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Glyphosate-based herbicides influence antioxidants, reproductive hormones and gut microbiome but not reproduction: A long-term experiment in an avian model☆

Suvi Ruuskanen [a, *], Miia J. Rainio [a], Carlos Gómez-Gallego [b, c], Otto Selenius [a, c], Seppo Salminen [c], Maria Carmen Collado [d], Kari Saikkonen [a], Irma Saloniemi [a], Marjo Helander [a]

[a] Department of Biology, University of Turku, Finland
[b] University of Eastern Finland, Finland
[c] Functional Foods Forum, University of Turku, Finland
[d] Department of Biotechnology, Institute of Agrochemistry and Food Technology, National Research Council (IATA-CSIC), Valencia, Spain

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ABSTRACT

Controversial glyphosate-based herbicides (GBHs) are the most frequently used herbicides globally. GBH residues in the wild, in animal and human food may expose non-target organisms to health risks, yet the developmental and cumulative effects of GBHs on physiology and reproduction remain poorly understood. We present the first long-term study on the effects of subtoxic GBH exposure (160 mg/kg) on multiple key physiological biomarkers (cellular oxidative status and neurotransmitters), gut microbiome, reproductive hormones, and reproduction in an avian model. We experimentally exposed in Japanese quail females and males (*Coturnix japonica*) to GBHs and respective controls from the age of 10 days–52 weeks. GBH exposure decreased hepatic activity of an intracellular antioxidant enzyme (catalase), independent of sex, but did not influence other intracellular oxidative stress biomarkers or neurotransmitter enzyme (acetylcholinesterase). GBH exposure altered overall gut microbiome composition, especially at a younger age and in females, and suppressed potentially beneficial microbes at an early age. Many of the microbial groups increased in frequency from 12 to 28 weeks under GBH exposure. GBH exposure decreased male testosterone levels both at sexual maturity and at 52 weeks of exposure, but did not clearly influence reproduction in either sex (maturation, testis size or egg production). Future studies are needed to characterize the effects on reproductive physiology in more detail. Our results suggest that cumulative GBH exposure may influence health and reproduction-related traits, which is important in predicting their effects on wild populations and global poultry industry.

1. Introduction

Glyphosate (N-[Phosphonomethyl]glycine) is the most used and currently one of the most controversial agrochemicals globally (Benbrook, 2016). Glyphosate residues have been increasingly reported in water, soil, and animal tissues (Bai and Ogbourne, 2016; Helander et al., 2012) and are therefore a topical concern for both the general public and decision makers. In the European Union (EU), the use and risks of glyphosate-based herbicides (GBHs) have been widely discussed. After much heated debate, GBHs were approved for use until 2022 (Szekacs and Darvas, 2018). Before the next EU decision is made on whether to renew the authorized use of glyphosate, more studies on the impact of glyphosate on ecosystems are urgently needed.

Glyphosate has long had a reputation for being non-toxic to animals, as it inactivates an enzyme (5-enolpyruvylshikimate-3-phosphate synthase, EPSPS) in the shikimate pathway (synthesizing essential amino acids), which is present only in plants and some microbes (Helander et al., 2012). However, evidence for the potential negative effects of glyphosate on development, and fitness is rapidly accumulating from various taxa, including annelids,

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* Corresponding author.
E-mail address: skruus@utu.fi (S. Ruuskanen).

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arthropods, mollusks, echinoderms, fish, reptiles, amphibians, and mammals (Szekacs and Darvas, 2018; Gill et al., 2018; Van Bruggen et al., 2018). GBHs may influence organismal health and reproduction via multiple pathways and numerous biological levels of organization, ranging from intracellular to physiological to whole organism level.

Firstly, glyphosate is likely to cause intracellular changes and cytotoxicity (Szekacs and Darvas, 2018). For example, GBHs influence mitochondrial activity and likely increase DNA damage (Ghisi et al., 2016; Tarazona et al., 2017). One key mechanism of cytotoxicity is oxidative stress, i.e., the imbalance between reactive oxygen species (ROS) and antioxidant defenses, which can in some cases lead to cell death (Halliwell and Gutteridge, 2015). In vertebrates, glyphosate has been found to increase oxidative stress and damage in fish and mammals (El-Shenawy, 2009; Modesto and Martinez, 2010).

Secondly, at the tissue and organismal levels, GBHs may disrupt neurotransmitter function and are likely to function as endocrine disruptors (Gill et al., 2018). For example, disrupters of brain acetylcholine esterase (ACHE) function can lead to neurological and behavioral changes and even influence learning abilities in rodents (Gallegos et al., 2018; Bali et al., 2019; Picciotto et al., 2012). As potent endocrine disrupters, GBHs are likely to influence the expression of steroidogenic acute regulatory proteins (StARs) (e.g. Walsh et al., 2000), which mediate the initial step of steroid hormone synthesis, affecting androgen hormone conversions via aromatase enzymes (e.g Defarge et al., 2016). Recent studies on mammalian models reported altered hormone levels (Nardi et al., 2017; Clair et al., 2012; Romano et al., 2010; Abarikwu et al., 2015; Manservisi et al., 2019; Ren et al., 2018) and the disruption of puberty, reproduction, and sperm traits (Romano et al., 2012; Hamdaoui et al., 2018; Alarcon et al., 2019). However, most extant studies on reproduction were conducted on adult animals and less is known about the effects of GBHs on reproductive maturation.

Thirdly, GBHs are likely to influence organisms via changes in microbial companions: The GBH-sensitive shikimate pathway is present in most bacteria, and its key enzyme, EPSPS, is susceptible to GBHs in many bacterial groups (e.g. Van Bruggen et al., 2018; Fei et al., 2013; Leino et al., 2020). Consequently, GBHs have recently been found to profoundly influence gut bacterial communities in a few model organisms and in vitro cultures (Aitbali et al., 2018; Shehata et al., 2013; Mesnage et al., 2020; Mao et al., 2018; Motta et al., 2018; Nielsen et al., 2018; Poppe et al., 2019), yet a comprehensive understanding across host taxa is lacking. As the gut microbiome is known to be indispensable for organism health (incl nutrient acquisition, immune function, metabolism), and potentially reproduction (Gould et al., 2018), altered gut bacterial communities due to GBHs could have unforeseen consequences on organism performance in wild populations and agricultural contexts.

We studied whether long-term exposure to GBHs influences key physiological responses (intracellular oxidative status and neurotrasmitters), gut microbiome, reproductive hormones and ultimately reproduction. We believe that such an approach is needed to comprehensively understand the potential influence of GBHs on health and reproduction. We conducted an experimental study using a rare, long-term experimental design. While short-term toxicology studies have dominated GBH research, there is now also emerging support for the potential chronic and cumulative effects of GBHs using environmentally relevant doses (Bai and Ogbourne, 2016; Greim et al., 2015; Mesnage et al., 2015); that said, the generality of such effects across taxa must be evaluated. We selected birds as our model organisms because, surprisingly, the effects of GBHs on any these traits have been almost completely ignored in this major vertebrate group (Bai and Ogbourne, 2016; Szekacs and Darvas, 2018; Mesnage et al., 2015). However, understanding the effects of GBHs on avian taxa is important to (i) predict the generality of GBH effects across vertebrates, (ii) understand the impact of GBHs on poultry, which are potentially exposed to GBH residues via feed (Efsa, 2018), and (iii) understand the impacts GBHs on wild bird populations feeding in GBH-contaminated areas.

We experimentally exposed Japanese quails (*Coturnix japonica*) chronically to dietary GBHs (RoundUp Flex, embedded within organic feed, N = 38, levels below the no-adverse-effect-level) or respective controls (organic feed only, N = 38) from 10 days after hatching to 52 weeks of age, to study both developmental and cumulative effects of GBHs. We predicted that (i) GBHs may increase intracellular oxidative stress and damage, (ii) GBHs may decrease neurotransmitter enzyme levels, (iii) GBHs may influence the gut microbiome, especially decreasing the abundance of bacterial groups with EPSPSs sensitive to GBHs, such as *Firmicutes* and *Lactobacillus*, and (iv) GBHs may decrease testosterone levels and ultimately delay reproductive maturation in both sexes, and decrease female reproductive investment. Tissue glyphosate concentrations were measured to evaluate tissue exposure load.

2. Material and methods

2.1. General experimental protocol

We performed a GBH exposure experiment in which Japanese quails were fed either GBH-contaminated feed (N = 38) or control feed (N = 38) from the age of 10 days to 52 weeks (see also Ruuskanen et al., 2020a, Ruuskanen et al., 2020b). The chicks originated from a randomly paired parental generation (ca. 1-year-old birds, N = 21 pairs) in Turku (Finland). The parental generation had no history of glyphosate exposure. Eggs from each pair were individually marked, and hatched individually in an artificial incubator (RcomMaru Max CT-190, South Korea). A small blood sample was collected at hatch for molecular sexing (Aljanabi and Martinez, 1997). The chicks were randomly and evenly divided into 2 groups from all parents, sexes, and hatch dates. Due to unknown reasons, the sex ratio of hatchlings was biased toward males; however, all hatched chicks were included in the experiment. The sample sizes were as follows: N(GBH, female) = 15, N(GBH, male) = 23, N(control, female) = 14, and N(control, male) = 24. Three individuals died because of injuries (aggressive pecking) during the experiment, but there was no mortality related to the treatments. The experiments were conducted under licenses from the Animal Experiment Board of the Administrative Agency of South Finland (ESAVI/7225/04.10.07/2017).

2.2. Treatment groups and preparation of the feed

The GBH-exposed group was fed organic feed (organic feed for laying chickens, "LuonnonPunaheltta," Danish Agro, Denmark) added with Roundup Flex® (480 g/l glyphosate, present as 588 g/l [43.8% w.w] of potassium salt of glyphosate, AXGD423115/7/2017 Monsanto, with surfactants alkylpolyglycoside [5% of weight] and nitrotryl [1% of weight]). The control group was fed the same organic feed with non-measurable glyphosate concentrations. A GBH product was selected over pure glyphosate to mimic the exposures in natural environments including exposure to adjuvants, as adjuvants may increase the toxicity of glyphosate (reviewed in Gill et al., 2018; Van Bruggen et al., 2018). However, with this experimental design we cannot distinguish the effect of pure glyphosate, the potential effects of adjuvants, or whether adjuvants they altered the effects of the active ingredient, glyphosate. The concentration of glyphosate in the GBH feed was aimed at 160 mg/kg feed. This concentration was ca. ½ of that calculated in grains

(available to granivorous birds) after GBHs are spread on a field containing grains (Eason and Scanlon, 2002). This concentration in the feed corresponds to a dose of 12–20 mg glyphosate/kg body mass/day in full-grown Japanese quails. The European Food Safety Authority (EFSA) has reported a NOAEL (No adverse effects level) of 100 mg/kg body mass/day for poultry (Efsa, 2018); therefore, our experiment tested a concentration below this threshold. Furthermore, a dose of 347 mg/kg did not negatively influence adult body mass in Japanese quails in a short-term experiment (Eason and Scanlon, 2002). The acute toxicity (LC50) of RoundUp Flex (via feed), reported by the manufacturer, was >4640 mg/kg for mallards (*Anas platyrhynchos*) and bobwhite quail (*Colinus virginianus*).

To validate the treatment levels, glyphosate concentrations were measured in 6 batches of food. Furthermore, residue levels were measured in excreta samples (fecal and urine material combined) after 52 weeks of exposure. Excreta of 4–6 randomly chosen individuals per treatment and sex were pooled. We analyzed glyphosate residues from 2 control pools (1 female and 1 male pool) and 3 glyphosate pools (1 female and 2 male pools). Glyphosate residues were measured via LC-MS/MS (certified laboratory, Groen Agro Control, Delft, Netherlands). The extraction was performed with a mixture of water and acidified methanol. The analyses were performed with a liquid chromatography coupled to a tandem quadruple mass spectrometer (LC-MS/MS). An ion exchange HPLC column was used for the separation. The separation was performed with a mix mode column using a gradient based on a mixture of water and acetonitrile. Two specific MRMs (i.e. multiple reaction monitoring) were used to identify the component and standard addition to quantify the concentration (see transitions Suppl. Table 1, and an example of the chromatograms, Suppl. Fig 1). Transition 1 was used as the quantifier and transition 2 was used as the qualifier. The detection limit was 0.01 mg/kg. The average of 6 batches of food was 164 mg/kg (SE 55 mg/kg). The average glyphosate concentration in 3 pools (4–5 individuals in each pool) of excreta samples (urine and fecal material pooled) was 199 mg/kg (SE 10.5 mg/kg). There were no glyphosate residues in the control pools (<0.01 mg/kg).

GBH feed was prepared every week to avoid potential changes in concentration caused by degradation. Diluted Roundup Flex® was mixed with the organic feed in a cement mill (Euro-Mix 125, Lescha, Germany). The feed was air-dried and further crushed with a feed crusher (Model ETM, Vercella Giuseppe, Italy) to a grain size suitable for the birds considering their age. The control feed was prepared using a similar method, but only water was added to the feed, and a separate cement mill was used (ABM P135 L, Lescha, Germany). After crushing, the dry feed was stored in closed containers at 20 °C in dry conditions. Separate equipment for feed preparation and storage were used for GBH and control feed to avoid contamination.

2.3. Rearing conditions

During the first 5 weeks, the quails were housed in 4 cages (1 m × 1 m, height 0.3 m, in a room with temperature +20 °C and photoperiod 16:8, equipped with an infrared heat lamp, ca. +38 °C, Kerbl, Germany) in groups of 19 animals. When the experiment began on Day 10 after hatching (see above), 2 cages were reserved for GBH treatment and 2 for control treatment. Feed (GBH or control), water, lime for chicks ("AitoPoikaskalkki", Nordkalk, Finland), and grit were available *ad libitum*. Water was supplemented with a multivitamin solution (Supreme Horse Care Multivitamin, Finland) in concentrations suggested for poultry by the manufacturer. From 5 to 12 weeks, the quails were housed in 12 floor cages (1 m × 1 m, height 0.5 m) with bedding (wood shavings) at +20 °C and 16:8 photoperiod. Each cage contained 5–7 birds, with males and females in separate cages. Birds within a treatment and sex were randomly allocated to cages. From the age of 12 weeks, quails were randomly assigned to male–female pairs within a treatment (i.e., 1 female and 1 male from GBH treatment) and housed in pairs in similar conditions (13 pairs in GBH treatment and 13 pairs in control treatment). The remaining males were reared in 4 large cages until 18 weeks, at which point they were euthanized via cervical dislocation to study the short-term effects of GBHs on liver oxidative biomarkers (see below).

2.4. Glyphosate residue analysis

Glyphosate residues were measured from liver samples after exposure for 52 weeks. To avoid contamination, tissue samples for glyphosate analysis were collected carefully with clean equipment to ensure they were not in contact with the skin or feathers of the birds, and the samples were processed rapidly. Liver samples from 3 control males and 3 control females were combined to obtain 2 pools of control samples, while 3 similar pools were obtained for GBH males and females (i.e., total of 9 males and females were sampled). Glyphosate concentrations from liver tissue were measured via LC-MS/MS (certified laboratory, Groen Agro Control, Delft, Netherlands) with a detection limit of 0.01 mg/kg.

2.5. Testosterone analysis

Blood samples (ca. 200 μl) were collected from the brachial vein to heparinized capillaries from males at maturation (age of 7 weeks) and after 52 weeks of GBH exposure to study both short-term and cumulative effects of GBHs on testosterone, a key reproductive hormone. The plasma testosterone concentration was analyzed using a commercial ELISA kit (ENZO Testosterone ELISA kit, ADI-900-065) according to manufacturer's instructions. This kit has been validated for and applied to many bird species (e.g. Abolins-Abols et al., 2018; and references therein, Clotfelter et al., 2004). We validated parallelism by analyzing pools of plasma in serial dilutions: The pools fell within 20% CV. The intraplate coefficient of variation was below CV 10% and inter-plate CV 2.6%.

2.6. Reproduction

The size of the cloacal gland correlates with the maturity and fertility (testis size) of the male Japanese quail (Biswas et al., 2007). Cloacal gland width and length (~0.1 mm) were measured weekly from 5 to 9 weeks using a digital caliper (Biltema), and the area in mm^2 (width × length) was used in further analyses. One person (VK) conducted all measurements. To track the start of egg-laying activity, i.e., reproduction in females, eggs were collected and weighed (~0.1g) from the cages every day after the birds started laying them. Eggs from individual females could not be identified since there were 6–7 females in each of the 4 aviaries (2 groups of females in both the control group and GBH group); thus, group means were analyzed.

2.7. Fecal microbiome sampling and analysis

Fecal microbiome samples were collected at 12 weeks of age (after reaching maturation) and 28 weeks of age, individually, from clean cages (cleaned with 70% EtOH) with mesh bottoms immediately after defecation. Samples were stored at −80 °C until analysis. DNA was extracted using the InviMag Stool DNA Kit/KFml (Stratec Molecular GmbH, Berlin, Germany) with slight alterations to the kit's instructions. For sample lysis, a sample preparation system, FastPrep −24 (serial no: 9010103, M.P. Biomedicals, Irvine, California, USA), was used. The DNA was harvested using an

automatic magnetic-particle purifying system, KingFisher (type: 700, Thermo Fischer Scientific Oy, Finland). A specific 16S rRNA gene region (V3–V4 region) was amplified following the Illumina protocols, and the libraries were sequenced using a 2 × 300 pb paired-end run (MiSeq Reagent kit v3). Sequencing was conducted at the FISABIO Sequencing and Bioinformatics Service (Valencia, Spain) using MiSeq. Samples with insufficient read counts (<1000 reads) were removed (N = 3, evenly distributed across the treatment groups and ages). Rare taxa (<0.01% abundance across all samples) were also removed. The quality filtered sequences were checked for chimera, and the non-chimeric sequences were processed using a DADA2 pipeline (in R) to produce an amplicon sequence variant (ASV) table. Taxonomy assignment was conducted with Silva database v. 132 (Quest et al., 2013).

2.8. Tissue sampling

At the age of 18 weeks (males only, N = 9 and 10 for GBH and CO, respectively) and 52 weeks (N = 13 females and males in GBH and CO, respectively), the birds were euthanized via cervical dislocation, after which they were dissected, i.e., their livers, brains, hearts, ovaries, and testes (if applicable) were removed and weighed. A sample of liver was snap-frozen in liquid nitrogen and further stored at −80 °C for oxidative status biomarker analyses. Whole brains were snap-frozen and further stored at −80 °C for ACHE analyses.

2.9. Oxidative status biomarkers and acetylcholinesterase

Oxidative status biomarkers were measured from livers after 18 (males only) and 52 weeks of exposure to GBHs. We measured multiple biomarkers of antioxidant status: antioxidant glutathione (tGSH), the ratio of reduced and oxidized glutathione (GSH:GSSG), and the activity of the antioxidant enzymes glutathione peroxidase (GPx), catalase (CAT), and lipid peroxidation, a measure of damage to lipids (using malonaldehyde, MDA, as a proxy). Of the measured biomarkers, the ratio of GSH:GSSG represents the overall oxidative state of cells, and consequently deviations in this ratio are often used as an indicator of oxidative stress (Halliwell and Gutteridge, 2015). GPx enzymes catalyze the glutathione cycle, whereas CAT directly regulates the level of ROS (Halliwell and Gutteridge, 2015). Lipid peroxidation measures the damage to lipids, and it is commonly measured with the thiobarbituric acid test (TBARS, Halliwell and Gutteridge, 2015). The methodology for measuring each biomarker is described in detail in Rainio et al., 2015; Espin et al., 2018, while the methods used for lipid peroxidation are detailed in Espin et al. (FAO, 2005) and in the supplementary material.

For the analysis of ACHE, whole brain samples were thawed and homogenized manually in PBS on ice; then, the samples were centrifuged and the supernatant was collected. ACHE was analyzed using a QuantiChromTM Acetylcholinesterase Assay Kit (DACE-100) following manufacturer's instructions. The results were calculated per mg protein (measured via BCA method).

2.10. Statistical analyses

Statistical analyses (except microbial community analyses) were conducted with SAS Enterprise Guide 7.1 and SAS 9.4. Residuals of the models were visually inspected to confirm normality and heteroscedasticity. Models were reduced sequentially by removing non-significant ($\alpha = 0.05$) interactions to allow for the interpretation of the main effects, and non-significant covariates were removed. Degrees of freedom were calculated with the Kenward–Roger method. Parental ID was included as a random effect in all applicable models to account for the non-independence of siblings. Non-significant terms were dropped sequentially from the final models, but the main effect of treatment was always kept in the models. Removed factors were re-introduced to the reduced models to confirm non-significance.

Body and organ masses, as well as *oxidative status, lipid peroxidation and ACHE* were analyzed using linear or generalized linear mixed models (LMMs or GLMMs) with fixed factors treatment, sex (if applicable) and their interaction, body mass as a covariate.

The analysis of *fecal microbiome* was conducted using Calypso. Fecal microbiome data were first normalized using the recommended Hellinger transformation (TSS combined with square root transformation) (Wang et al., 2017). The data was centered to 0 and scaled to range −2 to 2, with variance of 1. Differences among ages across the treatments were analyzed using mixed effect linear regressions, taking into account the repeated measurements from each individual. Given that the two ages seemed to show different patterns, the differences among treatment groups within an age group (and sex) in terms of composition and abundance were analyzed separately using multivariate methods (RDA) and ANOVAs, respectively. The sample sizes for the microbiome analyses are the following for the 12 week and 28 week measurements, respectively: Female CO = 11, 12; Male CO = 11, 11; Female GBH = 11, 12 and Male GBH = 10, 11.

Traits related to reproduction, i.e. *testosterone* concentration was analyzed using a LMM with fixed factors treatment, age (7 vs 52 weeks) and their interaction, body mass as a covariate and individual ID nested in parental ID, and hatch date as random intercepts. *The size of the cloacal gland* (cm^2) of males was analyzed using LMMs with fixed factors treatment, age (continuous, measured weekly from 5 to 9 weeks) and their interaction, and body mass as a covariate. Age (class) was included as a repeated effect, with an autoregressive (ar1) covariance matrix structure. *Egg production* (during the 3 weeks after the first egg appeared) was analyzed using GLMMs (Poisson distribution, logit link function) with fixed factors age (continuous, 52-73 days) treatment, and their interaction. *Egg mass* was analyzed using a LMM with the same fixed factors. Since eggs were not assigned to individual females, random effects could not be used in the models of egg production or mass. The group to which a female belonged (4 groups) was included as a random intercept to account for potential differences due to group structure or condition.

3. Results

3.1. Glyphosate residues are found in key organs, yet organ mass is not affected

Glyphosate and its metabolite (AMPA) residues were found in the liver, with concentrations 3 times higher in females than in males (Fig. 1). However, after 18 or 52 weeks of exposure, there was no difference in the body mass, or in the mass of the liver, heart, or brain among the 2 groups, in either females or males ($F < 0.25$, $p > 0.6$, Table 1), suggesting no overall effects on body composition.

3.2. Chronic exposure to GBHs has direct effects on physiology

GBHs decreased hepatic activity of a key intracellular antioxidant enzyme, CAT, ca 7% at 52 weeks of exposure to GBHs (independently of sex, $F_{1, 46} = 5.94$, $p = 0.019$, Suppl. Tables 2 and 3). There were no differences in other intracellular oxidative biomarkers or damage to lipids across between groups (Suppl. Tables 2 and 3). Contrary to our predictions, we found no effects of GBHs on brain ACHE enzyme activity in either sex (treatment $F_{1,42} = 0.68$, $p = 0.41$, treatment × sex $F_{1,43} = 0.0$, $p = 0.97$).

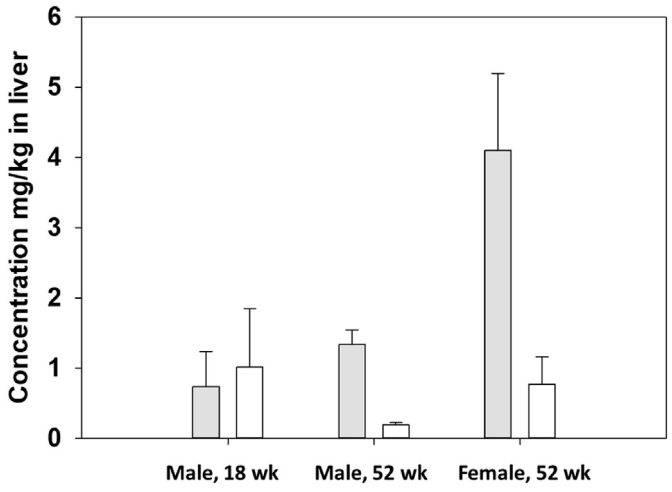

Fig. 1. Hepatic concentrations of glyphosate (grey) and its metabolite AMPA (white) of Japanese quails (average ± SD, mg/kg) exposed to dietary GBHs for 18 and 52 weeks. N = 3 (3 pools of 3 individuals)/data point.

3.3. GBHs perturb avian gut bacterial communities

Overall richness, richness estimation (Chao1 index) (Fig. 2), and abundance of many bacterial orders increased with age in the GBH group, while no change was observed in the control group (age from 12 to 28 weeks, Fig. 5, Suppl. Fig 2,3). The abundance of *Firmicutes*, the most abundant phyla, decreased, while the abundance of *Actinobacteria* increased in the GBH exposure group with age (Fig. 3). Such a pattern likely arose because GBHs influenced microbial groups predominantly at the younger age. To verify the differences within each age category, and to test for potential sex differences, we performed analyses separately by age and sex. We showed that GBH exposure shifted the community composition (RDA) at the age of 12 weeks (Fig. 4a1), predominantly in females (Fig. 4a2, a3), but not at the age of 28 weeks (Fig. 4b1–3). The ADONIS technique using Bray–Curtis distance matrix also showed similar, small differences in beta diversity across the treatment groups at the age of 12 but not 28 weeks (12 weeks: R2 = 0.0475; p = 0.0467; 28 weeks: R2 = 0.0242; p = 0.37). The main ASVs driving separations between GBH and control groups in the RDA at 12 weeks belong to phylum Firmicutes, Actinobacteria, Patescibacteria and Proteobacteria, including species such as *E. cecorum* and *Enorma massiliensis* (Figs. 3–5, Suppl. Figs. 2–3). Furthermore, we showed for the first time in a non-mammalian vertebrate that *Lactobacillus*, a likely beneficial bacterial group, was affected by GBHs: its abundance tended to decrease (P = 0.056) with cumulative GBH exposure (from week 12 to week 28, Fig. 5).

3.4. GBHs and reproduction

Reproductive maturation of males (changes in the size of the cloacal gland, a proxy for testis size) did not differ between the GBH and control treatment groups (interaction treatment × age: $F_{1,182} = 0.55$, p = 0.45, Suppl. Fig. 4). Male testis size did not differ among the groups at 18 or 52 weeks of age ($F_{1,13.9} = 3.78$, p = 0.073, Table 1). Yet, GBHs drastically decreased testosterone levels both at puberty (ca 57% decrease) and after 52 weeks of exposure (ca 25% decrease) (treatment $F_{1,47} = 4.67$, p = 0.036, treatment × age $F_{1,46} = 1.05$ p = 0.31, Fig. 6).

The commencement of reproduction in females, i.e., the start of egg laying, was 4 days later in the GBH group compared to the control group (Suppl. Fig. 5), yet there was no statistically significant difference in the number of eggs produced in the first 3 weeks (treatment $\chi^2 = 3.44$, p = 0.063, total N(GBH) = 110, N(control) = 135 eggs, N = 12 females in each group). Egg mass or ovary mass did not differ among the treatment groups (egg mass: $F_{1,233} = 0.46$, p = 0.50; Marginal means (g) average ± SE: GBH group: 10.47 ± 0.15; control group 10.11 ± 0.15; ovary mass $F_{1,13.9} = 0.16$, p = 0.69, Table 1).

4. Discussion

To our knowledge, this is the first long-term study on potential cumulative effects of GBHs in avian taxa. Our results suggest that GBHs influence multiple levels of biological organization, and potentially health, but not directly reproduction. As predicted, GBHs decreased the activity of a hepatic intracellular oxidative biomarker, yet a key brain neurotransmitter enzyme, acetylcholinesterase, was not affected. GBHs decreased male testosterone levels. GBHs altered microbiome composition, especially at a younger age and in females. However, reproductive maturation of males, egg production in females, body mass or organ masses were generally not affected.

GBHs residues were found in the liver, and the levels were higher than measured in previous short-term studies in the liver of poultry, even with a similar dose (FAO, 2005). This suggests that there is a risk that GBH residues may accumulate and eventually move up the food chain. The higher levels in females than males may be explained by increased feed consumption among actively egg-laying females, which are thus potentially exposed to higher dietary GBH load and potentially more vulnerable to dietary GBH contamination.

GBH exposure influenced key physiological biomarkers as they decreased antioxidant CAT activity, although showed no apparent effects on oxidative damage. GBH-induced oxidative stress and associated damage has been characterized across taxa, but responses vary greatly depending on the chemical formulation, species, and duration of exposure (e.g. Gill et al., 2018). The decreased

Table 1
Organ masses in Japanese quails after 18 and 52 weeks of exposure to glyphosate-based herbicide (GBH) or control (CO) groups: [a]p = 0.078 for testis mass; 18 weeks N = 10 and 9 for GBH and CO, respectively. At 52 weeks N = 13 per group per sex. Only females that retained a fully developing egg in their ovaries are included. Averages (SE in brackets) in grams or mg are shown.

Sex	Treat	18 weeks of exposure		52 weeks of exposure				
		Liver (g)	Testis (g)	Liver (g)	Brain (mg)	Heart (g)	Ovaries (g)	Testis (g)
Male	GBH	2.65 (0.08)	4.64 (0.25)	3.03 (0.17)	766 (13)	1.73 (0.08)		5.70 (0.51)[a]
	CO	2.24 (0.20)	4.85 (0.40)	3.21 (0.19)	801 (19)	1.92 (0.07)		4.90 (0.19)
Female	GBH			6.48 (0.61)	761 (27)	1.77 (0.10)	22.8 (0.67)	
	CO			6.26 (0.29)	731 (13)	1.88 (0.12)	23.6 (0.53)	

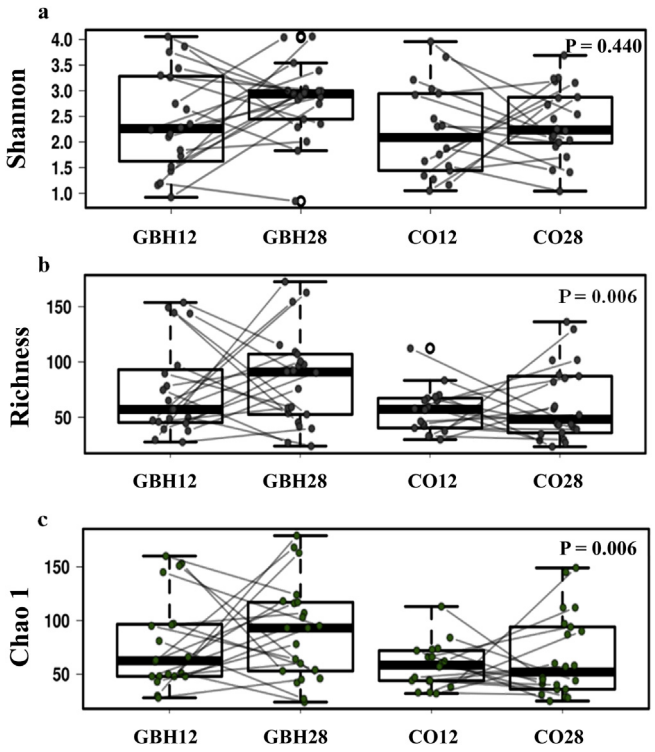

Fig. 2. Changes in (a) diversity (Shannon index), richness (b) and estimated richness (Chao1 index) with time under GBH exposure and control treatment (CO) at the age of 12 weeks (GBH and CO12) and 28 weeks (GBH and CO12). Significant differences between treatments is based on multilevel analysis (Mixed Effect Linear Regression).

antioxidant activities as reported here could lead to the accumulation of damage to proteins and DNA, a phenomenon which should be further investigated.

In contrast to our predictions, we did not find any effects of GBHs on neurotransmitter enzyme, acetylcholinesterase (ACHE). Previous studies in various organophosphate agrochemicals have identified decreased ACHE production in response to exposure (reviewed in Eyer et al., 1995), which could lead to neurological and behavioral changes and even influence learning abilities in rodents (Modesto and Martinez, 2010; Gallegoset al., 2018; Bali et al., 2019; Picciotto et al., 2012). We can speculate that either the GBH-exposure levels were not sufficient to induce such a change, or there may be species-specific differences in the response.

As predicted, we found large effects of GBHs on gut microbiome. Dysbiosis of gut microbial communities has been linked to various health problems across taxa (Das and Nair, 2019; Simon et al., 2016). Our results suggest that GBH exposure influences gut microbial community richness and abundance of known key taxa (e.g. *Lactobacillus* spp). The effects were strongest at a young age and in females, as there was a statistically significant difference in microbiome composition (RDA) between the treatment groups at week 12 (but not week 28) only in females, but not in males. Our results agree with the hypothesis that developing organisms are generally more vulnerable to environmental stressors and pollutants. Also other environmental stressors have been reported to cause stronger effects on the microbiome early in life (Dong and Gupta, 2019). The stronger effect in females may be explained by the higher sensitivity of females to GBHs or a higher exposure level (Fig. 1): GBH residues in tissues were higher in females than in males, most likely due to the fact that egg-laying females generally consume more feed than males. These results also support in vitro studies on poultry gut bacteria, where *Lactobacillus* spp. were found to be moderately to highly susceptible to GBHs (e.g. Shehata et al., 2013). Furthermore, recent (short-term) in vivo studies in rats, mice, and even bees similarly showed that (maternal) exposure to GBHs reduces *Lactobacillus* (Aitbali et al., 2018; Shehata et al., 2013; Mesnage et al., 2020; Mao et al., 2018; Motta et al., 2018; Nielsen et al., 2018)—yet in our study, a trend was only seen after cumulative GBH exposure. These studies imply that, across taxa, GBHs can have consequences on key beneficial microbial partners.

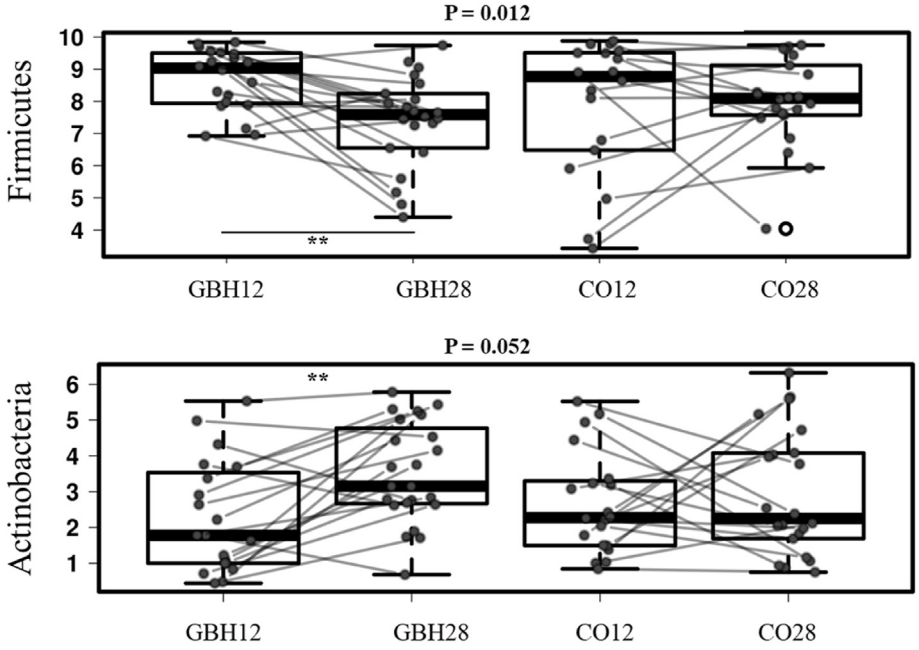

Fig. 3. Changes in the abundance of *Firmicutes* and *Actinobacteria* phyla with time under GBH exposure (GBH) and control treatment (CO) at the age of 12 weeks and 28 weeks. Significant differences between treatments is based on multilevel analysis (Mixed Effect Linear Regression). Asterisk indicate significant differences in time inside the same treatment group after ANOVA analysis at the level of $p < 0.001$. Sample sizes are indicated in the methods-section.

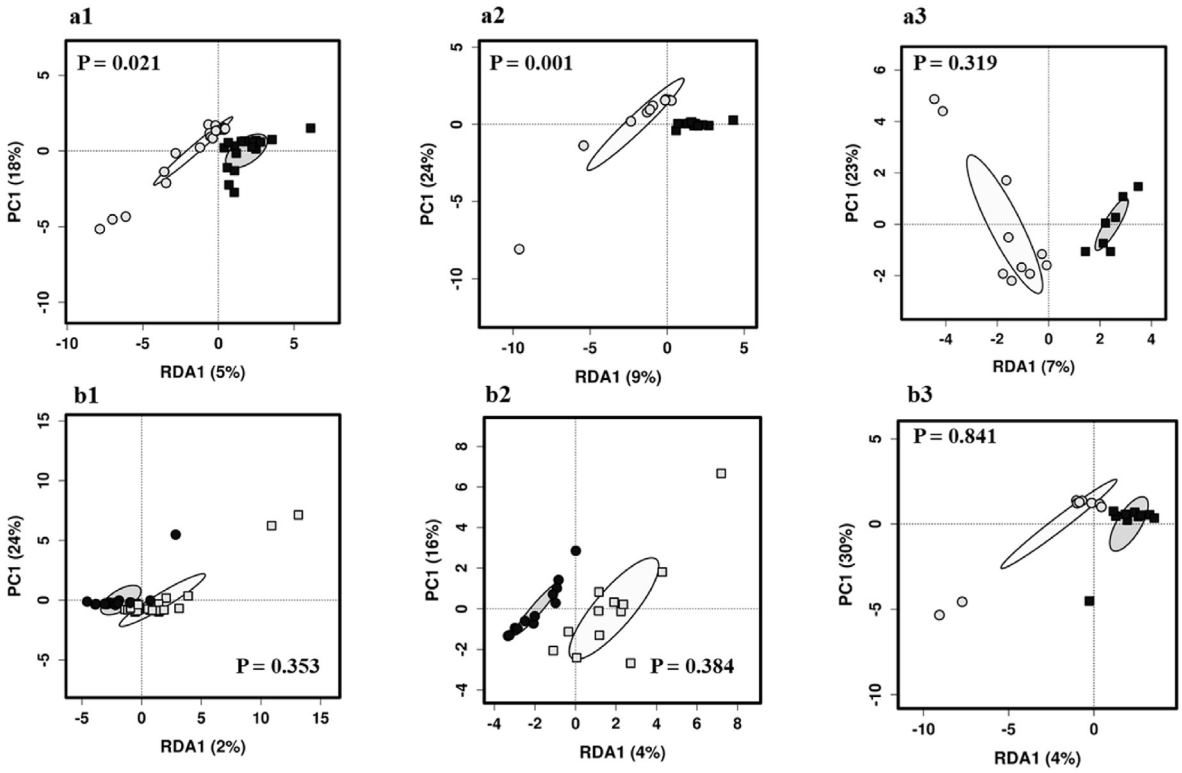

Fig. 4. RDA analyses after 12 weeks of GBH exposure (a) or after 28 weeks of exposure (b): (1) data on females and males combined, (2) females only, (3) males only (black = GBH exposure, white = control treatment).

The changes in microbial communities may be explained by (i) different sensitivities of bacterial groups to GBHs, as it is well known that in some bacterial genus/species, their EPSPS (class I) enzyme is sensitive to GBHs; whereas in others, EPSPS (class II) is resistant to GBHs (e.g. Leino et al., 2020). Second, (ii) the changes may also be due to shifts in community composition following a reduction in the abundance of some key bacterial groups and potentially altered species interactions. It has been suggested that the decrease in *Lactobacillus* by GBHs could lead to increases to pathogenic bacteria, like *Clostridia*, as found in vitro in poultry microbiome studies (Shehata et al., 2013; Okamoto et al., 2018). Supporting such processes, we found that the abundance of *Enterococcus cecorum,* an emerging pathogen in poultry (Jung et al., 2018), was higher at the age of 12 weeks in the GBH group compared to the control group (Fig. 5). Furthermore, increased GBH use has recently been reported to also increase resistance to glyphosate in some pathogenic *Salmonella* strains (Poppe et al., 2019). Thus, in the long run, both phenomena are likely to increase individuals' pathogen load, and GBH exposure may lead to increased susceptibility to diseases, which can in turn have severe ecological and economical costs in wild populations and poultry industry. All in all, given the essential role of the gut microbiome on multiple aspects of physiology and behavior (McFall-Ngai et al., 2013), it is very likely that GBH-induced changes in microbial communities will lead to detrimental effects on the host.

To understand the ecological relevance and potential for population-level consequences of agrochemicals on non-target taxa in wild populations, it is critical to study traits directly linked to reproduction in both males and females. We did not find evidence that GBHs influence reproductive maturation (cloacal gland development, start of egg-laying), reproductive organ gross morphology or reproduction itself (egg production), yet further studies should address the potential effects on e.g. testis and ovary histology in more detail. However, this is the first report to show that GBHs influence a reproductive hormone, testosterone, in an avian model. Our results corroborate recent findings in mammalian studies (Walsh et al., 2000; Defarge et al., 2016; Nardi et al., 2017; Clair et al., 2012; Romano et al., 2010; Abarikwu et al., 2015; Manservisi et al., 2019). The exact mechanisms underlying the decreased testosterone concentrations remain to be discovered, but alternatives include, for example, (i) altered hepatic metabolism for steroid hormone degradation and (ii) altered hormone synthesis and conversion due to altered regulatory proteins (such as StAr) and aromatase enzyme expression in response to GBH exposure. Given that testosterone has pleiotropic functions, e.g., on animal behavior and reproduction (activity, territoriality, sexual ornaments, sexual behavior, sperm quality), GBH-related reduction in testosterone, especially if cumulative, could have unforeseen consequences on both wild animal populations and in animal husbandry in the long term. For female reproduction, recent studies in mammals have indicated that subchronic exposure to GBHs impairs folliculogenesis and ovary and uterus development, decreases estrogen secretion, and induces changes in histomorphology (such as cell necrosis vacuolization of follicles, atretic follicles) (Ren et al., 2018; Hamdaoui et al., 2018; Alarcon et al., 2019), implying that GBHs function as an endocrine and reproductive disruptor for females. Thus, characterizing the potential effects of GBHs on female reproductive hormones also in avian models would be a fruitful avenue. We previously reported that glyphosate residues are found in eggs (0.7 mg/kg, Ruuskanen et al, 2020b), but there was only a tendency for negative effects of parental GBH exposure on embryo development, thus further studies are needed to characterize any potential effects on female reproduction and for example estradiol levels.

We hypothesize that the changes in microbiome may be associated with changes in oxidative stress, reproductive hormone

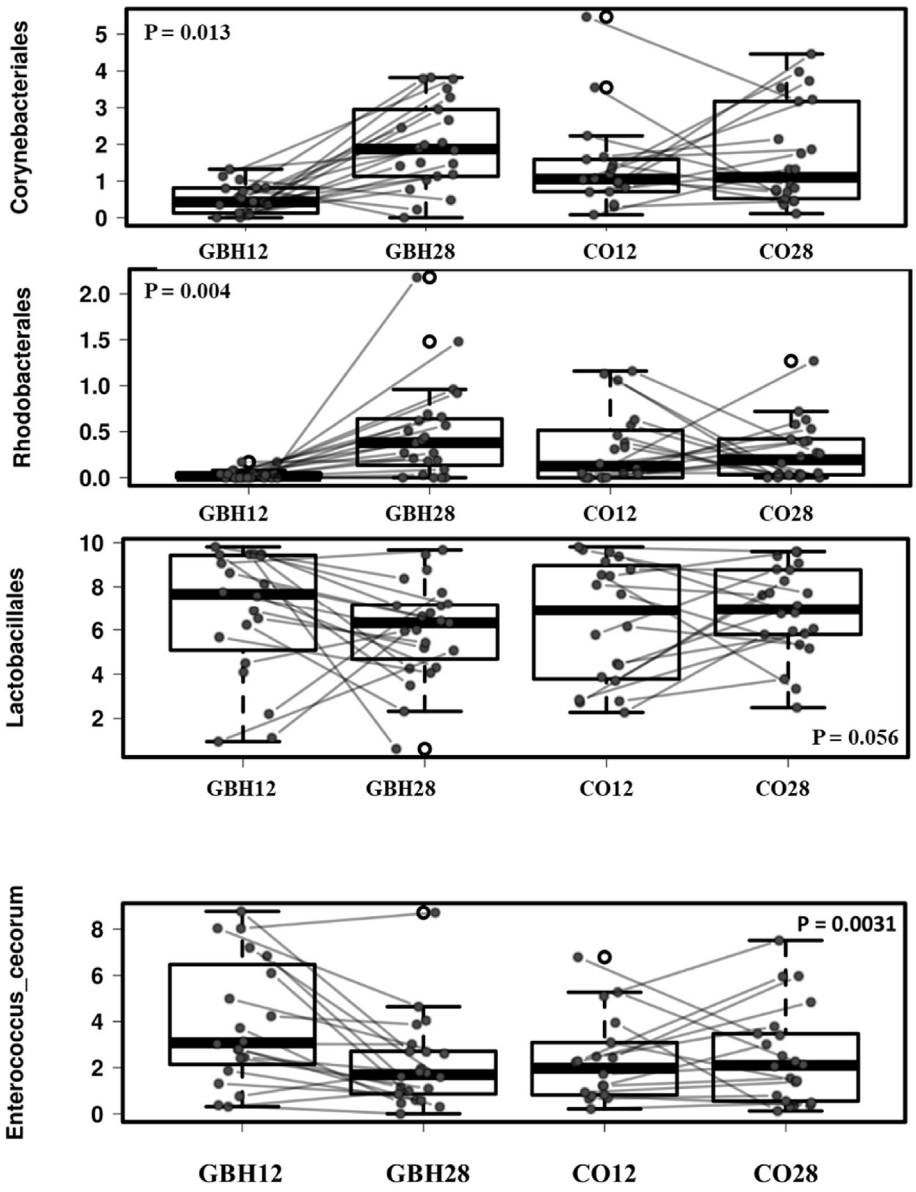

Fig. 5. Changes in abundance at order level with time under GBH exposure (GBH) and control treatment (CO) at the age of 12 weeks and 28 weeks. Significant differences between treatments are based on multilevel analysis (Mixed Effect Linear Regression).

levels and reproduction itself (yet the latter remained a tendencys in this study). For example, some beneficial microorganisms may produce antioxidant compounds and reduce oxidative stress (e.g. Wang et al., 2017). Concerning reproduction, work with invertebrate models has demonstrated that gut microbiome dysbiosis (especially the lack of symbionts) can negatively influence reproduction (Rosengaus et al., 2011; Sison-Magnus et al., 2013; Gould, 2018). Furthermore, germ-free mice showed altered testis development (AL-Asmakh et al., 2014), probiotic treatment increased testosterone in mice (Poutahidis et al., 2014) and in humans, testosterone and estradiol levels were recently found to be correlated with microbiome composition (Shin et al., 2019). This suggests that gut microbiome is likely linked to reproduction hormone levels, reproductive organ traits and even reproductive investment, yet the causal effect of gut microbiome composition to reproduction (nor the effect of glyphosate via this link) has not been studied in vertebrates up to date.

Importantly, the results can only be interpreted concerning the exposure and dosages used. The exposure level in this experiment was lower than European Food Safety Authority NOAEL for poultry for pure glyphosate (100 mg/kg body mass/day, Efsa, 2018), suggesting that commercial products may have effects below the recommended levels. The maximum allowed residue levels vary across countries: for example in US the allowed levels in animal feed can exceed the concentrations used here (in US up to 400 mg/kg, US Environmental Protection Agency, 2010), yet within EU the maximum levels for grains are lower than used here (20 mg/kg in EU, Efsa, 2018). A previous study has estimated that the exposure load of wild species foraging on fields recently treated with GBHs could reach ca 300 mg/kg (Eason and Scanlon, 2002), but we are currently completely lacking information on the realized exposure levels in wild birds – which is needed to assess the validity of the findings in wild populations.

Finally, when interpreting the results, one needs to take into

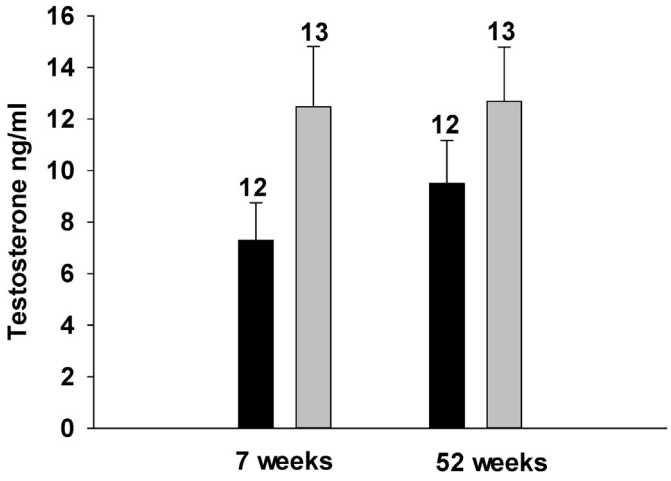

Fig. 6. Male plasma testosterone concentration (ng/ml, average ± SE) in GBH exposed (black) and control (grey) groups at the age of 7 and 52 weeks.

account that as a GBH product was used, our findings may be explained by effects of pure glyphosate, any of the adjuvants or a combination of both. A large portion of previous literature has reported that the effects of glyphosate commercial formulations may increase the toxicity of glyphosate and some of additives can be toxic by themselves (Bai and Ogbourne, 2016; Szekacs and Darvas, 2018; Gill et al., 2018; Van Bruggen et al., 2018; Mesnage et al., 2018).

In conclusion, we present the first long-term study on the potential effects of GBHs on physiology and reproduction in an avian model. We comprehensively investigated the impact of cumulative GBH exposure on multiple physiological pathways and reproduction in both females and males. Our results highlight that GBHs can modulate key physiological pathways, antioxidant status, testosterone, and the microbiome (even if body or organ mass exhibits no large effects), yet effects on reproduction were not detected. Therefore, the effects of glyphosate may not always be visible with traditional, especially short-term, toxicology testing, and such testing may not fully capture the risks related to GBHs on non-target taxa. In general, because of such subtle and potentially delayed effects, it may be difficult to link any recurrent changes in the health of wild populations or in production animals to glyphosate exposure. Our results largely support recent findings in rodent models and thus suggest the generality of such effects via multiple physiological pathways across taxa. Understanding the multitude of effects of GBHs is important for predicting their potential impacts on wild vertebrate populations, agriculture, and animal husbandry.

5. Data availability statement

The datasets generated and analyzed during this study are available at submission (supplementary data) and will be available in an open repository upon acceptance.

Author contributions

SR, MR, KS, MH, IS, and SS designed the study. SR, VK, OS, and MR conducted the data collection. SR, OS, MR, MCC, and CGG conducted the laboratory analyses. SR, MR and CGG conducted statistical analyses. SR drafted the ms and all authors contributed to manuscript preparation.

CRediT authorship contribution statement

Suvi Ruuskanen: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Miia J. Rainio:** Investigation, Methodology, Supervision, Validation, Formal analysis, Writing - review & editing. **Carlos Gómez-Gallego:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Software, Visualization, Writing - review & editing. **Otto Selenius:** Investigation, Methodology, Writing - review & editing. **Seppo Salminen:** Conceptualization, Funding acquisition, Methodology, Writing - review & editing. **Maria Carmen Collado:** Data curation, Formal analysis, Investigation, Methodology. **Kari Saikkonen:** Conceptualization, Funding acquisition, Writing - review & editing. **Irma Saloniemi:** Conceptualization, Funding acquisition, Writing - review & editing. **Marjo Helander:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115108.

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