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# Chronic dietary exposure to a glyphosate-based herbicide alters ovarian functions in young female broilers

Freville Mathias,* Estienne Anthony ,* Ramé Christelle,* Lefort Gaëlle ,* Piégu Benoît ,* Chahnamian Marine,† Froment Pascal ,* and Dupont Joëlle†,1

*Centre National de la Recherche Scientifique, Institut Français du Cheval et de l'Equitation, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, Université de Tours, Physiologie de la Reproduction et des Comportements, UMR85, Nouzilly F-37380, France; and †Unité Expérimentale du Pôle d'Expérimentation Avicole de Tours UEPEAT 1295, Nouzilly F-37380, France

**ABSTRACT** Glyphosate (**GLY**)-based herbicide (**GBH**) formulations are widely used pesticides in agriculture. The European Union recently decided to extend the use of GLY in Europe until 2034. Previously, we showed that chronic dietary GBH exposure in adult hens resulted in a reversible increase in early mortality in chicken embryos. In this present study, we investigated the GBH effects on metabolism and ovarian functions by using a transcriptomic approach in vivo in young female broilers and in vitro in ovarian explant cultures. We exposed 11-day-old female broilers to 13 mg GLY equivalent/kg body weight/d (GBH13, n = 20), 34 mg GLY equivalent/kg body weight/d (GBH34, n = 20), or a standard diet (control [CT], n = 20) for 25 d. These 2 GBH concentrations correspond to approximatively one-eighth and one-third of the no observed adverse effect level (**NOAEL**) as defined by European Food Safety Authority in birds. During this period, we evaluated body weight, fattening, food intake, and the weight of organs (including the ovaries). Chronic dietary GBH exposure dose dependently reduced food intake, body weight, and fattening, but increased oxidative stress and relative ovary weight. We analyzed the ovarian gene expression profile in CT, GBH13, and GBH34 broilers with RNA sequencing and showed that differentially expressed genes are mainly enriched in pathways related to cholesterol metabolism, steroidogenesis, and RNA processing. With quantitative polymerase chain reaction and western blotting, we confirmed that GBH decreased ovarian STAR and CYP19A1 messenger RNA and protein expression, respectively. Furthermore, we confirmed that GBH altered steroid production in ovarian explants. We have identified potential regulatory networks associated with GBH. These data provide valuable support for understanding the ovarian transcriptional regulatory mechanism of GBH in growing broilers.

**Key words:** glyphosate, young broiler, food intake, ovary, transcriptomic approach

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## INTRODUCTION

Glyphosate (**GLY**) or *N*-(phosphonomethyl)glycine, sold in the form of GLY-based herbicides (**GBH**), is an active herbicide used widely in agriculture throughout the world. Due to its extensive use, GLY has been frequently detected in environmental compartments such as the soil (Silva et al., 2018), groundwater and surface water (Geng et al., 2021), the air (Ramirez Haberkon et al., 2021), and cereals (Xu et al., 2019). Glyphosate has been considered harmless to animals because of its capacity to inhibit the Shikimate pathway present exclusively in plant cells and in some microorganisms (Schönbrunn et al., 2001). However, several studies have shown adverse effects after GBH administration in various mammalian models, including endocrine disruption (Walsh et al., 2000; Romano et al., 2010; Gill et al., 2018). Furthermore, in plants and animals, GLY is metabolized into aminomethylphosphonic acid (**AMPA**), which is also suspected to exert toxic effects (Martins-Gomes et al., 2022). Although evidence for the carcinogenicity and toxicological effects of GLY has been found and debated, the European Union decided to extend the use of GLY in Europe for 10 yr until 2034 (Cressey, 2015; Tarazona et al., 2017; Casassus, 2023).

Although the adverse effects of GBHs for human health have been particularly scrutinized and debated in



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[1]Corresponding author: joelle.dupont@inrae.fr

the public discourse, they are also suspected to have great ecotoxicological effects. Scientists are particularly concerned with the reduction in bird populations. A 2015 study showed that European birds have been in strong decline for at least 30 years (Inger et al., 2015). While biodiversity collapses are multifactorial phenomena, agricultural activities and particularly more widespread pesticide use are suspected to be critical factors (Rigal et al., 2023). Moreover, according to the European Food Safety Authority (**EFSA**), GLY and AMPA are highly persistent in soils: Their half-life can be several hundred days, depending of the aerobic/anaerobic condition of the soil (Martins-Gomes et al., 2022). GLY residues are also detectable in water and food (Gill et al., 2018; Fogliatto et al., 2020) and are therefore likely to be transferred to birds. Moreover, studies have concluded that GBHs can threaten birds. However, these studies claim that the threat would come from a lack of food due to the harmful effects of GBHs on the invertebrates in the diet of adult birds (Gill et al., 2018). Nonetheless, GBHs can decrease the liver antioxidant capacity and testosterone levels in Japanese quail (Ruuskanen et al., 2020a), and parental exposure might also affect embryo development (Ruuskanen et al., 2020b). A previous study from our laboratory on adult broiler hens showed that chronic dietary GBH intake significant increased embryo mortality and developmental defects (Estienne et al., 2022). Although these deleterious effects were reversible, the progeny of hens temporarily exposed to a GBH were more likely to fatten, even with a balanced diet (Estienne et al., 2023).

Because adverse effects have been observed in adult birds, we aimed to assess the effect of dietary GBH exposure in young broilers. We exposed 11-day-old female broilers to one of 2 dietary doses of GBH—13 or 34 mg GLY equivalent/kg body weight/d—for 25 d. We monitored their food intake, body weight, and fattening; the relative weight of the ovaries, abdominal adipose tissue, and spleen; the plasma GLY and AMPA concentrations; and oxidative stress markers. In addition, we evaluated the effect of the 2 GBH doses on genes involved in ovary steroidogenesis with RNA sequencing (RNA-seq) and reverse transcription−quantitative polymerase chain reaction (RT-qPCR). Finally, we exposed ovarian explants from 10-day-old broilers to GBH and evaluated progesterone and estradiol production.

## MATERIAL AND METHODS

### Ethical Issues

All experimental procedures were performed in accordance with the French National Guidelines for the care and use of animals for research purposes (certificate of authorization to experiment on living animals: APAFIS number 21549-2019071809504554v3, approval date November 6, 2021, Ministry of Agriculture and Fish Products, and a notice from the ethics committee of Val de Loire N°19).

### Animals

Sixty female broilers of the commercial ROSS 308 breed were obtained at 1 d of age from a local hatchery (Boye Accouvage La Villonniere, La Boissière en Gatine, France) and reared at "Pôle Expérimental Avicole de Tours" (INRAE, Nouzilly, France) according to the standard breeding conditions. At 11 d of age, the broilers were divided into 3 groups of 20 and subjected to the following dietary treatments for 25 d: a low Gallup Super 360 dose with 13 mg GLY equivalent/kg body weight/d (the GBH13 group), a high Gallup Super 360 dose with 34 mg GLY equivalent/kg body weight/d (the GBH34 group), or a regular diet without GBH (the CT group). Blood samples were collected to quantify the plasma GLY and AMPA concentrations and oxidative stress. The broilers were weighed on d 1 (D1), D9, D16, and D25 of the experiment. Ultrasonography was performed on D9 and D25 to measure the thickness of the abdominal adipose tissue. On D25, the broilers were killed by electrical stunning and bled out, as recommended by the ethical committee. Then, biological samples were collected (Figure 1).

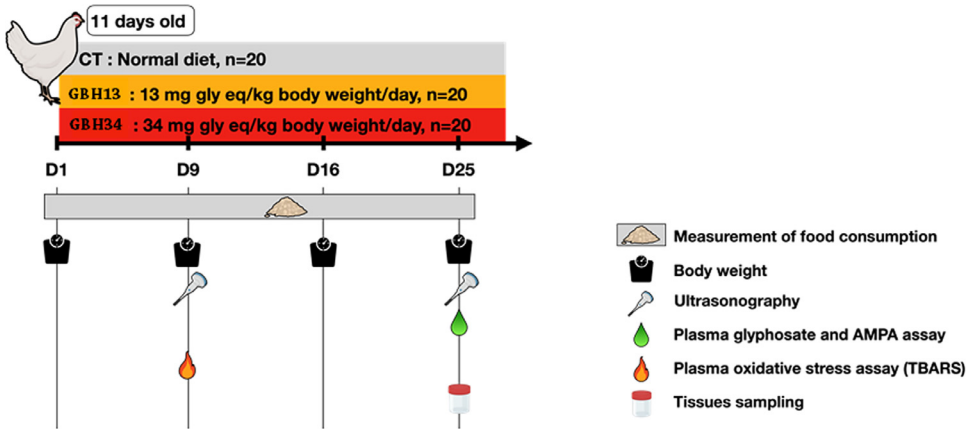


**Figure 1.** Experimental design. Eleven-day-old female broilers were exposed to glyphosate (**GLY**)-based herbicide (**GBH**) in the diet for 25 d, corresponding to a dose of 13 mg GLY equivalent/kg body weight/d (the GBH13 group, n = 20) or 34 mg GLY equivalent/kg body weight/d (the GBH34 group, n = 20). The control group (CT, n = 20) received a normal diet. Food intake was determined each day. On d 1 (D1), D9, D16, and D25 of the experiment, the broilers were weighed; on D9 and D25, an ultrasonographic examination was used to estimate the abdominal adipose tissue thickness; and on D25, blood was collected for oxidative stress assessment and the GLY and aminomethylphosphonic acid (**AMPA**) assays. All the animals were euthanized to collect tissues on D25.

### Diet Composition

The CT food had 0.001 mg GLY/g food and 0.0001 mg AMPA/g food; the GBH13 food had 0.086 mg GLY/g food and 0.0027 mg AMPA/g food; and the GBH34 food had 0.215 mg GLY/g food and 0.0068 mg AMPA/g food. The GLY and AMPA concentrations in food were determined by Phytocontrol company (Nîmes, France). Gallup (Gallup Super 360) was obtained from Axereal (Monnaie, France) and contains 360 g GLY/L (485.8 g isopropylamine salt/L). Control diet composition is shown in the Supplementary Table 1.

### Biological Sample Collection

Blood samples were collected from the occipital sinus into heparin tubes on D9 and D25 from 10 broilers per group. Blood samples were centrifuged (5,000 *g*, 10 min, 4°C) and stored at -20°C before use for the thiobarbituric acid reactive substances (**TBARS**), GLY, and AMPA assays. Tissues samples were obtained on D25.

### Plasma Thiobarbituric Acid Reactive Substances Assay

Lipid peroxidation, as determined through measuring the amount of MDA (malondialdehyde) that reacts with 2-thiobarbituric acid, was used to estimate oxidative stress (Armstrong and Browne, 1994). Blood samples were collected into EDTA-treated tubes, then gently shaken and kept and handled on wet ice. The plasma was separated by centrifuging the blood samples at 1,000 × *g* for 10 min at 4°C, then transferred to 1.5 mL microcentrifuge tubes and stored at −80°C. The TBARS values of the EDTA-treated plasma were measured using the modified method of Grotto et al. (2007). A standard curve for 1,1,3,3-tetramethoxypropane was used, and the concentration was expressed as mmol MDA/mL solution.

### GLY and AMPA Assays

The plasma GLY and AMPA concentrations were measured after a derivatization reaction using 9-fluorenylmethyl chloroformate (**FMOC-Cl**), in collaboration with Dr. S El Balkhi (Service de Pharmacologie, Toxicologie et Pharmacovigilance, Limoges, France), as described previously (Serra et al., 2021).

### Feed Conversion Ratio

The FCR was calculated for each animal on D9, D16, and D25 with the following formula:

$$FCR = \frac{Total\ feed\ consummed\ between\ two\ days\ (g)}{Weight\ gain\ between\ two\ days(g)}$$

### Relative Tissue Weight

On D25, the ovaries, abdominal adipose tissue, and spleen were weighed and their relative weight (ratio) was determined with the following formula:

$$Ratio = \frac{Tissue\ weight\ (g)}{Body\ weight\ (g)}$$

### RT-qPCR

Total RNA from CT, GBH13, and GBH34 ovaries and ovarian explants (n = 8 per group) were extracted using the TRIzol RNA Isolation Reagent (Invitrogen by Life Technologies, Montigny le Bretonneux, France) and an ULTRATURAX instrument for grinding, according to the manufacturer's recommendations. The RNA concentration and purity (based on the A260/A280 ratio) were determined with a Nanodrop spectrophotometer. Two micrograms of total RNA was reverse transcribed into complementary DNA (**cDNA**) in a 20-$\mu$L reaction containing 0.5 mM of each deoxyribonucleotide triphosphate (dATP, dTTP, dGTP, and dCTP), 2 M RT Buffer, 15 $\mu$g/$\mu$L of oligodT, 0.125 U of ribonuclease inhibitor, and 0.05 U of Moloney murine leukemia virus reverse transcriptase incubated at 37°C for 1 h. Quantitative polymerase chain reaction was performed in a 13 $\mu$L reaction containing 3 $\mu$L of cDNA and 8 $\mu$L of SYBR Green Supermix 1X Reagent (Bio-Rad, Marnes-la-Coquette, France), and 250 nM of specific primers (Invitrogen by Life Technologies, Montigny le Bretonneux, France); the primer sequences are shown in Table 1. Each sample was run in duplicate in a 384-well plate on a MyiQ real-time PCR detection system (Bio-Rad, Marnes-la-Coquette, France). The thermal cycling protocol was: incubation at 50°C for 2 min; denaturation at 95°C for 10 min; and 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. For each target gene, expression was calculated according to primer efficiency (E) and quantification cycle (Cq), where expression = E − Cq. Then, the relative expression of the target gene was determined by using the geometric mean expression of 3 reference genes (*GAPDH, ACTB*, and *EEF1α*).

### Western Blotting

Total protein was extracted from CT, GBH13 and GBH34 ovaries with lysis buffer (Tris HCl 1 M [pH 7.4], NaCl 0.15 M, EDTA 1.3 mM, EGTA 1 mM, VO43 23 mM, NaF 0.1 M, $NH_2PO_4$ 1%, and Triton 0.5%) and an ULTRATURAX instrument for grinding. The lysates were centrifuged (16,000 *g*, 20 min, 4°C), and the supernatant was collected and kept on ice. A bicinchoninic acid (**BCA**) protein assay kit (Interchim, Montluçon, France) was used to measure the protein concentration. Eighty micrograms of protein was mixed with 5 × Laemmli buffer and denatured at 95°C for 5 min. The protein was loaded on a sodium dodecyl

**Table 1.** Primer sequences used for RTqPCR analysis.

| Gene | Primer F | Primer R | Reference |
|---|---|---|---|
| *GAPDH* | ACGGATTTGGTCGTATTGGG | TGATTTTGGAGGATCTCGC | (Grandhaye et al., 2020) |
| *EEF1α* | AGCAGACTTTGTGACCTTGCC | TCACATGAGACAGACGGTTGC | |
| *β-actin* | ACGGAACCACAGTTTATCATC | GTCCCAGTCTTCAACTATACC | |
| *StAR* | TGCCATCTCCTACCAACA | CATCTCCATCTCGCTGAAG | (Estienne et al., 2020) |
| *HSD3β* | TACTGCTGGAAGAAGATGAG | CAAGGTGTCAATGATGGAAG | |
| *CYP11A1* | TGAATATCATCAGCCCCCGC | GTAGGGCTTGTTGCGGTAGT | |
| *CYP19A1* | TGTTCCATCACGCTATTT | GATTCTTGTTTGGGCTTC | (Jin et al., 2020) |
| *Catalase* | ACTGCAAGGCGAAAGTGTTT | GGCTATGGATGAAGGATGGA | |
| *GPX1* | AACCAAATTCGGGCACCAG | CCGTTCACCTCGACTTCTC | |
| *COLEC12* | GCGCCAAGATGAAAGACGAT | GCCACCTGTCACATTGTCCA | |
| *HMGCR* | ATGTCAGGAGTGCGACAACT | CGTCCTTCACGACTCTCTCG | (Jiang et al., 2020) |
| *THRSP* | GTTCTGACCGACCTCACCAA | GTGGGACTTGGCACAGGAAT | |

sulfate−polyacrylamide gel (12%) and separated by electrophoresis. The separated protein was transferred onto a nitrocellulose membrane. The membrane was incubated in Tris-buffered saline with 0.05% Tween 20% and 5% nonfat milk for 30 min at room temperature to block nonspecific protein binding. Then, the membrane was incubated overnight at 4°C with the appropriate primary antibody diluted 1:1,000: 3beta-hydroxysteroid-dehydrogenase (HSD3B1, Thermo Fisher Scientific, Illkirch-Graffenstaden, France, reference PA5-106895), cytochrome P450 11a1 (CYP11A1, Thermo Fisher Scientific, reference PA5-37359), steroidogenic acute regulatory (STAR, Thermo Fisher Scientific, reference PA5-21687), P450 aromatase (CYP19A1, Serotec, Varilhes, France), and vinculin (Sigma, Saint Quentin Fallavier, France, reference V9131). The next day, the membrane was incubated for 90 min at room temperature with horseradish peroxidase−conjugated anti-rabbit or anti-mouse IgG (Bio-Rad, Marnes-la-Coquette, France) diluted 1:5,000. An enhanced chemiluminescence substrate (Western Lightning Plus-ECL, Perkin Elmer, Villebon-sur-Yvette, France) and the G-box SynGene (Ozyme, St Quentin en Yvelines, France) and GeneSnap software (Ozyme) were used to visualize the protein bands. The GeneTools software (version 4.01.02; Syngene) was used to quantify the bands. The results are expressed as the signal intensity of the protein of interest after normalization with the vinculin signal intensity (arbitrary units).

## RNA Extraction, Library Preparation, and RNA-seq

Total RNA from CT, GBH13, and GBH34 ovaries (n = 5 per group) was extracted with the RNeasy Midi Kit (Qiagen, Courtaboeuf, France) and purified with a DNA-free kit (Invitrogen by Life Technologies, Montigny le Bretonneux, France), according to the manufacturer's recommendations. The total RNA quality was assessed with an Agilent Bioanalyzer 2100, using a RNA 6000 Pico Kit (Agilent Technologies, Les Ulis, France). Directional RNA-seq libraries were constructed using the TruSeq Stranded Total RNA library prep kit (Illumina, Évry-Courcouronnes, France), following the manufacturer's instructions, using 750 ng of total RNA. After the Ribo-Zero step, the samples were checked with an Agilent Bioanalyzer for proper ribosomal RNA depletion. The quality of the final libraries was assessed with an Agilent Bioanalyzer, using an Agilent High Sensitivity DNA Kit. The libraries were pooled in equimolar proportions and sequenced using 2 single-read 75 base pair (bp) runs on an Illumina NextSeq500 instrument, using NextSeq 500 High Output 75 cycles kits.

## Sequence Data Filtering

Demultiplexing was done (bcl2fastq2 V2.15.0) and adapters were removed (Cutadapt1.3); only reads longer than 10 bp were kept for analysis.

## Read Mapping and Gene Identification

The reads were analyzed by using the nfcore/RNASeq pipeline (https://nf-co.re/rnaseq/doi:10.5281/zenodo.1400710). Following quality and adapter trimming with trim_galore, the reads were mapped to the GRCg7b *Gallus gallus* genome (GCF_016699485.2) using STAR. Various tools integrated into the pipeline, including RSeQC, QualiMap, and dupRadar, were employed to assess sample quality and mapping. Transcripts were identified with geneIDs by using the gene transfer format, and the number of reads for each messenger RNA (**mRNA**) was quantified with the SALMON software tool. The corresponding filtered annotation file contained 18,023 protein-coding genes. The raw sequencing data were available in the EMBL database with the accession number PRJEB72648 (https://www.ebi.ac.uk/ena/browser/view/PRJEB72648).

## Differential Expression Analysis

Normalization of read counts (using the TMM method) and differential expression analysis were performed with the edgeR R/Bioconductor package (Robinson et al., 2010). Genes with a false-discovery rate (**FDR**)-adjusted $P$-value < 0.05 (Benjamini−Hochberg method; do i: 10.1111/j.2517-6161.1995.tb02031.x) and absolute log fold-change (|logFC|) > 0.5 were considered to be statistically significant. Upregulated genes showed

higher expression in the GBH13 or GBH34 group compared with the CT group (|logFC| > 0.5). Downregulated genes showed lower expression in the GBH13 or GBH34 group compared with the CT group. AVenn diagram was prepared with the ggvenn package in R to summarize the number of upregulated and downregulated genes.

### Gene Network Analysis

The Gene Ontology (GO) enrichment analysis was carried out by using the R/Bioconductor gprofiler2 package (for biological process, molecular function, and cellular component). The Kyoto Encyclopedia of Genes and Genomes (**KEGG**) enrichment analysis employed the WikiPathways networks (Kolberg et al., 2023). In addition, similar GO terms were grouped based on their semantic similarity with the rrvgo package in R (Sayols et al., 2023). Finally, the genes associated with each GO term are illustrated using network graphs, which were generated with the enrichplot package in R.

### In Vitro Ovarian Explant Culture

Ovaries from 80 chicks (10 d old) were collected by dissection after slaughtering and kept in saline solution for about 30 min at room temperature before use. For each culture, 16 ovaries were pooled and cut into small pieces (1 mm in diameter). The equivalent of 2 ovaries was cultured in each well of a 96-well culture plate for 24 h with various GBH concentrations in Dulbecco's Modified Eagle's Medium (**DMEM**; Sigma-Aldrich, l'Isle-d'Abeau Chesnes, France) with 1% fetal bovine serum. Conditioned culture medium was collected and stored at -20°C and ovarian explants were collected and stored at -80°C before use for hormonal assays and RT-qPCR, respectively. Five independent cultures were performed (one culture per week for 5 weeks).

### Progesterone and Estradiol Enzyme-Linked Immunosorbent Assays

The progesterone and estradiol concentrations in culture medium from ovarian explants were measured after 48 h of treatment using ELISAs (Cayman Chemicals, Montluçons, France) based on a previous study (Canepa et al., 2008). The intra- and inter-assay coefficients of variation were <10% and <8%, respectively. The results are expressed as the concentration of progesterone and estradiol (ng/mL) per 100 $\mu$g of protein. The data were obtained from 5 independent cultures.

### Statistical Analysis

GraphPad Prism version 10 (GraphPad Software, San Diego, CA) was used for all analyses. The data are presented as the mean ± the standard error of the mean. All data are reported as boxplots (minimum to maximum, with all points shown). Outliers were identified with the ROUT method and removed. The data were analyzed with 1- or 2-way analysis of variance (**ANOVA**) followed by Tukey's honestly significant difference test to compare the means of each group. For the 2-way ANOVA, the 2 factors were GBH concentration (**GBH**) and time effect. A $P$-value < 0.05 was considered as statistically significant.

## RESULTS

### Chronic Dietary GBH Exposure Dose Dependently Reduced Food Intake in Young Female Broilers

The amount of food consumed by the young female broilers over time is shown in Figure 2A; this information allowed us to calculate the estimated amount of GLY and AMPA ingested each day (Figures 2B and 2C, respectively). Of the 3 groups, the CT broilers ate the most (75 ± 1.6% of the given food), followed by the GBH13 broilers (55.2 ± 1.2% of the given food) and the GBH34 broilers (31.5 ± 2.0% of the given food). There was a significant difference between the 3 groups on D3 of the experiment (Figure 2A; $P < 0.001$). Despite the different food intake, the GBH34 broilers ingested 1.5 to 2 times more GLY and AMPA than the GBH13 broilers ($P < 0.0001$). Body weight gain followed the same pattern of food intake: The CT broilers gained significantly more weight than the GBH13 broilers on D9, D16, and D25 (Figure 2D; $P < 0.0001$). The GBH34 broilers had a significantly thicker abdominal fat layer on D9 and D25 (Figure 2E; $P < 0.0001$). Moreover, the GBH13 broilers had a higher body weight and were fatter than the GBH34 broilers (Figures 2D and 2E; $P < 0.0001$). The FCR was significantly higher in the GBH34 broilers than in the GBH13 and CT broilers at all 3 time points (P < 0.0001 on D9 and D25, and P = 0.0004 on D16; Figure 2F) whereas the GBH13 broilers had a higher FCR than the CT broilers only on D9 (Figure 2F; $P = 0.02$).

### Plasma GLY and AMPA Concentrations

On D25, the plasma GLY concentration was significantly higher in the GBH34 broilers than in the GBH13 broilers (Figure 3A; $P < 0.0001$). However, there was no significant difference in the plasma AMPA concentration between the GBH13 and GBH34 broilers, although there was a trend for a higher concentration in the GBH34 broilers (Figure 3B; $P = 0.055$). Plasma oxidative stress, based on the TBARS assay, was significantly increased in the GBH13 and GBH34 broilers (Figure 3C; $P < 0.0001$).

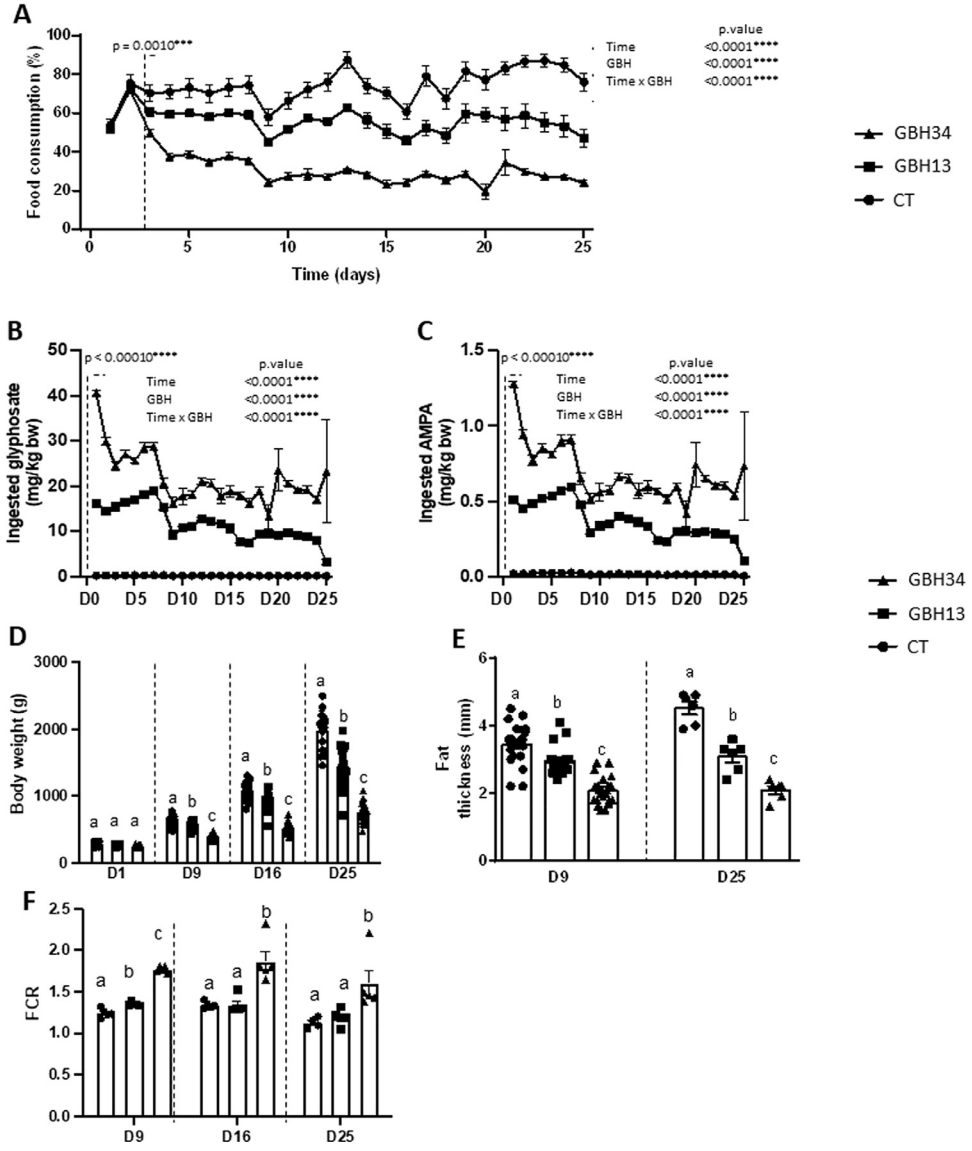


**Figure 2.** (A) The effect of chronic dietary exposure to 2 glyphosate (**GLY**)-based herbicide (**GBH**) concentrations on food intake in growing broilers. Eleven-day-old female broilers were exposed to GBH in the diet for 25 d, corresponding to a dose of 13 mg GLY equivalent/kg body weight/d (the GBH13 group, n = 20) or 34 mg GLY equivalent/kg body weight/d (the GBH34 group, n = 20). The control group (CT, n = 20) received a normal diet. The daily food consumption is shown as a percentage of the total food given (mean ± standard error of the mean). The *P*-values for the time (days), GBH exposure, and the time × GBH interaction are presented. The daily concentration of ingested equivalent (B) GLY and (C) AMPA (mg/kg body weight) in the CT, GBH13, and GBH34 broilers (n = 20 per group). The results are presented as mean ± the standard error of the mean. The *P*-values for the time (days), GBH exposure, and the time × GBH interaction are presented. The evolution of (D) body weight (g) on d 1 (D1), D9, D16, and D25 of the experiment; (E) fat thickness (mm) on D9 and D25; and (F) the feed conversion ratio (FCR) on D9, D16, and D25 in the CT, GBH13, and GBH34 broilers (n = 20 per group). The results are presented as the mean ± the standard error of the mean. For each day, different letters indicate significant differences determined with 1-way analysis of variance followed by Tukey's honestly significant difference test for pairwise comparisons ($P < 0.05$).

## *Chronic Dietary GBH Exposure Modified the Relative Ovary and Abdominal Adipose Tissue Weights*

The GBH34 broilers had a significantly heavier relative ovary weight compared with the CT and GBH13 broilers (Figure 4A; $P = 0.001$). On the other hand, the relative abdominal adipose tissue weight was significantly lower in the GBH34 broilers compared with the GBH13 and CT broilers (Figure 4B; $P < 0.0001$). The relative ovary and abdominal adipose tissue weights were similar between the CT and GBH13 broilers. The relative spleen weight was not significantly different between the CT, GBH13, and GBH34 groups (Figure 4C).

## *Alteration of the Ovarian Transcriptome by Chronic Dietary GBH Exposure in Young Broilers*

RNA-seq analysis revealed that the ovarian transcriptome was strongly affected by the chronic dietary GBH exposure. Compared with the CT ovaries, there were

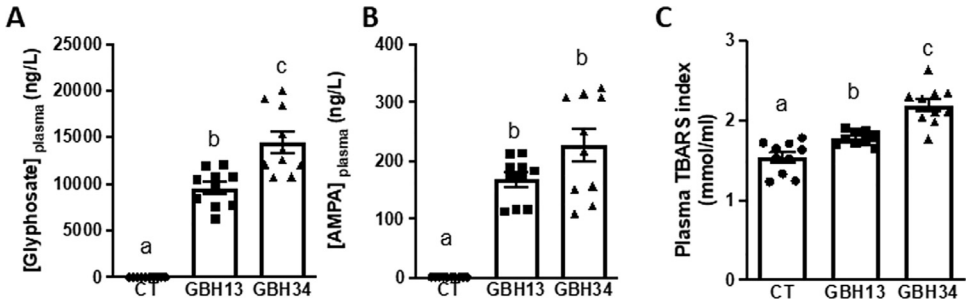


**Figure 3.** The plasma (A) glyphosate (**GLY**, ng/L) and (B) aminomethylphosphonic acid (**AMPA**, ng/L) concentrations and the thiobarbituric acid-reactive substances (**TBARS**) index in the control (**CT**), GBH13, and GBH34 broilers on d 25 of the experiment. Eleven-day-old female broilers were exposed to GBH in the diet for 25 d, corresponding to a dose of 13 mg GLY equivalent/kg body weight/d (the GBH13 group, n = 10) or 34 mg GLY equivalent/kg body weight/d (the GBH34 group, n = 10). The CT group (n = 10) received a normal diet. The results are presented as the mean ± standard error of the mean. Different letters indicate significant differences determined with one-way analysis of variance followed by Tukey's honestly significant difference test for pairwise comparisons ($P < 0.05$).

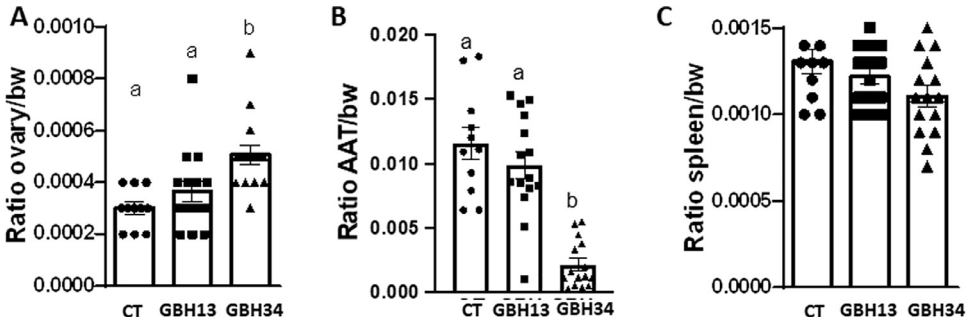


**Figure 4.** The weight of the (A) ovaries, (B) abdominal adipose tissue, and (C) spleen relative to the body weight in the control (**CT**), GBH13, and GBH34 broilers on d 25. Eleven-day-old female broilers were exposed to GBH in the diet for 25 d, corresponding to a dose of 13 mg GLY equivalent/kg body weight/d (the GBH13 group, n = 20) or 34 mg GLY equivalent/kg body weight/d (the GBH34 group, n = 20). The CT group (n = 20) received a normal diet. The results are presented as the mean ± the standard error of the mean. Different letters indicate significant differences determined with 1-way analysis of variance followed by Tukey's honestly significant difference test for pairwise comparisons ($P < 0.05$).

935 differentially expressed genes (**DEG**) in the GBH34 ovaries, 253 DEGs in the GBH13 ovaries, and 295 common DEGs in the GBH13 and GBH34 ovaries (Figure 5). All DEGs, up- and down-regulated genes was provided as Supplementary Table 2.

The GO enrichment analysis for the CT vs. GBH34, CT vs. GBH13, and GBH13 versus GBH34 comparisons are presented in Figures 6A, 7A, and 8A, respectively. The DEGs with |logFC| > 0.5 for the CT versus GBH34, CT versus GBH13, and GBH13 versus GBH34 comparisons are presented in Figures 6B, 7B, and 8B, respectively. For biological process, the most abundant GO terms for the GBH34 versus CT comparison were related to sterol and cholesterol metabolic processes and axoneme assembly (Figure 6A). Genes involved in the steroidogenesis process such as STAR (a cholesterol carrier) and hydroxy-delta-5-steroid dehydrogenase enzyme (**HSDB1**) were significantly reduced in the GBH34 compared with the CT ovaries (Figure 6B). However, radial spoke head 1 homolog (**RSPH1**), which is involved in primary ciliary dyskinesia and contributes to axoneme assembly, and hormone-sensitive lipase, HSL (**LIPE**) showed higher expressed in GBH34 ovaries (Figure 6B). For the GBH13 versus CT comparison, the most abundant GO terms were RNA processing and metabolic processes (Figure 7A). Genes involved in these processes showed higher expression in the GBH13 ovaries compared with the CT ovaries. However, we also found a significant reduction in a steroidogenic enzyme, CYP19A1, in the GBH13 ovaries (Figure 7B). The most important GO terms observed for the GBH34 versus GBH13 comparison were related to synapse and trans-synaptic signaling, secretory vesicle, and calcium ion-regulated exocytosis of neurotransmitters (Figure 8A). In addition, we observed an increase in the axonogenic processes and a decrease in cholesterol processes with a significant reduction in STAR expression (Figure 8B)

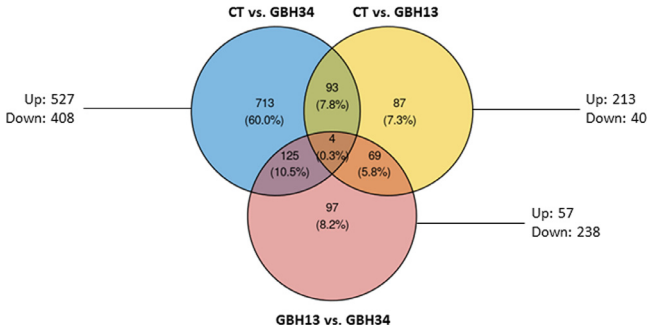


**Figure 5.** Venn diagram of differentially expressed genes in the ovaries of control (**CT**), GBH13, and GBH34 broilers on d 25. Eleven-day-old female broilers were exposed to GBH in the diet for 25 d, corresponding to a dose of 13 mg GLY equivalent/kg body weight/d (the GBH13 group) or 34 mg GLY equivalent/kg body weight/d (the GBH34 group). The CT group received a normal diet.

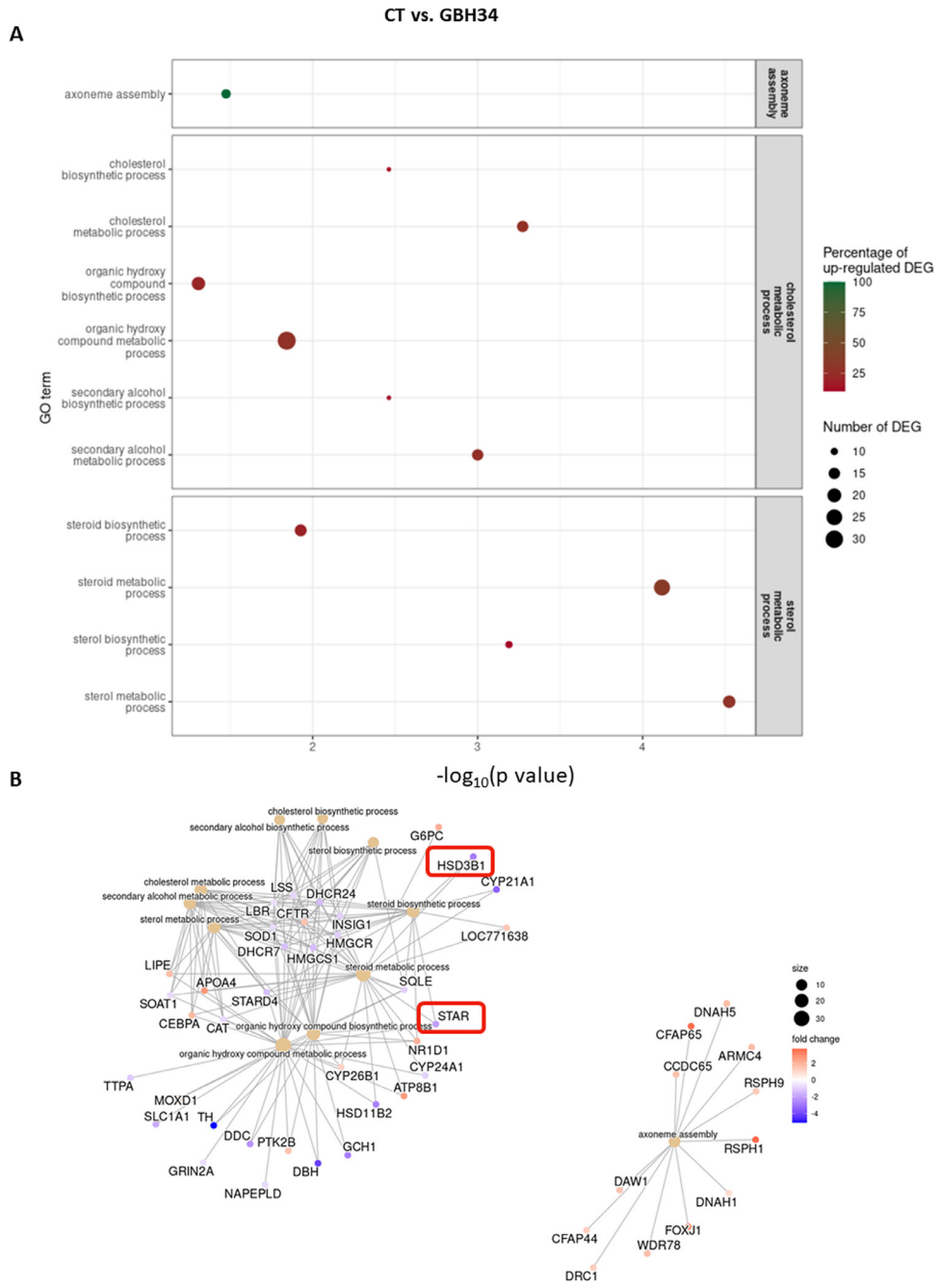


**Figure 6.** (A) The top 3 enriched gene ontology (**GO**) terms of differentially expressed genes (**DEG**) in the ovaries for the control (**CT**, n = 5) vs. GBH34 (n = 5) comparison on d 25 of the experiment. The size of the dots is positively correlated with the number of DEGs in the pathway. (B) Gene interaction network of the DEGs in the CT and GBH34 ovaries. The network nodes and edges represent gene and gene−gene associations, respectively. The red and purple nodes indicate the upregulated and downregulated genes, respectively. The size of the dots is positively correlated with the number of DEGs in the pathway.

## *Alteration of the Ovarian Expression of Steroidogenic Factors and Other Genes by Chronic Dietary GBH Exposure in Young Broilers*

We next validated our RNA-seq data by evaluating the mRNA and protein expression of steroidogenic factors and other genes after chronic dietary GBH exposure. As shown in Figure 9A, the GBH13 and GBH34 ovaries showed significantly reduced mRNA expression of *CYP11A1* ($P < 0.0001$), *CYP19A1* ($P < 0.0001$), and *HSD3B1* ($P = 0.004$). Moreover, the GBH34 ovaries showed markedly reduced *STAR* mRNA expression ($P = 0.012$). Similarly to the mRNA expression, CYP19A1 and STAR protein expression was significantly reduced to levels almost undetectable in the GBH13 ovaries (Figure 9B; $P = 0.010$ and $P = 0.011$, respectively). The CYP19A1 and STAR protein levels

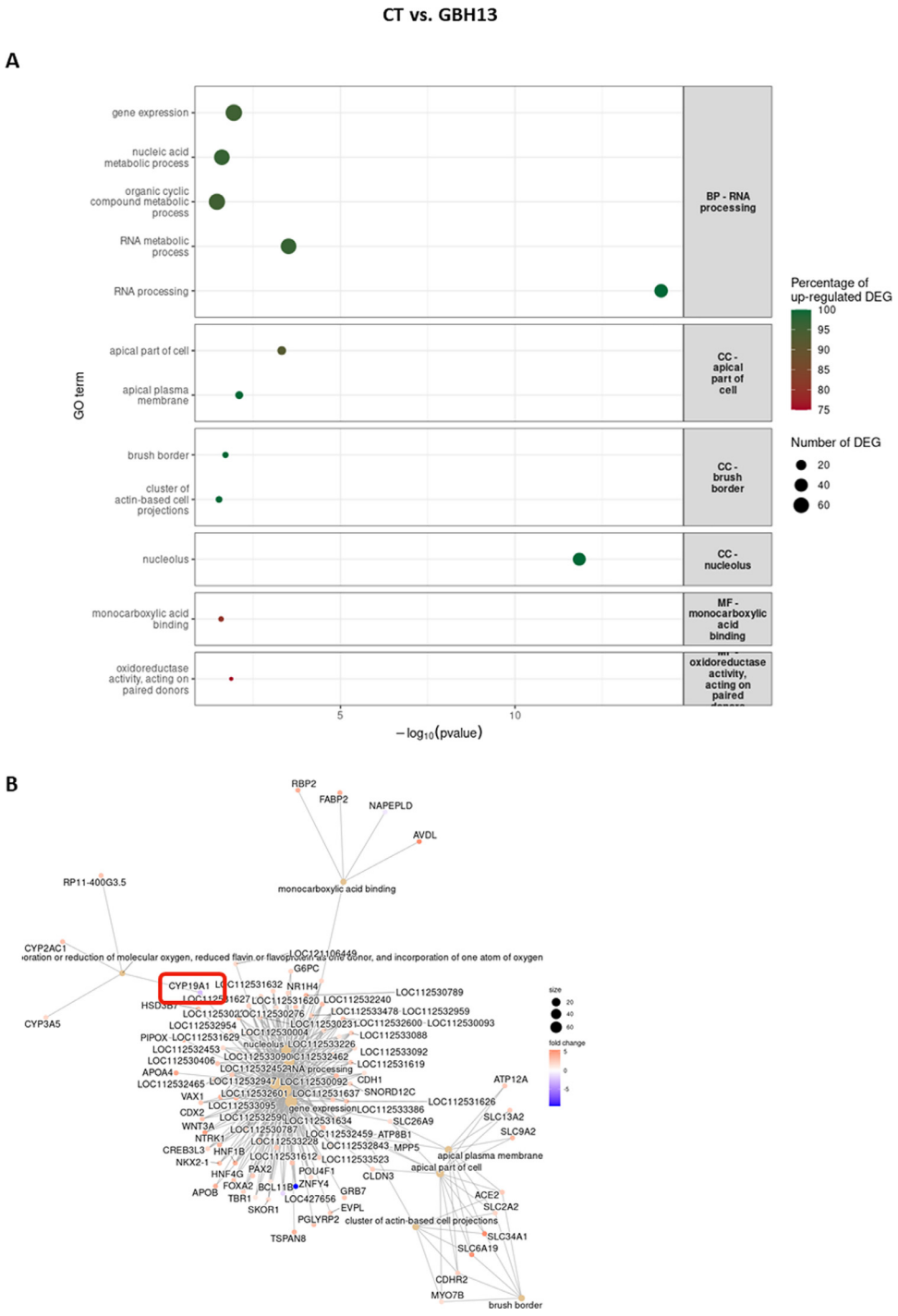


**Figure 7.** (A) The top 6 enriched gene ontology (**GO**) terms of differentially expressed genes (**DEG**) in the ovaries of the control (**CT**, n = 5) vs. GBH13 (n = 5) comparison. The size of the dots is positively correlated to the number of DEGs in the pathway. (B) Gene interaction network of the DEGs in the CT and GBH13 ovaries. The network nodes and edges represent gene and gene−gene associations, respectively. The red and purple nodes indicate the upregulated and downregulated genes, respectively. The size of the dots is positively correlated with the number of DEGs in the pathway.

tended to be partially restored in the GBH34 ovaries (CT vs. GBH34, $P = 0.2$ and $P = 0.09$, respectively). CYP11A1 protein expression was not significantly different between the CT, GBH13, and GBH34 ovaries ($P = 0.3$). Nonetheless, it appeared to be higher in the GBH34 ovaries and lower in the GBH13 ovaries (the measured protein level was near zero for most samples). The HSD3B1 protein level was significantly increased in the GBH34 ovaries ($P = 0.0014$). RT-qPCR also validated the RNA-seq data by showing changes in the GBH13 and GBH34 ovaries compared with the CT ovaries for other genes, including 2 genes that encode lipid metabolism regulators (*HMGCR* and *THRSP*), 2 genes that encode antioxidant enzymes (*CAT* and *GPX1*), and a gene that encodes a protein involved in immunity (*COLEC12*) (Table 2).

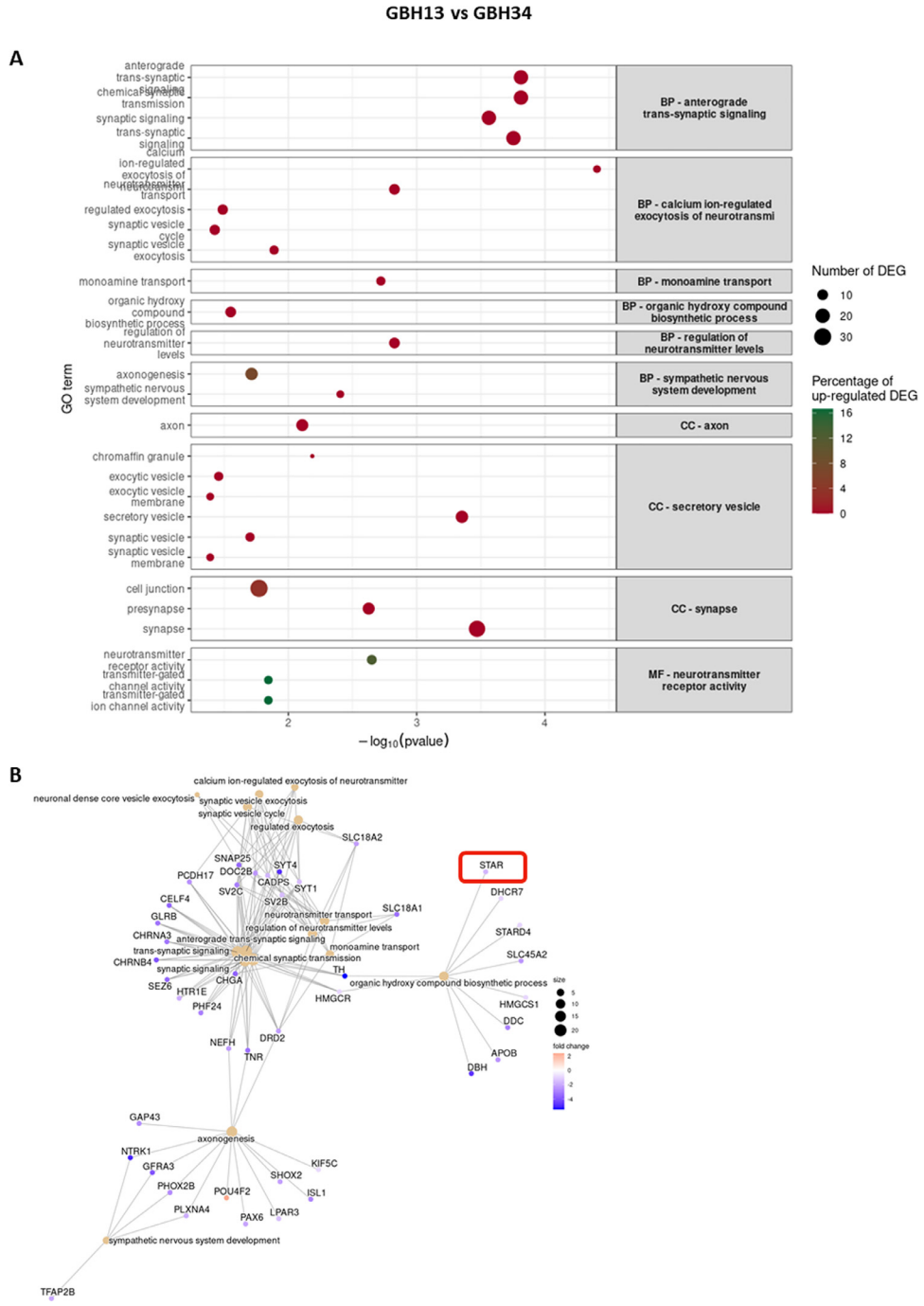


**Figure 8.** (A) The top 10 enriched gene ontology (**GO**) terms of differentially expressed genes (**DEG**) in the ovaries of the GBH13 (n = 5) vs. GBH34 (n = 5) comparison. The size of the dots is positively correlated to the number of DEGs in the pathway. (B) Gene interaction network of the DEGs in the CBH13 and GBH34 ovaries. The network nodes and edges represent gene and gene−gene associations, respectively. The red and purple nodes indicate the upregulated and downregulated genes, respectively. The size of the dots is positively correlated with the number of DEGs in the pathway.

### *Reduced Steroid Production by GBH-Treated Ovarian Explants From Young Broilers*

We investigated the effects of GBH treatment on steroid production in ovarian explants from young broilers. GBH dose dependently reduced progesterone (Figure 10A; $P < 0.0001$) and estradiol (Figure 10B; $P < 0.0001$) release by ovarian explants. Moreover, GBH treatment dose dependently reduced *STAR, HSD3B1*, and *CYP19A1* mRNA expression ($P < 0.0001$ for all 3 transcripts), but had no effect on *CYP11A1* mRNA expression ($P > 0.05$). Thus, the decrease in steroid secretion in response to GBH may be due to a reduction in *STAR, HSD3B1* and *CYP19A1* expression.

## DISCUSSION

We have shown for the first time that chronic dietary GBH exposure alters metabolic and ovarian functions in growing broilers. Indeed, GBH exposure dose dependently reduced food intake, body weight, and fattening,

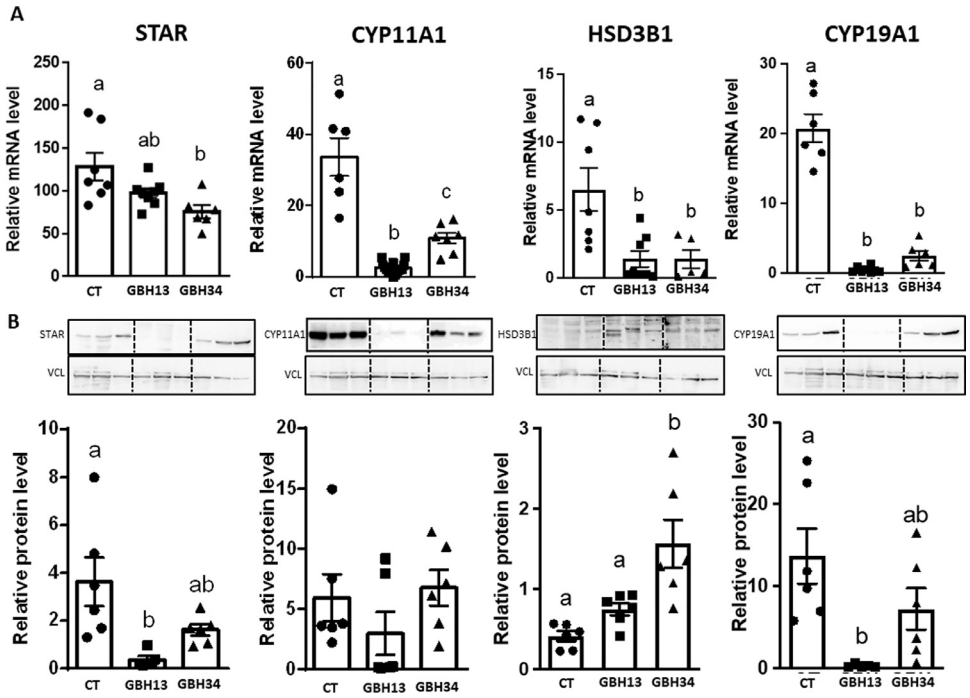


**Figure 9.** The (A) messenger RNA (mRNA, n = 8 per group) and (B) protein (n = 6 per group) expression of steroidogenic factors (STAR, HSD3B1, CYP11A1 and CYP19A1) in control (CT), GBH13, and GBH34 ovaries on d 25. Eleven-day-old female broilers were exposed to GBH in the diet for 25 d, corresponding to a dose of 13 mg GLY equivalent/kg body weight/d (the GBH13 group) or 34 mg GLY equivalent/kg body weight/d (the GBH34 group). The CT group received a normal diet. The results are presented as the mean ± the standard error of the mean. Different letters indicate significant differences determined with 1-way analysis of variance followed by Tukey's honestly significant difference test for pairwise comparisons ($P < 0.05$).

and increased plasma GLY, AMPA, and oxidative stress. Furthermore, the relative ovary weight was increased in GBH-treated broilers. Ovarian transcriptomic analyses using RNA-seq showed that steroidogenesis, sterol, and cholesterol metabolic processes were altered in response to GBH exposure. We validated the RNA-seq data with RT-qPCR of whole ovaries as well as in ovarian explants treated with GBH.

In the present study, the CT, GBH13, and GBH34 broilers received the same amount of food during the experiment; however, we found that the more GBH the food contained, the less the broilers consumed. Although the GBH34 broilers consumed less food than the GBH13 broilers, we estimated that they ingested more GLY and AMPA. These calculations were confirmed by the plasma GLY and AMPA concentrations. We noted that the CT broilers gained the most weight and fat of the 3 groups. The reason why the GBH13 broilers gained less weight and fat was likely due to the reduced food intake. The GBH34 broilers, who ate the least amount of food, showed the lowest weight and fat gain during the experiment. We propose 2 hypotheses to explain the rejection of the diet containing GBH: 1) GBH gives the food an unpleasant tase, so the broilers eat less; and 2) GBH produces a feeling of satiety, so the broilers eat less. GBH enrichment is known to decrease food consumption in birds (Evans & Batty, 1986). When that study was published, the authors also could not explain the exact reason for this observation. It is quite surprising that very few studies have focused on the rejection of GLY-containing food in animals, given that palatability is a crucial parameter of feeding behaviors. We tend to prefer our second hypothesis given that all 3 broiler groups ate the same amount of food during the first days of the experiment, suggesting that the taste of the food did not disturb them (of note, the broilers were not subjected to food restriction before the experiment began). Only the relative ovary weight was affected by the addition of GBH: The ovaries were heavier in the GBH34 broilers. A previous study showed that oral administration of

**Table 2.** Relative expression of various genes in hens' ovaries (CT, n = 8, GBH13, n = 8, GBH34, n = 8).

| | | CT | GBH13 | GBH34 | *P* value |
|---|---|---|---|---|---|
| Oxidative stress | *Catalase* | $0.69 \pm 0.10^{a}$ | $0.14 \pm 0.01^{b}$ | $0.46 \pm 0.11^{a}$ | 0.0002 |
| | *GPX1* | $6.97 \pm 0.81^{a}$ | $4.45 \pm 0.53^{b}$ | $4.01 \pm 0.41^{b}$ | 0.0076 |
| Immunity | *COLEC12* | $0.19 \pm 0.04^{a}$ | $0.02 \pm 0.003^{b}$ | $0.10 \pm 0.02^{b}$ | 0.0003 |
| Lipid metabolism | *HMGCR* | $9.84 \pm 3.56^{a}$ | $0.11 \pm 0.01^{b}$ | $1.56 \pm 0.86^{b}$ | 0.0119 |
| | *THRSP* | $7.84 \pm 2.90^{a}$ | $0.01 \pm 0.004^{b}$ | $1.13 \pm 0.69^{ab}$ | 0.0187 |

Results are presented as mean ± SEM of the several target gene mRNA, expression relative to the geometric mean of 3 housekeeping genes expression (*GAPDH, EEF1α* and *β-actin*); Letters indicate statistical significance. CT, control; GBH13, [glyphosate equivalent] = 13 mg/kg body weight/d, GBH34, [glyphosate equivalent] = 34 mg/body weight/d.

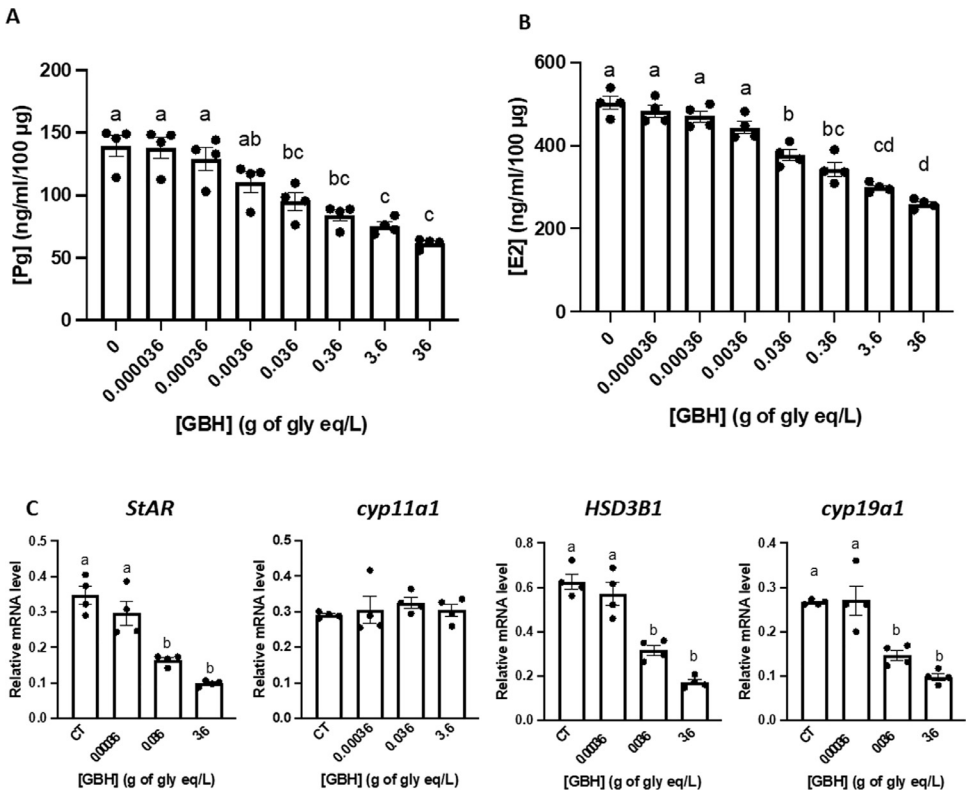


**Figure 10.** The effect of glyphosate-based herbicide (**GBH**) on in vitro steroid production by chicken ovarian explants. Conditioned culture medium was collected after 24-h exposure to GBH (0.000036−36 g glyphosate/L), and the (A) progesterone (Pg) and (B) estradiol (E2) concentrations were measured with enzyme-linked immunosorbent assays. (C) Total RNA was extracted from ovarian explants, and *HSD3B1, STAR, CYP11A1*, and *CYP19A1* messenger RNA (**mRNA**) expression was determined. The data are expressed as the mean ± the standard error of the mean of 5 replicates (one replicate is representative of approximately 2 ovaries for each condition). Bars with different letters are significantly different ($P < 0.05$).

GBH or GLY alone could modulate ovarian steroidogenic enzymes transcription in pregnant mice (Ren et al., 2018). In that study, the authors administered GBH to the animals through the drinking water. Consequently, their water consumption was lower, but not their food consumption. Still, their body weight was negatively affected by the treatment. This means that the growth reduction we observed in young broilers could be due to reduced feed intake as well as energy imbalance caused by the GBH ingestion. This energy imbalance could ultimately lead to the steroidogenic disruptions we detected, given that lipids and sterols play key roles in steroidogenesis.

*In vitro* exposure of ovarian explants to increasing GBH concentrations seemed to trigger similar dynamics, as it reduced the estradiol and progesterone concentrations in the culture medium. This indicates the likely involvement of another mechanism in the steroidogenic impairment in the GBH13 broilers. Another possible cause could then be the induction of oxidative stress, which would impair steroidogenesis. Oxidative stress induction by GBH has widely been discussed (Kwiatkowska et al., 2014; Owagboriaye et al., 2019; Turkmen et al., 2019). Here, *catalase* and *GPX1* transcripts levels were significantly reduced after GBH administration, suggesting an increase in hydrogen peroxide and other organic hydroperoxides. Considering the important role of lipids in steroidogenesis, lipid peroxidation could explain the reduction in steroid synthesis, as steroidogenic enzymes are very sensitive to this phenomenon (Takayanagi et al., 1986). We detected reduced *HMGCR* and *THRSP* transcript levels; these genes encode proteins that are involved in lipid and sterol dynamics.

The higher ovary weight in the GBH34 broilers prompted us to perform RNA-seq to evaluate changes in transcripts in response to the GBH exposure. In the GBH34 broilers, most of the genes affected were related to cholesterol and sterol metabolic process, such as *HSD3B1, HSD11B2*, and *STAR*, which were downregulated in the GBH34 broilers compared with the CT broilers. We confirmed these results with RT-qPCR. This analysis also showed that the *CYP11A1* and *CYP19A1* transcripts were downregulated in the GBH13 and GBH34 broilers, but with a stronger effect in the GBH13 broilers. The CYP19A1, CYP11A1, and STAR protein levels followed the same U-shaped pattern as their mRNAs. However, in the GBH34 broilers, the HSD3B1 protein level was surprisingly high, while its mRNA level was low. This type of non-monotonic U-shaped curve is reminiscent of what we can observe for some endocrine-disrupting chemicals (**EDC**; Vandenberg, 2014). Numerous studies have noted that GLY could be an EDC (Muñoz et al., 2021). Our results are support this view: GLY seems to act as an EDC in young broiler ovaries.

In a previous study, we showed that GBH is weakly biotransformed in adult female broiler liver (Fréville et al., 2022). Dietary GBH administration did not modulate the transcription of hepatic biotransformation genes, suggesting that it is eliminated unchanged in the urine and feces. Researchers have reached similar conclusions with other models (Williams et al., 2000; Panzacchi et al., 2018). Nonetheless, we observed that chronic dietary GBH exposure dose dependently altered the mRNA expression of several cytochrome P450s. A study showed that in ovo administration of GLY and/or GBH could affect the mRNA expression of some cytochrome P450s in young broiler embryos (Fathi et al., 2020). Some of the overexpressed cytochrome P450 are known to be linked to pollutant families. For example, *CYP1A4* transcription is known to be induced by dioxin-related compounds (Head & Kennedy, 2007; Kubota et al., 2008). Of note, GBHs comprise GLY as well as other products that help GLY exercise its herbicidal properties. Although some of them have been well described, like polyethoxylated tallow amines (**POEA**; Martins-Gomes et al., 2022), the exact recipe for most GBHs remains confidential. Therefore, we cannot designate a single compound as responsible for the effects we detected. However, we do know the exact GLY concentrations to which the broilers were exposed: 0.086 and 0.215 mg/kg food for the GBH13 and GBH34 groups, respectively. These concentrations are notably lower than what is found in cereals after GBH spread on fields (Eason & Scanlon, 2002). Thus, it is not incongruous to think that the effects we observed in broilers could be observed in wild birds. Nevertheless, GLY is not very persistent in soil, as its half-life in agricultural soil varies from 4 to 19 d, depending on the nature of the soil (Al-Rajab & Schiavon, 2010). Any conclusions drawn from our results must be balanced by the fact that wild animals are not actually exposed to a constant dose of GBH through their diet. Moreover, not all of the observed effects can be attributed to GBH or starvation-induced disorders; they are probably a combination of both.

## CONCLUSIONS

We showed that chronic dietary GBH alters feeding behavior in young female broilers, leading to a reduction in growth performance and an increase in plasma oxidative stress. These alterations were accompanied by a significant reduction in ovarian steroidogenesis, suggesting that GBHs are potential EDCs. Future work should focus on exploring the mechanisms leading GBHs to alter hormones dynamics. The ovarian transcriptomic data from young female broilers subjected to chronic dietary GBH exposure identified potential regulatory networks associated with GBH. These data provide valuable support for understanding the ovarian transcriptional regulatory mechanism of GBHs in growing broilers.

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## DISCLOSURES

The authors declare no conflicts of interest.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2024.103767.

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