52)}$	$61.3_{(0.52)}$	$60.3_{(0.53)}$	$61.1_{(0.55)}$	-	0.32 ^{ns1,ns2,ns3}
FCR, g feed/g egg[3,4]						
Age 22	$2.08_{(0.038)}$	$2.15_{(0.027)}$	$2.17_{(0.047)}$	$2.15_{(0.066)}$	-	- ^{ns1,ns2,ns3}
Age 30	$1.83_{(0.014)}$	$1.81_{(0.019)}$	$1.84_{(0.026)}$	$1.81_{(0.015)}$	-	- ^{ns1,ns2,ns3}
Age 48	$1.92_{(0.029)}$	$1.89_{(0.013)}$	$1.91_{(0.026)}$	$1.90_{(0.012)}$	-	- ^{ns1,ns2,ns3}

[1] Glyphosate amended per kg standard diet feed; CON: 0 mg, GM_{20}: 20 mg Glyphomax HL, IPA_{20}: 20 mg isopropylamine (IPA) salt of glyphosate, IPA_{200}: 200 mg IPA salt of glyphosate.
[2] Period 1: 22–29 weeks of age; Period 2: 30–48 weeks of age.
[3] Standard errors of means and contrast testing obtained by bootstrapping with 1000 replications.
[4] FCR was determined directly as ADFI divided by egg mass.
[5] $^{ns1}p_{adj} \geq 0.05$, $^{*}p_{adj} < 0.05$, $^{**}p_{adj} < 0.01$, $^{***}p_{adj} < 0.001$ (IPA_{20} vs CON); $^{ns2}p_{adj} \geq 0.05$, $^{¤}p_{adj} < 0.05$, $^{¤¤}p_{adj} < 0.01$, $^{¤¤¤}p_{adj} < 0.001$ (IPA_{200} vs CON); $^{ns3}p_{adj} \geq 0.05$, $^{\#}p_{adj} < 0.05$, $^{\#\#}p_{adj} < 0.01$, $^{\#\#\#}p_{adj} < 0.001$ (GM_{20} vs IPA_{20}).
[6] Main effect of glyphosate treatment in the additive model (i.e., excl. W × T or P × T).
[7] After allocation at arrival at 18 weeks of age, weight was 1.27 kg (SEM = 0.008) in all treatments.

this level were selected. The measured levels of glyphosate in the amended diets were close to the intended levels but that of the control diet (CON) was not entirely zero, indicating that feed free of glyphosate is difficult to obtain (Table 2). We are not aware of reports on actual glyphosate residue levels in poultry feed, except for a recently published work from our lab, reporting levels between 0.004 and 0.19 mg per kg in diets for conventional broiler breeders (Foldager et al., 2021). The EU-defined MRL for glyphosate is obviously not typically observed in commercial settings, thus the MRL may be regarded as a theoretical upper limit.

The origin of glyphosate and AMPA in the CON diet (Table 2) is not known because the feed ingredients were either organic or not treated with glyphosate. However, glyphosate is metabolised in plants (Duke, 2011), thus the high amount of AMPA relative to glyphosate indicates that the diets contained material from plants exposed to glyphosate during a growth phase where pesticide uptake is possible. Exposure could be foliar uptake from airborne glyphosate-containing aerosols through spray application in neighbouring fields (Tzanetou and Karasali, 2020) or root uptake from a soil pool (Simonsen et al., 2008) of which foliar uptake from the airborne aerosols most likely is the quantitatively important source. Glyphosate could also originate from glyphosate-containing aerosols ending up on mature grain or soybean crop not purposely treated with glyphosate. Glyphosate is a systemic herbicide that is absorbed in GR crops that are treated during growth, while it remains on the surface of ripe crop when used for pre-harvest desiccation of weeds. Amending glyphosate to the diets, as done in the present experiments, therefore imitates desiccation, however we are not aware of data indicating differences in bioavailability depending on glyphosate being absorbed or remaining on the plant surface.

The glyphosate and AMPA levels in blood serum reflected the levels in the diet. Glyphosate was found in eggs of hens from all four treatment groups and was around 100 fold higher in yolk than in white, while AMPA was below LLOQ in yolk and white except for AMPA in yolk for IPA_{200}. A higher concentration of glyphosate in egg yolk compared to egg white was also reported by FAO (2005). Under normal practical circumstances (Foldager et al., 2021), layers will be fed diets with glyphosate residues much below 20 mg/kg that is the MRL for common feed crops. Thus our data implies that although glyphosate residues was found in eggs from hens on the CON diet with 0.03 mg glyphosate per kg feed, residues in eggs will in general be well below 50 ng/g, which is the MRL for eggs in the EU. This implication is supported by the work of Foldager et al. (2021), where glyphosate was reported to be below LLOQ in yolk and white from organic as well as conventional eggs obtained from groceries.

The performance data for the layers in the present experiment deviated somewhat from those listed in the Lohmann LSL-Lite layers management guide (Lohmann Breeders, 2021). In week 18, 20 and 31, the layers had a higher average body weight (1.27, 1.45 and 1.66 kg,

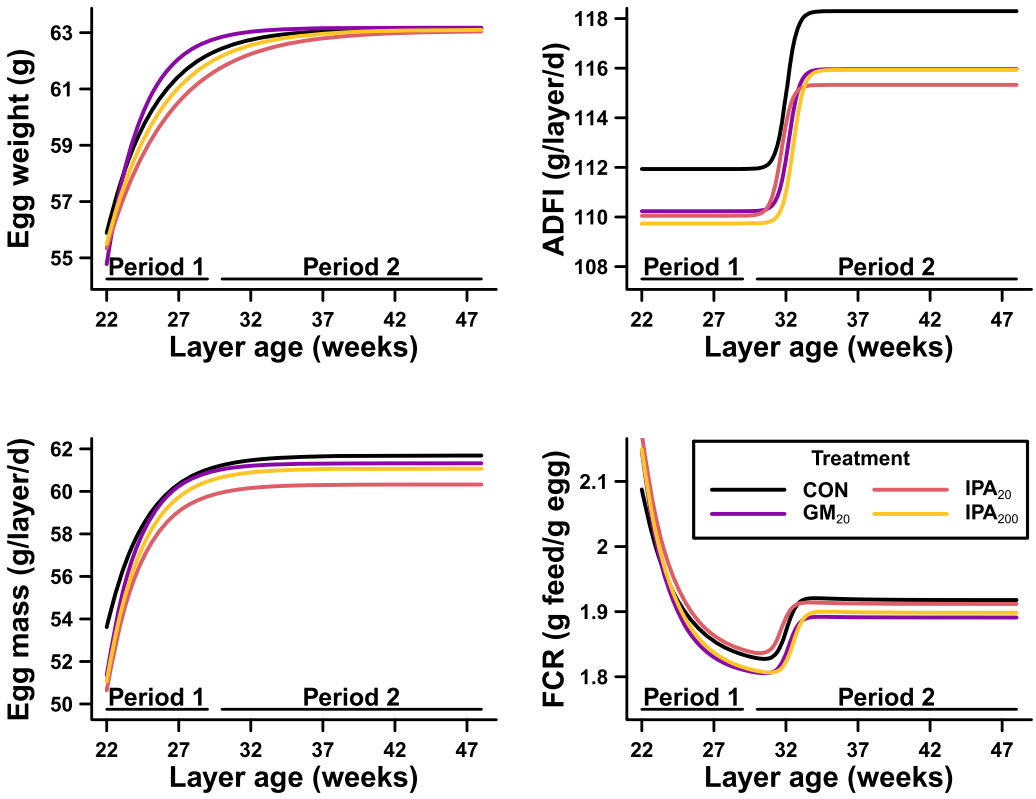

Fig. 1. The estimated curves from the models described in the statistical analyses section for average egg weight, ADFI, average daily egg mass and feed conversion ratio (FCR).

Table 4
Percentage of cracked, pecked, wind and dirty eggs from layers aged 22 to 48 weeks. Layers were fed standard diets amended glyphosate as indicated (treatment). Back-transformed EM-means (SEM) from mixed effects logistic regressions including period[1] (P), glyphosate treatment (T) and their interaction (P × T) as fixed effects. Week was included as random effect and an AR1 correlation structure (compound symmetry for wind eggs) to handle correlation among observations over weeks from the same cage.

	Glyphosate treatment[2]				*P*-value	
	CON	GM_{20}	IPA_{20}	IPA_{200}	P × T	T[3]
Cracked eggs, %[4]					0.28	0.17
Period 1	$0.28_{(0.094)}$	$0.35_{(0.111)}$	$0.41_{(0.122)}$	$0.27_{(0.094)}$		
Period 2	$0.48_{(0.109)}$	$0.37_{(0.089)}$	$0.82_{(0.173)}$	$0.71_{(0.153)}$		
Pecked eggs, %[4]					0.18	0.46
Period 1	$0.15_{(0.070)}$	$0.07_{(0.041)}$	$0.20_{(0.083)}$	$0.09_{(0.048)}$		
Period 2	$0.16_{(0.054)}$	$0.12_{(0.045)}$	$0.16_{(0.057)}$	$0.26_{(0.083)}$		
Wind eggs, %[4]					0.39	0.90
Period 1	$0.19_{(0.101)}$	$0.20_{(0.109)}$	$0.17_{(0.092)}$	$0.11_{(0.067)}$		
Period 2	$0.02_{(0.013)}$	$0.02_{(0.014)}$	$0.04_{(0.024)}$	$0.02_{(0.015)}$		
Manure[5] dirty eggs, %					0.73	0.83
Period 1	$15.2_{(1.77)}$	$16.1_{(1.85)}$	$14.1_{(1.71)}$	$13.7_{(1.69)}$		
Period 2	$16.1_{(1.04)}$	$16.9_{(1.07)}$	$17.2_{(1.09)}$	$16.4_{(1.05)}$		

[1] Period 1: 22–29 weeks of age; Period 2: 30–48 weeks of age.
[2] Glyphosate amended per kg standard diet feed; CON: 0 mg, GM_{20}: 20 mg Glyphomax HL, IPA_{20}: 20 mg isopropylamine (IPA) salt of glyphosate, IPA_{200}: 200 mg IPA salt of glyphosate.
[3] Main effect of glyphosate treatment in the additive model (i.e., excl. P × T interaction).
[4] Proportion of total number of eggs incl. cracked, pecked and wind eggs.
[5] Thin, light manure residue.

respectively) than expected (1.25, 1.37 and 1.63 kg). However, in week 48 the layers had an average weight of 1.64 kg when 1.66 kg is expected and this lower weight could be due to a higher than expected egg production. Egg mass was consistently higher than expected in week 31 and week 48 with our layers producing an average of 60.8 and 61.4 g per layer per day respectively compared to the expected egg mass of 56.7 and 58.7 g per layer per day. The rate of lay was also higher in week 31 and 48 with an average of 97.7 and 96.8%, respectively compared to the expected rate of 95.8 and 93.8%. Thus, our layers consistently performed better than expected as compared to the Lohmann LSL-Lite layers management guide.

Our results show that layer performance was not affected by the dietary inclusion of glyphosate from Glyphomax HL or IPA as neither average weight per layer, ADFI, egg mass nor FCR were significantly different across treatment groups. Eason and Scanlon (2002) also found that glyphosate added to feed at different concentrations (sprayed on floor-fed grain to apparent glyphosate levels of 347, 1388 and 3470 mg/kg) did not influence the body weight in quails. In agreement with our results, Ruuskanen et al. (2019) also found no effect on egg mass of adding 200 mg glyphosate per kg feed.

Rate of lay of IPA_{20} was significantly lower than control in period 2. We have no explanation for this effect of glyphosate and because a difference was not observed when the glyphosate concentration was ten times higher. A non-monotonic dose response may be a possibility, however only two points and the negative control included in the response curve makes it difficult to argue for this possibility; a false positive may be another possibility. During egg collection, eggs dirty from light manure were counted to monitor treatment effects on diarrhoea-like conditions and thus as an indicator of impaired gut health. However, there were no significant treatment differences in the frequency of dirty eggs, indicating that no severe perturbations of the gut microbiota had occurred. In agreement, Krogh et al. (2021) observed no effect on faeces score in weaned pigs fed diet levels of glyphosate

Table 5
Indicators of animal welfare measured from layers 48 weeks of age. Layers were fed standard diets amended glyphosate as indicated (treatment). The total sum of the six body scores (i.e., 6–24) was examined by a mixed effects Poisson regression, whereas Poor (total sum ≤ 12) and Good (total sum ≥ 18) plumage were analysed by mixed effects logistic regressions. Results shown as back-transformed EM-means (SEM) with diet treatment as fixed effect and cage as random effect.

	Glyphosate treatment[1]				
	CON	GM_{20}	IPA_{20}	IPA_{200}	P-value
Total sum of scores (mean)[2]	$16.1_{(0.58)}$	$16.1_{(0.58)}$	$15.3_{(0.57)}$	$15.8_{(0.57)}$	0.78
Poor plumage, total ≤ 12 (prob.)	$0.005_{(0.0084)}$	$0.010_{(0.0154)}$	$0.020_{(0.0297)}$	$0.007_{(0.0105)}$	0.87
Good plumage, total ≥ 18 (prob.)	$0.35_{(0.082)}$	$0.26_{(0.073)}$	$0.18_{(0.058)}$	$0.22_{(0.066)}$	0.40

[1] Glyphosate amended per kg standard diet feed; CON: 0 mg, GM_{20}: 20 mg Glyphomax HL, IPA_{20}: 20 mg isopropylamine (IPA) salt of glyphosate, IPA_{200}: 200 mg IPA salt of glyphosate.
[2] Sum of six body scores (tail, breast, cloaca, back, neck, wings), i.e. between 6 and 24.

Table 6
Novel object test. Layers were fed standard diets amended glyphosate as indicated (treatment). Data are presented as total number of cages by category.

	Glyphosate treatment[1]				
	CON	GM_{20}	IPA_{20}	IPA_{200}	P-value[2]
Layer age 29 weeks					0.23
Fearful	4	4	6	1	
Mixed	2	2	0	2	
Unfearful	2	2	2	5	
Layer age 39 weeks					0.67
Fearful	3	4	5	3	
Mixed	3	1	1	4	
Unfearful	2	3	2	1	
Change from age 29 to 39 weeks					0.37
More fearful	2	3	1	5	
Unchanged	4	3	6	3	
Less fearful	2	2	1	0	

[1] Glyphosate amended per kg standard diet feed; CON: 0 mg, GM_{20}: 20 mg Glyphomax HL, IPA_{20}: 20 mg isopropylamine (IPA) salt of glyphosate, IPA_{200}: 200 mg IPA salt of glyphosate.
[2] Fisher's exact test.

Table 7
Serum minerals at 49 weeks of age. Serum was extracted from blood samples of three randomly selected layers from each cage. Layers were fed standard diets amended glyphosate as indicated (treatment). Results are shown as EM-means (SEM) from linear mixed effects models with diet treatment as the fixed effect of interest and cage as random effect. For the micro minerals (Mn, Fe, Cu and Zn), log-transformation was applied to not violate the assumption of normal distribution and for these, estimates and SEM are shown after back-transformation.

	Glyphosate treatment[1]				
	CON	GM_{20}	IPA_{20}	IPA_{200}	P-value
Mn, µg/L	$166_{(9.6)}$	$183_{(10.6)}$	$185_{(10.7)}$	$185_{(10.7)}$	0.45
Fe, µg/L	$478_{(17.6)}$	$454_{(16.8)}$	$505_{(18.6)}$	$482_{(17.6)}$	0.23
Cu, µg/L	$310_{(9.8)}$	$323_{(10.0)}$	$329_{(10.2)}$	$309_{(9.6)}$	0.35
Zn, µg/L	$4859_{(191.7)}$	$5042_{(198.9)}$	$5086_{(203.6)}$	$5166_{(203.8)}$	0.69
Mg, mg/L	$33.1_{(0.87)}$	$34.0_{(0.87)}$	$34.9_{(0.87)}$	$34.0_{(0.87)}$	0.48
Ca, mg/L	$286_{(15.6)}$	$303_{(15.6)}$	$322_{(15.6)}$	$301_{(15.6)}$	0.39

[1] Glyphosate amended per kg standard diet feed; CON: 0 mg, GM_{20}: 20 mg Glyphomax HL, IPA_{20}: 20 mg isopropylamine (IPA) salt of glyphosate, IPA_{200}: 200 mg IPA salt of glyphosate.

similar to the present study.

Farmer observations indicated that dietary glyphosate might negatively affect layer welfare (Sørensen et al., 2014). In the present study, welfare was assessed through plumage scoring and the novel object test. These assessments were found not to be significantly affected by glyphosate, however more animal welfare indicators need to be investigated for a general conclusion regarding welfare.

The ability of glyphosate to form complexes with metal cations did not affect the micro (Mn, Fe, Cu, and Zn) and macro (Mg and Ca) mineral status in layers as there were no significant differences in blood serum levels of these minerals across treatment groups. Eggshell quality

Table 8
Egg characteristics at the end of study (49 weeks of age). Egg weight, shell weight and shell thickness were measured. Layers were fed standard diets amended glyphosate as indicated (treatment). Results are shown as EM-means (SEM) from linear mixed effects models with diet treatment as the fixed effect of interest and cage as random effect. Analyses of shell weight and shell thickness were furthermore adjusted for egg weight and included heterogeneous treatment-dependent variances.

	Glyphosate treatment[1]				
	CON	GM_{20}	IPA_{20}	IPA_{200}	P-value
Egg weight, g	$63.6_{(0.94)}$	$64.9_{(0.94)}$	$65.5_{(0.91)}$	$63.6_{(1.00)}$	0.35
Shell weight, g	$6.58_{(0.072)}$	$6.49_{(0.082)}$	$6.51_{(0.060)}$	$6.42_{(0.139)}$	0.66
Shell thickness, µm	$430_{(4.6)}$	$423_{(5.3)}$	$426_{(3.6)}$	$422_{(8.3)}$	0.75

[1] Glyphosate amended per kg standard diet feed; CON: 0 mg, GM_{20}: 20 mg Glyphomax HL, IPA_{20}: 20 mg isopropylamine (IPA) salt of glyphosate, IPA_{200}: 200 mg IPA salt of glyphosate.

(weight and thickness) was also not affected by treatment, which is in agreement with results of Ruuskanen et al. (2019) who found no effect of adding 200 mg glyphosate per kg feed on shell mass. These results indicate that potential chelation of minerals by glyphosate does not impair egg layer mineral status.

The presented results using preselected contrasts, indicating no clear adverse effects of glyphosate or magnification by the adjuvants in the used GBH on layer performance and mineral status, are in good accordance with resent results obtained with weaning pigs in a similar experimental setup (Krogh et al., 2021). Additionally, Schnabel et al. (2017) found no effects on performance and health characteristics in lactating dairy cows with a glyphosate intake of 79 mg per day. However, having reflected over the preselected contrast tests and the stated potential explanations for the effect of IPA_{20} on rate of lay, i.e. non-monotonic dose response or false positive, we decided to make post hoc contrast tests for the overall effect of glyphosate by comparing the three treatment groups with diets amended 20 or 200 mg glyphosate per kg feed to the group receiving the control diet. This indicated that dietary glyphosate concentrations above 20 mg/kg will unfavourably affect rate of lay by 0.9%-point.

5. Conclusion

In conclusion, there were no clear adverse effects of glyphosate-amended diets on performance, welfare indicators and mineral status of Lohmann LSL-Lite layers even at a level approximately 10x higher than the MRL for soybeans and other common feed crops (20 mg glyphosate per kg feed). However, post hoc contrasts suggested that diet glyphosate concentrations above 20 mg/kg may reduce rate of lay by 0.9%-point. Moreover, the layers exposed down to 0.03 mg of glyphosate per kg feed produced eggs with residues of this pesticide.

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CRediT authorship contribution statement

Amanda D. Carlsvig: Writing – original draft, Writing – review & editing, Investigation. **Leslie Foldager:** Writing – original draft, Writing – review & editing, Formal analysis, Software, Data curation, Visualization. **Sanna Steenfeldt:** Writing – review & editing, Investigation. **Ole Højberg:** Writing – review & editing, Conceptualization. **Natalja P. Nørskov:** Writing – review & editing, Investigation. **Martin T. Sørensen:** Writing – original draft, Writing – review & editing, Conceptualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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