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The impact of glyphosate at regulatory "safe" levels on reproductive health: cellular and molecular disruptions on male germ line

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ABSTRACT

Glyphosate, a widely used herbicide in both agricultural and non-agricultural practices, has become pervasive in the environment, leading to significant human and animal exposure. Despite growing evidence of its potential endocrine-disrupting and reproductive toxicity, European regulatory agencies continue to affirm its safety. This study examines the effects of glyphosate on male reproductive health by exposing adult zebrafish to dietary glyphosate at doses within European safety thresholds. After 21 days of exposure, testicular samples were analysed using a combined approach involving transcript analysis, targeted metabolomics and proteomics, epigenetics, as well as immunohistochemistry. At 0.5 mg/kg body weight(bw)/day (the acceptable daily intake, ADI), glyphosate impaired germ cell differentiation and triggered cell-specific changes in histone acetylation within the male germline. Higher exposure levels of 50 mg/kg bw/day (the no observed adverse effect level, NOAEL) induced metabolomic and proteomic disruptions linked to impaired steroidogenesis, DNA damage in germ cells, and alterations in testicular architecture, culminating in reduced reproductive capacity. Interestingly, minimal testicular effects observed at 5 mg/kg bw/day highlighted the non-monotonic dose–response relationship to glyphosate. These findings unveil critical molecular and cellular disruptions caused by glyphosate and emphasize its potential reproductive risks, even at doses considered "safe" by regulatory standards. This research contributes to ongoing discussions surrounding sustainable agricultural practices and public health policies, calling for a re-evaluation of glyphosate safety thresholds.

1. Introduction

Glyphosate (GLY), or N-(phosphonomethyl)glycine, is the primary active component of glyphosate-based herbicides (GBHs), such as Roundup®. GLY acts on the shikimate pathway by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) required for the synthesis of aromatic amino acids in plants, fungi, and microorganisms, thus causing their physiological detriment when applied (Leino et al., 2021). This mechanism has supported the development of an agro-industrial model centered on the use of GLY-resistant transgenic crops, particularly in South American countries such as Brazil, where surface water concentrations of glyphosate have been reported to reach up to 110 μg/L (Brovini et al., 2021). In Europe, GLY is also widely used in both cropping systems and non-agricultural areas, accounting for approximately one third of all herbicide applications (Antier et al., 2020; Fogliatto et al., 2020). As a result, both GLY and its primary metabolite, the aminomethylphosphonic acid (AMPA), have been detected in European surface waters in the range of <5 ng/L to 10 μg/L for GLY and <5 ng/L to 134 μg/L for AMPA (Grandcoin et al., 2017; Poiger et al., 2017; Richmond, 2018). GLY residues (ranging from 0.0012 to 0.139 mg/Kg) have been also measured in cereals, beverages (drinking water, wine…), honey as well as in fruits and vegetables (Zoller et al., 2018),

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resulting in human exposure either by food and/or water intake (Grau et al., 2022). Besides, occupational and indirect exposure can also occur via inhalation and dermal contact when spraying, handling, and manufacturing (Gandhi et al., 2021).

Owing to its widespread use and its presence in edible products, exposure to GLY has become a public health concern. Understanding the specific effects of GLY on non-target species is essential for informed risk assessment and regulatory decisions to minimize unintended environmental consequences (Tarazona et al., 2017). In that regard, there is a growing number of scientific studies demonstrating the toxicity of this compound in non-target species including insects, aquatic organisms, and mammals (Stanley and Preetha, 2016). Since GLY has been proven to interfere with the hormone synthesis, transport, expression, and activation of hormone receptors as well as to induce epigenetic modifications in the hormone-responsive cells, it has been considered an endocrine-disrupting chemical (EDC), as recently reviewed (Muñoz et al., 2021). The disruption of steroidogenesis and/or hormone signaling pathways has been associated with the reproductive toxicity of GLY in mammalian and non-mammalian species (Serra et al., 2021). Moreover, some studies revealed that exposure of gestating females can lead to the transgenerational inheritance of offspring pathology and sperm epimutations (Ben Maamar et al., 2021; Kubsad et al., 2019). As a result of the species-specific effects, the window of exposure, the concentrations, and the type of substances (pure GLY or GLY-based herbicides, GBHs) to which animals are exposed, many controversies persist in the scientific literature regarding the potential impact of these compounds on steroidogenesis, spermatogenesis, sperm quality, and offspring development (de Araújo-Ramos et al., 2021; Serra et al., 2021).

The safety debate surrounding GLY intensified when, in 2015, the International Agency for Research on Cancer (IARC) classified it as 'probably carcinogenic to humans' (Kudsk and Mathiassen, 2020). In contrast, the European Chemicals Agency (ECHA) reported in 2022 that GLY 'does not meet the scientific criteria to be classified as a carcinogenic, mutagenic or reprotoxic substance' (Agency, 2022). Moreover, in the last report of July 2023, the European Food Safety Authority (EFSA) concluded that 'GLY does not meet the criteria for endocrine disruption' (Álvarez et al., 2023). Based on this last assessment, the European Commission decided in November 2023, after months of wrangling, to renew the approval of GLY for 10 years (Implementing Regulation, 2023). It is noteworthy that agencies could have reached different conclusions due to the consideration of different bodies of scientific evidence and methodologies (Finger et al., 2023). Yet, the economic concerns in such decisions cannot be neglected, considering that GLY use has relevant implications for the agricultural production system (Finger et al., 2023). Therefore, the identification of data gaps underscores the importance of ongoing scientific investigations to comprehensively assess the potential risks posed by GLY to non-target species and ecosystems.

In this scenario, we hypothesize that adult male exposure to dietary GLY at doses within the safety limits established by the EFSA [acute reference dose (ARfD)] and acceptable daily intake (ADI) of 0.5 mg/kg of body weight per day (mg/kgbw/d) and no observed adverse effect level (NOAEL) of 50 mg/kgbw/d) (Álvarez et al., 2023) could impact cellular and molecular processes involved in spermatogenesis and male breeding capacity. To test this, we specifically assessed the effects of glyphosate alone, aiming to isolate its biological mechanisms, minimize potential interference from additives in herbicide formulations, and establish a baseline for regulatory and safety assessments. Zebrafish was chosen as model species due to its remarkable reproductive capacity and absence of reproductive seasonality, the extensive identification and study of genes related to gonadal development and gametogenesis (Zha et al., 2024), and its high genetic homology to humans (Howe et al., 2013). These characteristics make zebrafish particularly suitable for assessing reproductive toxicity and predicting human toxicological risks (Zhao et al., 2024). While numerous studies have investigated the effects of waterborne exposure to glyphosate alone on zebrafish development (reviewed by (Ames et al., 2022; Lanzarin et al., 2023), to the best of our knowledge, only two studies have explored its toxicity in zebrafish male reproduction (Lopes et al., 2014; Uren Webster et al., 2014). The effects of exposure via diet, however, remain largely unexplored. This is particularly important as persistent organic pollutants, including herbicides, insecticides, and industrial chemicals, can bioaccumulate in the food chain of aquatic ecosystems (Tye and Masino, 2019) and they are also present in diverse dietary products (reviewed by (Muñoz et al., 2023)). Therefore, using a multidisciplinary approach, we investigated the reprotoxic effects of dietary exposure to "safe" glyphosate doses in male zebrafish.

2. Methods

2.1. Animal maintenance and glyphosate exposure

Adult zebrafish (*Danio rerio*, AB wild-type strain) were randomly divided into four experimental groups: one control group and three glyphosate-treated groups. The control group (CTRL) was fed commercial dry food (TetraMin Granules; Tetra, Melle, Germany), while the treated groups were fed the same dry food enriched but with glyphosate. The daily glyphosate doses for the treated groups were 0.5 mg/kgbw (GLY0.5, EFSA ADI), 5 mg/kg bw (GLY5), and 50 mg/kgbw (GLY50, EFSA NOAEL) (Álvarez et al., 2023). The trial was conducted under semi-static conditions at 27 °C, with a 10/14 light/dark cycle, in 30L glass tanks, each housing 30 males. The entire exposure experiment was independently repeated three times. Following 21 days of exposure, all fish, except for breeding males, were euthanized using 300 mg/L tricaine. Whole-body weight was recorded before organ dissection. Gonads were excised, weighed, and either stored at −80 °C for proteomic, metabolomic, and PCR analyses or fixed in Bouin's solution (Sigma-Aldrich, Milan, Italy) for histological, immunohistochemical, and TUNEL assay analyses. The gonadosomatic index (GSI) was calculated as follows: GSI = (gonad weight (mg)/total body weight (mg)) × 100. All procedures involving animal exposure and manipulation were conducted according to the University of Calgary Animal Care Protocol (AC15-0183) for the care and use of experimental animals, and every effort was made to minimize suffering. All experimental samples and individuals used for the different analysis performed were randomly selected.

2.2. Feed intake

Prior to feeding, each tank housing 30 males, was siphoned to remove any eventual residual feed from previous administrations as well as fecal matter. The feed was then administered, and after 30 min, any uneaten feed was collected, dried at room temperature, and weighed. The food intake was calculated as the percentage ratio between the ingested food (determined by subtracting the residual food from the administered food) and the administered feed, using the following formula: Food intake (%) = [(Administered food − Residual food)/Administered food] × 100. This trial was repeated in triplicate on different groups of fish within each experimental group.

2.3. Testicular histological and immunohistochemical evaluation

Routine histology analysis was carried out in 4 μm-section of testicular tissue, with 6 samples from each experimental group. The sections were stained with hematoxylin and eosin. For each testis, 3 sections were analyzed, with 2 images per section captured using a Zeiss Axio Imager optical microscope (Zeiss, Germany) at 400x magnification. The proportion (%) of different testicular cell types was quantified using Fiji ImageJ software.

For immunohistochemistry, 5 deparaffinized testicular sections from each experimental group were subjected to antigen retrieval by boiling in 10 mM sodium citrate with 0.05 % Tween-20 (pH 6) in a water bath

for 20 min. The sections were air-dried and permeabilized with 1 % triton X-100 in PBS for 20 min at RT, followed by 3 washes with PBS for 3 min each. Blocking was performed using 3 % (w/v) bovine serum albumin (BSA) in 0.05 % (v/v) Tween-20 in PBS for 90 min. After blocking, the sections were incubated overnight at 4 °C with primary antibodies: anti-Pcna (1:100, sc-56, Santa Cruz), anti-Ddx4 (1:200; ab209710, Abcam), anti-H3K9ac (1:200, ab10812, Abcam), and anti-H4K12ac (1:200, ab46983, Abcam). This was followed by three PBS washes (3 min each). Sections were then incubated for 1 h at 37 °C with secondary antibodies: Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488; ab150077, Abcam, Cambridge, UK) and Goat Anti-Mouse IgG H&L (Alexa Fluor® 568; ab175473, Abcam, Cambridge, UK), both diluted 1:500. The slides were washed three times with PBS, and mounted with DAPI-Aqueous Fluoroshield (ab104139, Abcam, Cambridge, UK) for nuclear staining. Images were captured using a Nikon A1R confocal microscope, and the levels of Ddx4, H3K9ac, and H4K12ac in testicular sections were analyzed using Fiji ImageJ Software.

2.4. Gene expression analysis

Testicular RNA was extracted from 5 individuals per experimental group using RNAzol® RT (Sigma-Aldrich, Milan, Italy), following the manufacturer's instructions. RNA concentrations were measured using a nanophotometer (Implen GmbH, Munich, Germany), and RNA quality was verified by running all samples in 1 % agarose gel stained with Xpert Green DNA Staining dye (Grisp, Porto, Portugal). All samples underwent DNase treatment (MBI Fermentas, Milan, Italy) according to the manufacturer's instructions, and RNA concentrations were reassessed with the nanophotometer.

For cDNA synthesis, 1 μg of total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Milan, Italy), following the manufacturer's guidelines. The resulting cDNA was stored at −20 °C until further analysis. cDNA samples were diluted 1:10, and quantitative real-time PCR (qRT-PCR) was performed with 100 ng of template per well, in duplicate, using SYBR Green (Bio-Rad, Milan, Italy) on a CFX thermal cycler (Bio-Rad, Milan, Italy). The thermal cycling protocol was as follows: an initial denaturation at 95 °C for 3 min, followed by 45 cycles of 95 °C for 20 s, annealing at the primer-specific melting temperature (Tm) for 20 s, and extension at 72 °C for 20 s. All the primers were used at a final concentration of 10 pmol/mL. Primer sequences and accession numbers are listed in Supplementary (Table S1). Ribosomal protein l13a (*rpl13a*) and ribosomal protein, large, p0 (*rplp0*) were used as internal reference genes, and relative expression levels were calculated using the formula: $2^{-\Delta Ct}$= Ct target gene-Ct reference gene.

2.5. Metabolomics analysis by targeted approach

For cell lysis, 5 tissue samples from each experimental group with a final wet weight of approximately 3–8 mg were homogenized using an Ultra-Turrax IKA T (IKA, Staufen, Germany). Homogenization was carried out in 300 μL of lysis buffer (1 % SDS, 6 M UREA, 2 mM EDTA, 100 mM AMBIC), supplemented with 5 μL of protease inhibitor (Merck, Darmstadt, Germany) and 200 μL of Milli-Q water. The homogenates were subjected to 15 min of sonication to enhance cell disruption. Subsequently, the samples were centrifuged at 11,200*xg* for 10 min to separate corpuscular tissue debris. The resulting supernatants were collected and used for both proteomics and metabolomics analyses.

To extract metabolites, four volumes of cold acetonitrile (ACN)/methanol (MeOH) (1:1, v/v; Merck, Darmstadt, Germany) were added to 150 μL of testes lysates. The mixture was centrifuged at 16,128*xg* for 20 min to recover the supernatant. The supernatant was then dried using a SpeedVac concentrator (Thermo Fisher, Waltham, United States) and resuspended in 150 μL of methanol containing 0.1 % formic acid (HCOOH) for subsequent liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis in multiple reaction monitoring (MRM) ion mode. For metabolomics analysis, a targeted approach was employed to investigate three distinct classes of molecules: steroid hormones, amino acids, and metabolites associated with purine metabolism.

Chromatographic separation was performed at 40 °C using a Kinetex 5 μm C18 column (100 × 2.1 mm; Phenomenex, Torrance, United States) with a flow rate of 0.2 mL/min and a gradient lasting 10.5 min. The coupled mass spectrometer was a 5500 QTRAP (AB Sciex, Toronto, Canada), equipped with an electrospray ionization (ESI) source and a triple quadrupole tandem mass analyzer (QqQ). The source parameters were set as follows: voltage, −4500 V; temperature, 500 °C; curtain gas, 20 psi; ion source gas 1, 60 psi; and ion source gas 2, 60 psi. Analyses were conducted in multiple MRM mode. Positive polarity was used for amino acid and steroid hormone analyses, while negative polarity was applied for purine intermediates. For steroid hormone analysis, solvent A consisted of 5 mM ammonium formate with 0.1 % formic acid (HCOOH) (Merck, Darmstadt, Germany), and solvent B was ACN/isopropanol (90:10, v/v) with 0.1 % HCOOH. The gradient elution profile was as follows: 0–1 min, 5 % B; 1–3 min, 15 % B; 3–9 min, 85 % B; 9–10 min, 95 % B; and 10–10.5 min, 95 % B. The MRM method for steroid hormone quantification was previously published by Luti et al. (Luti et al., 2021). For amino acid analysis, solvent A was 5 mM ammonium formate with 0.1 % HCOOH, and solvent B was ACN with 0.1 % HCOOH. The gradient elution was programmed as follows: 2–5 min, 2–20 % B; 5–8 min, 20–80 % B; 9–10 min, 95 % B. MRM-MS analysis was performed in positive ion mode, and the monitored precursor-product ion (*m/z*) transitions, along with collision energy and other parameters, were as previously described (Lane, 2019). For purine metabolism intermediates, solvent A consisted of 0.1 % HCOOH, and solvent B was MeOH with 0.1 % HCOOH. The gradient elution program and MRM transitions were based on protocols reported by McCloskey and Ubhi (McCloskey, 2015).

Skyline software (3.7. 64-bit version; MacCoss Lab Software. University of Washington, version 23.1.0.455) was used for data interpretation and a report containing peak areas of MRM transitions was obtained as output (MacLean et al., 2010). The MetaboAnalyst 5.0 online platform (University of Alberta, Alberta, Canada) was used to perform Principal Component Analysis (PCA) and Partial Least Square Discriminant Analysis (PLS-DA) models for metabolomics and proteomics data. Model quality was evaluated using R2 and Q2 values, with thresholds of >0.5 indicating acceptable model performance.

2.6. Proteomics analysis by targeted approach

For proteomics analysis, an aliquot of the recovered supernatant, as described above, was used. Protein concentration was determined using the Bradford assay with BSA (Merck, Darmstadt, Germany) as a standard. Absorbance measurements were recorded at λ = 595 nm. For each sample, 100 μg of proteins were precipitated by adding four volumes of cold acetone (Merck, Darmstadt, Germany). The samples were centrifuged at 11,200*xg* for 10 min, and the resulting pellets were recovered, dried, and stored at −20 °C for subsequent analysis.

The protein pellets were dried under vacuum and resuspended in 50 μL of denaturing buffer (6 M Urea, 25 mM ammonium bicarbonate [AMBIC]). To reduce cysteines, 12.5 μL of 100 mM dithiothreitol (DTT) (Merck, Darmstadt, Germany), prepared in denaturing buffer, was added to each sample and incubated at 60 °C for 1 h. After cooling, the reduced cysteines were alkylated by adding 30 μL of 120 mM iodoacetamide (IAM) (Merck, Darmstadt, Germany) and incubating in the dark at room temperature for 1 h. To complete the alkylation process, 12.5 μL of 100 mM DTT was added and incubated at room temperature for 1 h. Enzymatic digestion was performed by adding 2 μL of trypsin (1 μg/μL; Merck, Darmstadt, Germany) to each sample at an enzyme-to-substrate ratio of 1:50 (w:w). Hydrolysis was carried out at 37 °C for 16 h in a thermostatic bath. Following digestion, a standardized desalting procedure was performed for all samples using stage tips containing three layers of 3 M Empore C18 membrane (Merck, Darmstadt, Germany). The

stage tips were pre-conditioned with 100 μL of 0.1 % formic acid (HCOOH), and peptides were eluted sequentially with 50 μL of 50 % acetonitrile (ACN) and 50 μL of 80 % ACN, both acidified with 0.2 % HCOOH. The peptide mixtures were subsequently dried under vacuum and resuspended in 30 μL of 2 % ACN with 0.2 % HCOOH. Samples were stored at −20 °C until analysis by LC-MS/MS.

Peptide mixtures were analyzed using a Xevo TQ-S mass spectrometer (Waters, Milford, United States) equipped with an ionKey interface coupled to an Acquity UPLC system (Waters, Milford, United States). For each run, 2 μL of the peptide sample was injected and separated on a BEH C18 peptide separation device (130Å, 1.7 μm, 150 μm × 50 mm) (Waters, Milford, United States) at a column temperature of 45 °C and a flow rate of 3 μL/min. The mobile phases consisted of buffer A (2 % ACN with 0.2 % HCOOH) and buffer B (98 % ACN with 0.2 % HCOOH). The gradient for the MRM method started with 7 % buffer B for 5 min, from 5 to 40 min reached 50 % buffer B to 95 % buffer B during the next 2 min. The column was finally re-equilibrated to initial conditions for 4 min. The parameters of the MS source were as follows: 3900 V as ion spray voltage, 150 °C interface heater temperature, 150 L/h gas flow with 7 bar nebulizer pressure. MRM mass spectrometric analyses were performed in positive ion mode for the run time with 5 points per peak and dwell times of 3 ms. The cone voltage was set to 35 V. A range of 300–1000 *m/z* was preferentially selected to choose the precursor or product ions for each target peptide.

All instrumental parameters, including the selected peptides for each protein target, precursor and product ions (*m/z*), collision energy (eV), and cone voltage (V), are detailed in Table S2. The LC-MS/MS method was developed using the Skyline software, which facilitated the selection of optimal transitions based on the amino acid sequences of the target proteins retrieved from their UniProt IDs. Skyline was also used for extracting MS parameters and visualizing the data.

2.7. TUNEL assay

DNA damage of germ cells was assessed in 5 testes per experimental group using the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics), according to the manufacturer's instruction with slight modifications, as previously described by González-Rojo and colleagues for zebrafish testes (González-Rojo et al., 2019). Both negative and positive controls were included; the positive control involved incubation of sections with 100 U/mL DNase I for 10 min at 28 °C. Testicular nuclei were stained with DAPI-Aqueous Fluoroshield (ab104139, Abcam, Cambridge, UK). The assay was performed in duplicate. Images were captured using a confocal microscope (A1R Nikon) and analysed with Fiji ImageJ software. The number of TUNEL-positive cells was quantified in each image field.

2.8. Assessment of fertility rate and embryo development

From 14 to 21 day of GLY treatment, 10 males from each experimental group were mated with untreated females. Each male was sequentially mated with two different females, maintaining a 1:1 ratio during each mating trial. Fertilized eggs were collected, counted, and cleaned before being transferred to egg water containing 0.038 mM $CaCO_3$, 0.446 mM $NaHCO_3$, 1.025 mM sea salt, and 0.005 % (vol/vol) methylene blue. The embryos were maintained at 28 °C. Embryo mortality was assessed at 3.3 h post-fertilization (hpf), and the hatching rate was evaluated at 72 hpf.

2.9. Statistical analysis

Univariate analysis of the metabolomics and proteomics data was conducted using volcano plots for metabolites and proteins with Variable Importance in Projection (VIP) scores >1, as determined by the PLS-DA models built in MetaboAnalyst 5.0. A significance threshold of $P < 0.05$ and a fold change >2 was applied to identify statistically significant differences between experimental groups. For metabolomics, metabolites with a VIP score >1 in each PLS-DA model were further analyzed through Pathway Analysis (MetPA) using the MetaboAnalyst 5.0 platform (Giommi et al., 2024).

Statistical analysis of the different analysis performed was performed using Graph Pad Prism V8.0.1. (GraphPad Software, Inc., San Diego, CA, USA). Normality of the data was tested by the Shapiro-Wilk test. For non-parametric data, a Kruskal-Wallis test was performed using Dunn's post hoc test ($P < 0.05$). For parametric data, an analysis of variance (ANOVA) with a Tukey's post hoc test was run ($P < 0.05$). Results are shown as mean ± SD.

3. Results

3.1. Feed intake

The analysis of feed intake confirmed that the fish consumed nearly all the provided food, with no statistically significant differences observed among the groups: CTRL (99.92 % ± 0.05), GLY0.5 (99.83 ± 0.16), GLY5 (99.86 ± 0.1), and GLY50 (99.86 ± 0.2).

3.2. Impact of GLY on spermatogenesis and testicular architecture

Exposure of adult males to GLY0.5 significantly increased the GSI compared to the control group (Fig. 1a). Regarding the testicular germ cell composition, an uncontrolled proliferation of both spermatogonia A and B (Sg A and B) was observed in the fish exposed to GLY0.5 (Fig. 1b, c). In contrast, the percentage of spermatozoa (Spz) was significantly lower in this experimental group compared to the control (Fig. 1f). No alterations of testicular structure were observed in the males exposed to GLY 5. In males treated with GLY 50 the percentage of Sg B was also significantly higher than in the control group (Fig. 1c).

These histological data are consistent with the results from the immunohistochemistry analysis: the percentage of DEAD-box helicase 4 (Ddx4)-positive germ cells (mainly SgA and B) (Ye et al., 2023) per testicular area was significantly higher in males exposed to both GLY0.5 and 50 compared to the control testes (Fig. 1h,j). Given the essential role of Ddx4 in germline development (Xu et al., 2022), its levels in proliferating cell nuclear antigen (Pcna)-positive cells were analyzed, as these cells are also Ddx4-positive cells (Ye et al., 2023). The protein levels of Ddx in SgA and SgB were slightly and significantly increased in males treated with the lowest and the highest dose of GLY, respectively, when compared to the control group (Fig. 1i,j).

3.3. Impact of GLY on zebrafish testicular gene expression and steroidogenesis

The assessment of gene expression in the testes showed few changes between CTRL and GLY-treated males (Fig. 2a). Exposure to the highest dose of GLY significantly upregulated the expression of the gene encoding anti-Mullerian hormone (*amh*) (Fig. 2b). Among the genes involved in steroid production, the expression of both steroidogenic acute regulatory protein (*star*) and cytochrome P450 family 11 subfamily A member 1 (*cyp11a1*) was significantly increased in the testes of males exposed to GLY 50 (Fig. 2b).

By performing mass spectrometry analysis (LC-MS/MS in MRM ion mode), six steroid hormones were measured in zebrafish testes. Hormone levels were expressed as pg/mg of wet tissue. The obtained data revealed that, consistent with the gene expression data, the highest dose of GLY enhanced steroidogenesis. Regarding sex steroids, pregnenolone and progesterone concentrations were higher in the testes of males exposed to both GLY5 and GLY50, while 17β-estradiol (E2) concentrations were only increased by the highest GLY dose (Fig. 2c,e,f). In contrast, exposure to GLY0.5 decreased the levels of 17α-OH-pregnenolone (Fig. 2d). No effects were observed in testosterone (T) testicular concentration or E2/T ratio after GLY exposure when compared to the

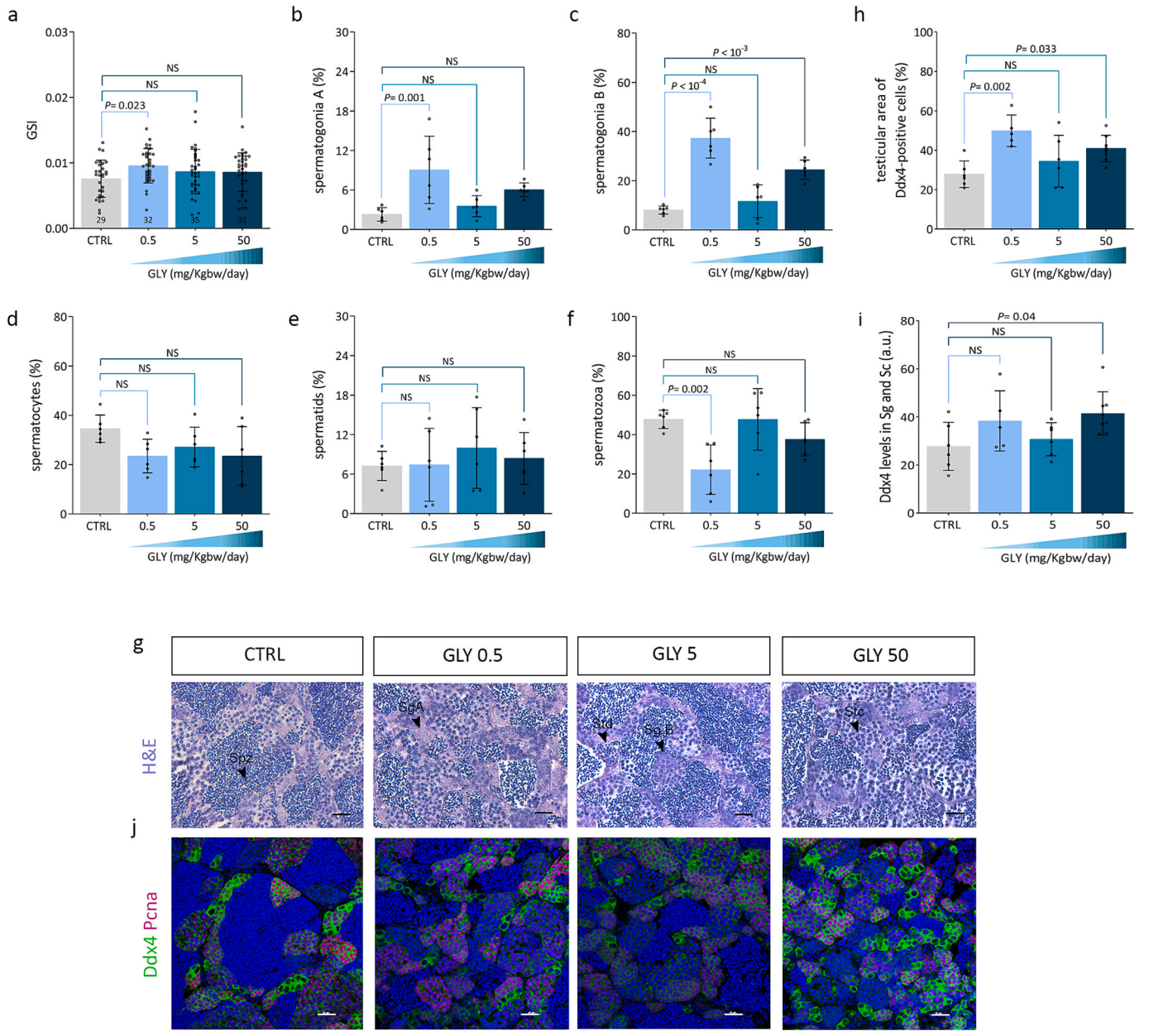

Fig. 1. Study of testicular architecture. **a** Gonadosomatic index (GSI) of CTRL and GLY-treated males, being the *N* indicated in the lower part of the corresponding bar. Proportion of each type of testicular cells: **b** spermatogonia A (Sg A), **c** spermatogonia B (SgB), **d** spermatocytes (Sct), **e** spermatids (Std) and **f** spermatozoa (Spz), $N = 6$. **g** Representative H&E-stained testicular sections from CTRL and males exposed to different doses of GLY (0.5, 5, 50), scale bar = 20 μm. **h** percentage of Ddx4-positive testicular cells (Sg and Sct), $N = 5$. **i** Ddx4 levels in Sg and Sct, $N = 5$. **j** Representative confocal images of Ddx4 immunohistochemical detection in Pcna-positive germ cells from CTRL and males exposed to different doses of GLY (0.5, 5, 50), scale bar = 20 μm.

control group (Fig. 2g,h). Concerning glucocorticoids, the concentration of cortisol in the testes of males exposed to both GLY5 and GLY50 was significantly higher when compared to the control group (Fig. 2i).

3.4. *Proteomic and metabolic changes triggered by GLY in zebrafish testes*

Targeted proteomics analysis was conducted using LC-MRM/MS, enabling the monitoring of 11 zebrafish proteins playing a pivotal role in zebrafish spermatogenesis and testicular physiology that were annotated in the UniProt database. This method tracked the total ion current of 804 transitions, representing precursor (*m/z*)–product ion (*m/z*) pairs associated with 136 peptides. The high specificity of the MRM approach was achieved by monitoring 3 to 6 transitions per target peptide, ensuring unambiguous assignment of peptide sequences (Illiano et al., 2018). Analyses for each biological replicate were performed in duplicate technical replicates. The peak areas recorded for each biological replicate were averaged to assess the differential abundance of each target protein across the four experimental groups. The results showed that neither PCA nor PLS-DA analyses resulted in a clear separation of the different experimental groups, as they were not distinctly resolved from one another (Supplementary Fig. S1 and Fig. 3a). Additionally, 2D PCA and PLS-DA models comparing individually GLY-exposed groups with the control (CTRL) revealed only partial discrimination between these groups (Supplementary Fig. S1a and Fig. 3a). However, the 3D representation of the PLS-DA model, which included all groups, demonstrated a clearer separation between the GLY-exposed groups and CTRL (Supplementary Fig. S2a). This observation was further supported by the 3D PLS-DA models comparing each GLY-

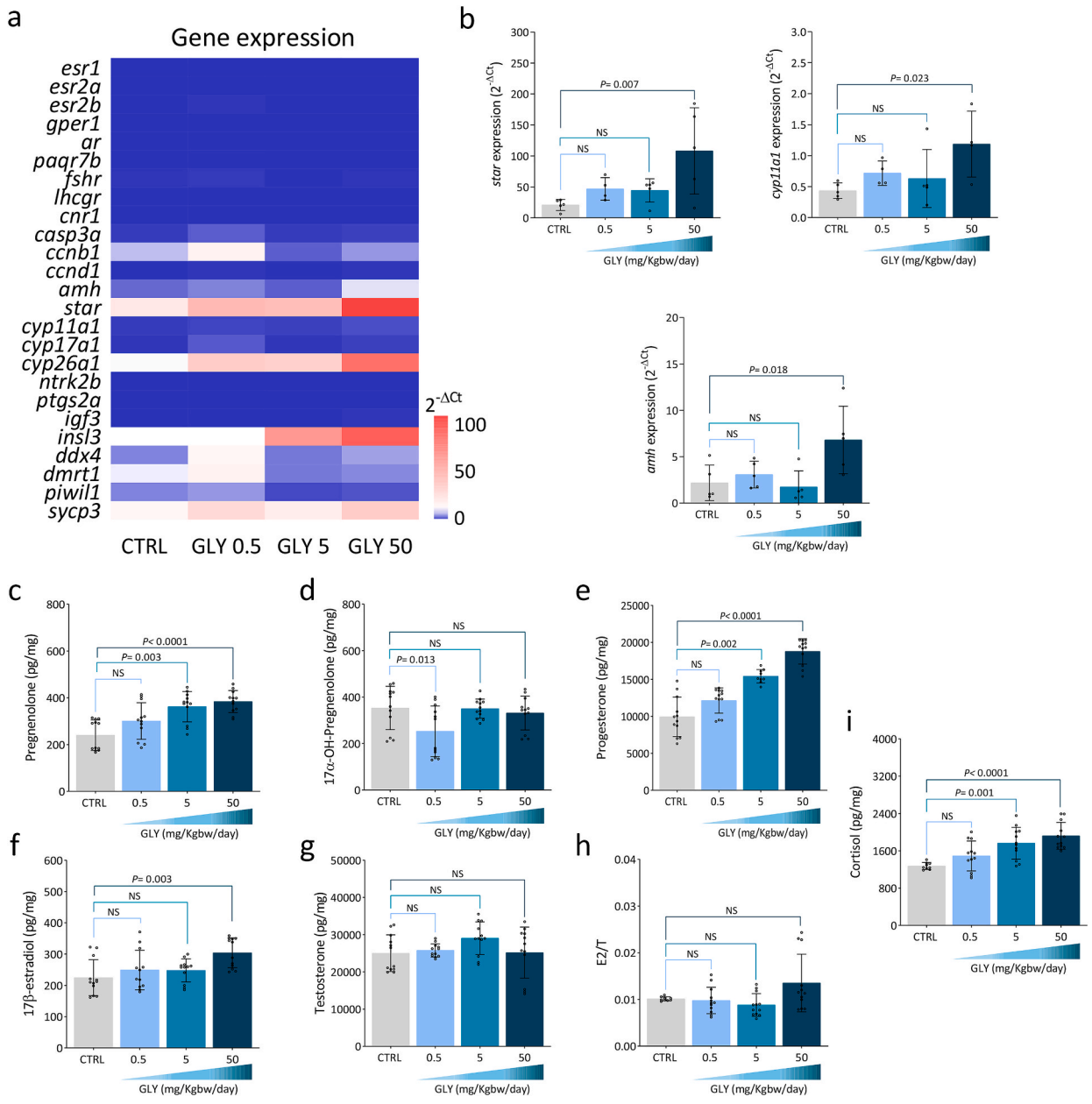

Fig. 2. Analysis of testicular gene expression and steroidogenesis. a Heatmap showing the expression ($2^{-\Delta Ct}$) of genes related to steroidogenesis and spermatogenesis in the testes of CTRL and GLY-exposed males, $N = 5$. **b** Histograms of *amh*, *star*, and *cyp11a1* differential expression in the testes of CTRL and GLY-exposed males, $N = 5$. **c** Histograms of pregnenolone, 17α-OH-pregnenolone, progesterone, 17β-estradiol (E2), testosterone (T) concentrations, and cortisol (pg/mg), as well as E2/T ratio, in the testes of CTRL and GLY-exposed males, $N = 12$.

exposed group individually with CTRL, which showed distinct separation for GLY0.5 (Supplementary Fig. S2b), and even more pronounced separation for GLY5 (Supplementary Fig. S2c) and GLY50 (Supplementary Fig. S2d). These results indicate that, while two principal components were insufficient for strong group discrimination, the inclusion of a third principal component provided clear differentiation among groups. From the PLS-DA models, proteins with VIP values >1 were identified and annotated, and subsequent univariate analyses were conducted based on these proteins. The univariate analysis revealed significant changes in only two proteins following GLY exposure compared to the control group (Fig. 3b): Gper1 levels were slightly reduced in the GLY0.5 group, while Sumo3 levels showed a significant decrease in the testes of males exposed to GLY50 (Fig. 3c).

A targeted metabolomic approach was employed to analyse specific testicular metabolites previously reported to be susceptible to GBHs (Qi et al., 2023). The results obtained were similar to the proteomic data. The 2D PCA and PLS-DA models comparing CTRL to each of the GLY-exposed groups did not indicate clear separation between CTRL and the experimental groups (Supplementary Fig. S1b and Fig. 3d). However, when a third component was incorporated into the analysis, the 3D PLS-DA representation revealed distinct separation between CTRL and the GLY-exposed groups (Supplementary Fig. S3a). This separation was most pronounced when comparing CTRL to GLY0.5 (Supplementary Fig. S3b), with less clear differentiation for CTRL versus GLY5 (Supplementary Fig. S3c) and CTRL versus GLY50 (Supplementary Fig. S3d). Metabolites with VIP values >1 from the PLS-DA models were annotated, and subsequent univariate analysis was performed using these metabolites. The univariate analysis highlighted significant changes only in purine metabolism (Fig. 3e), where increased levels of cAMP, AMP, and GMP were observed in the testes of males exposed to

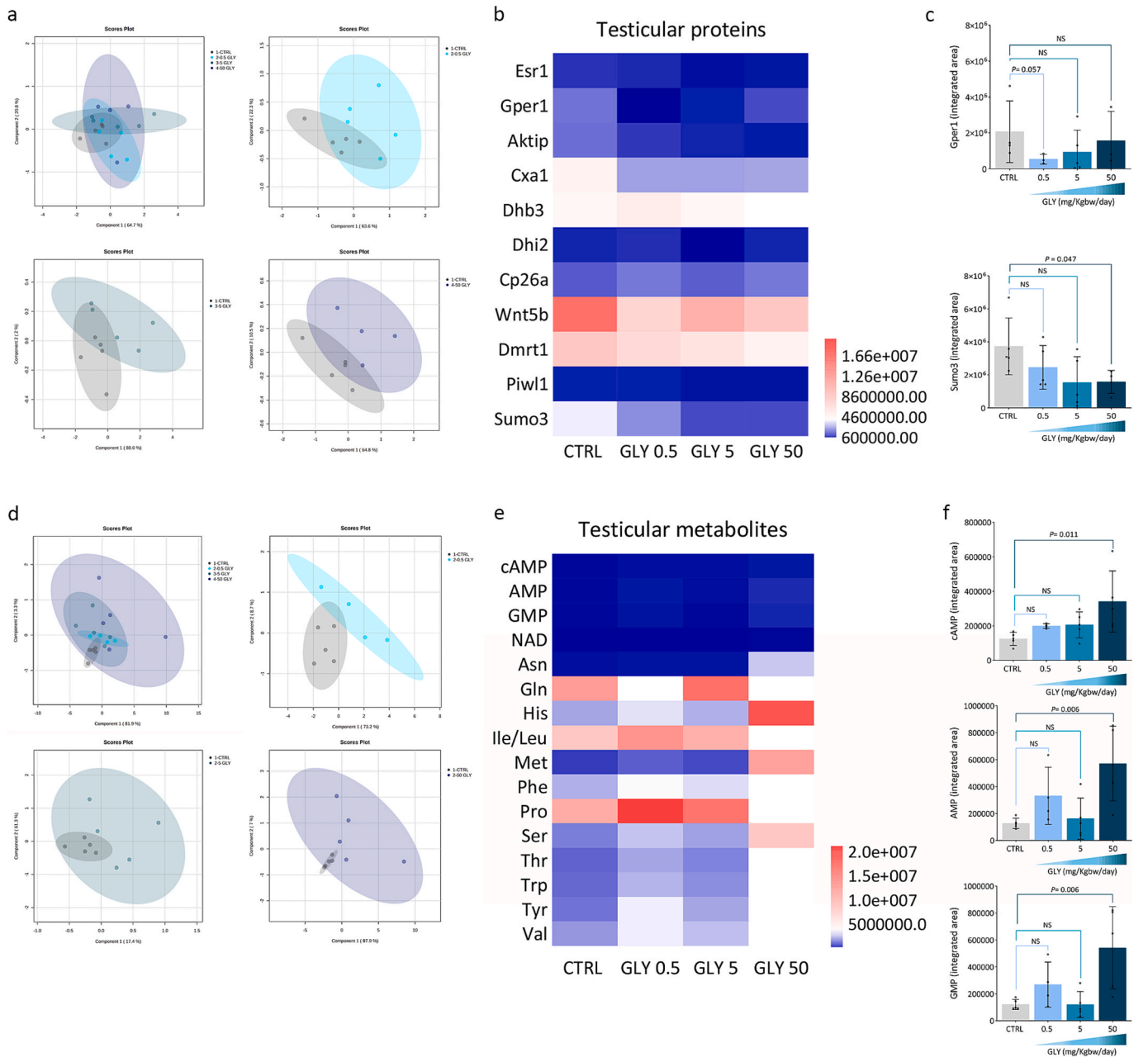

Fig. 3. Testicular targeted proteomics and metabolomics. a PLS-DA score plot of the proteomic analysis of all groups, GLY0.5 vs. CTRL, GLY5 vs. CTRL, and GLY50 vs. CTRL, $N = 5$ for all groups except for GLY50 in which $N = 4$. **b** Heatmap showing the differences in protein levels in the testes of CTRL and GLY-exposed males used in the following univariate analyses, $N = 5$for all groups except for GLY50 in which $N = 4$. **c** Histograms of Gper1 and Sumo3 differential levels in the testes of CTRL and GLY50-exposed males, $N = 5$ for all groups except for GLY50 in which $N = 4$ **d** PLS-DA score plot of the metabolomic analysis of all groups, GLY0.5 vs. CTRL, GLY5 vs. CTRL, and GLY50 vs. CTRL, $N = 5$. **e** Heatmap showing the differences in metabolite levels in the testes of CTRL and GLY-exposed males with a Variable Important in Projection (VIP) score of ≥ 1 and used in the following univariate analyses, $N = 5$. **f** Histograms of cAMP, AMP, and GMP differential levels in the testes of CTRL and GLY50-exposed males, $N = 5$.

the highest dose of GLY (Fig. 3f).

3.5. GLY triggers genotoxic and epigenotoxic effects in germ cells

The results from the TUNEL assay showed that the DNA damage in testicular cells of males exposed to the highest dose of GLY was significantly increased when compared to the control group (Fig. 4a).

The epigenetic toxicity of GLY in testicular cells was specifically analysed in two histone acetylation marks (H3K9ac and H4K12ac) in Pcna-positive cells (Sg and Scts) and haploid cells (Stds and Spz) separately. Immunohistochemical analysis revealed that exposure to GLY0.5 slightly decreased H3K9ac levels in Sg and Sct (Fig. 4b,d) and significantly increased H4K12ac levels in haploid cells (Fig. 4c,d).

3.6. GLY impairs male breeding capacity

Regarding breeding capacity, male exposure to GLY0.5 and 5 did not affect fecundity or hatching rates. However, mortality at 3.3 hpf (mid-blastula transition) of the embryos was increased from 0 % in batches from the CTRL group to 6 % ± 1.41 in batches from males treated with GLY 5. In contrast, the highest dose of GLY had a drastic effect on breeding capacity: none of the ten matings resulted in the production of

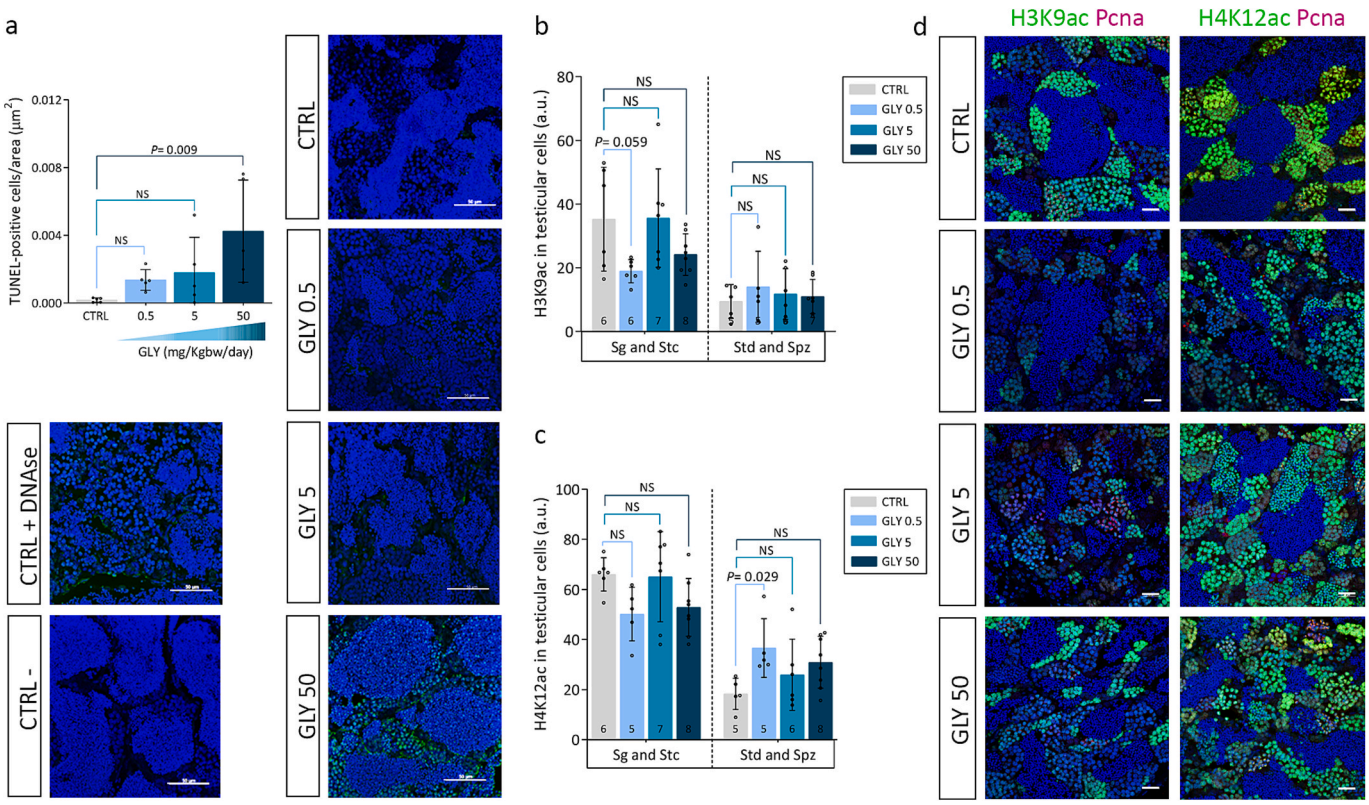

Fig. 4. Genotoxic and epigenotoxic effects of GLY. a Number of TUNEL-positive cells in testicular sections from CTRL and GLY-treated males, $N = 5$. Representative confocal images of TUNEL assay in testicular sections of each experimental group, including the technical positive and negative controls (nuclei marked in blue and apoptotic cells in green), scale bar = 50 µm. **b,c** Levels of H3K9ac and H4K12ac, respectively, in Pcna-positive cells (Sg and Scts) and haploid cells (Stds and Spz) in CTRL and males exposed to different doses of GLY (0.5, 5, 50), being the *N* indicated in the lower part of the corresponding bar. **d** Representative confocal images of H3K9ac and H4K12ac immunohistochemical detection in germ cells from CTRL and males exposed to different doses of GLY (0.5, 5, 50), scale bar = 20 µm.

F1 embryos (Table 1).

4. Discussion

In recent years, substantial research has focused on analysing the impact of GLY alone and/or GBHs on male reproduction across animal species (Serra et al., 2021). However, to the best of our knowledge, no studies have specifically examined the reproductive effects of adult male exposure to GLY via diet, at the ADI set by the EFSA. This highlights a significant gap in the current literature, as understanding the reproductive impacts at exposure levels considered "safe" is essential for comprehensive risk assessment and informed regulatory decisions. To address this, we exposed adult male zebrafish to dietary GLY at doses ranging from the ADI up to the NOAEL (Álvarez et al., 2023) over a 21-day period (chronic exposure). We assessed the effects of glyphosate alone to isolate its specific mechanisms of action, eliminating potential confounding variables associated with different herbicide formulations, and providing a standardized reference useful for regulatory and safety assessments. Consequently, we primarily compared our results with available data on exposure to glyphosate alone or to both glyphosate and GBHs. However, studies assessing the effects of GBHs alone on male reproduction were also considered when specific literature on glyphosate exposure was unavailable.

Table 1
Male breeding capacity of CTRL and GLY-exposed males. Number of fertilized eggs per male in 5 matings per experimental group ($N = 5$), except for GLY 50 in which 10 matings were performed ($N = 10$). Data are presented as mean ± SD. Percentage of embryo mortality and hatching rate of 3 different batches per experimental group ($N = 3$), except for the GLY 50 in which no embryos were obtained. Asterisk indicates statistically significant results when compared to the control group.

	CTRL	GLY 0.5	GLY 5	GLY 50
Number of fertilized eggs per male	110 ± 74	115.67 ± 38.56	46.67 ± 50.33	0
Embryo mortality at 3.3 hpf (%)	0	0	6 ± 1.41 *	–
Hatching rate (%)	90.46 ± 14.87	85.43 ± 20.85	82 ± 25.46	–

In this study, exposure to GLY0.5 significantly increased the GSI. No previous studies have analysed the impact of GLY on the GSI in adult zebrafish. However, in mice, perinatal exposure (from embryonic day 10.5 to 20 days post-partum) to the same dose of GLY decreased the relative testes weight once these males reach the adulthood (Pham et al., 2019), highlighting the importance of the window of exposure. In addition to higher GSI, in GLY0.5 group, our histological data and Ddx4 immunodetection revealed an increase in the percentage of spermatogonia. The exposure to GLY50 not only increased the Ddx4 positive cells, but also led to higher testicular levels of this protein, a RNA helicase playing an essential role in germline development, which serves as a marker of spermatogonia (Xu et al., 2022; Ye et al., 2023). Most studies examining the effects of GLY and GBHs on male reproduction, primarily focus on the overall testicular architecture, rather than analyzing the frequency of testicular cells (Gorga et al., 2021; Johansson et al., 2018; Owagboriaye et al., 2017; Uren Webster et al., 2014). In fact, the percentage of Ddx4-postive cells has been just studied in the first wave of mouse spermatogenesis after perinatal exposure to GLY, but no changes were reported at any of the tested doses (the same three as herein studied). On the other hand, when analysing the testes of mice perinatally exposed to GLY5 later in development, these authors found an

increase in undifferentiated Sg (Pham et al., 2019).

As for spermatozoa, studies in mammals have reported a lack of alterations in sperm concentration after adult rat exposure to GLY (2 and 50 mg/kgbw/d) (Liu et al., 2022). In our study, the expanded pool of type A spermatogonia, observed in the GLY0.5 group, suggested an overall reduced spermatogenic activity, further confirmed by the decrease in the percentage of spermatozoa. However, this outcome was not detected in the testes of males exposed to GLY50 where the increased percentage of type B spermatogonia, likely triggered by higher testicular cortisol levels, ensured the progression of spermatogenesis. In that regard, a previous study on zebrafish spermatogenesis has reported the ability of cortisol to exert a paracrine effect on testicular function stimulating spermatogonial differentiation in an androgen-independent manner and promoting meiosis and spermiogenesis, thus ultimately increasing the number of spermatozoa in the testes (Tovo-Neto et al., 2020).

To elucidate the mechanisms by which GLY affects spermatogenesis, we employed a targeted multi-omics approach. Our proteomic analysis revealed a slight decrease in Gper1 levels in the testes of males exposed to GLY0.5. In previous studies, the loss of GPER in peritubular testicular cells has been associated with impaired spermatogenesis in adult primates (Sandner et al., 2014), whereas serum GPER levels have been positively correlated with sperm count in human samples (Barut et al., 2020). Although the role of this protein in zebrafish spermatogenesis is not yet fully understood, our results suggest that alterations in testicular Gper1 levels may be critical for male germ cell differentiation in this species, similar to observations in zebrafish exposed to bisphenol A (González-Rojo et al., 2019). In males exposed to GLY50, the metabolomic analysis showed a significant increase in cAMP testicular levels. While various pathways, such as TGF-alpha, influence steroid hormone production, the cAMP-dependent regulation of StAR expression is the primary pathway for steroidogenesis (Manna et al., 2006). Consistent with this pathway, males exposed to GLY50, with higher testicular levels of cAMP, exhibited an overexpression of both *star* and *cyp11a1*, which in turn led to higher levels of pregnenolone, progesterone, and E2. In fish, spermatogenesis is highly dependent on steroid hormones with estrogens, androgens and progestins exerting distinct but critical roles (Delbes et al., 2022; Hatef and Unniappan, 2019). Specifically, in an *ex vivo* zebrafish testis culture, E2 exposure has been shown to induce significant histological changes, notably increasing the amount of spermatogonia (de Castro Assis et al., 2018), a result we also observed in the testes of males exposed to GLY50.

Endocrine disruptors are also known to induce genotoxic and epigenotoxic effects in male germ cells, thus disrupting male reproduction and jeopardizing the development of future generations (Lombó and Herráez, 2021). Concerning genotoxicity, previous studies have reported reduced sperm DNA integrity in zebrafish following exposure to 10 mg/L GLY for 24 or 96 h (Lopes et al., 2014). Likewise, we evidenced a high increase in the DNA damage of testicular cells in males exposed to GLY50, which could be linked to the reduced testicular levels of Sumo3. Sumo proteins are involved in the stress response during spermatogenesis (Shrivastava et al., 2010), and they play a crucial role in p53-mediated apoptosis under genotoxic stress (Park et al., 2014). The high level of DNA fragmentation likely contributes to the observed reproductive impairment, as males exposed to GLY50 were completely unable to breed. Moreover, other factors could also contribute to this infertility, such as reduced ejaculated sperm or altered courtship behaviour, either of which could prevent successful fertilization, such as already reported in other fish species (Sánchez et al., 2017; Sánchez, 2021). This is particularly relevant considering the significantly higher cortisol levels found in GLY50-exposed males, which may induce anxiety-like behaviours that likely interfere with normal courtship.

While maintaining genome integrity is critical for successful reproduction (Bhargava et al., 2020), many adverse effects induced by environmental factors cannot be attributed solely to DNA sequence changes (Skinner, 2014). Spermatogenesis is particularly vulnerable due to the intense epigenetic remodelling required for germ cell maturation. In this regard, histone post-translational modifications not only balance active and repressed chromatin states but also govern diverse processes such as DNA replication and repair, chromosome maintenance, and early embryo development (Luense et al., 2016). In the present study, we analysed two specific histone modifications in testicular cells: the acetylation of lysine 9 in histone H3 (H3K9ac) and lysine 12 in histone H4 (H4K12ac) in both Pcna-positive and negative cells. Our findings showed that exposure to GLY0.5 altered these epigenetic marks, decreasing H3K9ac in spermatogonia and spermatocytes while increasing H4K12ac in spermatids and spermatozoa. Interestingly, previous studies in zebrafish testes evidenced BPA-induced changes in H4K12ac could be reversed by incubation with a GPER antagonist, indicating the involvement of this receptor in alterations of this histone acetylation mark (González-Rojo et al., 2019). Therefore, the decrease in Gper1 we observed in the testes of males exposed to GLY0.5 could also contribute to the increase in H4K12ac observed in the haploid cells of these males. Similarly, in the context of GLY exposure, perinatal treatment with 350 mg/kgbw/d GBH led to a decrease in H3ac and an increase in H4ac levels at the ERα promoter in uterine samples (Lorenz et al., 2019). To our knowledge, this is the first study to assess changes in histone acetylation in male germ cells following adult GLY exposure, which also suggests the potential for transgenerational effects. Given that, in rats, exposure to 25 mg/kgbw/d GLY from day 8 to 14 of gestation resulted in transgenerational inheritance of other sperm epimutations up to the F3 generation (Ben Maamar et al., 2021; Kubsad et al., 2019), further research is essential to determine whether the epimutations observed in the present study may be transmitted to subsequent non-exposed generations.

In conclusion, the present study provides important insights into the reproductive hazards of adult exposure to GLY doses considered safe by EFSA. Both the ADI and NOAEL doses caused structural alterations in testicular tissue; however, only the NOAEL dose impaired zebrafish breeding capacity. Notably, the mechanisms driving these toxic effects differed by dose: epigenetic changes were more evident following GLY0.5 exposure, while steroidogenic alterations and genetic toxicity were more pronounced with GLY50. Additionally, the non-monotonic response to GLY exposure is underscored by the minimal effects observed at the intermediate GLY5 dose, highlighting complex dose related responses in male reproductive health.

While this study provides valuable insights into the reproductive toxicity of glyphosate, some limitations should be considered. Although overall feed intake was stable at the group level, some degree of individual variation in feed consumption may naturally occur, reflecting real-world ecological conditions, where individuals in a population are not uniformly exposed to contaminants due to differences in feeding behaviour. One major challenge is the pleiotropic nature of glyphosate effects, which makes it difficult to pinpoint a single pathway underlying its reprotoxic effects in males. Additionally, although targeted metabolomics and proteomics offered specific molecular insights, they provide a limited view of broader systemic changes. Further studies are also needed to explore whether the observed epigenetic modifications in male germline cells could have transgenerational effects. Finally, to gain a comprehensive understanding of the reproductive effects of GLY and its degradation products, future studies should also investigate AMPA, the primary environmental metabolite of glyphosate, due to its presence in aquatic ecosystems at relevant concentrations and its potential impact on spermatogenesis.

IA in scientific writing

During the preparation of this work the authors used ChatGPT4.0 in order to improve the readability and language of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Author statement

M.L., C.G., O.C. conceived the project and designed the experiments. **M.L., C.G., F.S., F.M., H.H.** performed the experiments and collected the samples. **M.L., C.G., A.A., G.P., A.I., S.S.** performed the analysis and interpreted the data. **A.A. and O.C.** were responsible for the funding acquisition. **M.L., C.G., G.P., O.C.** wrote the manuscript and supervised the work. All authors read and approved the manuscript.

CRediT authorship contribution statement

Marta Lombó: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Christian Giommi:** Writing – original draft, Validation, Formal analysis, Data curation, Conceptualization. **Angela Amoresano:** Validation, Funding acquisition, Formal analysis. **Gabriella Pinto:** Writing – original draft, Validation, Formal analysis, Data curation. **Anna Illiano:** Formal analysis, Data curation. **Fiorenza Sella:** Formal analysis, Data curation. **Stefania Serpico:** Formal analysis, Data curation. **Hamid Habibi:** Data curation. **Francesca Maradonna:** Data curation. **Oliana Carnevali:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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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. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2025.109544.

Data availability

Data will be made available on request.

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