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Reproductive toxicity of roundup®-treated feed on broiler breeder roosters and the amelioration of these deleterious effects with inclusion of humic acids in feed.

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Glyphosate-based herbicides (GBHs) such as RoundUp® are a staple of modern crop production, and as a result, residues of their ingredients are typically found in animal feeds. GBH ingredients have repeatedly been shown to impact the male reproductive health of various animals, but at present, the impact of GBH exposures on reproductive health have not been investigated in broiler breeder roosters. This study sought to determine the effect of these exposures on roosters as well as the ability of humic acids (HA) to counteract the effect of GBH exposure. Through 18 weeks of treatment with Roundup®-added or HA-added feeds compared against a common broiler breeder mash, negative effects of Roundup® exposure were seen on testis morphology as well as sperm quality. Increased exposure to Roundup® ingredients resulted in increased vacuolation of seminiferous tubule epithelium. Exposure to Roundup® impacted assessments of sperm quality including sperm mobility, viability and count during the experimental trail. HA supplementation served as a promising adsorptive additive by improving both morphology and sperm quality during the trial. Roundup® exposure was shown to have a negative influence on broiler breeder rooster reproductive health while HA improved reproductive health. The use of HA as an absorbent additive in broiler breeder feeds shows promise in improving reproductive efficiency in broiler breeders.

Introduction

Crops genetically modified for tolerance to glyphosate (*N*-(phosphonomethyl)glycine), a common herbicide, have become increasingly popular since their introduction by the Monsanto Company in 1996 (Benbrook, 2012; Coup & Capel, 2015). In present day production, herbicide-tolerant corn and soy varieties comprise 90 and 94%, respectively, of the acres of these crops planted in the United States, and glyphosate-tolerant (GT) varieties account for roughly 50% of corn and soy grown in the United States (USDA, 2013; Kniss, 2018; USDA, 2018). These GT cultivars are typically exposed to glyphosate-based herbicides (GBHs) throughout their production period, and due to their tolerance to glyphosate, crop production is unaffected (Arregui et al., 2004; Green, 2018). Glyphosate is still incorporated into the tissues of the GT plant, but a modification of enolpyruvylshikimate-3-phosphate synthase, the target enzyme of glyphosate, allows the GT plant to remain healthy (Feng, Chiu & Sammons, 2003; Arregui et al., 2004). This results in chemical residues of glyphosate typically being found in the tissues of these crops postharvest in addition to the presence of common GBH inert ingredients on the surface of the product (Duke, Rimando, Pace, Reddy & Smeda, 2003; Arregui et al., 2004; Cuhra, 2015).

Glyphosate is generally considered a moderate toxicity chemical, however low dose negative impacts on mammalian male reproductive systems have been reported (Dallegrave et al., 2007; Henderson et al., 2010; Abarikwu, Akiri, Durojaiye & Adenike, 2015; Cuhra, 2015; Owagboriaye et al., 2017; Cai et al., 2020; Nerozzi et al., 2020). Glyphosate is especially damaging when exposures occur in combination with its co-formulant polyoxyethylene tallow amine, as is the case when Roundup® (RU) is used (Defarge et al., 2016; Defarge, Spiroux de Vendomois & Seralini, 2018; Mesnage, Benbrook & Antoniou, 2019; Nerozzi et al., 2020). The modes of toxic action for glyphosate and its metabolite, aminomethylphosphonic acid (AMPA), in an animal model are unknown, but they are reported to produce oxidative damage (Abarikwu et al., 2015; Myers et al., 2016; Cai et al., 2020).

Humic acids (HA), a class of organic acids derived from humic substances, have been shown to be strong neutralizers of glyphosate in solution *in vitro* studies (Piccolo, Celano & Conte, 1996; Shehata, Kühnert, Haufe & Krüger, 2014; Mazzi & Piccolo, 2012; Van Oosten, Pepe, De

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Pascale, Silletti, & Maggio, 2017). Though typically used as soil additives to act as biostimulants, HAs are theorized to neutralize glyphosate in media through adsorption (Piccolo et al., 1996; Arroyave, Waiman, Zanini & Avena, 2016; Van Oosten, Pepe, De Pascale, Silletti, & Maggio, 2017). This ability to sequester glyphosate from solution is due to the large molecular surface area and flexibility of humic acids, allowing for a high capacity for hydrogen bonds (Piccolo et al., 1996). *In vitro*, HAs have been shown to adsorb as much as 4.5 mg glyphosate per gram of HA (Piccolo et al., 1996).

Specific to male reproductive physiology, exposure to the ingredients of RU has been reported to result in endocrine disruption, abnormal morphologies of reproductive tissues and dysfunctional sperm production at RU or glyphosate exposure levels no higher than 5 mg/kg bodyweight in murine studies, although, the endocrine effects of RU exposure are widely disputed (Dallegrave et al., 2007; Abarikwu et al., 2015; EFSA, 2017; Owagboriaye et al., 2017; Nerozzi et al., 2020). An investigation of RU toxicity in drakes reported endocrine disruption and abnormal testis and epididymal morphologies with treatment with RU at 5 mg/kg bodyweight, indicating similar reproductive toxicity in avian models (Oliveira, Telles, Hess, Mahecha & Oliveira, 2007). This is despite glyphosate being listed as having very low toxicity in avian models, both in overall toxicity and reproductive toxicity (Henderson et al., 2010; Cuhra, 2015). Recently, Ruuskanen et al. (2020) demonstrated that chronic, subtoxic exposure (12–20 mg glyphosate/kg body wt/day) for 52 weeks impacted the gut microbiome and lowered plasma testosterone but did not impact testes size or egg production in Japanese quail.

In the same years that GT crops and GBHs have tandemly grown in popularity and use, broiler breeder populations have displayed a steady decline in fertility, due to a number of issues. The fault of much of this issue is attributed to the broiler breeder paradox, the reality of losses in fertility resulting from intense selective pressure for rapid growth (Decuypere et al., 2010; Hocking, 2014). While the reproductive consequences of intense selection are without contest, there is little to no evidence to suggest that it is the only contributor to the observed, gradual loss in fertility of broiler breeders. Given what is known about the toxic nature of RU ingredients towards male reproductive tissues and the likelihood of exposure to these toxicants through GT feed crops, the effect of RU exposure on the reproductive health of broiler breeder roosters should be addressed. The objectives of this study were 1) to explore the effects of RU exposure through feed on the reproductive health of broiler breeder roosters and 2) to test the efficacy of HAs as a remedy for RU residues found in GT feed crops.

2. Materials and methods

2.1. Chemicals

The Roundup PRO® Concentrate Herbicide formulation (Monsanto, St. Louis, MO, USA) was used for treatment of feed with glyphosate-based herbicide. This RU formulation consists of 50.2% isopropylamine salt of glyphosate, 13.0% surfactant and 36.8% inert ingredients. A GBH product was selected over pure glyphosate to better mimic the exposures in natural environments including exposure to adjuvants as adjuvants may increase the toxicity of glyphosate (Gill, Sethi, Mohan, Ditta & Girdhar, 2018). With this experimental design, the effect of pure glyphosate, the potential effects of adjuvants or whether adjuvants alter the effect of glyphosate cannot be determined. Furthermore, organic feed, free of potential RU exposure, could not be adequately sourced to serve as the control. The HA was purchased as a water-soluble powder at 90% HA and 10% fulvic acid and inert soil contents (Earthworks Health, Norfolk, NE, USA).

2.2. Animals

120 Cobb 500 broiler breeder roosters were grown to target weight and photostimulated (14L:10D) at 15 weeks of age. At 25 weeks of age, roosters were randomly assigned to individual cages. Prior to treatment, roosters were allowed water *ad libitum* and given 120 g broiler breeder developer mash (BBDM) each day at the start of their lighted period. The roosters were reared and maintained according to the rules set forth by the University of Georgia's Institutional Animal Care and Use Committee.

2.3. Experimental design

Treatments were administered through feed. Feed was mixed fresh every two weeks for one of four treatments: 1) control BBDM, 2) BBDM treated with 1.25 mL RU/kg feed, 3) BBDM treated with 2.50 mL RU/kg feed and 4) BBDM treated with 1.25 mL RU/kg feed and 0.30% (w/w) HA. A HA treatment was used to evaluate HA as a method for counteracting RU exposures. Dosages of RU were chosen based on previous study of RU exposures in drakes (Oliveira et al., 2007), and the HA dosage was chosen based on previous study of *in vitro* neutralization of glyphosate with HA (Piccolo et al., 1996; Arroyave et al., 2016). This concentration in the feed would correspond to a dose of 12–31 mg glyphosate/kg body weight/day in broiler breeder roosters, which is far below the European Food Safety Authority's (EFSA) reported NOAEL (No adverse effects level) of 100 mg/kg body mass/day for poultry (EFSA, 2017).

After being allowed 2 weeks for habituation to individual cages, roosters began treatment at 27 weeks of age. Roosters were randomly assigned to 1 of 4 experimental groups of 30 birds. Throughout the treatment period, roosters were allotted 120 g treatment feed, individually weighed on an Ohaus Scout (Ohaus Corp, Parsippany, NJ, USA), and allowed water *ad libitum* at the start of the light period each day. Treatment lasted for 18 weeks. At the end of treatment, all experimental groups were returned to the control BBDM diet for a 4-week long recovery period in order to observe the permanence of any effects observed with respect to RU or HA treatment. Eight roosters per treatment were weighed every 2 weeks to verify homologous bodyweights between treatments.

2.4. Feed preparation

All feed treatments were prepared every 10 days to avoid potential changes in concentration because of degradation. Briefly 20 kg of BBDM feed was weighted out in a large mixer bowl. Feed, 4 kg, was removed and placed in a small mixer with treatment (RU and/or HA) and mixed for 25 min. Following mixing, the treated sample was then slowly added back to the large feed sample and mixed for another 30 min before being bagged and stored at 20 °C.

2.5. Feed and fecal analysis

Feed ($n = 6$) and fecal ($n = 6$) glyphosate and AMPA levels were assessed by HPLC analysis. Sample extraction and HPLC analyses were performed by the UGA Laboratory for Environmental Analysis. Samples, collected every 3 weeks during treatment, were stored at −80 °C, thawed, and then homogenized before extraction. For each sample collection, 5 g of feed or fecal material was transferred to 50 mL centrifuge tubes and were extracted with 10 mL of 0.6 KOH. The samples were shaken for 2 h and then centrifuged at 3500 rpm for 30 min. 1 ml of the supernatant was transferred to a 1.5 mL centrifuge tube and 80 μL was added to adjust the pH to 9 before derivatization with 9-fluoromethyl chloroformate for HPLC analysis. The limits of quantification (LOQ) were 0.5 ppm for both glyphosate and AMPA in the extracted samples. The limits of detection (LOD) were 0.03 ppm for glyphosate and 0.04 ppm for AMPA in both feed and fecal extracted samples.

2.6. Histology

At the end of the treatment period, 5 roosters per experimental group were euthanized by CO_2 gas asphyxiation for sampling of reproductive tracts ($n = 5$). Reproductive tracts were immediately fixed in 10% buffered formalin and stored at room temperature (RT). For each sample, an approximately 4 mm thick sample was taken from the transverse equator of one testis. Samples were dehydrated, embedded in paraffin, sectioned transversely at approximately 5 μm thickness, hematoxylin and eosin stained and mounted by the UGA College of Veterinary Medicine Histology Laboratory.

2.7. Morphometry

Hematoxylin and eosin-stained testes ($n = 5$) sections were imaged using a TH4 100 microscope (Olympus Corp., Tokyo, Japan) at 10X. For each sample, 25 images were taken, with each image centered on a single, transverse section of a seminiferous tubule. Morphometrical analysis was performed as described by Montoto, Arregui, Sánchez, Gomendio and Roldan (2012) with the following modifications. A horizontal and vertical measure of the tubule diameter was taken of each tubule along with 2 horizontal and 2 vertical measures of the epithelial height. Mean epithelial height was calculated relative to each tubule's mean radius. Measurements were obtained using ImageJ v1.52k (National Institutes of Health, Bethesda, MD, USA). Seminiferous tubules were also assessed for number of individual vacuoles present.

2.8. Sperm quality

Roosters were habituated to weekly semen collection by the dorso-abdominal massage method as described by Burrows and Quinn (1937) for 2 weeks prior to treatment. For the duration of the treatment and recovery period, semen was sampled every week from three roosters per treatment. Every other week, at least 0.50 mL semen was collected from the same three roosters and transported in glass tubes. Semen samples in 500 μL aliquots were twice washed by centrifugation at 1000 x *g*. After the first wash, semen was reconstituted in 1.5 mL phosphate-buffered saline (PBS; pH 7.4), and after the final wash, semen was reconstituted to its original volume of 0.50 mL with motility buffer (MB: 111 mM NaCl, 25.0 mM glucose, 4.00 mM $CaCl_2$, 50.2 mM TES; pH 7.4). Washed sperm samples were assessed for cell count by hemacytometer counting as previously described (Freund & Carol, 1964).

Sperm viability was assessed by the eosin-nigrosin vital staining assay as previously described by Chalah and Brillard (1998) with the following modifications. Sperm samples were prepared in 50 μL aliquots at a concentration of 1.0×10^8 cells/mL by diluting in MB in a microcentrifuge tube. Eosin-nigrosin stain (2.5% (w/v) eosin, 5% (w/v) nigrosin) was mixed 1:1 into the sample by pipetting followed by vortexing. Samples were incubated in the stain at RT for 5 min. After incubation, 20.0 μL stained sample was added to a glass slide, smeared, air-dried and cover-slipped. Slides were photographed under an Olympus TH4 100 microscope and counted for total sperm and total dead sperm. Percentage viability was calculated by number of live sperm out of a minimum of 100 cells counted per sample.

Sperm mobility was assessed by the Accudenz assay, a method previously validated for the objective quantification of chicken sperm mobility (Froman & McLean, 1996), with the following modifications. A 30% (w/v) Accudenz (Accurate Chemical & Scientific Corp., Westbury, NY, US) stock solution was prepared in a 3 mM KCl, 5 mM TES solution (pH 7.4) and diluted 1:4 in MB to prepare a working 6% Accudenz solution. The Accudenz solution was preheated to 41 °C, and 1.00 mL aliquots were added to polystyrene cuvettes kept at 41 °C. Sperm samples were prepared at a concentration of 5.0×10^8 cells/mL, and 100 μL sperm sample overlaid onto Accudenz solution prepared in cuvettes. Samples were incubated in cuvettes at 41 °C for 10 min. Absorbance was read at 550 nm using a DU 530 spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA). The Accudenz assay was performed in duplicate.

2.9. Testosterone assessment

At weeks 0, 9 and 18 of treatment as well as at the end of the 4-week recovery period 4 roosters per experimental group were sampled for blood ($n = 4$). Blood samples were collected from the wing vein into a sterile syringe and injected into EDTA coated tubes. Blood samples were centrifuged at 3000 x *g* for 10 min at 4 °C to collect plasma. Plasma samples were stored at −20 °C. Plasma samples were subjected to diethyl ether extraction for separation of steroid content, and concentrations of plasma testosterone were determined by a testosterone ELISA kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA).

2.10. SDS-PAGE and western blotting

At end of treatment and after 4 weeks of recovery, 5 roosters per experimental group were euthanized by CO_2 and their reproductive tracts harvested, placed in PBS and immediately stored at −80 °C ($n = 5$). Testes tissues were later thawed and an approximately 1 cm^3 sample cut from the caudal end of the testis. Samples were suspended 1:5 in lysis buffer (50 mM NaCl, 10 mM Tris base, 1 mM EGTA, 1 mM EDTA and 1% (v/v) Triton X) and homogenized with a 10 mm X 115 mm saw-tooth generator probe (VWR International, Radnor, PA, USA) for 2 repetitions of 30 s at medium power followed by 1 min on ice. Samples were then sonicated by an Artek Model 150 sonic dismembrator (Thermo Fisher Scientific, Waltham, MA, USA) at 60% output for 5 repetitions of 15 s followed by at least 1 min on ice. Sonicated samples were then centrifuged at 12,000 x *g* for 30 min at 4 °C and supernatant collected. Protein was quantified using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with a bovine serum albumin standard. Measurements of absorbance were taken at 750 nm with a Spectronic 200 spectrophotometer (Thermo Fisher Scientific) per the manufacturer's instructions. Lysates were stored at −80 °C.

Testis protein lysates were thawed, and concentrations adjusted to 1.2 mg/mL. Samples were diluted 1:1 in 2X sample buffer (4% SDS, 20% glycerol, 120 mM Tris–HCl, 200 mM dithithreitol, 0.02% bromophenol blue; pH 6.8) and then denatured at 95 °C for 5 min. In duplicate lanes, 50 μL protein samples were loaded into Mini-PROTEAN® TGX Stain-Free™ 10% precast gels (Bio-Rad) alongside Precision Plus Protein™ All Blue Standards (Bio-Rad). SDS-PAGE was performed on the gels in Tris-glycine-SDS running buffer (25 mM Tris base, 192 mM glycine and 0.1% SDS; pH 8.3) at 70 V for 10 min followed by 120 V until completion in a Mini-PROTEAN® Tetra Cell system (Bio-Rad). Gels were UV-activated using a ChemiDoc™ MP Imaging System (Bio-Rad) and then transferred to Immun-Blot® polyvinylidene difluoride membranes (Bio-Rad) in Towbin transfer buffer (25 mM Tris base, 192 mM glycine and 20% (v/v) methanol) via wet-blot transfer system (Bio-Rad) at 100 V for 1 hr. Membranes were washed in Tris-buffered saline with Tween (TBST: 20 mM Tris base, 150 mM NaCl and 0.1% (v/v) Tween 20; pH 7.4) and then blocked in 5% (w/v) skim milk in TBST for 30 min. Membranes were imaged for total protein normalization using the Chemidoc™ MP Imaging System. Blocked membranes were probed with a polyclonal human anti-androgen receptor IgG antibody produced in goat (product no. PA1–9005; Thermo Fisher Scientific) diluted 1:1000 in TBST overnight at 4 °C under slow rocking. Membranes were washed in TBST and then probed for 1 hr at RT in horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG secondary antibody (product no. A16005; Thermo Fisher Scientific) diluted 1:10,000 in TBST, followed by washing in TBST. Membranes were subjected to visualization by Clarity™ Western ECL Substrate (Bio-Rad) and images developed on the Chemidoc™ MP Imaging System. Membranes probed with only secondary antibody at RT for 1 hr served as secondary controls. Abundances of androgen receptor were quantitated by normalizing the densities of the androgen receptor protein bands to that of the total loaded protein per lane as measured by

stain-free imaging technology. Calculations of relative androgen receptor abundances were performed using Image Lab™ Software (Version 5.2.1; Bio-Rad).

2.11. Statistical analysis

Statistical analyses were performed with R 3.5.1 software (The R Foundation for Statistical Computing, Vienna, Austria). Differences in bodyweight, tissue morphology, sperm parameters, androgen receptor abundance and plasma testosterone between experimental groups were analyzed by one-way ANOVA. Differences in bodyweights and sperm parameters with respect to treatment and length of treatment were assessed by two-way repeated measures ANOVA. Differences in bodyweight and sperm parameters between treatment groups after the recovery period were compared by one-way ANOVA. Significant differences were compared by Tukey's honest significant difference test, with differences considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Treatment validation

To validate treatment with differing levels of GBH, feed and fecal samples were assessed by HPLC for glyphosate and AMPA levels. Control, untreated BBDM was found to contain glyphosate and AMPA residues merely as a result of the production practices used to produce the feed crops (Table 1). BBDM treated with 1.25 mL RU/kg and 2.50 mL RU/kg had glyphosate levels increase by 66% and 189%, respectively, relative to the control BBDM (Table 1). AMPA levels were increased in a similar but less dramatic trend. BBDM treated with HA and 1.25 mL RU/Kg exhibited a decrease in glyphosate by 13% relative to the control BBDM and 91% compared to the BBDM with 1.25 ml RU/kg; AMPA levels, however, were increased by 27% when compared to that of the control (Table 1). Glyphosate was only found in fecal samples collected from roosters treated with 1.25 mL RU/kg and 2.50 mL RU/kg (Table 2). AMPA levels were similar in fecal samples between all groups except BBDM treated with HA and 2.50 mL RU/kg, which exhibited AMPA increased 76% relative to that of the control treatment (Table 2).

3.2. Body weight

Rooster bodyweights were recorded to ensure that differences in growth rate did not confound comparisons of differences in reproductive health. Throughout the experiment, no significant differences were observed in rooster bodyweights between experimental groups (data not shown).

3.3. Tissue morphology

Tissue morphology was assessed to discern mode of effects of RU and HA treatment on reproductive tissues. No significant effects were observed on epithelial height at end of the treatment period or the end of the recovery period. At the end of the treatment period, a dose dependent increase was seen on seminiferous tubule vacuolation with respect to RU exposures ($p < 0.05$), and, in a similar pattern, treatment with HA resulted in a non-significant decrease in tubule vacuolation when compared with the control (Fig. 1).

Table 1
Glyphosate and aminomethylphosphonic acid (AMPA) residues in treatment feeds as measured by HPLC. Values represented as mean ± SEM ($n = 6$).

Treatment	Glyphosate (ppm)	AMPA (ppm)
Control	10.75 ± 2.68	1.30 ± 1.30
1.25 mL RU/kg	18.19 ± 1.45	1.533 ± 0.19
2.50 mL RU/kg	31.05 ± 2.57	1.642 ± 0.95
0.30% HA + 1.25 mL RU/kg	9.30 ± 1.88	1.685 ± 0.78

Table 2
Glyphosate and aminomethylphosphonic acid (AMPA) residues as measured in fecal samples by HPLC. Values represented as mean ± SEM ($n = 6$).

Treatment	Glyphosate (ppm)	AMPA (ppm)
Control	0.00 ± 0.00	0.14 ± 0.14
1.25 mL RU/kg	3.17 ± 0.18	0.13 ± 0.13
2.50 mL RU/kg	3.92 ± 0.26	0.14 ± 0.14
0.30% HA + 1.25 mL RU/kg	0.00 ± 0.00	0.59 ± 0.16

3.4. Sperm parameters

Sperm count, viability, mobility, and semen volume were measured throughout the treatment and recovery periods to assess the effects of treatments on sperm performance in individual roosters over time. Sperm mobility exhibited a clear response to treatment with either increased exposure to RU or added HA. Throughout the treatment period, sperm mobility decreased with a dose-dependent response to added RU, while sperm mobility increased with HA-treatment of feed ($p < 0.05$) (Fig. 2). Significant differences were not observed in sperm mobility between groups after 4 weeks of recovery (Fig. 2). As with sperm mobility, sperm count was improved with the addition of HA throughout the treatment period (Fig. 3). The addition of 2.5 mL/kg RU significantly reduced sperm count, compared to the control, during the first 6 wks of treatment. Following the recovery period from treatment, no differences were detected for sperm count among the roosters. Measurements of sperm viability were only detected from weeks 7 – 12 of treatment (Fig. 4), with high RU (2.5 mL/kg) leading to a decrease in the percentage of viable sperm and the addition of HA increasing the percent of viable sperm. The HA roosters maintained a numerically higher percentage of viable sperm until the end of recovery, when there were no differences among the roosters. Semen volume in RU roosters, both 1.25 mL/kg and 2.5 mL/kg treatments, was reduced during the last 12 weeks of treatment (Fig. 5). However, following 4 weeks recovery, there was no difference in semen volume among the roosters.

3.5. Endocrine effects

In order to determine the influence of any endocrine-related effects on reproductive health, plasma testosterone levels were assessed by ELISA at start of treatment (week 0), mid-point of treatment (week 9), end of treatment (week 18) and after 4 weeks of recovery (week 22). Androgen receptor abundance was assessed by western blotting at end of treatment and end of recovery.

Unfortunately, plasma testosterone levels were significantly higher in the RU-added groups prior to treatment ($p < 0.05$, Fig. 6). However,

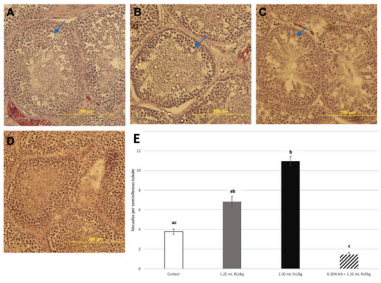

Fig. 1. Effect of RoundUp® (RU) and humic acid (HA) treatments on tubule vacuolization. Cross sections of a seminiferous tubule of roosters treated with A) a control ration, B) 1.25 mL RU added/kg feed, C) 2.50 mL RU added/kg feed and D) 0.30% HA + 1.25 mL RU/kg feed added. E) Degrees of seminiferous tubule vacuolation post-treatment (week 18). Blue arrows indicating representative seminiferous tubule vacuole. Values represent mean ± SEM of relative epithelial height ($n = 5$). [a-c]Indicates significant difference in means, $p < 0.05$.

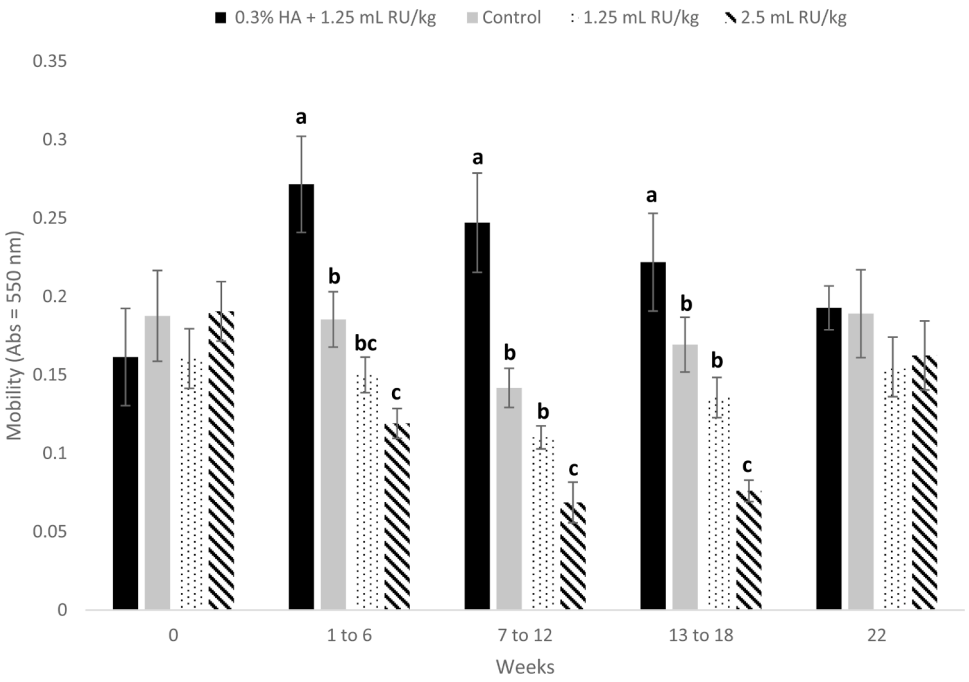

Fig. 2. Effect of RoundUp® (RU) and humic acid (HA) treatments on sperm mobility. A) Sperm mobilities at the start of treatment ($n = 4$), first 6 weeks of treatment ($n = 24$), second six weeks of treatment ($n = 24$), final 6 weeks of treatment ($n = 24$), and end of the recovery period ($n = 4$) are shown. Values represent mean ± SEM of weekly sperm mobility assessments. [a-c]Indicates significant difference in means, $p < 0.05$.

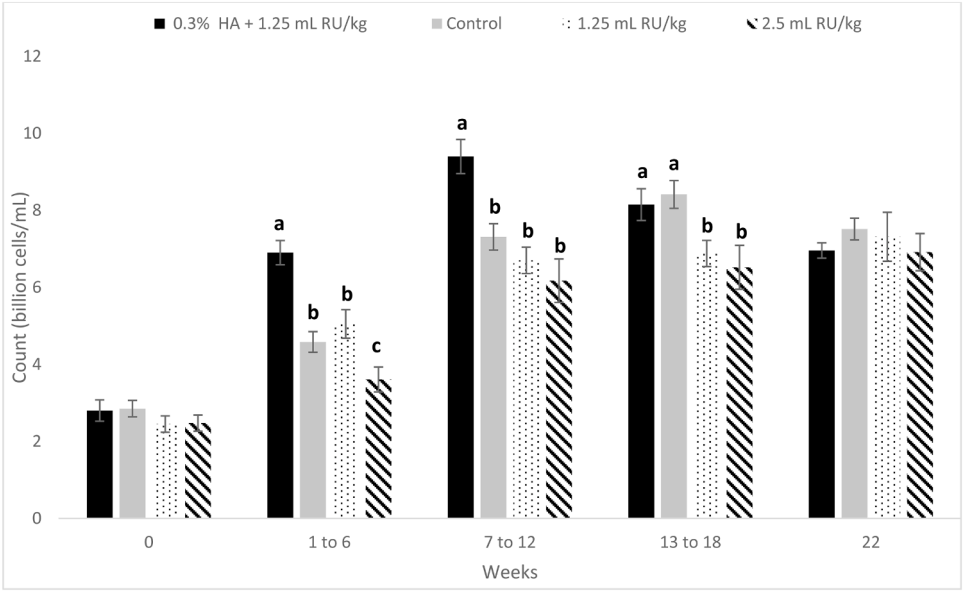

Fig. 3. Effect of RoundUp® (RU) and humic acid (HA) treatments on sperm cell count. Sperm cell counts at the start of treatment ($n = 4$), first 6 weeks of treatment ($n = 24$), final 6 weeks of treatment ($n = 24$) and end of the recovery period ($n = 4$) are shown. Values represent mean ± SEM of cell counts. [a-b]Indicates significant difference in means, $p < 0.05$.

midway through treatment, testosterone levels in the HA treatment were more than double that of the control group and those treated with the higher dose of added RU ($p < 0.05$). No significant differences were observed at the end of treatment nor after recovery (Fig. 6). Androgen receptor abundance was not significantly different between any groups at the end of treatment; however, following recovery androgen receptor abundance was significantly lower in the 2.5 mL RU/kg treatment (Fig. 7).

4. Discussion

The objectives of this work were to explore the effects of RU exposure through feed on the reproductive health of broiler breeder roosters and to test HAs potential to neutralize RU residues found in feed. Here in this study, we show that exposure to increased levels of RU negatively impact the health of the seminiferous epithelium of the testes and sperm mobility, while treatment of feed with HA improves health of the seminiferous epithelium and sperm quality in roosters.

While no effect is seen on epithelial height of the seminiferous

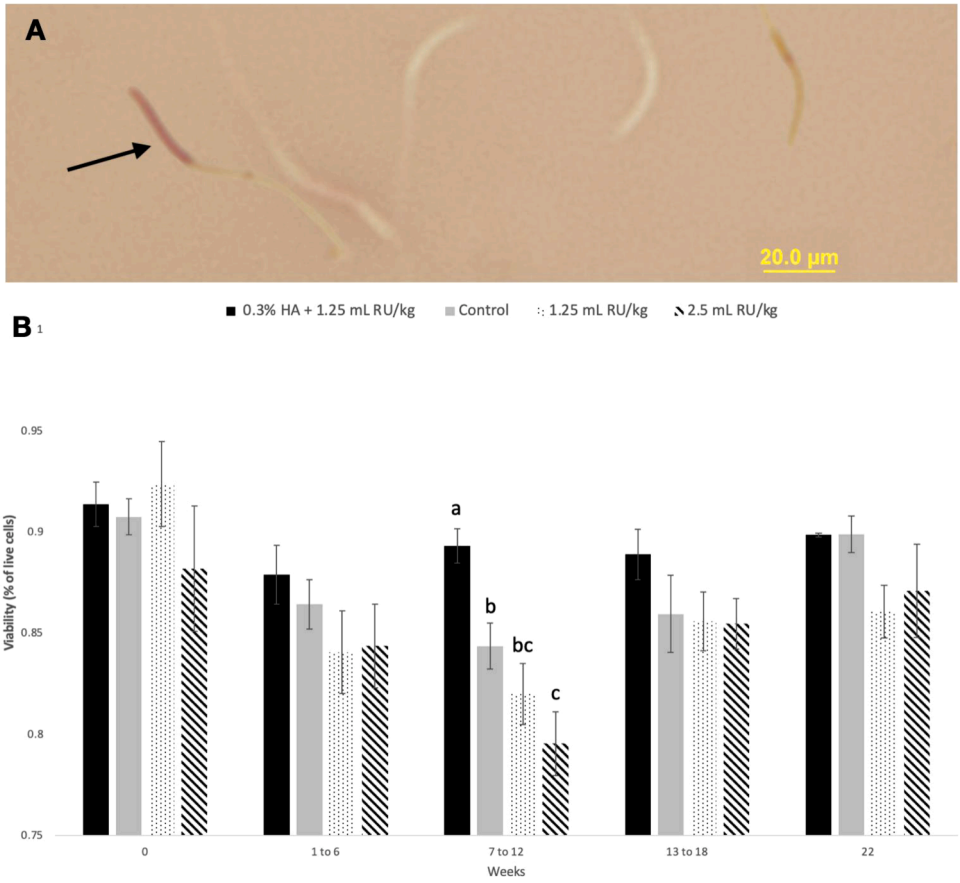

Fig. 4. Effect of RoundUp® (RU) and humic acid (HA) treatments on sperm cell viability. A) Eosin-nigrosin staining for sperm cell viability. The black arrow indicates stained, dead sperm. B) Sperm cell viabilities at the start of treatment ($n = 4$), first 6 weeks of treatment ($n = 24$), final 6 weeks of treatment ($n = 24$) and end of the recovery period ($n = 4$) are shown. Values represent mean ± SEM of cell viability assessments. [a-c] Indicates significant difference in means, $p < 0.05$.

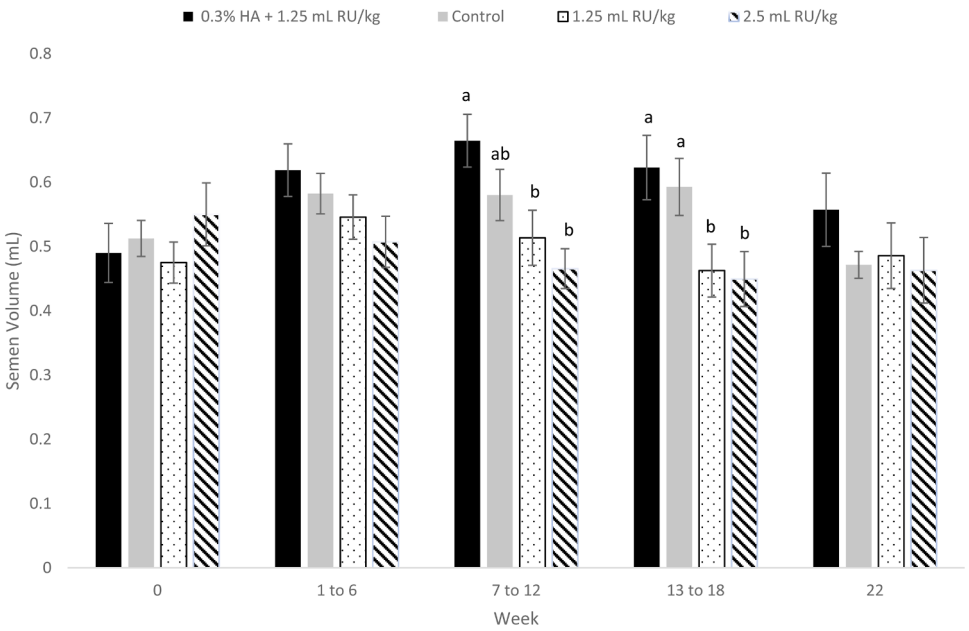

Fig. 5. Effect of RoundUp® (RU) and humic acid (HA) treatments on semen volume. A) Semen volumes following manual ejaculation at the start of treatment ($n = 4$), first 6 weeks of treatment ($n = 24$), second six weeks of treatment ($n = 24$), final 6 weeks of treatment ($n = 24$), and end of the recovery period ($n = 4$) are shown. Values represent mean ± SEM of weekly sperm volume measurements. [a-c]Indicates significant difference in means, $p < 0.05$.

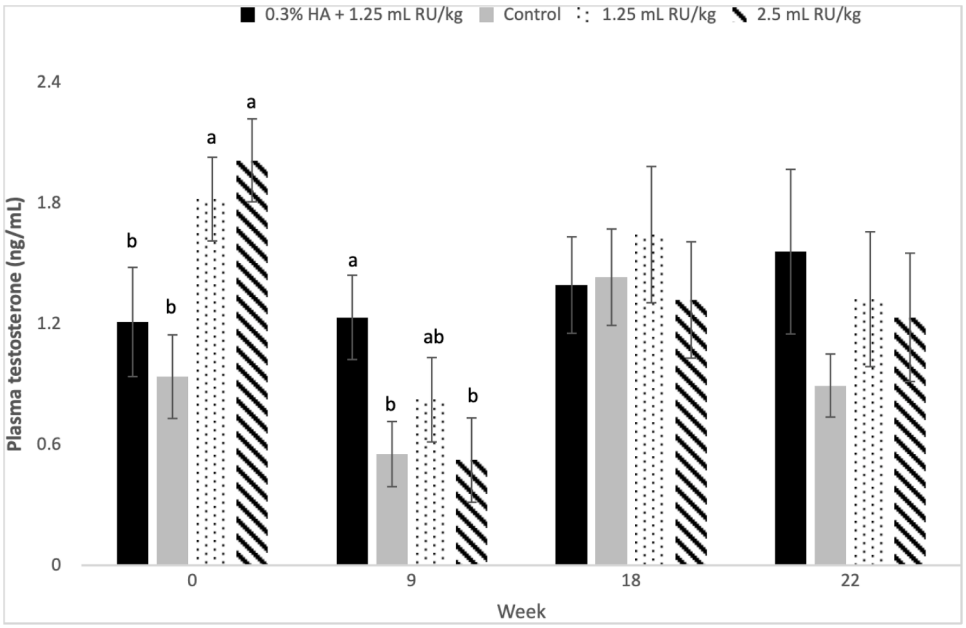

Fig. 6. Effect of RoundUp® (RU) and humic acid (HA) treatments on plasma testosterone levels. Plasma testosterone levels at start of treatment (week 0), mid-point of treatment (week 9), end of treatment (week 18) and end of the recovery period (week 22) are shown. Values represent mean ± SEM of plasma testosterone levels ($n = 4$). [a-b]Indicates significant difference in means, $p < 0.05$.

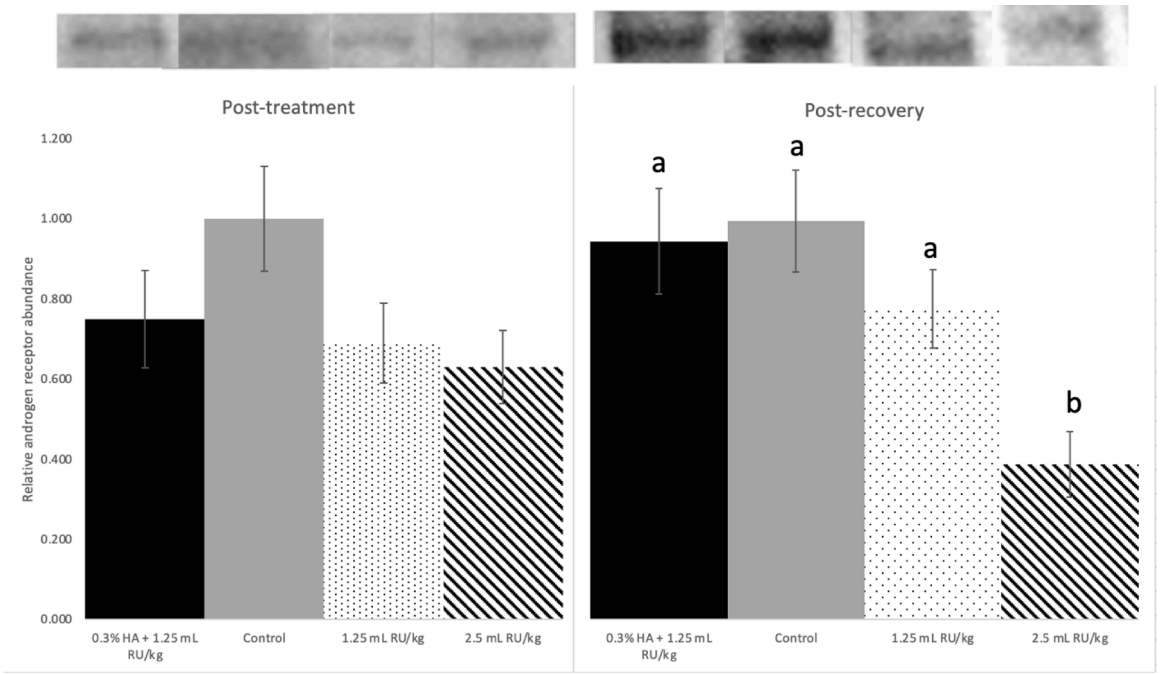

Fig. 7. Effect of RoundUp® (RU) and humic acid (HA) treatments on relative abundance of androgen receptor protein. Relative abundance of androgen receptor protein at post-treatment (week 18) and post-recovery (week 22) are shown. Values represent mean ± SEM of androgen receptor abundance relative to that control tissues ($n = 5$). Androgen receptor abundances are normalized relative to total loaded protein per lane as measured by stain-free technology. [a-b]Indicates significant difference in means, $p < 0.05$.

tubules, a clear pattern of effect can be seen by increasing effective glyphosate levels on the degree of vacuolation in the seminiferous epithelium. Vacuolation was more dramatic in roosters exposed to higher levels of RU, and it was decreased in those fed with HA included to counteract glyphosate. This supports previous findings that show marked increases in vacuolation of the seminiferous tubules as a result of exposure to Roundup (Owagboriaye et al., 2017). Furthermore, vacuolation of the seminiferous epithelium and germ cells has been previously shown with glyphosate exposure in both *in vitro* studies and *in vivo* studies of the multigenerational effects of glyphosate exposure (Dallegrave et al., 2007; Jiang et al., 2018; Kubsad et al., 2019). This type of vacuolation of the seminiferous epithelium is typically indicative of Sertoli cell damage. The Sertoli cells are fairly resilient to toxic insults, relative to germ cells; however, their level of exposure to any such insults is highest, as these cells form the blood-testis barrier. With prolonged exposure and Sertoli cell damage, damage to germ cells typically

follows (Casey, 2001). Recently, Gorga et al. (2020) reported that the adverse effect of glyphosate and RU on male reproductive function is not a result of Sertoli cell metabolism but results from the alteration of the permeability of the Sertoli cell junction barrier. Subsequent studies should investigate the timing of the onset of vacuolization due to RU exposure. The current study looked at a cumulative effect at the end of the study when the roosters were near the end of their reproductive prime and the overall quality of testis micromorphology tends to decrease even under normal physiologic conditions (Avital-Cohen et al., 2013).

A pattern of effects like that seen with seminiferous epithelium vacuolation was seen regarding sperm mobility, with sperm mobility increasing with HA treatment and decreasing with increasing measured glyphosate levels (Fig. 2). This effect on sperm mobility was consistent during the experimental trail; however, following the recovery period, all differences in sperm mobility were removed. Sperm mobility is historically considered the most important determinant of broiler breeder rooster fertility (Froman, Feltmann, Rhoads & Kirby, 1999; Sun et al., 2019). Sperm motility has formerly been shown to be negatively affected with glyphosate treatment at *in vivo* levels as low as 3.6 mg/kg bodyweight in murine studies and with *in vitro* treatment of human sperm at 360 ppb (Abarikwu et al., 2015; Anifandis et al., 2017; Owagboriaye et al., 2017, 2018). Our results show an effect on sperm mobility with chronic *in vivo* treatment of feed with just over 18 ppm glyphosate residues. Based on the average weight of roosters throughout the experimental period and the feed ration allotted, that is an estimated treatment level of 0.443 mg/kg bodyweight. In connection with sperm motility, GBH has been found to impair mitochondrial activity at low concentrations (Bailey et al., 2018; Burchfield et al., 2019; Vanlaeys, Dubuisson, Seralini & Travert, 2018; Nerozzi et al., 2020; Ferramosca et al., 2021). Although not measured in the current study, previous reports also link the decrease in sperm mobility to increased production of testicular free radicals and reactive oxygen species (ROS) because of RU exposure (Owagboriaye et al., 2017). Fortunately, the effects of exposure appear to be temporary, as the recovery period removed all differences between groups. Although not as consistent as the changes in sperm mobility; sperm count (Fig. 3, wks 13–18), sperm viability (Fig. 4, wks 7–12) and semen volume (Fig. 5, wks 13–18) were all negatively impacted by RU. As reviewed by (Cai et al., 2020), these measured parameters on sperm (count, viability) and semen volume have previously been reported to be impacted by both glyphosate and GBHs. Each of these measures are associated with avian fertility, but the impact on motility, at least for broiler breeders, would be the most detrimental to rooster reproductive fitness (Sun et al., 2019). Fortunately, the removal of differences in reproductive health between groups with respect to treatment indicates that the effects of exposure to RU may be temporary, and intervention in broiler breeders already impacted by exposure to RU ingredients may improve their reproductive health.

The endocrine effects shown in this study are not strong and may have been impacted by the small sample size ($n = 4$, Fig. 6& 7). The significant differences observed in plasma testosterone follow no pattern, and significant differences were observed prior to treatment. The only effect seen on androgen receptor abundance was a decrease in the high RU-feed (2.5 mL RU/kg), but this only occurred after recovery on control feed (Fig. 7). Our findings are different from previous studies in the avian model that showed a decrease in testosterone (Oliveira et al., 2007; Ruuskanen et al., 2020) and androgen receptor expression in testes (Oliveira et al., 2007) as a result of RU exposure. However, the full scope of the effect on plasma testosterone and androgen receptor abundance in the testes, if any, may have not been captured in this experimental design. In the literature there are conflicting reports on the effects of glyphosate and GBHs on reproductive endocrinology. Although there are several reports concerning the endocrine disrupting effects of GBHs (Abarikwu et al., 2015; Clair, Mesnage, Travert, & Seralini, 2012; Dallegrave et al., 2007; Oliveira et al., 2007; Owagboriaye et al., 2017; Pandey & Rudraiah, 2015; Romano, Romano, Bernardi, Futado, & Oliveira, 2010), these studies have been subject to speculation. The European Food Safety Authority (EFSA) performed a peer review (2017) of studies investigating endocrine disruption caused by glyphosate. The EFSA concluded that glyphosate does not cause any endocrine disruption, criticizing the experimental design of many of the studies. More studies are needed to measure the potential impact of GBH exposure on birds and agriculturally important farm animals (Mesnage et al., 2019).

As reviewed by Arif et al. (2019), humic acids have been shown to increase feed efficiency and weight gain in broilers and improve egg weight, egg mass and egg production in laying hens. HA has also been shown to increase antioxidant activity in broilers reared under normal and stress conditions (Vaskova et al., 2018) and lower *E. coli* counts (Diaz Carrasco et al., 2018) and coccidian oocysts in excreta (Domínguez-Negrete et al., 2019). In addition to neutralization of the antimicrobial effect of glyphosate, Shehata et al. (2014) showed that feed supplemented with humic acid (0.2%) lead a significant reduction in glyphosate accumulation in several broiler tissues (intestine, lung, spleen, liver). Reports demonstrate that both HA and GBH impact the gut microbiome in poultry (Diaz Carrasco et al., 2018; Arif et al., 2019; Ruuskanen et al., 2020). As there is a large overlap between the gut microbiome and the reproductive microbiome in avian species (Shterzer et al., 2020), the alteration of the gut microbiome due to GBH exposure may be of consequence to the reproductive microbiome and impact reproductive performance. Further research is also needed to determine the mechanisms by which HA supplementation improved reproductive outcomes, rather it was solely RU neutralization (Van Oosten, Pepe, De Pascale, Silletti, & Maggio, 2017) or other means known to improve outcomes in broilers (Arif et al., 2019).

5. Conclusions

This study displayed that exposure to GBH ingredients through animal feeds, at legally allowed levels, can significantly influence the reproductive health of broiler breeder roosters. Both the gross histopathology of the rooster testis and sperm mobility, the most important component of rooster sperm quality, are seriously impacted by exposure to GBH ingredients. This study was the first work to display this effect of GBH ingredient exposure at such low levels in broiler breeder roosters. The addition of HA reduced the effect of these residues in feed and provided no additional negative effects on reproductive health or growth of the rooster. Further study should be performed to ensure that the benefits seen with HA supplementation are related to its ability to neutralize glyphosate residues in the feed, rather than due to a mode of action outside of its relationship to glyphosate. Additionally, further study should investigate the effects of GBH ingredient exposure on both male and female broiler breeders, their microbiome, and their overall reproductive efficiency to determine the impact that GBH exposure imposes on commercial broiler chick production.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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