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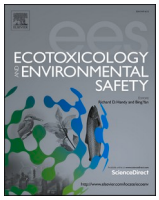

Chronic dietary exposure to a glyphosate-based herbicide results in reversible increase early embryo mortality in chicken

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ABSTRACT

Glyphosate (Gly) is the active molecule of non-selective herbicides used in conventional agriculture. Some evidence shows that exposure to Glyphosate-Based Herbicides (GBH) can affect both male and female fertility in animal models. However, few data exist on birds that can be easily exposed through their cereal-based diet. To our knowledge, there are no current studies on the effects of chronic dietary exposure to GBH and the potential reversibility on the fertility and embryo development in chickens. In our protocol, hens (32 weeks-old) were exposed to GBH (47 mg kg-1/day-1 glyphosate equivalent corresponding to half of the No-Observed-Adverse-Effect-Level (NOAEL) as defined by European Food Safety Authority in birds, GBH group (GBH), n = 75) or not (Control group (CT), n = 75) for 6 weeks. Then, both CT and GBH groups were fed for 5 more weeks without GBH exposure. During these two periods, we investigated the consequences on the egg performance and quality, fertilization rate, embryo development, and viability of offspring. Despite the accumulation of Gly and its metabolite aminomethylphosphonic acid (AMPA) in the hen blood plasma, the body weight and laying rate were similar in GBH and CT animals. We observed from the 4th day of exposure an accumulation of Gly (but not AMPA) only in the yolk of the eggs produced by the exposed hens. After artificial insemination of the hens followed by eggs incubation, we showed a strong significant early embryonic mortality level in GBH compared to CT animals (78 ± 2 % vs 2.5 ± 0.3 %, $p < 0.0001$) with embryo death mainly occurring on the third day of incubation. By using computed tomography (CT) and magnetic resonance imaging (MRI) tools, we noted a significant delay in the embryo development of GBH survivors at 15 days with a reduction by half of the embryo volume and some disturbances in the calculated volumes of the embryonic annexes. At 20 days of incubation, we showed a reduction in the length of the tibia and in the volume of the soft tissues whereas the skeleton volume was increased in GBH chicks. The vast majority of these phenotypes disappeared two weeks after an arrest of the GBH maternal dietary exposure. Taken together, the dietary chronic exposure of broiler hens to GBH at a Gly equivalent concentration lower than NOAEL induces an accumulation of Gly in the egg yolk resulting in severe early embryonic mortality and a delayed embryonic development in survivors that were abolished two weeks after the end of GBH exposure.

1. Introduction

Glyphosate (Gly), or N- (phosphonomethyl) glycine, is widely used as an active ingredient in herbicides that are common in conventional agriculture products such as Roundup (RU) (Muñoz et al., 2021). Glyphosate-based herbicides (GBHs) are mainly composed of 36–48 % of Gly, water, salt, and other coformulants such as polyoxyethylene tallow amine (POEA), a hydrophilic ethoxylated alkylamine, and of contaminants such as heavy metals or polycyclic aromatic hydrocarbons (PAHs), and quaternary ammonium (Defarge et al., 2018, 2016; Mesnage et al., 2019). Gly has a broad spectrum and can act on various weed species (Agostini et al., 2020). In animals and plants, Gly is metabolized

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into CO_2 and amino-methyl-phosphonic acid (AMPA) by the glyphosate oxidoreductase enzyme (Mesnage et al., 2015). A review reported the persistence and toxicity of Gly metabolites and coformulants present in herbicide formulations, and verified the animal toxicity induced by Gly, its metabolite AMPA and GBH, evaluated in various experimental models (Martins-Gomes et al., 2022). Moreover, a study conducted in xenopus and chicken embryos showed that Gly is able to influence the retinoic acid signaling and the Sonic hedgehog (Shh) signaling in vertebrates species during embryonic development leading to marked alterations in cephalic and neural crest development and shortening of the anterio-posterior axis (Paganelli et al., 2010). Some recent evidence shows that Gly and GBHs have deleterious effects on vertebrates at different levels, such as the central nervous system, liver, kidney, blood, or reproductive tract (Agostini et al., 2020; Gillezeau et al., 2019; Ingaramo et al., 2020; Jarrell et al., 2020; Martinez and Al-Ahmad, 2019). At the female reproductive tract level, GBHs has been suspected to be an endocrine disruptor by altering normal hormonal functions. In mammals, steroidogenesis is disturbed, with an induction of a delay of puberty onset (Dallegrave et al., 2007) and perturbations in estradiol (E2) and progesterone (P4) production in female rodents (Hamdaoui et al., 2018; Ren et al., 2018), swine (Gigante et al., 2018) and bovine ovarian cells (Perego et al., 2017a, 2017b). A recent review discussed the potential role of GBHs as an endocrine-disrupting chemical (EDC) (Ingaramo et al., 2020). Moreover, it appears that GBHs and/or AMPA can also behaves as an inhibitor of cell growth and induces cell apoptosis in in vitro studies conducted on human ovarian cells (Muñoz et al., 2021).

Few studies have been performed on avian species, whereas wild and domesticated birds are strongly threatened by exposure to pesticides through their rich cereal diet. In Japanese quails, a long-term exposure to GBHs, with a concentration of Gly below the one that causes observable adverse effects (NOAEL; 100 mg/kg/body weight/day(European Food Safety Authority (EFSA), 2018)), showed that Gly and AMPA could accumulate within the liver and reduce the plasma testosterone (T) levels during puberty in males (Ruuskanen et al., 2020a). Moreover, GBHs appear to induce epigenetic effects, with an impact on the phenotype of the offspring when the parents are exposed (Ruuskanen et al., 2020b). In quails, Ruuskanen et al. revealed evidence that Gly accumulated within the egg and that GBHs caused lipid damages within the brains of embryos and induced a poor embryo development (Ruuskanen et al., 2020b). In adult mallards (*Anas platyrhynchos*), an exposition to GBHs for 15 days induced a reduction in plasma T and E2 levels. Moreover, authors observed modification in epididymis morphology associated with a reduced thickness of the epithelium of the seminiferous tubules and interstitial tissue (Oliveira et al., 2007).

Focusing on the chicken model, in 1980, Batt et al. (Batt et al., 1980) demonstrated that eggs immerged for 5 s in GBH solution at 0 %, 1 % and 5 % concentration on day 0, 6, 12 or 18 of incubation had a normal embryo development with no impact on hatching rate nor chicken development. Authors concluded that external application of GBH on the eggshell has no lethal effects on the developing embryo. Other studies focused on the effects of *in ovo* direct exposure of chicken embryos to GBHs showed that GBHs induced teratogenic effects on embryo development (Lehel et al., 2021; Paganelli et al., 2010) and embryo mortality (Fathi et al., 2019; Lehel et al., 2021). At molecular level, it has been demonstrated that Gly disrupted cytochrome P450 enzymes in the liver and small intestine (Fathi et al. (2020) and increased reactive oxygen species (ROS) production (Fathi et al., 2020, 2019). Our laboratory previously demonstrated that a chronic dietary exposure of roosters to a GBH altered sperm parameters and induced metabolic disorders in the offspring probably through epigenetic effects (Serra et al., 2021). An observational study conducted on broiler breeder egg production highlighted a negative association between residues of Gly commonly found in food for broiler breeders and egg hatchability while no association was found between residues of Gly in the food and laying rate (Foldager et al., 2021). According to our knowledge, no studies were conducted on the consequences of chronic dietary GBH exposure on the fertility of the female chicken and on the offspring development. Therefore, the understanding of the putative effects of Gly or GBHs on bird fertility and embryo development needs to be investigated. Based on the literature and on the results of our laboratory, we hypothesized that dietary GBH exposure can alter female fertility and the embryo development. Thus, the objectives of this study were to investigate the impacts of chronic dietary GBH exposure on the fertility and the embryo development and their potential reversibility in the reproductive hens.

2. Material and methods

2.1. 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: 6 November 2021, Ministry of Agriculture and Fish Products, and a notice of ethics committee of Val de Loire N◦19).

2.2. Animals

All animals (150 female chicks and 10 male chicks of the commercial breed ROSS 308) were obtained at 1 day of age from a local hatchery (Boye Accouvage La Villonniere 79310 La Boissière en Gatine, France) and reared at "Pôle Expérimental Avicole de Tours" (INRAE, Nouzilly, France) until 32 weeks old according to the traditional breeding conditions. At 32 weeks old, hens were divided into two homogenous groups: control (n = 75) and dietary GBH exposed (n = 75). For each group of hens, animals were distributed by 5 in 15 pens, each pen with an area of 3 m^2. Roosters (n = 10) were reared together. They were included in the study for sperm collection followed by artificial insemination. The design of the experiment is summarized in Fig. 1. Seventy-five hens were exposed for 6 weeks to GBH via the food (GBH group, 46.8 mg/kg bw/day), and 75 hens were fed with a regular diet without GBH (noted control or CT) (week 1 to week 6 of the protocol). The diet for the GBH group consists of regular broiler diet mixed with Gallup 360 in our laboratory in accordance with the directives of the "Directions Départementales de la Protection des Populations" (Departmental Directorate for the Protection of Populations). The mix was carried out by a technician with certification for the handling of phytosanitary products "Certiphyto", as recommended by the French law. After that, all animals (CT hens and ex-GBH hens) were fed with a regular diet (week 7 to week 11 of the protocol). During this protocol, blood samples of hens were collected to quantify plasma Gly and AMPA concentrations. At week 4, hens were artificially inseminated with sperm from control roosters that were fed with regular diet (not GBH exposed). The next day and for 3 weeks (weeks 4, 5, and 6), eggs were collected. At week 7, exposure to GBH was stopped, and twelve CT hens and twelve GBH hens were slaughtered to recover biological samples. At week 9, hens were artificially inseminated again with sperm from control roosters. The next day and for 3 weeks (weeks 9, 10, and 11), eggs were collected. At the end of each week (weeks 4, 5, and 6 and then week 9, 10, and 11), eggs fertilized with the sperm of CT roosters were incubated for 21 days. Before and during the whole experiment, we did not observe any significant differences between CT and GBH (week 6: CT: 3762 ± 18.9 g and GBH: 3699 ± 18.9 g) and CT and ex-GBH (week 11: CT: 3996 ± 20.5 g and GBH: 3920 ± 31.7 g) hens in terms of body weight. All animals were killed by electrical stunning and bled out, as recommended by the ethical committee.

2.3. Diets composition

Hens (32 weeks old) received a restricted laying diet according to

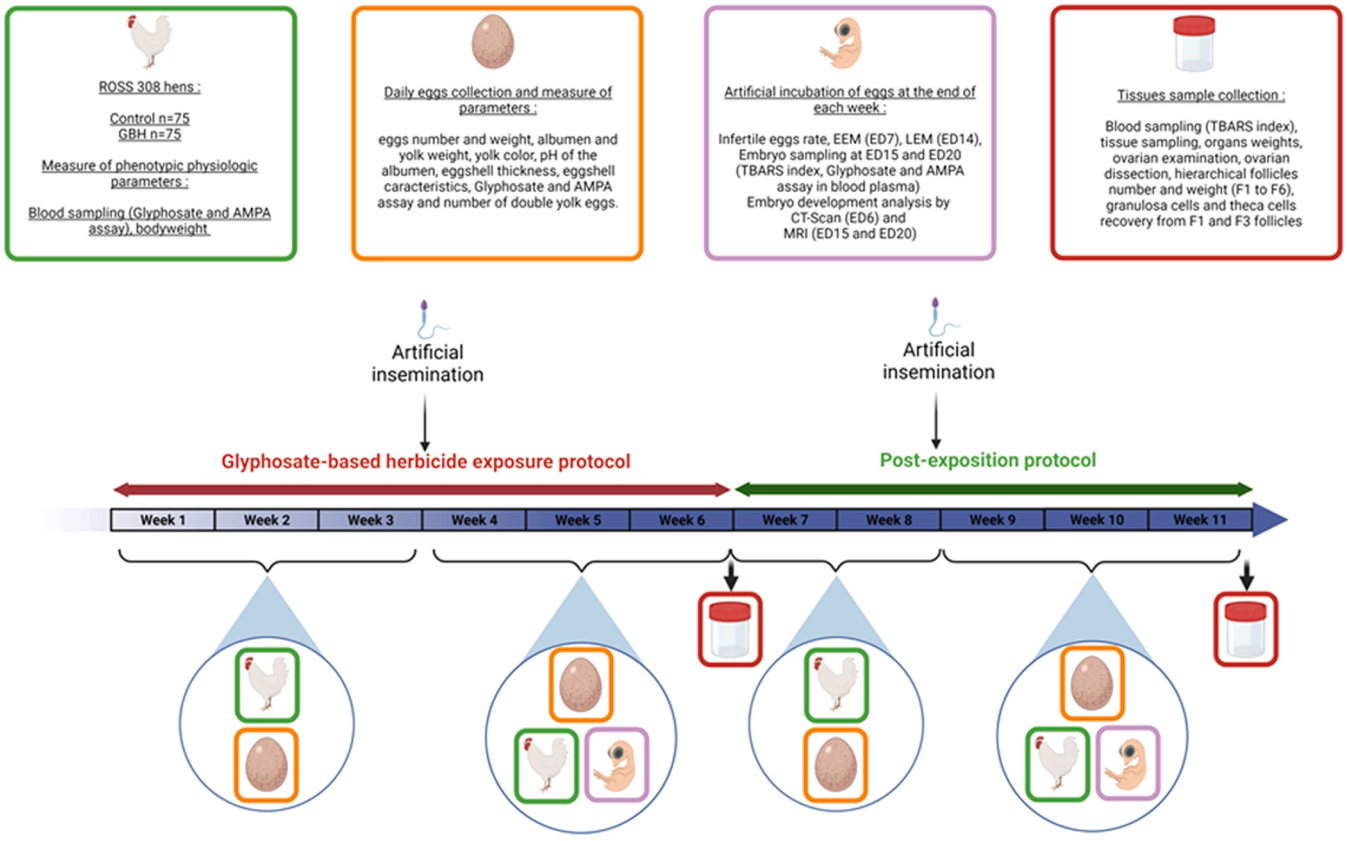

Fig. 1. Experimental design: The timeline is represented in weeks. (ED: Embryonic Day, EEM: Early Embryonic Mortality (at ED7), LEM: Late Embryonic Mortality (at ED14), MRI: Magnetic Resonance Imaging, CT-Scan: Computed Tomography Scanner F1: Hierarchical Follicle 1; F3: Hierarchical Follicle 3) 150 32 week old hens ROSS 308 were included in the study. Seventy five GBH hens were exposed for 6 weeks to GBH via the food (46.8 mg/kg bw/d), and seventy five CT hens were fed with a regular diet without GBH. Then, GBH was removed from the diet and animals were followed for 5 more weeks (week 7 to week 11). During the protocol (D0, D21, D38 during exposure and D3, D6 and D27 after exposure), blood samples were collected to quantify glyphosate (Gly) and its metabolite AMPA within the blood plasma. At week 4 and week 9, hens were artificially inseminated with sperm from control roosters. The next day and for 3 weeks (weeks 4, 5, and 6 during exposure and weeks 9, 10, and 11 after exposure), eggs were collected daily, egg parameters were measured (number, total weight, albumen and yolk weight, pH of the albumen, eggshell thickness and characteristics, Gly and AMPA assay, and number of double yolk eggs) and egg were artificially incubated for 21 days at the end of each week collection. Infertile eggs rate, EEM (ED7) and LEM (ED14) were assessed by candling. Embryo analysis by CT-Scan (ED15 and ED21), MRI (ED15 and ED21), and embryo sampling was performed (ED15 and ED21) at week 6 during exposure and week 11 after exposure. At birth, all chicks (n = 745 for CT and n = 5 for GBH during exposure and n = 571 for CT and n = 523 for Ex-GBH during post-exposition protocol) were counted and weighted, sex was determined, and chicks from each group were euthanized to collect samples and weigh organs. At the end of exposure (end of week 6) and at the end of the post-exposition protocol (end of week 11), hens were euthanized for tissue sampling and measurements (n = 9 for CT and GBH at week 6; n = 12 for CT and Ex-GBH at week 11).

Hendrix Genetics recommendation. They were fed with either GBH exposed feed (n = 75) or control feed (n = 75) from the first week of protocol to week 6 (Fig. 1). The control feed contained low measurable Gly and AMPA concentrations (0.21 mg/kg feed for Gly and undetectable levels for AMPA as determined by Phytocontrol, Nimes, France) (See Spplemental data 1 for details (Estienne, 2022)). The concentration of Gly in the GBH feed was 1250 mg/kg feed for Gly and 0.30 mg/kg feed for AMPA, as determined by Phytocontrol. Gallup super 60, called GBH within the text, was obtained from Axereal (Monnaie, France); it contained 360 g/L Gly (485.8 g/L isopropylamine salt). Hens were food-restricted as recommended by the provider. Knowing that the hens weighed on average 3.45 kg over the exposure period and their food consumption was on average 130 g/day, the concentration in the feed thus corresponded to a dose of 47 mg Gly/kg body weight/day. The European Food Safety Authority (EFSA) has reported a NOAEL of 100 mg/kg body weight/day for poultry, with a maximum residue level (MRL) of 2.28 mg/kg bw/d (European Food Safety Authority (EFSA), 2018). Therefore, our experiment tested a concentration of approximately 47 % of the NOAEL threshold. From week 7 to week 11, all animals were fed with the control feed (CT n = 63 and ex-GBH n = 63).

2.4. Measurement of egg production and quality

The eggs from each pen were collected twice a day, counted and weighted using a balance (Ohauss, Pionner). The numbers of normal and double (two yolks) eggs were collected and counted. The weight of the albumen, egg yolk, and dehydrated shell were determined. The pH of the albumen was also measured. Albumen and yolk samples were used for Gly and AMPA assays (Phytocontrol, Nimes, France) as well. As GBHs appear to impair calcification process in vertebrates, including the chicken species (Díaz-Martín et al., 2021; Kubena et al., 1981), we decided to analyse eggshell properties. Using an Instron instrument (Instron, UK527), we analyzed the eggshell elastic modulus (N/mm2) and the toughness (N/mm3) of the eggs. The egg shape, eggshell percent, index, and thickness were also measured. These parameters were assessed for 30 eggs of each animal group during GBH exposure. The fatty acid profile in the egg yolk was also determined. The total lipids were extracted from the yolk of the eggs from CT and GBH hens (week 3) after homogenization of the samples (n = 30 for each group) with a chloroform/methanol mixture. Lipids were extracted gravimetrically into methanol:chloroform (1:2). The fatty acid composition was determined by gas chromatography (Autosystem; Perkin Elmer, St Quentin en Yvelines, France) after transmethylation of lipids.

2.5. Fertility and embryo development parameters

For artificial insemination, we used the semen of 10 CT roosters, collected and pooled to have a single sample. For each experiment, the hens were inseminated with 2 × 108 spermatozoa, at the 3rd and 8th week of the protocol (Fig. 1). Eggs were collected and counted daily for 3 weeks following the artificial insemination and incubated every 7 days. We assessed the number of unfertilized eggs, early (EEM) and late (LEM) embryonic mortality by breaking eggs and candling on the 7th (EEM) and 14th (LEM) day of incubation. As previously described (Barbe et al., 2020; Mellouk et al., 2018), the different percentages (EEM, LEM, hatchability, hatchability of fertile eggs and fertility) were calculated using the following formula:

% of unfertilized eggs = (number of unfertilized eggs / number of incubated eggs) x 100 % EEM = number of EEM/(number of incubated eggs – unfertilized eggs) x 100 % LEM = number of LEM/(number of incubated eggs – unfertilized eggs + number of EEM) x 100 % Hatchability of incubated eggs = (number of hatched chicks / number of incubated eggs) x 100 % Hatchability of fertilized eggs = (number of hatched chicks/number of fertilized eggs) x 100.

2.6. Measurement of progesterone, androstenedione, testosterone and oestradiol deposition in egg yolk

As previously described (Barbe et al., 2020), we assessed steroids concentration from 30 egg yolks per group. Briefly, after thawing, the yolk was mixed and 0.5 g of each yolk was diluted with 1.5 mL of distilled water and vortexed for 30 s. Steroids were extracted by adding 3 mL of diethyl-ether to 300 μL of the mixture, followed by vortexing for 30 min and stored overnight at −20 °C. The steroid-containing diethyl ether phase was then decanted after freezing the tubes in nitrogen for 10 s. After centrifugation (1500 g, 15 min), the organic solvents were then evaporated under air flow, and the extracts taken up in 600 μL of phosphate buffer. Steroid hormones were then measured in the extracts using ELISA (oestradiol, testosterone and androstenedione) and EIA (progesterone) assays. For progesterone, the EIA assay was performed as described by Canépa et al. (2008) (See Supplemental material and methods for details) (Estienne, 2022). The sensitivity of the assay was 0.4 ng/mL. Oestradiol and testosterone concentrations were determined using commercial ELISA assays from Cayman Chemicals (reference number 501890 for oestradiol, and 582701 for testosterone) and the sensitivity of these assays was 0.01 ng/mL. Androstenedione levels were analyzed using an ELISA assay from Abcam (reference number ab108672) and the sensitivity of the assay was 0.01 ng/mL. The intra-assay and inter-assay coefficients of variation (CV) for each assay averaged <10 %.

2.7. Hens and embryos biological samples

Blood samples from 150 hens were collected from the occipital sinus into heparin tubes at different days during the experiment (days 0 and 21 of week 3 and day 38 of week 6 during GBH exposure and day 3 of week 7, day 8 of week 8, and day 27 of week 10 corresponding to the protocol after the end of GBH exposure). Blood samples were centrifuged (5000g for 10 min at 4 °C) and stored at −20 °C before the use for Gly and AMPA assays. Tissue samples (granulosa and theca cells) were obtained at different times of the experiment (week 6 of the protocol after the beginning of GBH exposure and week 11 of the protocol after the end of GBH exposure) by dissection after slaughtering. Organs were also weighted during dissection. At ED15 and ED21, eggs were randomly selected (n = 20 for CT and GBH groups at week 6, n = 20 for CT and Ex-GBH groups at week 11) and opened to access the embryo. The embryos were euthanized by decapitation and dissected in order to recover tissue samples and to weigh the organs.

2.8. Body weight, carcass traits, and embryo and chick quality

At ED15 and ED21 (0-day post-hatch), after euthanasia, the carcasses were dissected, and different parts including the heart, residual yolk sac (vitelline vesicle), digestive tract (proventriculus, gizzard, and intestine), liver, and brain were collected and weighed. Chicks quality was assessed as reported by Tona et al. (Tona et al., 2004).

2.9. Measurement of Gly and AMPA in blood plasma and eggs

In blood plasma of hens and embryos, Gly and AMPA were measured after a derivatisation reaction using FMOC-Cl (9-fluorenylmethyl chloroformate), in collaboration with Dr. S El Balkhi (Service de Pharmacologie, Toxicologie et Pharmacovigilance, Limoges, France) as previously described (Serra et al., 2021) (See Supplemental material and methods for details) (Estienne, 2022). Gly and AMPA concentrations in yolk and albumen eggs were determined by Phytocontrol company (Nïmes, France).

2.10. TBARS assay in blood plasma and yolk eggs

The determination of lipoperoxidation in plasma samples and yolks was performed by measuring the level of thiobarbituric acid reactive substances (TBARS) in the samples using a spectrophotometer at 532 nm, following the technique described by Lapenna et al. (Lapenna et al., 2001) and the results are represented in nmol malondialdehyde (MDA)/mL plasma or /kg yolk.

2.11. Determination of weight and number of follicles

At weeks 6, and 11, 12, and 9 hens per group respectively were selected and euthanized and the preovulatory follicles, classified from F1 to F6 (Waddington and Walker, 1993) were collected, hierarchically defined and weighed. Granulosa cells and theca cells were collected from preovulatory follicles 1 (F1) and 3 and 4 (F3/4).

2.12. Theca and granulosa cells recovery followed by in vitro culture

After ovarian dissection and follicles classification, theca layer were mechanically removed from the surface of the follicles and kept in fresh PBS. Granulosa cells layers were also collected and washed twice with PBS to remove remnant yolk. Then, cells were dispersed in 0.3 % collagenase type A (Roche Diagnostic, Meylan, France) in F12 medium containing 5 % fetal bovine serum (FBS), at 37 °C. Cells were pelleted by centrifugation, washed twice with fresh medium and counted in a haemocytometer. The viability of granulosa cells was estimated by Trypan Blue exclusion. Cells were cultured in a medium composed of DMEM supplemented with 100 U/mL penicillin, 100 mg/L streptomycin, 3 mmol/L L-glutamine and 5 % FBS. The cells were initially cultured for 24 h with no treatment. After overnight serum deprivation, cells were stimulated with IGF1 (10^{-8} M), LH (10^{-8} M), IGF1 +LH (10^{-8} M) or left untreated. All cultures were maintained under a water-saturated atmosphere of 95 % air/ 5 % CO2 at 37°C. After 48 h of in vitro culture conditioned media were recovered for progesterone assy. Finally, samples of uncultured theca and granulosa cells were pelleted before snap freezing for RT-qPCR experiments. All samples were kept at −80 °C before analysis.

2.13. Measurements of the expression of steroidogenic genes in granulosa and theca cells

Total RNA from granulosa and theca cells of preovulatory follicle F1 and F3/4 of the twelve animals per group (CT vs GBH) and nine animals per group (CT vs Ex-GBH) was extracted by homogenization in the TRIzol tissue reagent using an ultraturax instrument, according to the manufacturer's recommendations (Invitrogen, by Life Technologies,

Villebon sur Yvette, France). The cDNA was generated by reverse transcription of total RNA (2 μg) in a mixture comprised of 0.5 mM of each deoxyribonucleotide triphosphate (dATP, dTTP, dGTP, dCTP), 2 M of RT Buffer, 15 μg/μL of oligodT, 0.125 U of ribonuclease inhibitor, and 0.05 U of Moloney murine leukemia virus reverse transcriptase (MMLV) for one hour at 37 °C. Real-time PCR was performed using the MyiQ Cycle Device (Bio-Rad, Marnes-la-Coquette, France), in a mixture with SYBR Green Supermix 1X Reagent (Bio-Rad, Marnes-la-Coquette, France), 250 nM specific primers (Invitrogen by Life Technologies, Villebon-sur-Yvette, France), 3 μL of cDNA diluted 1:5 in water) for a total volume of 11 μL. The samples were set up in duplicate on the same plate according to the following procedure: after an incubation of 2 min at 50 °C and a denaturation step of 10 min at 95 °C, samples were subjected to 40 PCR cycles (30 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C). The primers used are shown in the Supplemental data 2 (Estienne, 2022). Then, the relative expression of the gene of interest was related to the relative expression of the geometric mean of the three reference genes (*GAPDH*, *ACTB,* and *EEF1a*). For each gene (*StAR*, *3βHSD*, *P450scc* and *CYP19A1*), expression was calculated according to primer efficiency (E) and quantification cycle (Cq), where expression = E-Cq. Then, relative expression of the target gene to the three reference genes was analyzed.

2.14. MRI analysis of incubated eggs

The MRI analysis was carried out using a 3 Teslas (T) MRI scanner (Siemens Magnetom® Verio, Erlangen, Germany) with a 70 cm inner-diameter and field gradient strength of 45 mT/m. The Radio Frequency (RF) coil was the rigid 'loop' type with an inner diameter of 7 cm. The egg could just fit inside the RF coil. MRI scans were made one by one on embryonated eggs (15 CT and 15 GBH eggs at ED15; 5 CT and 5 GBH eggs at ED21) which were placed in normal position on a cardboard egg tray per incubation day studied (ED15 and E20) and these eggs have been randomly selected alongside the live eggs checked by candling in the control group and the treated group. The eggs were incubated at 37.5 °C and 53 % relative humidity at INRAE. Thereafter, the embryonated eggs were cooled for 1 h at 4 °C followed by 10 min at − 20 °C prior to MRI scanning to minimize embryonic movement, which can lead to artefacts on the MR images (Bain et al., 2007). T2 (spin-spin interaction or transverse relaxation time) weighed images were taken within 8 min 57 s. For the purpose of this investigation, when high-contrast/high-resolution images were a pre-requisite, T2-weighted imaging was carried out using the SPACE (Sampling Perfection with Application-optimized Contrasts) pulse sequence because they produced high signal contrast between the organs of interest, making them readily identifiable. The Repetition Time (TR) and Echo Time (TE) were respectively 1860 ms and 140 ms, flip angle = 140 degrees, and bandwidth of 296 Hz/Px. The Matrix was 192 × 192, the Field of View (FOV) was 70 mm × 70 mm, with a slice thickness of 0.35 mm making a voxel size of 0.36 × 0.36 × 0.35 mm^3. 144 slices were required to cover the entire egg. Meanwhile MRI acquisitions, the viability of all the embryos could be checked thanks to presence of artefacts due to the heart beating.

2.15. Computed Tomography Scanner (CT-Scan) analysis of incubated eggs

The clinical computed tomography machine used was a Somatom Définition AS128 (Siemens, Germany). Selected live eggs at ED6 (15 CT, and 15 GBH eggs), ED15 (15 CT, and 15 GBH eggs) and ED21 (5 CT, and 5 GBH eggs) were placed on a cardboard egg tray. The X-ray source was set at 80 kV and 400 mA/s. The image acquisition mode was 16 cm × 16 cm with 512 pixels matrix size and with a slice thickness of 0.4 mm making a resolution of 312,5 μm. A I26 Safire reconstruction filter was used for an optimal *in ovo* tissues characterization.

2.16. CT-Scan and MRI images analysis

For CT-Scan and MRI analysis, the Digital Imaging and Communication in Medicine (DICOM) images were converted to the Neuroimaging Informatics Technology Initiative (NIfTI) format. Analyses of all images were carried out using ITK SNAP software (Researchers at UPenn and UNC, General Public License), which is a free post-processing software generally used to segment 3D medical image structures. The segmentation of surface growth type was done automatically and then corrected manually. It was then possible to edit the volume of each segmented structure in mm^3.

2.17. Statistical analysis

The GraphPad Prism® software (version 8) was used for all analyses. All data are reported as means ± standard error of mean (SEM). Bartlett's test was run to test the homogeneity of variance, and normal distribution was verified by the Shapiro–Wilk test. We performed one t-test or one-way analysis of variance (ANOVA) or two-way ANOVAs to compare the different means, when appropriate. When the one-way ANOVA indicated significant effects, the means were analyzed by using the Fisher's test. When the two-way ANOVA indicated significant effects, the means were analyzed by using Tukey HSD's tests. Letters correspond to the ordinary one-way or two-way ANOVA significance ($p < 0.05$), followed by Fischer's and Tukey's multiple comparisons test, respectively; Stars (*) correspond to the unpaired t-test significance ($p < 0.05$).

3. Results

3.1. Plasma and yolk egg Gly and AMPA concentrations

Before the start of GBH exposure, Gly and AMPA were undetectable in the plasma of all hens (day 0). After 21 days of exposure (week 3), the Gly concentrations of the two molecules significantly increased at week 3 in the plasma of GBH hens at 1500 ($p < 0.0001$) and 20 ng/mL ($p < 0.0001$), respectively. Three days after the end of GBH exposure (week 7), both concentrations decreased ($p < 0.0001$ for Gly and $p = 0.0001$ for AMPA at week 7) until day 27 of cessation exposure (week 10) (Fig. 2A and B). We then measured Gly concentrations in the yolk of the GBH group eggs during the first week of exposure to this herbicide. Gly concentrations remained undetectable or very low for the first 4 exposure days and began to increase significantly on day 5 until reaching a maximum on day 8 (Fig. 2C) ($p < 0.05$). During week 3, we found that Gly accumulation within the yolk was important (0.6 ng/mL), whereas it was very low within the albumen (Fig. 2D) ($p < 0.01$). At the end of exposure to the GBH, we noticed a constant and significant decrease of Gly concentrations from 0.9 ng/mL to 0.15 ng/mL in the yolk of eggs from the GBH group during the next 8 days (Fig. 2E) ($p < 0.05$). We then assessed the oxidative stress of hens by measuring the lipoperoxidation in plasma samples and yolks with the level of thiobarbituric acid reactive substances (TBARS). Results showed a significant increase of oxidative stress in hens' plasma from the GBH group compared to the CT group ($p < 0.001$). A similar result was obtained when measuring oxidative stress within eggs with a significant increase of TBARS index in eggs from the GBH group compared to eggs from the CT group ($p < 0.0001$) (Fig. 2F).

3.2. Effects of GBH on hen ovarian functions

At both slaughters (weeks 6 and 11), we found no difference in the ovarian parameters such as the number of hierarchical follicles, weight of the ovary (without hierarchical follicles), or hierarchical follicular weight (F1 to F6) (Supplemental data 3 (Estienne, 2022)). Moreover, RT-qPCR experiments performed on granulosa cells and theca samples from F1 and F3 follicles exhibited no difference of gene expression for

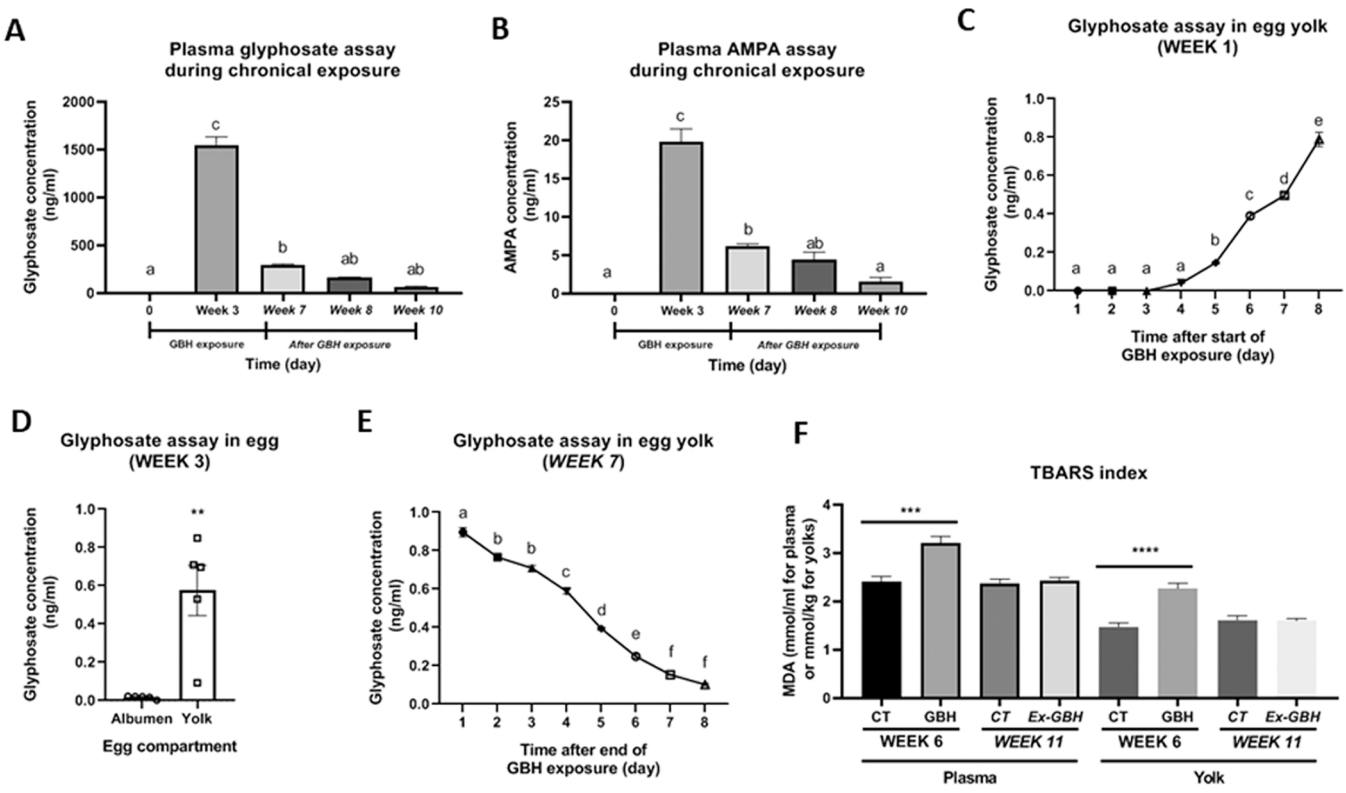

Fig. 2. Determination of glyphosate (Gly) concentrations, AMPA concentrations and TBARS index in the blood plasma and the egg compartments. A) Gly assay in the blood plasma during the whole protocol (ng/mL) (n = 10 per group). B) AMPA assay in the blood plasma during the whole protocol (ng/mL) (n = 10 per group). C) Gly assay in egg yolks from Glyphosate Based Herbicide (GBH) group during the first week of exposure to GBH (WEEK 1) (ng/mL) (n = 10 eggs per day). D) Gly assay in GBH egg yolks and albumen during the third week of exposure to GBH (week 3) (ng/mL) (n = 5 per condition). E) Gly assay in egg yolks from GBH group during the first week of post-exposition to GBH (WEEK 7) (ng/mL) (n = 10 eggs per day). F) TBARS index in blood plasma and egg of broiler hens during GBH exposure (WEEK 6, CT n = 10 versus GBH n = 10) and after GBH exposure (*WEEK 11*, CT n = 10 versus Ex-GBH n = 10). Results are presented as means ± SEM. Lowercase letters correspond to the ordinary one-way ANOVA significance ($p < 0.05$), followed by Tukey's multiple comparisons test comparing the evolution of the concentration of Gly or AMPA at different days; Stars (*) correspond to the unpaired t-test significance ($p < 0.05$) corresponding to the comparison between albumen and the yolk compartment for Gly concentrations and between the CT and Ex-GBH for TBARS index in plasma and eggs. **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

StAR, *3βHSD*, *CYP19A1*, and *P450scc* (Fig. 3A, B, C, D, F, G, H and I). We also performed in vitro granulosa cells (GC) cultures from F1 follicles and stimulated the cells with LH (10^{-8} M) alone, IGF1 alone (10^{-8} M), or both hormones in combination to check their responses in terms of progesterone secretion. Results showed that GC significantly increased their secretion of progesterone within the culture medium when stimulated with LH alone, IGF1 alone, or with LH and IGF1, but we observed no difference between CT and GBH GC and between CT and Ex-GBH GC (Fig. 3E and J). Focusing on eggs parameters, we didn't find any differences of eggs number/hen/day from week 1 to week 6 of the protocol (GBH exposure) between CT and GBH. Even after exposure arrest, the eggs number/hen/day from week 7 to week 10 remained identical between CT and Ex-GBH hens (Supplemental data 4A (Estienne, 2022)). During the whole experiment, the number of double yolk eggs (double ovulation) was not significantly different between the groups (CT versus GBH then CT versus ex-GBH). We only observed a significant decrease of double yolk eggs in the GBH group during week 2 (Supplemental data 4B (Estienne, 2022)). Egg yolk steroid concentrations including measurements of progesterone, estradiol, androstenedione and testosterone (Supplemental data 4C (Estienne, 2022)), and egg weights were similar between the groups during the experiment (Supplemental data 5 (Estienne, 2022)). Moreover, we analyzed lipid composition within the egg yolks sampled at week 3 and found no difference between CT and GBH eggs (Supplemental data 6 (Estienne, 2022)). Finally, other egg parameters were analyzed during week 6, such as egg weight, yolk weight, albumen weight, albumen pH, egg shape, eggshell percent, index, thickness, toughness, and elasticity. For all parameters, we only found a significant increase in eggshell percent, index, and thickness in the GBH group compared to the CT group ($p < 0.05$) (Table 1).

3.3. Effect of GBH exposure on fertility parameters

After artificial insemination, egg daily collection and artificial incubation, infertile egg rate, EEM, and LEM during and after GBH exposure were determined by candling at ED7 and ED14; dead embryos were analyzed to determine the embryonic stage when death occurred. Concerning the unfertilized rate, the data were similar between the CT and GBH groups during the GBH exposure (week 4–6). After GBH exposure (week 9–11), we noticed a slight but significant increase in the rate of unfertilized eggs in the Ex-GBH group (34.55 ± 3.12 %) compared to the CT group (23.20 ± 2.80 %) at week 10 only. EEM and LEM rates were significantly increased in the GBH group compared to the CT group during GBH exposure (Table 2). Hatching of incubated eggs and hatching of fertile eggs were significantly reduced in the GBH group compared to the CT group during the same period (Table 2). However, all of these latter parameters were unchanged between the two groups after GBH exposure (Table 2). Dead embryo analysis during GBH exposure showed that embryonic death, observed by disappearance of blood vessels, occurred mostly during ED3 ($p < 0.0001$) (Fig. 4A). We also assessed Gly and AMPA concentrations in embryo plasma and found that concentrations of both molecules were significantly increased in the GBH group compared to the CT group only during GBH exposure (for Gly: ED15, $p < 0.0001$; ED21, $p < 0.001$; for AMPA: ED15, $p < 0.001$; ED21, $p < 0.05$) (Fig. 4B and C). However, the

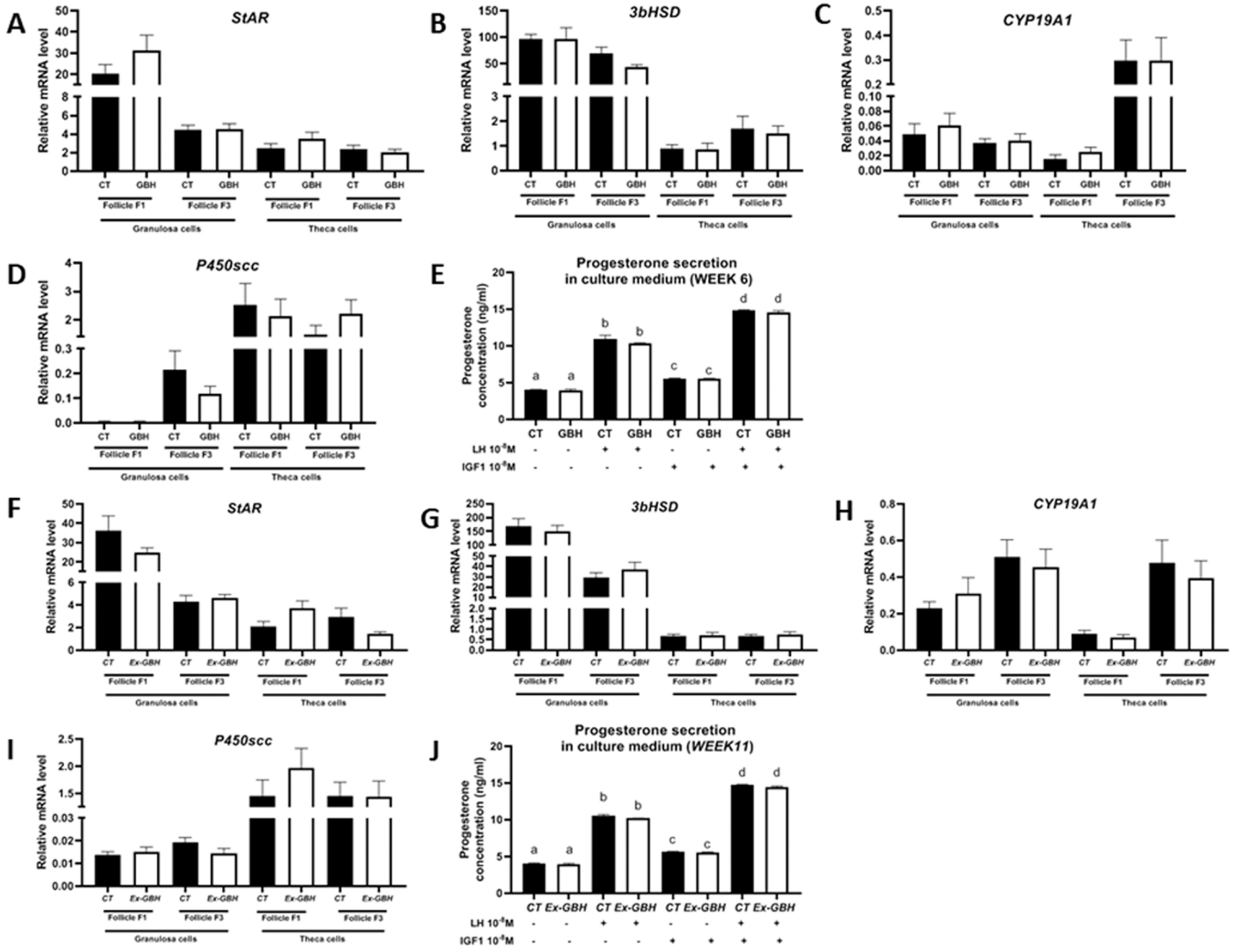

Fig. 3. Levels of A) *StAR*, B) 3*βHSD*, C) *CYP19A1*, and D) *P450scc* mRNA expression in granulosa cells and theca layers from broiler hens during Glyphosate Based Herbicide (GBH) exposure (WEEK 6, CT versus Ex-GBH). mRNA was extracted from granulosa cells of 12 preovulatory F1 follicles and 12 F3 follicles for each group. E) Effect of GBH on in vitro steroidogenesis during exposure (WEEK 6) in response to IGF1 and LH alone or in combination in hen primary granulosa cells. Granulosa cells from preovulatory follicles 1 (F1) from different groups of animals (CT versus GBH) (n = 3 per group) were seeded for 24 h and after overnight serum starvation, granulosa cells were incubated with IGF1 (10^{-8} M), LH (10^{-8} M) or both for 48 hr. The culture medium was then collected. Basal progesterone level and the response to IGF1 or/and LH were assessed. Results are presented as means ± S.E.M. P-values from ordinary one-way ANOVA followed by Tukey's multiple comparisons test comparing the CT versus the GBH were considered as significant if $p < 0.05$. Lowercase letters correspond to unpaired t-test significance ($p < 0.05$). Levels of F) *StAR*, G) *3βHSD*, H) *CYP19A1*, and I) *P450scc* mRNA expression in granulosa cells and theca layers from broiler hens after Glyphosate Based Herbicide (GBH) exposure (WEEK 11, CT versus Ex-GBH). mRNA was extracted from granulosa cells of 12 preovulatory F1 follicles and 12 F3 follicles for each group. J) Effect of GBH on in vitro steroidogenesis during exposure (WEEK 11) in response to IGF1 and LH alone or in combination in hen primary granulosa cells. Granulosa cells from preovulatory follicles 1 (F1) from different groups of animals (CT versus Ex-GBH) (n = 3 per group) were seeded for 24 h and after overnight serum starvation, granulosa cells were incubated with IGF1 (10^{-8} M), LH (10^{-8} M) or both for 48 hr. The culture medium was then collected. Basal progesterone level and the response to IGF1 or/and LH were assessed. Results are presented as means ± SEM. P-values from ordinary one-way ANOVA followed by Tukey's multiple comparisons test comparing the CT versus the Ex-GBH were considered as significant if $p < 0.05$. Lowercase letters correspond to unpaired t-test significance ($p < 0.05$).

results were similar between the two groups after GBH exposure with undetectable levels of AMPA for CT and ex-GBH embryos.

3.4. Focus on the effect of GBH exposure on embryo development and offspring general condition

In order to study the effect of GBH exposure on embryo development, we analyzed the developing embryo *in ovo* by non-invasive medical imaging with CT-Scan analysis (ED15 and 21) combined with MRI analysis (ED15 and 21). At ED15, CT-Scan images (illustrated on Fig. 5A) showed a significant increase in the ratio between the total egg volume and the albumen volume in the GBH group compared to the CT group ($p < 0.0001$) (Fig. 5C) and a significant decrease in the ratio between the total egg volume and the vitellus volume in the GBH group compared to the CT group ($p < 0.0001$) (Fig. 5D) whereas the air chamber volume remained unchanged between the two groups (Fig. 5B). MRI analysis at ED15 showed a significant decrease in the volumes of the embryo, eyes of the embryos, vitelline vesicle, and amnios from GBH eggs compared to CT eggs ($p < 0.05$) (Fig. 5E, F, G and J). We noticed a significant increase in the albumen volume of GBH eggs compared to CT eggs ($p < 0.001$) (Fig. 5H) while the allantoic volume appeared not significantly different between the two groups (Fig. 5I). In Fig. 5K a sagittal image of a CT embryo analyzed *in ovo* by MRI at ED15 with the different colourized compartments (albumen in blue, amnios in dark blue, vitellus in light blue, allantoic in green, eyes in pink and embryo body in purple) is shown. In Fig. 5L, we performed a three

Table 1
Quality of eggs from broiler hens during GBH exposure (WEEK 6, CT n = 30 versus GBH n = 30). Total egg, yolk, and albumen were weighted. Albumen pH was determined. Eggshell characteristics were measured with Instron. Results are presented as means ± SEM. P-values of the effect of the GBH exposure (during exposition) were considered as significant if $p < 0.05$ (t-test). Lowercase letters correspond to the unpaired t-test significance ($p < 0.05$) corresponding to the comparison between CT and GBH eggs.

Egg parameters	GBH Exposure	
	Control (WEEK6 n = 30)	GBH (WEEK6 n = 30)
Egg weight (g)	62.20 ± 3.58	62.56 ± 3.77
Yolk weight (g)	18.40 ± 0.18	18.03 ± 0.20
Albumen weight (g)	34.87 ± 0.33	34.18 ± 0.33
Albumen pH	8.36 ± 0.03	8.30 ± 0.02
Egg shape (index)	0.79 ± 0.02	0.79 ± 0.03
Eggshell percent	8.65 ± 0.50[a]	8.85 ± 0.60[b]
Eggshell index	7.32 ± 0.40[a]	7.58 ± 0.50[b]
Eggshell thickness (mm)	0.31 ± 0.02[a]	0.32 ± 0.02[b]
Eggshell toughness (N/mm^3)	469.30 ± 65.17	441.81 ± 47.71
Eggshell elasticity (N/mm^2)	18428.13 ± 1418.18	18595.73 ± 1825.99

dimensional reconstruction of the same embryo (purple) with its vitelline vesicle (yellow). Finally, in Fig. 5M we provided a picture of a GBH embryo on the left (a) and a CT embryo on the right (b) at ED15, permitting a qualitative appreciation of the reduction in size of the GBH embryo, as well as its vitelline vesicle, compared to the CT embryo and vitelline vesicle. At ED21, CT-Scan imaging (illustrated in Fig. 6A), permitted the analysis of the ratio between the total egg volume, soft tissues, and skeleton volumes showing a significant decrease of the soft tissue volume ($p < 0.05$) and an increase in the skeleton volume ($p < 0.05$) in embryo from the GBH group compared to the CT group (Fig. 6B and C). At the same embryonic stage, MRI imaging illustrated in Fig. 6D (sagittal view) and in Fig. 6E (3 dimensional reconstruction of the egg content) demonstrated a significant reduction of the tibia length of embryos from the GBH group compared to the CT group ($p < 0.01$) (Fig. 6H) whereas eyes volume and gizzards length remained similar between the two groups (Fig. 6F and G). All these measurements were performed for CT and Ex-GBH embryos during weeks 9, 10 and 11 but no significant differences were found between the two groups (data not shown).

Furthermore, we also carried out a dissection of embryos at ED15 and ED21 in order to weigh several organs. At ED15, measurements made during GBH exposure showed a significant reduction in the weight of embryo, yolk sac, liver, brain, digestive tract and heart ($p < 0.0001$ for all parameters, Table 3) of GBH embryos associated with a similar total egg weight for GBH eggs compared to CT group. At the same stage, after exposure to GBH, we found only a significant increase in brain weight of embryos from the Ex-GBH group compared to the CT (Table 3). At ED21, measurements showed a significant reduction of brain and liver weights ($p < 0.05$ for both parameters) in embryos from the GBH group (Table 3). Then, after GBH exposure, we only measured an increase of brain weight in Ex-GBH chicks when compared to CT chicks (Table 3). Finally, after birth chicks were checked and a Tona score was individually given. During and after GBH exposure, CT chicks exhibited a significantly higher Tona score when compared to chicks from the GBH group ($p < 0.01$ and $p < 0.001$, respectively) (Table 3).

4. Discussion

To our knowledge, the present work is the first to assess the chronic dietary effects and the reversibility of GBHs on the fertility, embryo development, and the quality of the offspring related to plasma and egg Gly concentration during the laying period in hens. In addition, we used innovative imaging techniques such Tomography (CT Scan) and MRI permitting to evaluate the evolution of different egg compartments in response to GBHs exposure without interrupting incubation and embryo development. The results obtained in the female broiler breeders model showed that a chronic diet exposure to 47 mg/kg day^{-1} Gly equivalent (that corresponds to half of NOAEL in poultry (European Food Safety Authority (EFSA), 2018) did not alter body weight, egg performance, fertilization level, and the steroid production by ovarian cells but strongly reduced the early embryo development. This was associated to an increase in Gly and AMPA concentration in egg yolk and potential oxidative stress in plasma and egg yolk. Moreover, all of these negative effects were reversible three weeks after GBH exposure in the diet. Coformulants are frequently included in the formulas of many herbicides and act by increasing the capacity of the active ingredients to penetrate the leaf cuticle. Some studies indicate that these coformulants

Table 2
Percentage of unfertilized eggs, early (EEM) and late (LEM) embryonic mortality and hatchability of incubated and fertile eggs in broiler hens during Glyphosate Based Herbicide (GBH) exposure (WEEK 4, 5 and 6, CT versus GBH) and after GBH exposure (*WEEK* 9, 10, and 11, CT versus Ex-GBH). Results are presented as means ± SEM. Effective are in parenthesis (n). P-values of the effect of the GBH exposure were considered as significant if $p < 0.05$. Lowercase letters correspond to unpaired t-test significance ($p < 0.05$).

		Unfertilized rate		EEM rate		LEM rate		Hatchability of incubated eggs		Hatchability of fertile eggs	
		Control	GBH	Control	GBH	Control	GBH	Control	GBH	Control	GBH
GBH exposure	Week 3	Artificial insemination									
	Week 4	7,81 ± 1.55 (n = 478)	9,25 ± 1.85 (n = 477)	2.62 ± 0.77[a] (n = 437)	78.80 ± 2.20[b] (n = 429)	0.70 ± 0.41[a] (n = 426)	10.58 ± 1.69[b] (n = 95)	86.91 ± 1.85[a] (n = 478)	0.57 ± 0.33[b] (n = 477)	97.54 ± 0.79[a] (n = 437)	8.57 ± 4.80[b] (n = 429)
	Week 5	22,14 ± 2.77 (n = 459)	29,27 ± 3.14 (n = 470)	11.52 ± 2.11[a] (n = 363)	81.25 ± 2.27[b] (n = 334)	0.89 ± 0.44[a] (n = 329)	11.16 ± 1.65[b] (n = 61)	67.81 ± 3.07[a] (n = 459)	0.19 ± 1.19[b] (n = 470)	98.32 ± 0.72[a] (n = 363)	10.00 ± 10.00[b] (n = 334)
	Week 6	88,37 ± 1.81 (n = 455)	90,03 ± 1.71 (n = 474)	44.52 ± 7.67[a] (n = 56)	83.33 ± 6.12[b] (n = 47)	0.00 ± 0.00[a] (n = 35)	15.63 ± 6.12[b] (n = 7)	7.32 ± 0.32[a] (n = 455)	0.24 ± 0.24[b] (n = 474)	99.96 ± 0.04[a] (n = 56)	0.00 ± 0.00[b] (n = 47)
After GBH exposure	Week 8	Artificial insemination									
	Week 9	6.34 ± 1.77 (n = 369)	6.99 ± 1.86 (n = 367)	4.01 ± 1.07 (n = 345)	3.01 ± 0.99 (n = 338)	1.27 ± 0.64 (n = 333)	0.97 ± 0.57 (n = 331)	85.00 ± 2.33 (n = 369)	83.44 ± 2.56 (n = 367)	95.54 ± 1.55 (n = 345)	93.27 ± 1.74 (n = 338)
	Week 10	23.20 ± 2.80[a] (n = 378)	34.55 ± 3.12[b] (n = 380)	12.53 ± 2.49 (n = 283)	9.38 ± 2.26 (n = 237)	1.64 ± 0.80 (n = 262)	1.93 ± 1.39 (n = 223)	64.35 ± 3.16 (n = 378)	56.69 ± 3.22 (n = 380)	95.71 ± 1.61 (n = 283)	99.03 ± 0.56 (n = 237)
	Week 11	90.29 ± 1.84 (n = 388)	93.87 ± 1.44 (n = 373)	26.00 ± 8.83 (n = 31)	20.59 ± 9.64 (n = 18)	4.00 ± 2.77 (n = 29)	0.00 ± 0.00 (n = 16)	6.77 ± 1.47 (n = 388)	4.96 ± 1.32 (n = 373)	99.95 ± 0.05 (n = 31)	99.87 ± 0.13 (n = 18)

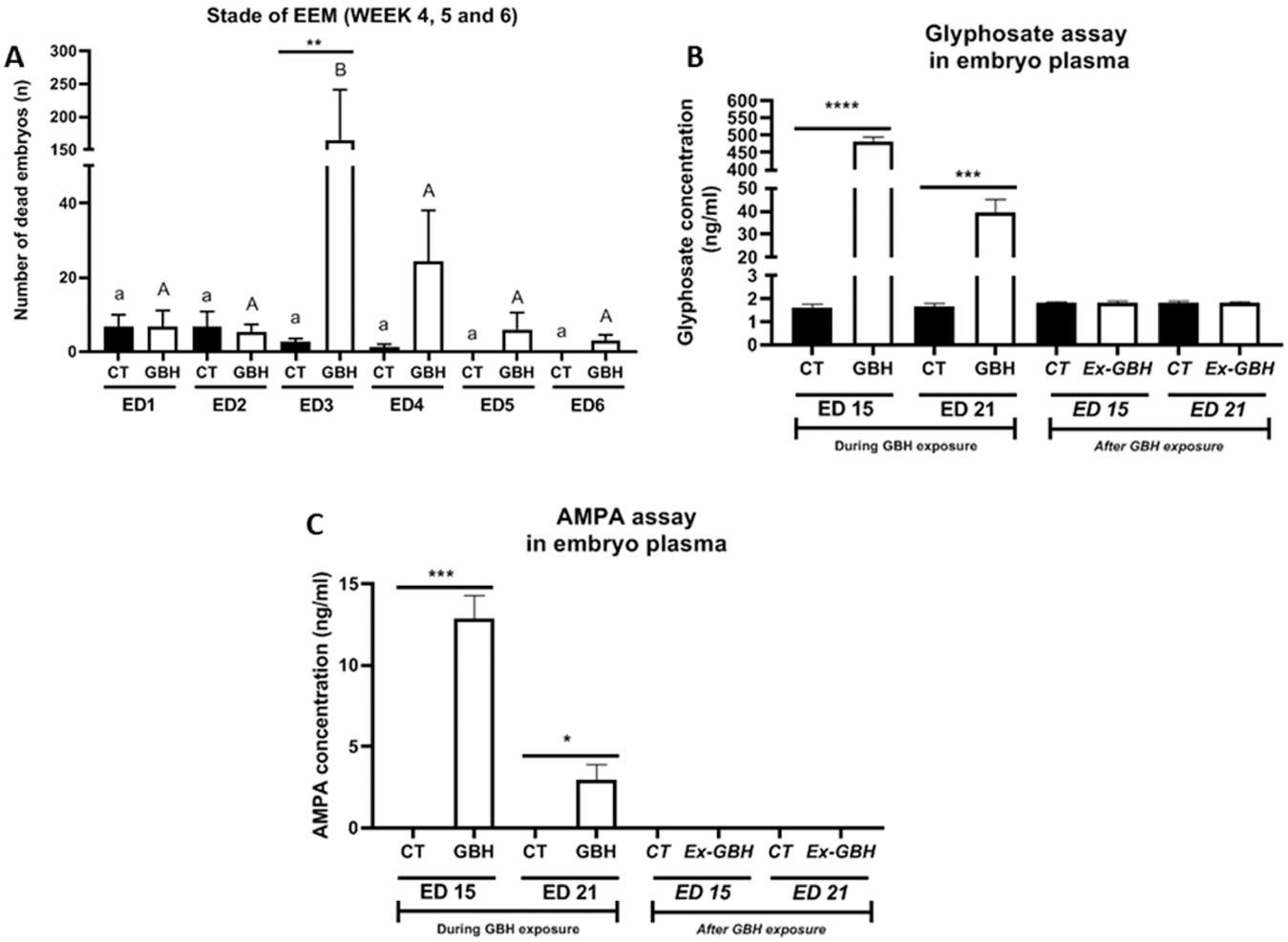

Fig. 4. Determination of embryonic stage for embryo mortality and glyphosate (Gly) and AMPA concentrations in the blood plasma of embryos. A) Embryonic stage determination for EEM in CT (black bars) and GBH (white bars) groups. B) Gly assay in the blood plasma of embryos at ED15 and ED21 for CT and GBH groups (WEEK 3) and for CT and Ex-GBH groups (*WEEK 11*) (ng/mL) (n = 4 per group). C) AMPA assay in the blood plasma of embryos at ED15 and ED21 for CT and GBH groups (WEEK 3) and for CT and Ex-GBH groups (*WEEK 11*) (ng/mL) (n = 4 per group). Results are presented as means ± SEM. Letters correspond to the ordinary two-way ANOVA significance ($p < 0.05$), followed by Tukey HSD's multiple comparisons test comparing the number of dead embryos in CT and GBH groups according to the embryonic stage; Stars (*) correspond to the unpaired t-test significance ($p < 0.05$) corresponding to the comparison between CT and GBH embryos (WEEK 3) and between CT and Ex-GBH embryos (*WEEK 11*) blood plasma for Gly and AMPA concentrations at ED15 and ED21. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$.

may be more toxic than glyphosate alone (Moore et al., 2012). Thus, we chose to determine the effect of GBH and not that of Gly alone.

4.1. GBH, metabolic functions and egg performance

Most of the studies that are available in avian species mainly focus on animal growth performances, welfare, and/or laying performances showing no or few adverse effects on measured parameters (Kubena et al., 1981). In our experiment, we chose to expose broiler hens to GBH via food with a concentration of Gly in the GBH food of 1250 mg/kg of food, which is about 3 times higher than the amounts found in cereals after GBHs are spread on fields (Eason and Scanlon, 2002). Moreover, this concentration is higher than the maximum residue level of 20 mg/kg of Gly found in several feed crops and defined by the European Union (European Food Safety Authority (EFSA), 2018). About egg quality, two studies conducted in quail with a parental dietary exposure to Gly (200 mg/kg of feed) showed that Gly does not alter egg production and quality (egg, yolk, or shell mass and egg hormone concentration) in females (Ruuskanen et al., 2020a, 2020b). These data are in good agreement with our data except for a significant increase in eggshell quality. Indeed, we demonstrated that statistical difference were observed, between CT and GBH, for eggshell percent (higher in GBH group) and consequently also for index, and eggshell thickness. This result is different from quail species as Ruuskanen et al. (Ruuskanen et al., 2020a) found no difference in eggshell quality. In accordance with results from other studies, our treated hens with GBH exhibited normal phenotype and no difference in terms of bodyweight and egg production compared to their CT counterparts. Indeed, a prospective study conducted in broiler breeder egg production companies revealed no association between residues of Gly in the food and the laying rate (Foldager et al., 2021).

4.2. GBH, ovarian functions and fertilization

In our study we observed that steroid concentrations in yolks were similar in GBH and CT groups, leading to the hypothesis that dietary GBH exposure doesn't affect ovarian function in laying hens. In agreement with our hypothesis, we demonstrated that in our condition's expression of steroidogenesis enzyme (*CYP11A1*, *CYP1A19*) and cholesterol carrier (*StAR*) in granulosa and theca cells was not in vivo affected by dietary GBH exposure. These data are opposite to the main data observed in vitro. A lot of studies showed that GBH or Gly inhibit

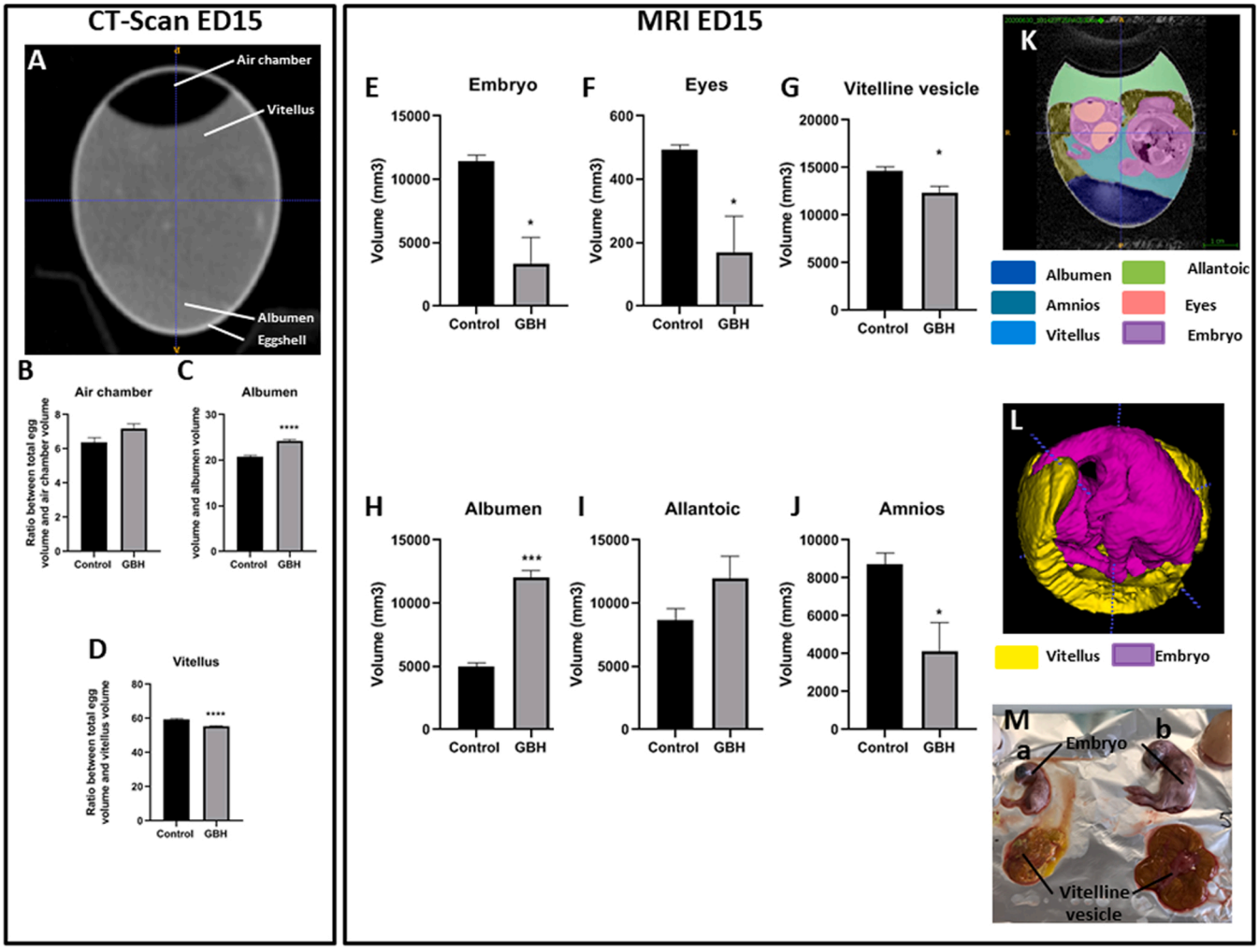

Fig. 5. Computerized tomography and Magnetic Resonance Imaging analysis of incubated eggs at ED15. Representative picture of A) analyzed incubated eggs at ED15 by CT-Scan; B) Ratio between the total egg volume and the air chamber volume, C) the albumen volume and D) the vitellus, respectively (n = 10 eggs per group). E) Volume of the embryo body, F) Volume of the eyes of the embryo, G) the vitelline vesicle, H) albumen, I) allantoic, and J) amnios determined by analysis of MRI images at ED15 (n = 4 per group). Representative picture of K) analyzed incubated egg at ED15. Structures are colorized with the albumen in blue, the amnios in dark blue, the vitellus in light blue, the allantoic in green, the eyes in pink, and the embryo body in purple. Representative picture of L) 3 dimensions reconstructed analyzed incubated egg at ED15 with the vitellus in yellow and the body of the embryo in purple. Qualitative photography M) of a GBH embryo (left) and of a CT embryo (right) with their respective vitelline vesicle showing their size difference. Results are presented as means ± SEM. Stars (*) correspond to the unpaired t-test significance ($p < 0.05$) corresponding to the comparison between CT and GBH eggs at ED15. **$p < 0.01$; ****$p < 0.0001$.

steroidogenesis, but these results were observed with higher doses of Gly (Alarcón et al., 2020; Fu et al., 2021; Perego et al., 2017b, 2017a; Ren et al., 2018; Wrobel, 2018). We went further in our investigations and demonstrated that after artificial insemination, the unfertile egg rate was not affected by the treatment, so GBH does not appear to affect egg fertilization (Uren Webster et al., 2014). However, Gly alone and one of its commercial formulation appears to affect oocyte maturation in xenopus (Slaby et al., 2020) and so, could affect fertility in some species.

4.3. GBH and embryo development and chick viability

Most studies concerning the effect of GBH on the embryo development in chicken species involved direct exposition to GBH with *in ovo* injections or egg immersion (Fathi et al., 2020, 2019; Lehel et al., 2021; Paganelli et al., 2010). Nevertheless, all these studies concluded that GBH have adverse effects on embryo development. Two studies conducted in quail with a parental exposure to Gly via food (200 mg/kg of food) showed poor embryonic development within the treated group compared to CT (Ruuskanen et al., 2020a). In a second study, the authors confirmed the same result (Ruuskanen et al., 2020b). But according to our knowledge and literature, no studies have been conducted on fertility and embryonic development in the case of parental exposure to GBH via the food in chicken.

Our results showed that Gly and AMPA were found within the plasma and eggs from hens treated with GBH. In eggs, it appears that the two molecules are mainly found in the yolk, whereas they have low concentrations or are undetectable in the albumen. Gly concentrations in egg yolk were detectable 4 days after the beginning of exposure before reaching a maximum of 8 days after the beginning of exposure, suggesting an accumulation. At the end of exposure, Gly concentrations decreased continuously until day 8. However, during the artificial incubation period, our results showed a dramatic early embryonic death occurring at ED3 in eggs from the GBH group. During ED3, corresponding to stage 20 according to Hamburger and Hamilton (1992), the embryo lies on its left side. There is the beginning of the blood circulation and the appearance of the cardiac structures which begin to beat. The vitelline membrane extends over the surface of the yolk (Hamburger and Hamilton, 1992). Thus, embryonic mortality at this stage could be due to a passage of Gly from the yolk to the embryo through its new blood circulation. Foldager et al., 2021 found a negative association

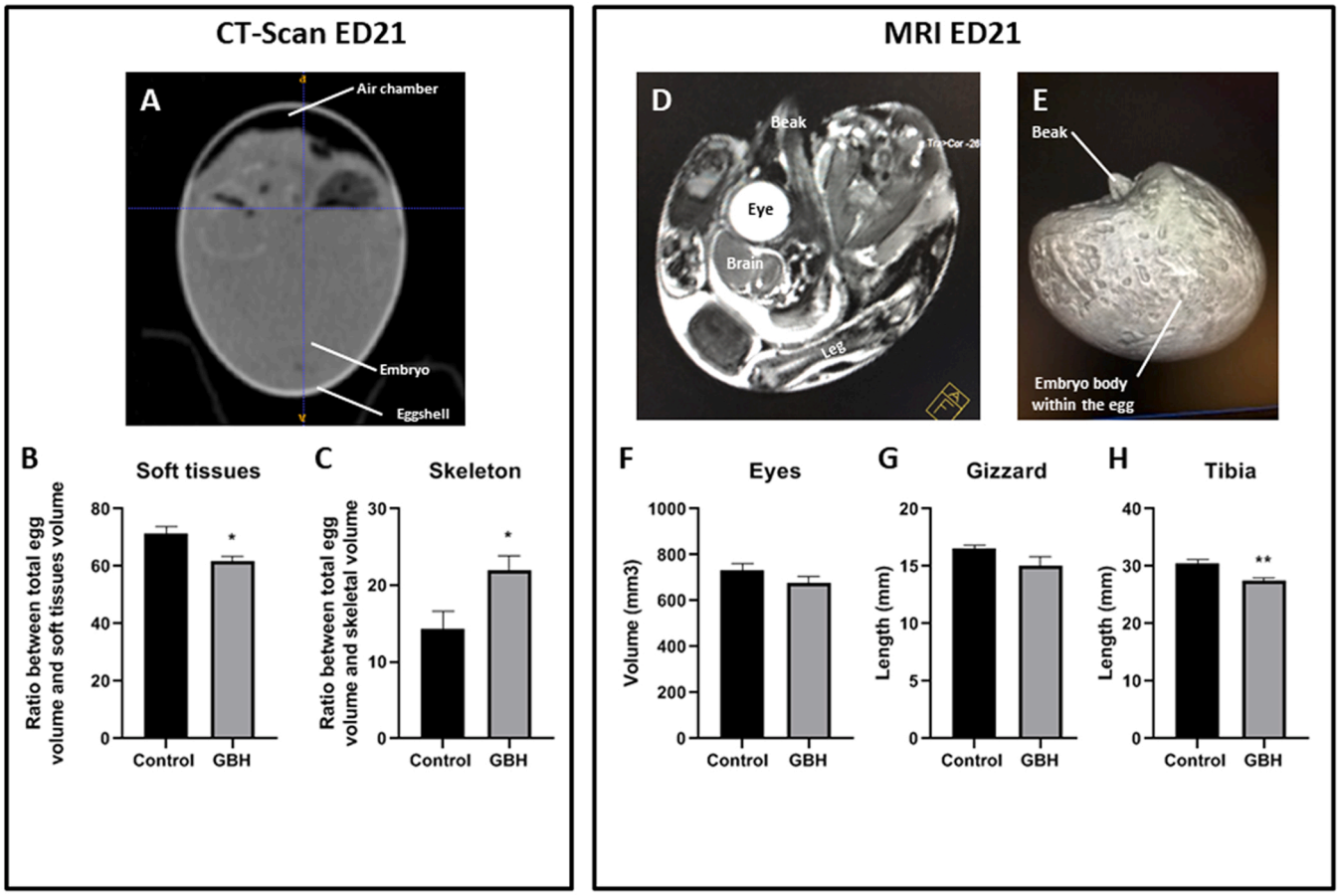

Fig. 6. Computerized tomography and Magnetic Resonance Imaging analysis of incubated eggs at ED21. Representative picture of A) computerized tomography analyzed incubated egg at ED21 (n = 5 per group); B) Ratio between the total egg volume, and C) the soft tissues and the skeleton volume, respectively. Representative pictures of D) and E) in 3 dimensions Magnetic Resonance Imaging analyzed incubated egg at ED21 (n = 4 per group); F) Volume of the eyes of the embryo, G) length of the gizzard and H) of the tibia. Results are presented as means ± SEM. Stars (*) correspond to the unpaired t-test significance ($p < 0.05$) corresponding to the comparison between CT and GBH eggs at ED6 and ED15. *$p < 0.05$; **$p < 0.01$.

between residues of Gly commonly found in food for broiler breeders and egg hatchability but give no information about the stage of embryonic death (Foldager et al., 2021). In contrary, Ruuskanen et al., 2020 only found a tendency for embryonic death in eggs from the GBH treated group in quail species (Ruuskanen et al., 2020b). This difference could be due to the dose used in the protocol and species specificities. This important EEM was also followed by a significantly higher LEM, meaning that surviving ED3 mass death embryos continue to be affected by GBH treatment during incubation. Our results about the eggshell quality could also explain this massive embryo loss. Indeed, eggshell thickness, elastic modulus, toughness and pore size are important parameters for embryo survival and could be affected by exposure to pesticides and thus alter embryo development. These observations have been already made in several wildlife species of oviparous animals (Beldomenico et al., 2007; Buck et al., 2020; Fry, 1995; Stoker et al., 2013; Tavalieri et al., 2020). We quantified Gly and AMPA in embryo plasma at ED15 and ED21 and found both molecules in the plasma of the embryo of the GBH group, indicating a passage of the two molecules from the yolk to the blood of embryo. In addition, we have studied in depth the development of the embryo by analyzing the eggs during incubation by non-invasive methods such as CT-Scan and MRI. These original results highlighted a significant delay in embryonic growth within the GBH group. This was associated with disturbances in the development of embryonic annexes during the stages studied with a larger yolk sac at ED6, followed by a smaller yolk sac at ED14 - 15 associated with a higher albumen volume in the GBH group. Moreover, embryos exhibited a significant reduction of organs weight (around 40 %) with a higher reduction of the digestive tract weight (60 %) when compared to the CT. During the dissection experiment, we made the same observation for yolk sacs and it also appeared that this structure was abnormal with spillage of its contents into the albumen compartment.

MRI analysis showed an important embryo growth retardation of GBH embryos compared to CT at ED15. Indeed, the total embryo and eye volumes were strongly reduced in the GBH group but these differences were abolished at ED21. This is explained by the fact that 15 embryos per group were analyzed by MRI at ED15, but among these 15 embryos, only 5 reached the ED21 stage. We noticed that the embryos exhibiting the highest growth retardation died during the incubation process and that only the embryos with the lowest growth retardation survived until ED21. Our observations on embryonic growth retardation are also in agreement with other studies showing that direct exposure of chicken embryos by injections of GBH *in ovo* induces histopathological alterations of several organs, imbalances of serum parameters and an oxidative stress (Fathi et al., 2020, 2019). Moreover, another study with GBH *in ovo* injection or egg immersion showed a reduction in the average body weight, and resulted in a higher incidence of congenital anomalies and embryo death (Lehel et al. (2021). At a molecular level, Paganelli et al. demonstrated that GBH impair retinoic acid and Shh signaling inducing teratogenic effects and embryonic death (Paganelli et al., 2010). This is consistent with the important role of these signaling pathways in embryo development in chicken species (Stainton and Towers, 2022). At ED21, CT-Scan results showed a significant increase of skeleton volume associated with a decrease of soft tissues volume in

Table 3
Eggs and embryos characteristics of broiler hens during Glyphosate Based Herbicide (GBH) exposure (red, week 6, CT versus GBH) and after GBH exposure (green, week 11, CT versus GBH) at ED15 and ED21. The weight of the eggs were determined at dissection. After dissection, embryo, vitelline vesicle, liver, brain, and digestive tract were weighted. After birth, chick's Tona scores were determined. Results are presented as means ± SEM. P-values of the effect of the GBH exposure were considered as significant if $p < 0.05$. Lowercase letters correspond to unpaired t-test significance ($p < 0.05$).

	GBH exposure		*After GBH* exposure	
ED15	**ED15 Control (WEEK6 n = 20)**	**ED15 GBH (WEEK6 n = 20)**	***ED15 Control (WEEK11 n = 20)***	***ED15 Ex-GBH (WEEK11 n = 20)***
Egg (g)	55.87 ± 0.70	54.60 ± 0.70	60.65 ± 0.92	60.24 ± 0.73
Embryo (g)	21.32 ± 0.24[a]	11.40 ± 1.18[b]	20.54 ± 0.50	20.94 ± 0.58
Vitelline vesicle (g)	13.08 ± 0.53[a]	6.063 ± 0.77[b]	15.24 ± 0.70	14.85 ± 0.61
Liver (g)	0.39 ± 0.014[a]	0.21 ± 0.02[b]	0.36 ± 0.01	0.36 ± 0.02
Brain (g)	0.58 ± 0.02[a]	0.34 ± 0.03[b]	0.52 ± 0.02[a]	0.59 ± 0.01[b]
Digestive tract (g)	1.14 ± 0.06[a]	0.48 ± 0.07[b]	1.02 ± 0.06	1.03 ± 0.07
Heart (g)	0.14 ± 0.01[a]	0.08 ± 0.01[b]	0.15 ± 0.01	0.14 ± 0.01
ED21	**E21 Control (WEEK6 n = 7)**	**E21 GBH (WEEK6 n = 4)**	***E21 Control (WEEK11 n = 40)***	***E21 Ex-GBH (WEEK11 n = 40)***
Chick (g)	41.09 ± 1.04	38.16 ± 2.25	43.90 ± 0.48	44.07 ± 0.39
Brain (g)	0.89 ± 0.03[a]	0.74 ± 0.05[b]	0.89 ± 0.02	0.88 ± 0.02
Digestive tract (g)	4.97 ± 0.09	4.33 ± 0.37	5.38 ± 0.09	5.39 ± 0.07
Heart (g)	0.33 ± 0.02	0.28 ± 0.01	0.33 ± 0.008	0.33 ± 0.008
Liver (g)	1.02 ± 0.03[a]	0.84 ± 0.05[b]	1.12 ± 0.02	1.15 ± 0.03
Vitelline vesicle (g)	2.97 ± 0.35	3.01 ± 0.92	2.71 ± 0.01	2.76 ± 0.02
Birth	**Control (WEEK6 and 7 n = 7)**	**GBH (WEEK6 and 7 n = 4)**	***Control (WEEK11 and 12 n = 100)***	***Ex-GBH (WEEK11 and 12 n = 87)***
Tona score	105.00 ± 2.17[a]	78.25 ± 9.93[b]	105.40 ± 0.50[a]	102.30 ± 0.67[b]

GBH group. Within the GBH group, by MRI, we noticed a smaller length of tibia bone. Previous studies in zebrafish species demonstrated that Gly exposure can induce bone disorders in embryos with craniofacial and locomotor disorders (Díaz-Martín et al., 2021). Moreover, it has been established that Gly and AMPA can alter estrogen receptor alpha osteopontin and bone sialoprotein. Exposed embryos present spine deformities as adults and these developmental alterations are likely induced by changes in protein levels related to bone and cartilage formation (Zhang et al., 2021). In xenopus species, cephalic malformations and abnormal development of the craniofacial skeleton have been observed in exposed embryos, which also showed higher retinoic acid activity (Bonfanti et al., 2018; Paganelli et al., 2010). These last results are completely new and skeletal abnormalities have to be investigated more deeply even if Gly has been shown to have an influence on bone weight, length, and mineral content in growing chicks exposed to Gly (Kubena et al., 1981). Finally, after birth, dissection permitted to put in evidence a smaller brain and liver in GBH chicks while the Tona score was significantly decreased in this same group compared to CT.

4.4. Reversibility of GBH effects on embryo development

In our experiment, we removed GBH from the diet of our animals for two weeks before repeating the fertilization experiments by artificially inseminating the hens with semen from unexposed roosters. For most measured parameters (measurement of egg production and quality; fertility and embryo development parameters; bodyweight, carcass traits, and embryo and chick quality), we found no significant difference between the CT and GBH groups, indicating reversibility of the adverse effects of GBH on these parameters. We only found that chicks from the GBH group at ED21 have a significantly heavier brain and a reduced Tona score compared to chicks from the CT group. This last result could indicate that GBH can have long-term effects on the offspring like our previous study on the effects of this GBH on rooster fertility which revealed epigenetic disorders measured on sperm DNA (Serra et al., 2021).

5. Conclusion

Our results showed that a chronic GBH diet of 47 mg/kg/day (half of the NOAEL in poultry) during the laying period in broiler breeder females strongly reduced eggshell quality and early embryo development where it did not affect egg performance, fertilization level, and ovarian steroidogenesis. Furthermore the surviving embryo had a delay of growth for most of the organs except for skeletal volume. These negative effects on the eggshell parameters and the embryogenesis were associated to an increase in Gly concentration and oxidative stress in egg yolks. Interestingly, they were totally abolished two weeks after the arrest of dietary GBH exposure. Further studies are required to investigate the effect of GBH on embryo and embryonic annexes development. Additionally, as indicated by our results on brain weight differences between CT and Ex-GBH group chicks, the long-term effects of this GBH need to be investigated, particularly with a focus on the epigenome of the offspring.

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CRediT authorship contribution statement

Anthony Estienne: Methodology, Validation, Formal analysis, Investigation, Visualization, Writing – original draft. **Mathias Fréville:** Methodology, Validation, Formal analysis, Investigation, Visualization, Writing – original draft. **Guillaume Bourdon:** Methodology, Writing – review & editing. **Christelle Ramé:** Methodology, Validation, Formal analysis, Writing – review & editing. **Marine Chahnamian**: Methodology, Validation, Formal analysis, Writing – review & editing. **Joël Delaveau:** Methodology, Validation, Formal analysis, Writing – review & editing. **Christophe Rat:** Methodology, Writing – review & editing. **Aurélien Brionne:** Methodology, Writing – review & editing. **Pascal Chartrin:** Methodology, Writing – review & editing. **Hans Adriaensen:** Methodology, Writing – review & editing. **François Lecompte:** Methodology, Writing – review & editing. **Pascal Froment:** Validation, Formal analysis, Investigation, Visualization, Data curation, Writing – original draft. **Joëlle Dupont:** Methodology, Validation, Formal analysis, Investigation, Visualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113741.

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