

Multi-generational exposure to glyphosate and glyphosate-based herbicides induces neurobehavioral alterations in Sprague-Dawley rats

I. Menghetti

1. Cesare Maltoni Cancer Research Center, Ramazzini Institute

I. Thiara

2. University of California

S. Panzacchi

1. Cesare Maltoni Cancer Research Center, Ramazzini Institute

L. Falcioni

1. Cesare Maltoni Cancer Research Center, Ramazzini Institute

R. Giovannini

1. Cesare Maltoni Cancer Research Center, Ramazzini Institute

M. Iuliani

1. Cesare Maltoni Cancer Research Center, Ramazzini Institute

M. Manservigi

1. Cesare Maltoni Cancer Research Center, Ramazzini Institute

S. Nanni

1. Cesare Maltoni Cancer Research Center, Ramazzini Institute

D. Sgargi

1. Cesare Maltoni Cancer Research Center, Ramazzini Institute

V. Strollo

1. Cesare Maltoni Cancer Research Center, Ramazzini Institute

M.J. Perry

3. College of Public Health, George Mason University

P. J. Landrigan

4. Program for Global Public Health and the Common Good, Boston College

D.R. Smith

drsmith@ucsc.edu

2. University of California

D. Mandrioli

1. Cesare Maltoni Cancer Research Center, Ramazzini Institute

Research Article

Keywords: Glyphosate, Neurobehavior, Roundup Bioflow, Ranger Pro, Anxiety

Posted Date: July 14th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10060520/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Environmental and occupational glyphosate exposure is associated with a variety of adverse health and neurobehavioral effects in children and adults, such as anxiety and autism spectrum disorders. Here we used a Sprague-Dawley rat model of transgenerational environmental exposure to glyphosate or two glyphosate-based herbicides (Roundup Bioflow and Ranger Pro), at targeted daily oral doses of 0, 0.5 (EU ADI), 5, or 50 (EU NOAEL) mg/kg bw/day of glyphosate, starting on gestational day 6 in F0 pregnant dams and continuing in F1 generation postnatally through pregnancy and lactation and postnatally in the F2 generation through 1 year of age. Neurobehavioral performance was assessed at 1 year of age using the open field test of behavioral activity, the balance beam test of gross motor coordination, the grip strength test of fore- and hindlimb neuromuscular tone, the passive avoidance test of short-term memory and the Irwin test to detect behavioral/physiological changes. Findings show that all three compounds tested altered behavioral reactivity and open field exploration consistent with increased anxiety in a dose-response fashion, though the Roundup Bioflow and/or Ranger Pro formulations caused greater anxiety-like behaviors than glyphosate alone; when sex-based differences were present, males were more affected than females. Moreover, Roundup Bioflow and/or Ranger Pro, but not glyphosate alone altered gross motor coordination and neuromuscular tone. There were no effects of any formulation on short term memory to avoid an aversive stimulus in the passive avoidance test. These results demonstrate that multi-generational exposure to glyphosate and glyphosate-based herbicides produces significant neurobehavioral impairments and increased anxiety-like behaviors in Sprague-Dawley (SD) rats at doses that are currently considered “safe”, substantiating concerns for the adverse health effects of environmental and occupational applications of these commonly used herbicides.

1 INTRODUCTION

Glyphosate [N-(phosphonomethyl)glycine] is a broad-spectrum organophosphate herbicide and the most widely used pesticide worldwide since its commercial introduction under the trade name Roundup Bioflow® in 1974.¹ Glyphosate-based herbicides (GBHs) are extensively applied in agriculture, residential landscaping, forestry, and public right-of-way maintenance, making environmental exposure potentially ubiquitous. Urinary biomonitoring studies in the United States and Europe have detected glyphosate or its primary metabolite aminomethylphosphonic acid (AMPA) in the majority of individuals tested, including pregnant women and children.^{2,3} Human environmental and occupational exposure to glyphosate and GBHs has been associated with a range of adverse health outcomes, including hepatotoxicity, nephrotoxicity, endocrine disruption, reproductive toxicity, and carcinogenicity.^{4–11}

Mounting evidence from epidemiological and pre-clinical studies have additionally implicated glyphosate in neurological and neurobehavioral disturbances, including cognitive impairment, increased anxiety and depressive symptoms, and autism spectrum disorders.^{12–22} Analysis of data from the National Health and Nutrition Examination Survey (NHANES) 2013–2014 found that urinary glyphosate levels were significantly associated with cognitive impairment and severe depressive symptoms in a representative

sample of US adults.¹⁵ Among adolescents in Ecuador's ESPINA cohort, urinary glyphosate, detected in 98.3% of 519 participants aged 11–17 years living near floriculture operations, was associated with lower performance on social perception tests.¹⁶ A companion analysis within the same ESPINA cohort demonstrated that seasonal inhibition of erythrocytic acetylcholinesterase activity, a biomarker of exposure to cholinesterase-inhibiting pesticides, including GBHs, between two agricultural spray seasons was significantly associated with increased depression symptoms in adolescents.¹⁷

Experimental animal studies have corroborated and extended the epidemiological evidence linking glyphosate exposure to neurological and behavioral dysfunction. Rodent models of gestational, early postnatal, and adult GBHs exposure have reported a consistent profile of neurobehavioral alterations, including heightened anxiety-like behavior in open field and elevated plus maze paradigms, impaired locomotor activity, depression-like behavior in tail suspension and splash tests, disrupted social behavior, and impaired learning and memory.^{14,18–21,23–27} Buchenauer et al. demonstrated in a mouse model that maternal glyphosate exposure during pregnancy and lactation at the EU no observed adverse effect level (NOAEL) dose of 50 mg/kg bw/day induced depression- and anxiety-like behavior and social deficits, most notably in adult female offspring.¹⁹ Neuromechanistically, GBHs exposure has been associated with a number of adverse effects, including oxidative stress and neuroinflammation in the prefrontal cortex, hippocampus, and cerebellum; disruption of monoaminergic neurotransmission including dopaminergic and serotonergic pathways regulating affect and cognition; acetylcholinesterase inhibition; glutamate excitotoxicity mediated through NMDA receptor activation; gut microbiome dysbiosis with downstream effects on tryptophan and serotonin metabolism; and, as recently characterized, NLRP3 inflammasome-mediated microglial pyroptosis.^{12,20,26}

Importantly, the neurobehavioral outcomes documented across these experimental and epidemiological studies — anxiety, depression, impaired cognition, disrupted motor function, social deficits — are directly relevant to the rising global burden of anxiety disorders, attention deficit and hyperactivity disorder, and neurodevelopmental conditions^{28,29}, underscoring the public health significance of identifying and characterizing their modifiable environmental determinants. The concept of the Developmental Origins of Health and Disease (DOHaD) provides an important theoretical framework for understanding glyphosate's potential neurobehavioral impacts.³⁰ The DOHaD paradigm holds that early-life environmental exposures — including those occurring in utero, during lactation, and in early childhood — can program physiological and neural systems in ways that confer lasting susceptibility to disease across the life course, and that such programming effects can be transmitted epigenetically across generations.³¹ For a herbicide as pervasive as glyphosate the DOHaD framework suggests that even exposures deemed toxicologically “safe” at current regulatory thresholds may carry unrecognized chronic transgenerational consequences for neurological health.

It is noteworthy that many pre-clinical studies demonstrating neurobehavioral impacts of glyphosate, while compelling, have limitations in translational relevance to real-world human exposure scenarios. Many of the existing animal studies have focused on short-duration, single-generation exposures

confined to discrete life stages — gestational, early postnatal, or adult — and have examined the parent compound glyphosate in isolation rather than as formulated commercial products.^{14,18,19} These design features diverge from the exposure realities faced by environmentally and occupationally exposed human populations, in whom glyphosate exposure often spans decades and may encompass multiple generations, as illustrated by the multigenerational ESPINA cohort in Ecuador.^{16,17} Furthermore, it is increasingly recognized that the commercial herbicide formulations to which humans are actually exposed — such as Roundup Bioflow and Ranger Pro — contain, in addition to glyphosate, a variety of adjuvants and surfactants that may substantially alter the toxicokinetic and toxicodynamic properties of the preparation.^{20,27} Several in vitro and in vivo studies have reported that commercial GBH formulations are more toxic than equimolar concentrations of glyphosate alone, suggesting that adjuvant components modify or amplify glyphosate's biological effects.^{18,20,24} The extent to which the neurobehavioral toxicity of commercial GBH formulations — with differing surfactant profiles — exceeds that of glyphosate alone across multiple generations of exposure remains poorly characterized, representing a critical knowledge gap for risk assessment and regulatory decision-making.

To address these knowledge gaps, the present study was conducted as the neurobehavioral arm of the Global Glyphosate Study (GGS), a multi-institutional investigation designed to provide the most comprehensive toxicological evaluation of glyphosate and GBHs to date (glyphosatestudy.org). The GGS is an integrated study designed to test a wide range of toxicological outcomes including carcinogenicity, neurotoxicity, multi-generational effects, organ toxicity, endocrine disruption, and prenatal developmental toxicity. Results from different arms of the study have been already published.^{10,32–35} In designing the GGS, it was hypothesized that exposure to GBHs may have different toxic effects than glyphosate alone; these co-formulants might increase glyphosate bioavailability and activity in mammals, including humans. Here, glyphosate and two commercial GBH formulations, the EU formulation Roundup Bioflow (MON 52276) and the US formulation Ranger Pro (EPA 524 – 517), were administered trans-generationally to SD rats at targeted glyphosate concentrations of 0, 0.5, 5, and 50 mg/kg bw/day – doses that encompass both the EU ADI (0.5 mg/kg bw/day) and the NOAEL (50 mg/kg bw/day) for glyphosate, and that correspond to regulatory thresholds at which neurobehavioral effects have now been documented in rodent models.^{19,25} Neurobehavioral outcomes were assessed in adult second generation (F2) offspring, including spontaneous locomotor activity and behavioral reactivity, gross sensorimotor coordination, forelimb and hindlimb neuromuscular tone, and short-term fear memory and emotional learning. By spanning three generations of continuous exposure, comparing glyphosate alone to two commercially relevant formulations differing in surfactant composition, and employing a broad neurobehavioral test battery in both male and female offspring, this study was designed to provide a uniquely translational assessment of the neurodevelopmental risks posed by glyphosate and GBHs exposure under conditions that better approximate the multi-generational human exposure scenarios of greatest public health concern.

2 METHODS

2.1 Test substances

The three glyphosate and GBHs test substances administered to female and male SD rats in drinking water were 1) glyphosate (CAS 38641-94-0 [N-(phosphonomethyl) glycine], purity 99%, Sigma-Aldrich, Milan, Italy), 2) the commercial GBH formulation Roundup Bioflow (MON 52276, containing 360 g/L of glyphosate acid in the form of 486 g/L isopropylamine salts of glyphosate (41.5%)), and 3) the commercial GBH formulation Ranger Pro [EPA registration n. 524–517, containing 356 g/L of glyphosate acid in the form of 480 g/L isopropylamine salts of glyphosate (41%)]. Solutions of these three test substances were prepared by the addition of an appropriate volume of tap drinking water, to obtain the desired corresponding concentration of glyphosate, in line with analyses of the solutions stability and concentrations of the test substance.^{10,35}

2.2 Animals and experimental design

2.2.1 Animals

All aspects of the animal experiments were performed according to Italian law regulating the use and treatment of animals for scientific purposes (Legislative Decree n. 26, 2014. Implementation of the directive n. 2010/63/EU on the protection of animals used for scientific purposes. G.U. General Series, n. 61, March 14th, 2014). All animal study procedures were performed at the Cesare Maltoni Cancer Research Centre of the Ramazzini Institute, Bentivoglio, Italy. The study protocol was approved by the Ethical Committee of the Ramazzini Institute and approved and formally authorized by the *ad hoc* commission of the Italian Ministry of Health (ministerial approval n. 945/2018-PR).

The Ramazzini Institute animal breeding facility was the supplier of the Sprague Dawley (SD) rats used in this study. Female breeder rats were placed individually in polycarbonate cages (42 × 26 × 18 cm; Tecniplast Buguggiate, Varese, Italy) with a single unrelated male (outbred) until evidence of copulation (presence of a vaginal copulation plug or sperm in a daily vaginal lavage) was observed. After mating, the male was removed, and females were housed separately during gestation and delivery. Newborns were housed with their dams until weaning (Post Natal Day, PND ~ 28). Weaned offspring were co-housed by sex and treatment group, with no more than three animals per cage. Cages were identified by a card indicating: study protocol code, experimental and pedigree numbers, and dosage group. A shallow layer of white fir wood shavings served as bedding (supplier: Giuseppe Bordignon, Treviso, Italy). During the experiment, animals had *ad libitum* access to an organic pellet feed (“Corticella bio” supplied by Laboratorio Dottori Piccioni Srl, Piccioni Laboratory, Milan, Italy). Animals were provided *ad libitum* fresh municipal tap water from glass bottles and water bottles were changed daily; tap drinking water was tested for possible glyphosate contamination in compliance with Directive 2008/105/EC, D.Lgs. 152/2006, Directive 2006/118/EC (active substances in pesticides, including their relevant metabolites, degradation and reaction products). The pelleted feed was tested for possible glyphosate contamination in compliance with Commission Regulation (EU) No 293/2013 (maximum residue levels (MRLs) < 0.01 mg/Kg). In addition, the cage bedding was screened for possible chemical contamination (metals,

aflatoxin, polychlorinated biphenyls, organophosphorus and organochlorine pesticides) by CONSULAB srl Laboratories (Treviso, Italy). Animals were maintained on cage racks at room temperature ($22^{\circ}\text{C} \pm 3^{\circ}\text{C}$) and 50% ($\pm 20\%$) relative humidity and checked daily. The light was artificial, and a light/dark cycle of 12 h was maintained.

2.2.2 Experimental Design

An overview of the experimental design is shown in Fig. 1. SD rat dams (F0) and relative pups (F1) were treated with either glyphosate or Roundup Bioflow or Ranger Pro diluted in drinking water to achieve the desired glyphosate concentration. The three concentrations selected for each test substance were 5, 50 and 500 mg/L of glyphosate in drinking water: the target exposure levels, based on mean water consumption (40 mL) and a mean body weight (400 g), corresponded to 0.5, 5 and 50 mg/kg bw/day. Control animals were administered drinking water *ad libitum*. The study design included a total number of 10 experimental groups, as follows: 1) Control group (drinking water), 2) glyphosate groups (3 doses, corresponding to 5, 50 and 500 mg/L), 3) Roundup Bioflow groups (3 doses corresponding to 5, 50 and 500 mg/L), and 4) Ranger Pro groups (3 doses corresponding to 5, 50 and 500 mg/L). Randomization was carried out in both F0, F1 and F2 animals. Body weight was taken into account in the randomization of F0 dams, prior to the start of treatment, considering that the weight distribution of the animals selected for the study should be as close as possible to $\pm 20\%$ of the average body weight (by sex) of all animals available for the study. F1 animals were randomly distributed in order to have no more than 1 male and 1 female per litter per group. F2 animals were randomly distributed in order to have no more than 2 males and 2 females per litter per group. The remaining animals of each litter were assigned to other arms of the GGS.

The F0 female breeders received the treatment through drinking water from GD 6, corresponding to early embryo implantation and start of organogenesis to the end of lactation, while the offspring (F1) continued to be exposed after weaning and during mating. After weaning ($\sim$ PND 28), F1 animals were randomly distributed in order to have no more than one male and one female per litter per treatment group. Each F1 experimental group was composed of 15 males and 15 females, belonging to the same treatment group as their exposed dams (F0). Female and male SD rats from the F1 adult arm were used to generate F2 offspring. Animals from the same treatment groups were matched outbreed and mated at 19 weeks of age. After weaning ($\sim$ PND 28) the offspring were assigned to the same treatment group of their parents, with no more than two males and two females from the same litter per treatment group. Each F2 experimental group of the Neurobehavioral arm of the study was composed of 12 males and 12 females. The total number of animals was 240 (120 males and 120 females). Treatment exposure continued in F2 offspring until terminal sacrifice at 1 year of age. Animals underwent behavioural testing over 51–53 weeks of age, as described below.

2.3 Behavioural testing

Adult F2 generation male and female Sprague-Dawley rats were evaluated for a suite of behavioral outcomes over age 51–53 weeks, with $n = 12$ animals per sex and exposure condition. Importantly,

whenever possible, only two males and two females littermate per exposure condition were assigned to particular behavioral outcome measure. The behavioral outcomes included 1) Open field test, 2) Forelimb/Hindlimb grip strength test, 3) Balance beam test, 4) Passive avoidance test, and 5) Irwin test, as described below.^{13,36–40}

2.3.1 Open Field Test

The open field test assesses a variety of behaviors in rodents, including general activity (amount of time the rat spends moving, rearing, grooming, etc.), exploratory behavior in a novel environment (e.g., distance traveled in defined wall and center zones of the open field), and anxiety (based on how much time/distance the rat spends near the walls of the arena compared to the center). A multiple unit open field maze (OFM) consisting of four activity chambers was used for this analysis. Each chamber measured 46 cm (length) x 46 cm (width) x 40 cm (height), and within each bin a perimeter ‘wall’ zone (perimeter open field within 13 cm of the wall) and internal ‘center’ zone (open field floor area 20 x 20 cm) was defined.

Open field activity was measured over a single 30 min testing duration and captured using the ANY-MAZE Video Tracking software (Ugo Basile). All open field activity assessments were performed under infrared light at night (9:00 PM – 3:00 AM) during the animals’ natural active (dark) cycle. The number of fecal boluses deposited in the open field apparatus were also assessed at the end of the test session, including: 1) Defecation boli, 2) Diarrhea (scored absent or present as liquid or non-formed stool), and 3) Increased urination with handling (scored as normal or increased).

A total of 28 different outcomes were collected over the 30 min open field test duration (see Supplemental Table S1). In addition, grooming behavior was assessed using offline analysis of videotapes in conjunction with a computer-assisted scoring system and a notation system for detailed descriptions of stereotyped grooming sequences (chain grooming and non-chain grooming).⁴¹ Specifically, the ANY-MAZE software allowed offline review of each open field test video to add additional scored behaviors. The observers received extensive training to accurately and reproducibly score grooming behavior. During training, agreement between observers on the number and timing of grooming events was 100%.

For scoring of open field grooming behavior in the ANY-MAZE software, each video was analyzed twice. In first analysis the quantity of grooming was scored to determine the number of grooming events, the duration (s) of each event, and the open field location (i.e., wall or center zone). In second analysis the quality of grooming was evaluated, since grooming activity in rodents is increased under conditions of both high and low stress, while stress-evoked grooming is generally characterized by interrupted and chaotic bouts that defy the cephalocaudal rule (i.e., the highly stereotyped, head-to-tail progression of self-grooming), and alters the behavioral microstructure of grooming. Each grooming event was categorized as syntactic-chain grooming or non-chain grooming. The observers identified the total number of syntactic-chain grooming events, considering that chain pattern proceeds in a cephalo-caudal

direction; incorrect transitions and incomplete or interrupted bouts were excluded. Grooming bouts were considered separated when the animals stopped and raised their heads. The total number of non-chain grooming events was obtained by subtracting the number of syntactic-chain grooming from the total number of grooming events.

2.3.2 Forelimb/Hindlimb grip strength test

Forelimb and hindlimb grip strength was measured using a grip strength meter (Bioseb, Inc.). The grip strength metal grid was positioned horizontally, and the rat was gently lowered and allowed to grab the grid with its two forelimbs or hindlimbs and was then gently pulled backwards in the horizontal plane until it lost its grip and was separated from the grid. The force applied to the grid before the rat lost its grip was recorded as the peak tension.

2.3.3 Balance beam test

The balance beam test is used to assess sensorimotor function and coordination during ambulation across the suspended narrow beam. The balance beam measured 100 cm (length) x 7 cm (width) and was suspended 100 cm above the padded floor. An enclosed darkened box was placed at the end of the beam for the animal to retreat into as a reinforcer to cross the beam. The completion time (the interval between the moment the rat was released to the moment it entered the darkened box) and the number of paw slips (forelimb/hindlimb descending more than 1.5 cm below the top surface of the beam) were recorded independently by two observers. Animals were tested with three consecutive trials and allowed 3 minutes to complete each trial. If the rat fell off the beam or took more than 3 minutes to transit the beam, the trial was recorded as incomplete. All trials were also videotaped for later scoring confirmation.

2.3.4 Passive avoidance test

The passive avoidance test is used to measure short-term and long-term memory, and emotional learning in laboratory animals, such as rodents. The test is based on negative reinforcement and involves training animals to inhibit a natural response, such as entering a darkened chamber, after experiencing an aversive stimulus. The primary outcome was the latency (i.e., time) it took for the animal to enter the darkened chamber following trial onset, which is used to evaluate the animal's ability to form associations between environmental contexts and aversive stimuli.

Here, a passive avoidance apparatus (step-through; Ugo Basile, Italy) was used to test the animals over two consecutive days. The apparatus consisted of two adjacent compartments separated by a sliding door; one compartment was brightly illuminated, while the other one was dark. Day 1 of testing was used for acquisition training, where each rat was placed in the center of the illuminated compartment facing the wall opposite the closed door. After a 4 second delay, the door was opened and the latency time of the rat entering the darkened compartment was recorded (darkened compartment entry was based on placement of all four paws in the compartment). As soon as the rat entered the darkened compartment, the door was closed and an inescapable, scrambled foot-shock (1.5 mA for 4 seconds) was delivered through the grid floor. Immediately after completion of this training trial, the rat was removed from the

dark compartment and put back in its home cage. Memory retention of the aversive foot shock stimuli was assessed on day 2, during which the same procedure was followed as for the day 1 acquisition session, except that no shock was applied through the grid floor of the darkened compartment. The time for each rat to enter the darkened compartment was measured as the step-through latency. If a rat did not enter the darkened compartment after 120 seconds elapsed, the maximum step-through latency of 120 seconds was recorded.

2.3.5. Irwin Test

The Irwin test in rats is an observational and functional screening battery used to detect behavioral/physiological changes. It is divided into two phases of observation: undisturbed and during handling. In the undisturbed observation each rat was observed in their cage and the following parameters were scored: 1) Body position; from a completely flattened position, lying on one side, sitting crouched to standing upright (flattened = -1, normal = 0, stand up = 1); 2) Arousal; while undisturbed in the test cage, the animal's state of arousal was assessed (depressed = -1, normal = 0, or excited = 1); 3) Piloerection; erection of hair—either ruffled or puff-ball appearance (normal = 0, slight = 1, moderate = 2, marked = 3, extreme = 4); 4) Palpebral closure (normal = 0, slight = 1, moderate = 2, marked = 3, closed = 4); 5) Exophthalmos: protruding palpebral opening, protrusion of one or both eyeballs (scored relative to the open control, absent = 0, present = 1); 6) Unusual behavior; forepaw treading, stereotypies, straub tail, head flicks, head shakes, circling, retropulsion, obstinate progression, wet-dog shakes (each behavior scored normal = 0, slight = 1, moderate = 2, marked = 3, extreme = 4); 7) Respiration (normal = 0, slow = -1, labored = -2, gasping = -3, arrested = -4, rapid = +1); 8) Convulsions (absent = 0, present = 1, and if present the type noted as clonic, tonic, or tonic-flexion); 9) Tremors (none = 0, slight body tremor = 1, moderate with slightly impaired locomotion = 2, marked with moderate to marked impaired locomotion = 3, and if present the type noted as exertion tremor, head only, tail only); 10) Writhing, abdominal stretching (none = 0, slight = 1, moderate = 2, marked = 3, extreme with sides consistently sucked in = 4); 11) Twitches (normal = 0, slight = 1, moderate = 2, marked = 3, severe = 4); 12) Lacrimation (scored if present or with chromodacryria as normal (no lacrimation) = 0, slight = 1, moderate = 2, marked = 3); 13) Salivation (none = 0; slight wet margins = 1; entire submaxillary region wet = 2); and 14) Fur appearance, presence of dull or unkempt fur (absent = 0, present = 1).

In the handling phase rats were handled to score the parameters below in the following order: 1) Touch response (passive = 0, irritable = 1, vocalizing when handled = 2, and increased urination/defecation = 3); 2) Vocalization (absent = 0, present = 1); 3) Abdominal tone (completely flaccid, with no return to normal tone when manipulated = -2, flaccid but with slow return = -1, slight resistance (normal) = 0, moderate resistance = +1, extreme resistance/hard = +2); 4) Limb tone, assessed limb resistance by pressing the plantar surface of each hind paw (slight resistance = 0, moderate = 1, marked = 2, extreme/stiff = 3); 5) Corneal reflex by lightly touching the eye (no eye blink reflex = -2, slow reflex = -1, normal reflex = 0, exaggerated reflex where animal continues to respond when stimulus is removed = +1); 6) Pinnae reflex when the ear is lightly touched (no reflex = -2, slow reflex = -1, normal reflex (a sharp head flick) = 0, exaggerated reflex (animal continues to respond when stimulus is removed) = +1); 7) Ipsilateral hind

limb toe pinch reflex (no reflex = - 2, slow reflex = - 1, normal (retraction of the paw) = 0, exaggerated reflex (animal continues to respond when stimulus is removed) = + 1); 8) Pupil size with light stimulus (decreased (miosis) = -1, normal = 0, increased (mydriasis) = + 1); 9) Righting reflex following release from tail suspension (no righting reflex = - 2, slow reflex = - 1, normal = 0, exaggerated reflex = + 1); and 10) Tail pinch response (no response to pinch = - 1, mild escape attention to pinch = 0, biting = + 1).

2.4 Statistical methods

The behavioral data were cleaned of statistical outliers with Tukey box plots, followed by analysis using mixed model analysis of variance using a 4 glyphosate and GBHs x 2 sex model design for each of the three different glyphosate-based formulations (i.e., glyphosate, Roundup Bioflow, and Ranger Pro). Fixed treatment effects included glyphosate and GBHs exposure (4 dose levels) and sex (2 levels). The random effect was rat ID to account for correlations within observations from the same animal. Significant main effects or interaction effects were followed by single degrees of freedom pair-wise contrasts of individual glyphosate and GBHs dose levels versus controls using the Student's two-tailed *t*-test to clarify the nature of the effects. The categorical outcomes of the Irwin test were analyzed using Chi square contingency analyses. All analyses used JMP software (JMP pro version 17, JMP Statistical Discovery, LLC), and the significance level was set at $p \leq 0.05$.

3 RESULTS

There were significant adverse effects of oral transgenerational glyphosate and GBHs exposure on multiple measures of behavior in a novel open field environment, motor coordination, and neuromuscular tone, but not on short-term memory. Roundup Bioflow and Ranger Pro formulations often cause greater effects than glyphosate alone. Moreover, when sex-based differences were present, males were more affected than females. Below we focus on and graphically present the results that most clearly differentiated one or more of the glyphosate and GBHs treatment groups from the controls and delineate the nature of the dysfunction caused by glyphosate and GBHs exposure.

3.1 Body weights of the behaviorally-tested animals were not affected by oral glyphosate-based herbicide exposure

Mixed model analyses of body weights of animals at the completion of oral glyphosate and GBHs exposure and the onset of behavioral testing showed a significant effect of sex in all three glyphosate and GBHs treatment groups ($p < 0.001$, males heavier), but no effect of glyphosate (main effect $p = 0.578$; glyphosate x sex interaction $p = 0.801$), or Roundup Bioflow (main effect $p = 0.097$; Roundup Bioflow x sex interaction $p = 0.288$) (see Supplemental Table 2 for summary of mixed model main effects). There was a significant effect of Ranger Pro exposure on body weight ($p = 0.0011$; Ranger Pro x sex interaction $p = 0.361$), but this effect was due to a significant difference between the male 0.5 mg/kg/d treatment group ($766 \text{ g} \pm 20.5 \text{ SE}$) being somewhat heavier than their 5.0 mg/kg/d counterparts ($656 \text{ g} \pm 20.5 \text{ SE}$, $p = 0.006$), with no other group differences emerging in either sex. Follow up analyses

showed that overall, males were significantly heavier ($715 \text{ g} \pm 8.88 \text{ SE}$, $n = 120$) than females ($436 \text{ g} \pm 5.36 \text{ SE}$, $n = 120$) ($p < 0.001$), as expected.

3.2 Glyphosate and GBHs exposure altered multiple measures of open field exploration behavior, consistent with increased anxiety

The open field test is commonly used to assess general activity and behavioral exploration in a novel environment, including the degree of anxiety-like behavior based on the animal's exploration of *a priori* defined wall-adjacent versus center zones in the open field. Here, we found that transgenerational glyphosate and GBHs exposure altered a broad array of measures of activity and exploration of the open field environment in a dose-dependent fashion from 0.5 mg/kg/d to 50 mg/kg/d, with the Roundup Bioflow and Ranger Pro formulations typically causing broader degrees of altered exploration than glyphosate alone, versus controls (Table 1; see Supplemental Table 1 for a complete summary of open field outcomes). Both males and females were affected by glyphosate and GBHs exposure, though when sex-based differences were present, males were typically more affected than females.

First, we found that there were no effects of any glyphosate/GBH formulation on the total distance traveled or the average speed of ambulation in the open field, indicating no glyphosate/GBH effects on general neuromotor activity, though there was a significant effect of sex on both of these outcomes (i.e., for total distance, treatment group p 's > 0.1 , sex p 's < 0.0001 , treatment $\times$ sex interactions p 's > 0.1 for mixed model analysis of each of the three glyphosate and GBH formulations) (Table 1, Fig. 2; see Supplemental Table 2 for mixed model results). However, there were significant effects of one or more of the glyphosate and GBH formulations on outcome measures reflecting the nature of behavioral exploration of the open field environment, including the ratio of total distance traveled in the wall zone to total distance overall, the time spent in the wall zone, the average duration of visits to the wall zone, and the time spent freezing or immobile in the wall zone—all consistent with increased anxiety-like behavior due to glyphosate and GBHs exposure (Table 1, Fig. 3).

Specifically, the proportion of distance traveled in the wall zone (i.e., ratio of wall distance to total distance) was significantly increased by glyphosate (main effect $p = 0.0058$, sex $p = 0.0089$, glyphosate $\times$ sex interaction $p = 0.311$), and by Ranger Pro (main effect $p = 0.0006$, sex $p = 0.220$, Ranger Pro $\times$ sex interaction $p = 0.454$) (Fig. 3A; Supplemental Table 2). Specific contrasts show that all three glyphosate doses in females increased the proportion of distance traveled in the wall zone ($p = 0.0131$, 0.0396 , and 0.0323 versus controls for the three doses, respectively), while for males only the highest glyphosate dose group traveled proportionally more in the wall zone compared to controls ($p = 0.0361$) (Fig. 3A). Ranger Pro exposure caused similar effects of increases in the proportion of distance traveled in the wall zone in the 5 mg/kg/d group (males, 0.0052 vs controls) and the 50 mg/kg/d group ($p = 0.0239$ and 0.0007 for males and females, respectively, versus controls). There were no effects of Roundup Bioflow exposure on this outcome in either females or males (main effect $p = 0.102$, sex $p = 0.016$, Roundup Bioflow $\times$ sex interaction $p = 0.722$) (Fig. 3A).

Consistent with the above, there was an effect of Roundup Bioflow (main effect $p = 0.0119$, sex $p = 0.299$, Roundup Bioflow x sex interaction $p = 0.131$) and Ranger Pro treatment (main effect $p = 0.0002$, sex $p = 0.761$, Ranger Pro x sex interaction $p = 0.0969$) to increase the time spent in the wall zone, as well as a main effect of glyphosate ($p = 0.0280$, sex $p = 0.0157$, glyphosate x sex interaction $p = 0.116$), Roundup Bioflow ($p = 0.0005$, sex $p = 0.0003$, Roundup Bioflow x sex interaction $p = 0.390$) and Ranger Pro ($p = 0.0499$, sex $p = 0.0218$, Ranger Pro x sex interaction $p = 0.540$) to increase the average duration of visits to the wall zone (Figs. 3B, C, Table 1). Specific contrasts show that all three Roundup Bioflow and Ranger Pro doses in males increased the time spent in the wall zone, compared to controls (for Roundup Bioflow, p 's = 0.049, 0.0004, 0.005 for the 0.5, 5, and 50 mg/kg/d dose groups, respectively, while for Ranger Pro p 's = 0.0068, 0.0002, 0.0047) (Fig. 3B). Females exposed to the highest Ranger Pro dose also spent more time in the wall zone compared to their female control counterparts ($p = 0.0027$). Contrasts also reveal longer average durations of visits to the wall zone in glyphosate-exposed males versus controls (p 's = 0.0429 and 0.0044 for the low and high dose groups, respectively), Roundup Bioflow-exposed males and females (males p 's < 0.0001 and 0.014 for the middle and high dose; females $p = 0.0464$ for the middle dose), and Ranger Pro-exposed males ($p = 0.005$ for the high dose versus controls) (Fig. 3C).

The animals' behavior while in the wall zone was also altered by glyphosate and GBHs exposure, with increased freezing time (i.e., time spent immobile following an abrupt cessation of movement) caused by exposure to each of the three glyphosate and GBH formulations (glyphosate main effect $p = 0.221$, sex $p = 0.401$, glyphosate x sex interaction $p = 0.0305$; Roundup Bioflow $p = 0.0034$, sex $p = 0.340$, Roundup Bioflow x sex interaction $p = 0.583$; Ranger Pro $p = 0.0003$, sex $p = 0.400$, Ranger Pro x sex interaction $p = 0.353$), and an increase in the overall time spent immobile in the wall zone (Roundup Bioflow $p = 0.0074$, sex $p = 0.0001$, Roundup Bioflow x sex interaction $p = 0.155$; Ranger Pro $p = 0.0008$, sex $p = 0.0009$, Ranger Pro x sex interaction $p = 0.138$) (Fig. 3D, E). Contrasts reveal that these treatment effects were driven primarily by Roundup Bioflow and Ranger Pro-exposed males, though the highest Ranger Pro dose also similarly affected females (i.e., for males all three Roundup Bioflow and Ranger Pro dose groups p 's < 0.05 versus control for both outcomes) (Fig. 3D, E).

Finally, we also evaluated grooming behavior in the open field, assessing both the time spent grooming and number of grooming 'touches' or events, grooming in specific zones of the open field, as well as the number of grooming episodes that were syntactic (i.e., a highly ordered stereotypical sequence of grooming behaviors occurring in a specific cephalocaudal pattern) versus non-syntactic. Results show that all three glyphosate and GBH formulations altered grooming behavior, primarily in males, with an increase in the number of grooming touches in the wall zone (all treatment effects $p < 0.05$, all sex, and glyphosate and GBHs x sex interactions p 's > 0.37), and a decrease in the proportion of grooming episodes that were syntactic (i.e., the ratio of syntactic grooming episodes to total grooming episodes) (i.e., all treatment effects p 's < 0.0001, all sex p 's < 0.0001, and all glyphosate and GBHs x sex interaction p 's < 0.020) (Table 1, Fig. 4, Supplemental Table 2). In particular, contrasts reveal that all three doses of glyphosate, Roundup Bioflow, and Ranger Pro in males significantly reduced the proportion of grooming episodes that were syntactic, compared to controls (glyphosate p 's < 0.0001, < 0.0001, and < 0.0001 for

the 0.5, 5, and 50 mg/kg/d dose groups, respectively; Roundup Bioflow p 's < 0.0001, 0.0107, and < 0.0001; and Ranger Pro p 's < 0.0001, < 0.0001, < 0.0001) (Fig. 4).

Altogether, we conclude that 1) glyphosate and GBHs exposure alters multiple behavioral measures in the open field test, consistent with increased anxiety; 2) the commercial GBH formulations of Roundup Bioflow and Ranger Pro produce greater neurobehavioral effects than glyphosate alone; and 3) these effects are generally more pronounced in males than females, though the particular glyphosate and GBHs dose-response and sex-susceptibility varies somewhat depending on the glyphosate and GBH formulations and outcome measure.

3.3 Glyphosate and GBHs exposure did not affect motor coordination on the balance beam test

The balance beam test is commonly used to assess general motor coordination, balance, and fine sensorimotor function in rodents. Here, we found that there were no effects of any glyphosate and GBH formulations on the time to cross the balance beam or the number of falls (p 's > 0.3) (Fig. 5; see Supplementary Table 2 for a summary of the mixed model treatment group and sex effects). There was a significant main effect of sex on the number of falls in the Roundup Bioflow (p = 0.0147) and Ranger Pro (p = 0.0319) treatment groups (though no treatment group $\times$ sex interaction, p 's > 0.49), with males experiencing more falls than females, though this is likely due to the significantly larger body size of the males compared to females (see section 3.1 above).

3.4 Modest evidence suggests that glyphosate and GBHs exposure altered neuromuscular tone in the Forelimb/Hindlimb Grip Strength test

The grip strength test is widely used to measure neuromuscular function in rodents by assessing the maximum muscle force of the forelimbs or hindlimbs to grip and hold onto the testing bar of the apparatus. Here, we found modest evidence that glyphosate exposure affected forelimb grip strength (treatment group main effect p = 0.0338), although specific contrasts revealed no significant differences between the controls and any glyphosate treatment group (p 's > 0.1) (Fig. 6; see Supplementary Table 2 for a summary of the mixed model main effects). In contrast, there were no main effects of any glyphosate and GBHs treatment group on hindlimb grip strength (p 's > 0.1), although there was a highly significant effect of sex on hindlimb grip strength in all three glyphosate and GBHs exposure groups (p 's < 0.0001), and a significant treatment group $\times$ sex interaction for the Roundup Bioflow and Ranger Pro groups (p 's = 0.0038 and 0.0017, respectively). Specific contrasts revealed a significantly lower hindlimb grip strength in the Roundup Bioflow 5 mg/kg/d males compared to their male control counterparts (p = 0.0498), and a near trending reduction in hindlimb grip strength in the Ranger Pro 5 mg/kg/d males versus their respective controls (p = 0.109) (Fig. 6). Overall, we conclude that the 5 mg/kg/d dose of Roundup Bioflow and Ranger Pro, but not glyphosate, reduced hindlimb grip strength compared to controls in male rats, indicating modest deficits in neuromuscular tone.

3.5 Glyphosate and GBHs Exposure did not affect short-term memory in the Passive Avoidance test

The passive avoidance test is commonly used to assess associative learning and memory in rodents by measuring the latency to enter an aversive dark compartment on a subsequent trial (i.e., test day 2) where on a previous trial (i.e., test day 1) the animal experienced a mild uncomfortable electric shock. Here, we found that there were no effects of any glyphosate and GBH formulations or dose on the difference in latency to enter the aversive compartment on test day 1 versus test day 2, indicating no effect of glyphosate and GBHs exposure on short-term memory. Specifically, there were no significant main effects of treatment group, sex, or treatment x sex interactions for any glyphosate and GBH formulations (p 's > 0.1) (see Supplementary Table 2 for summary of mixed model ANOVA main effects).

3.6 Irwin test shows no overt physiological effects of glyphosate or GBHs exposure

As noted above, the Irwin test in rats is an observational and functional screening battery used to evaluate neurobiological, physiological, and behavioral states and responses, with scoring occurring in two distinct phases of observation – undisturbed and during handling. Here we evaluated 14 outcomes in the undisturbed observational phase (body position, arousal, piloerection, palpebral closure, exophthalmos, unusual behavior, respiration, convulsions, tremors, writhing, twitches, lacrimation, salivation, and fur appearance), and 10 outcomes in the handling phase (touch response, vocalization, abdominal tone, limb tone, corneal reflex, pinnae reflex, ipsilateral hind limb toe pinch reflex, pupil size with light stimulus, righting reflex, tail pinch response). There were no significant effects of glyphosate or GBH formulations on any phase 1 or phase 2 Irwin test outcome (p 's > 0.05; data not shown).

4 DISCUSSION

Glyphosate is the most widely used herbicide globally, and biomonitoring data consistently detect glyphosate or its metabolites in humans.^{1–3} Despite the pervasiveness of environmental exposures, which in many cases approaches regulatory acceptable daily intake levels,² the behavioral and neurological impacts of glyphosate/GBHs exposure remain incompletely understood. Here, we show that multi-generational exposure to glyphosate and both GBH formulations (Roundup Bioflow and Ranger Pro) produced dose-dependent effects on multiple open field outcomes indicating increased anxiety-like behavior, with GBH formulations causing broader and larger effects than glyphosate alone. In addition, Roundup Bioflow and Ranger Pro, but not glyphosate alone, modestly reduced hindlimb grip strength in males. In contrast, there were no effects of any compound on gross motor coordination, general motor activity, or associative memory. Notably, these effects, which were observed at target doses currently considered “safe” by regulatory agencies, underscore the importance of adjuvants and surfactants in commercial glyphosate formulations in enhancing their toxicological profile. Altogether, these findings raise concern that current regulatory thresholds may not be protective against neurobehavioral harm caused by GBH formulations across generations. These effects are discussed in detail below.

4.1 Glyphosate and GBHs exposure increase anxiety-like behavior in the open field test

Multi-generational oral exposure to glyphosate, Roundup Bioflow, or Ranger Pro led to dose-dependent increases in anxiety-like behaviors in the open field test. Moreover, when sex differences were present, males were in general more affected than females. These effects are based on significant increases in i) the ratio of wall-zone to total distance traveled, ii) time spent in the wall zone, iii) average duration of wall-zone visits, iv) time spent freezing in the wall zone, and v) total time immobile in the wall zone, which together constitute a coherent profile of thigmotaxis and behavioral inhibition consistent with heightened anxiety (Table 1, Figs. 2 and 3). Consistent with this, assessment of an anxiety-sensitive behavior of grooming microstructure shows that all three formulations increased the number of grooming touches in the wall zone and significantly reduced the proportion of syntactic (chain) grooming episodes in males (Fig. 4). In contrast, there were no measurable effects of any glyphosate or GBH formulations on total distance traveled or average speed of ambulation in the open field, thereby ruling out a general neuromotor activity effect and supporting specificity of the behavioral change to anxiety-related exploration patterns. Finally, while effects were observed in all three glyphosate/GBHs treatment groups, they were generally more common and/or severe in the Roundup Bioflow and Ranger Pro treatment groups, implicating an important role of the commercial co-formulant adjuvants and surfactants in enhancing the more severe anxiety effects.

These findings are consistent with and substantially extend prior rodent model studies showing that glyphosate or GBHs exposure increases anxiety-like behavior. For example, De Castro Vieira Carneiro et al. showed that in utero exposure to glyphosate at the EU ADI (0.3 mg/kg/d) increased anxiety-like behavior (marble-burying) in mice offspring.²⁵ Similarly, Buchenauer et al. reported that low-dose maternal GBH (0.5 and 50 mg/kg/d of glyphosate) exposure in mice during pregnancy and lactation produced anxiety-like behavior (open field test) in adult female offspring.¹⁹ Both of these studies are notable for their reported effects following relatively low exposures consistent with the present study and below current regulatory thresholds. Further, a number of other studies, albeit typically using much higher glyphosate/GBH exposure levels (e.g., 50–500 mg/kg/d) of various durations within a single generation and life stage, have similarly reported increased anxiety-like behaviors in rodent models using a variety of test paradigms such as the open field and elevated plus maze.^{18,23,24,26} In contrast, one study reported reduced anxiety, based on more open-arm explorations in an elevated plus maze, in rat offspring following perinatal GBH exposure.¹⁴ Altogether, these animal model studies suggest that the specific anxiety-related outcomes may depend upon the dose and life stage of glyphosate/GBH exposure, further underscoring the strength and relevance of the present study's environmentally-relevant exposure regimen and multi-generational design.

Epidemiological studies, while entirely observational, have provided evidence of altered neurobehavior and cognitive deficits associated with glyphosate/GBH (or other acetylcholinesterase-inhibiting pesticides) exposure. For example, the Suarez-Lopez et al. ESPINA cohort study found that greater

seasonal inhibition of acetylcholinesterase between pesticide spray seasons was significantly associated with higher depression scores, with stronger effects in girls.¹⁷ However, no significant association with anxiety per the MASC-2 scale was found in this study, contrasting with the present animal findings and possibly reflecting the adolescent age, specific biomarker used, or population-level exposure characteristics. Further, other studies reported that urinary glyphosate was positively associated with cognitive impairment and depression¹⁵ and lower social perception test performance¹⁶ in humans, suggesting broader CNS effects consistent with the present behavioral findings, though anxiety specifically was not assessed in those studies either.

There is some mechanistic convergence across the animal model studies pointing to serotonergic disruption, gut-brain axis perturbation, neuroinflammation, and dopaminergic impairment as potential principal pathways mediating GBH-induced anxiety. De Castro Vieira Carneiro et al. found that the heightened anxiety-like behavior caused by low dose glyphosate exposure co-occurred with prefrontal cortical neuroinflammation (elevated microglia, astrocytes, TNF- α)²⁵; Buchenauer et al. showed that the anxiety-like behavior in adult female offspring mice from mothers exposed low-dose GBH during pregnancy and lactation was associated with reduced tph2 expression and DNA hypermethylation of tph2 in the hippocampus, and altered gut microbiota¹⁹. A number of other studies, all of which used higher glyphosate/GBH doses compared to the studies above or the present study, reported that the anxiety-like behaviors produced by GBH exposure were accompanied by reduced serotonin immunoreactivity in the dorsal raphe, basolateral amygdala, and mPFC,¹⁸ gut microbiota dysbiosis,²⁴ or neuroinflammation evidenced by hippocampal NLRP3 inflammasome-mediated microglial pyroptosis and blood-brain barrier disruption.²⁶

4.2 Glyphosate and GBHs exposure do not impact gross motor coordination, but GBHs exposure produces modest reductions in neuromuscular tone

Here we found that there were no effects of any glyphosate or GBH formulations or dose on the time to cross or the number of falls in the balance beam test. There was a significant effect of sex on balance beam falls (more falls in males) in the Roundup Bioflow and Ranger Pro treated groups, but this outcome is likely confounded by the greater body mass of males rather than a treatment-related motor coordination effect, since there was no treatment group x sex interaction for number of falls in either group (p 's > 0.49). We did, however, observe modest evidence of forelimb grip strength reduction with glyphosate (treatment main effect p = 0.034), though no individual dose contrasts versus controls reached significance (Fig. 5). Further, the Roundup Bioflow 5 mg/kg/d dose significantly reduced hindlimb grip strength in males (p = 0.050), with a near-trending effect in Ranger Pro 5 mg/kg/d males (p = 0.110). There were no effects on hindlimb grip strength in females in the Roundup Bioflow and Ranger Pro groups, and no effect on hindlimb grip strength overall for glyphosate alone. Consistent with these latter findings, the Irwin test showed no changes in limb tone or abdominal tone, consistent with the absence of broad clinical neuromuscular toxicity. Collectively, these findings indicate that the primary neuromuscular effect of GBHs exposure is modest and sex-specific, pointing toward subtle

neuromuscular junction or peripheral motor neuron effects rather than overt muscle pathology, and that such effects are more apparent with formulated GBHs than with glyphosate alone.

Prior studies, albeit limited in number, have similarly shown that glyphosate or GBH exposure reduces muscle strength. Nozdrenko et al. reported that adult rats chronically exposed to oral glyphosate (10 µg/kg/d for 30 days) exhibited reduced soleus muscle contraction power to ~ 41% of control, with elevated oxidative stress markers, demonstrating direct glyphosate effects on muscle mechanics at a lower dose and shorter duration than used here.⁴² Also, Barbosa et al. found that offspring mice exposed to GBH over maternal gestation and lactation exhibited reduced neuromuscular junction density and increased fibrosis in the soleus muscle, consistent with the findings of Nozdrenko et al. and the reduced forelimb grip strength seen in the present study.⁴³

Importantly, these animal model studies are generally consistent with several epidemiological studies reporting associations between higher glyphosate/GBH exposures and reduced hand grip strength. For example, using NHANES data from 1,466 adults, Fang et al. reported that higher urinary glyphosate levels were associated with lower combined hand-grip strength, with the effect stronger in adults ≥ 60 years and those with BMI 25–30.⁴⁴ Further, no significant association was found with lean muscle mass, paralleling the present study's finding of neuromuscular tone effects without gross motor coordination impairment. Similarly, Jauregui-Zunzunegui et al. used NHANES data from 2,132 adults to show that each doubling of urinary glyphosate levels was associated with – 0.55 kg reduction in dominant-hand grip strength and with elevated odds of clinically low grip strength (OR 1.27–1.43).⁴⁵ Related to these outcomes, Yang et al. used NHANES data to show a positive association between urinary glyphosate levels and serum neurofilament light chain (a biomarker of axonal damage linked to low grip strength), thus providing a potential mechanistic bridge between GBH exposure and peripheral neuromuscular impairment that is consistent with the grip strength reduction observed in GBH-exposed males in the present study.⁴⁶

4.3 Associative memory for aversive events is not impaired by multi-generational glyphosate or GBHs exposure

There were no significant effects of any glyphosate or GBHs formulation or dose on step-through latency in the passive avoidance test (i.e., no treatment main effects, no sex effects, no treatment × sex interactions; all p's > 0.10). Animals in all exposure groups showed normal retention of the conditioned aversive association between the darkened compartment and the foot shock on Day 2. One interpretation of this outcome is that the hippocampal and amygdaloid circuits underlying associative fear memory, which are central to passive avoidance performance, are not meaningfully disrupted by multi-generational oral glyphosate or GBHs exposure at these doses. This null finding is noteworthy given the anxiety-like behavioral profile observed in the open field discussed above, where animals show exaggerated fear-related avoidance in the novel open field context (thigmotaxis, freezing) but retain normal associative memory for a specific aversive event. This contrast may reflect differential sensitivity of contextual vs. cue-based fear circuitry, or may indicate that the anxiety-like phenotype in the open field

reflects heightened reactivity to novelty/uncertainty rather than a generalized memory or fear-learning deficit.

The absence of a glyphosate/GBHs effect on associative memory of aversive events in the present study contrasts somewhat with findings from prior studies in the literature. For example, subchronic and chronic oral GBH exposure (250–500 mg/kg/d) in adult male mice significantly impaired both short-term (2h) and long-term (24h) passive avoidance memory.^{47,48} In subsequent studies, Ait Bali et al. reported that subchronic GBH exposure (250–500 mg/kg/d) in adult male mice impaired performance on the Barnes Maze forward probe (spatial memory) and particularly the reversal probe (cognitive flexibility), suggesting that spatial memory and cognitive flexibility may be more sensitive endpoints than aversive associative memory.⁴⁹ It is noteworthy, however, that the doses used in those studies are 5–10× higher than the maximum dose used in the present study, and were administered only in adult animals rather than across generations—two factors that may explain the divergent findings. More recently, Barnett et al. reported that mice prenatally exposed to a low glyphosate dose approximating dietary exposure (i.e., 0.01 mg/kg/d) committed more radial arm maze working memory errors, suggesting that working spatial memory may be impaired at lower doses than those required to disrupt passive avoidance.⁵⁰

While epidemiological studies have not reported outcomes comparable to the passive avoidance test tapping memory associated with aversive events, studies have reported associations between GBH exposure and other aspects of memory function. Most notably, after controlling for co-exposure to multiple other pesticides Fuhrmann et al. found that higher annual glyphosate exposure was significantly associated with lower scores on the Benton Visual Retention Test (a visual/recognition memory measure) in Ugandan smallholder farmers.⁵¹ More recently, a birth cohort study by Jenkins et al. found that at 12 months of age, urinary levels of the glyphosate metabolite AMPA were negatively associated with receptive communication, and by 24 months of age urinary levels of glyphosate and AMPA were associated with cognitive score deficits (attention, memory, perception subdomains), fine motor performance, and personal-social domains; no significant associations were found at 6 months of age.⁵² This evidence of prenatal exposure-associated cognitive impacts at 24 months (including memory subdomains) at environmentally relevant biomarker concentrations is consistent with a developmental neurotoxic effect, though the memory domain affected (verbal/receptive cognitive milestones) differs from the aversive associative memory tested in the passive avoidance paradigm used in the present study.

4.4 Study Limitations and Strengths

The study possesses some possible limitations, including: i) The continuous multi-generational exposure design, while a strength in its ecological validity, precludes determination of specific developmental windows that may be most critical for the behavioral outcomes observed. ii) The study was designed as a behavioral assessment arm of the Global Glyphosate Study and did not include neurochemical, neuroinflammatory, or neuroepigenetic endpoints in the behaviorally-tested animals. As a result, the molecular mechanisms underlying the observed anxiety-like behavior and grip strength changes cannot

be directly characterized in these animals, though they may be inferred from the mechanistic studies in the literature (discussed above). And iii) the behavioral assessments used here, while relatively comprehensive, do not tap other domains of cognitive and behavioral function such as spatial learning and memory, attention and impulse control, and social interaction that some studies have suggested are affected by glyphosate/GBHs exposure.

This study also possesses several notable strengths. These include: i) The multi-generational exposure paradigm is a comprehensive design that better approximates the multigenerational exposure scenario in environmentally-exposed humans, and it aligns with the DOHaD framework's emphasis on early-life programming. To our knowledge, this is among the first studies to assess neurobehavioral outcomes in the F2 generation following continuous oral GBHs exposure, enabling detection of effects that may emerge or be amplified across generations. ii) By spanning from the EU ADI (0.5 mg/kg/d) to the NOAEL (50 mg/kg/d), all three target dose levels tested here reflect exposures at or below current regulatory safety thresholds, making the findings directly relevant to risk assessment and regulatory decision-making. iii) The inclusion of both the pure compound (glyphosate) and two commercially relevant GBH formulations with distinct surfactant profiles (EU Roundup Bioflow / MON 52276; US Ranger Pro / EPA 524 – 517) is an important strength. The finding that GBH formulations in general produce broader and larger behavioral effects than glyphosate alone provides direct evidence that co-formulants modify toxicity in ways that may not be captured by regulatory assessments focused on glyphosate alone. iv) The behavioral assessments, which encompassed anxiety-related behaviors, gross motor coordination, neuromuscular tone, short-term associative memory, and general observational neurology, provide a multidimensional profile of neurobehavioral outcomes that are considered relevant for human diseases, including autism spectrum disorders⁵³. And v) the inclusion of both male and female offspring allows characterization of sex-dependent vulnerability, revealing that males were more affected than females in several outcomes—a finding with implications for understanding sex differences in neurobehavioral susceptibility to GBHs exposure.

4.5 Conclusions

Glyphosate and GBHs are among the most pervasive environmental chemical exposures in use today, and biomonitoring evidence shows widespread human exposure including in pregnant women and children. Here we show that multi-generational oral exposure to glyphosate and commercial GBH formulations in Sprague Dawley rats at target doses that span and do not exceed current EU regulatory limits (i.e., ADI–NOAEL) produces a coherent profile of anxiety-like behaviors, with GBH formulations causing greater effects than glyphosate alone and males showing greater susceptibility than females. Neuromuscular tone is modestly reduced by GBH formulations in male rats, while gross motor coordination and associative memory for an aversive event are unaffected. The finding that commercial GBH formulations consistently produce larger or broader neurobehavioral effects than glyphosate alone at equivalent glyphosate concentrations underscores the toxicological importance of adjuvant components in real-world herbicide formulations, and suggests that risk assessments based solely on glyphosate may underestimate the neurobehavioral risk of actual GBHs exposure to humans.

Collectively, these findings substantiate growing concerns about the neurobehavioral safety of glyphosate-based herbicides, including autism spectrum disorders, and the adequacy of current regulatory thresholds to protect human populations. They also provide strong support for incorporating multi-generational neurobehavioral assessments in regulatory evaluation of GBHs.

Abbreviations

ADI	Acceptable Daily Intake
EU	European Union
GBH	Glyphosate-Based Herbicide
GGs	Global Glyphosate Study
IARC	International Agency for Research on Cancer
NOAEL	No Observed Adverse Effect Level
NTP	National Toxicology Program
POEA	polyoxyethylene tallow amine
PND	Post Natal Day
RI	Cesare Maltoni Cancer Research Center, Ramazzini Institute
SD	Sprague-Dawley

Declarations

Authors' contributions

IM conceptualization, data curation and formal analysis, investigation, visualization, writing the original work, reviewing and editing

IT investigation, formal data analysis

SP conceptualization, data curation and formal analysis, investigation, visualization, reviewing and editing

LF investigation

RG investigation

MI investigation

MM investigation

SN investigation, visualization, reviewing and editing

DS formal analysis

VS investigation

MJP reviewing and editing the work

PJL conceptualization, reviewing and editing the work

DRS conceptualization, formal data analysis, data interpretation, writing the original work

DM conceptualization, investigation, visualization, writing the original work, reviewing and editing, supervision, project administration and resources for study conduct

Competing interests

The authors declare that they have no competing interests

Funding

The Global Glyphosate Study was supported by the Ramazzini Institute (Italy), the Heartland Health Research Alliance (USA), the Boston College (USA), the Institute for Preventive Health (USA), the Fondazione Carisbo (Italy), the Fondazione del Monte di Bologna e Ravenna (Italy), the Associazione Federide (Italy), the Coop Reno (Italy) and the Coopfond Fondo Mutualistico Legacoop (Italy).

Acknowledgements

The authors would like to acknowledge the memory of Dr. Luciano Bua and his major contributions to this work. The authors would like to honor the memory of Giulia Maroni and Daniele Rimondi, two free and generous spirits that left too soon and supported our research.

Ethics approval and consent to participate

Animal study underwent ethics approval.

Clinical Trial: Not applicable

References

1. Benbrook CM. Trends in glyphosate herbicide use in the United States and globally. *Env Sci Eur*. 2016;28(3).
2. Connolly A, Coggins MA, Koch HM. Human biomonitoring of glyphosate exposures: state-of-the-art and future research challenges. *Toxics*. 2020;8(60).
3. Dias J, Yen L, Alcon F, et al. Large scale biomonitoring of glyphosate and AMPA by analysis of human and animal feces and comparison with urine. *Environ Int*. 2026;207:110021. doi:10.1016/j.envint.2025.110021
4. In: *International Agency for Research on Cancer (IARC). Monographs on the Evaluation of Carcinogenic Risks to Humans*. Vol 112. IARC; 2015.
5. Zhang L. Exposure to glyphosate-based herbicides and risk for non-Hodgkin lymphoma: a meta-analysis and supporting evidence. *Mutat Res Rev Mutat Res*. 2019;781:186-206.
6. Maddalon A, Galbiati V, Colosio C, Mandić-Rajčević S, Corsini E. Glyphosate-based herbicides: evidence of immune-endocrine alteration. *Toxicology*. Published online July 2021. doi:10.1016/j.tox.2021.152851.
7. Milesi MM, Lorenz V, Durando M, Rossetti MF, Herbicide VJG. Reproductive Outcomes and Multigenerational Effects. *Front Endocrinol Lausanne*. 2021;12(672532). doi:10.3389/fendo.2021.672532.
8. Stone AM, Camp OG, Biernat MM, Bai D, Awonuga AO, Abu-Soud HM. Re-evaluating the use of glyphosate-based herbicides: implications on fertility. *Reprod Sci*. 2025;Apr;32(4):950-964. doi:10.1007/s43032-025-01834-6.
9. Marino M, Mele E, Viggiano A, Nori SL, Meccariello R, Santoro A. Pleiotropic outcomes of glyphosate exposure: from organ damage to effects on inflammation, cancer, reproduction and development. *Int J Mol Sci*. 2021;22;22(22):12606. doi:10.3390/ijms222212606.
10. Panzacchi S, Tibaldi E, Angelis L. Carcinogenic effects of long-term exposure from prenatal life to glyphosate and glyphosate-based herbicides in Sprague-Dawley rats. *Env Health*. 2025;10;24(1):36. doi:10.1186/s12940-025-01187-2.
11. Muñoz JP, Silva-Pavez E, Carrillo-Beltrán D, Calaf GM. Occurrence and exposure assessment of glyphosate in the environment and its impact on human beings. *Env Res*. 2023;15;231(Pt 3):116201. doi:10.1016/j.envres.2023.116201.
12. Costas-Ferreira C, Durán R, Faro LRF. Toxic effects of glyphosate on the nervous system: a systematic review. *Int J Mol Sci*. 2022;21;23(9):4605. doi:10.3390/ijms23094605.
13. Moser VC, Morris-Schaffer K, Richardson L JR, A.A. Glyphosate and neurological outcomes: a systematic literature review of animal studies. *J Toxicol Env Health B Crit Rev*. 2022;19;25(4):162-209. doi:10.1080/10937404.2022.2083739.
14. Gallegos CE, Bartos M, Bras C, Gumilar F, Antonelli MC, Minetti A. Exposure to a glyphosate-based herbicide during pregnancy and lactation induces neurobehavioral alterations in rat offspring.

NeuroToxicology. 2016;53:20-28. doi:10.1016/j.neuro.2015.11.015

15. Hsiao CC, Yang AM, Wang C, Lin CY. Association between glyphosate exposure and cognitive function, depression, and neurological diseases in a representative sample of US adults: NHANES 2013-2014 analysis. *Env Res*. 2023;237(116860).
16. Chronister BNC. Urinary glyphosate, 2,4-D and DEET biomarkers in relation to neurobehavioral performance in Ecuadorian adolescents in the ESPINA cohort. *Env Health Perspect*. 2023;131(107007).
17. Suarez-Lopez JR, Nguyen A, Klas J, et al. Associations of Acetylcholinesterase Inhibition Between Pesticide Spray Seasons with Depression and Anxiety Symptoms in Adolescents, and the Role of Sex and Adrenal Hormones on Gender Moderation. *Expo Health*. 2021;13(1):51-64. doi:10.1007/s12403-020-00361-w
18. Ait Bali Y, Ba-Mhamed S, Bennis M. Behavioral and Immunohistochemical Study of the Effects of Subchronic and Chronic Exposure to Glyphosate in Mice. *Front Behav Neurosci*. 2017;11:146. doi:10.3389/fnbeh.2017.00146
19. Buchenauer L. Maternal exposure of mice to glyphosate induces depression- and anxiety-like behavior in the offspring via alterations of the gut-brain axis. *Sci Total Env*. 2023;905(167034).
20. Madani NA, Carpenter DO. Effects of glyphosate and glyphosate-based herbicides like Roundup™ on the mammalian nervous system: A review. *Environ Res*. 2022;214:113933. doi:10.1016/j.envres.2022.113933
21. Pu Y, Ma L, Shan J, Wan X, Hammock BD, Hashimoto K. Autism-like Behaviors in Male Juvenile Offspring after Maternal Glyphosate Exposure. *Clin Psychopharmacol Neurosci*. 2021;19(3):554-558. doi:10.9758/cpn.2021.19.3.554
22. Gonzalez B, Bartels K, Talyn B. Scoping review of the relationship between glyphosate-based herbicide exposures and autism spectrum disorder. *Food Chem Toxicol*. 2025;204:115621. doi:10.1016/j.fct.2025.115621
23. Baier CJ, Gallegos CE, Raisman-Vozari R, Minetti A. Behavioral impairments following repeated intranasal glyphosate-based herbicide administration in mice. *Neurotoxicol Teratol*. 2017;64:63-72. doi:10.1016/j.ntt.2017.10.004
24. Ait Bali Y, Ba-M'hamed S, Elhidar N, Nafis A, Sora N, Bennis M. Glyphosate based- herbicide exposure affects gut microbiota, anxiety and depression-like behaviors in mice. *Neurotoxicol Teratol*. 2018;67:44-49. doi:10.1016/j.ntt.2018.04.002
25. De Castro Vieira Carneiro CL, Chaves EMC, Neves KRT, et al. Behavioral and neuroinflammatory changes caused by glyphosate: Base herbicide in mice offspring. *Birth Defects Res*. 2023;115(4):488-497. doi:10.1002/bdr2.2138
26. Shu S, Chen X, Ren J, Yu X, Zhang H, Yu Y. Glyphosate Induces Anxiety-Like Behaviors in Mice via Activating NLRP3-Mediated Hippocampal Microglia Pyroptosis. *J Appl Toxicol*. 2025;45(8):1572-1582. doi:10.1002/jat.4793

27. Mesnage R, Benbrook C, Antoniou MN. Insight into the confusion over surfactant co-formulants in glyphosate-based herbicides. *Food Chem Toxicol*. 2019;128:137-145.
28. Derbyshire E, Maes M. The Role of Choline in Neurodevelopmental Disorders—A Narrative Review Focusing on ASC, ADHD and Dyslexia. *Nutrients*. 2023;15(13):2876. doi:10.3390/nu15132876
29. Zhou J, Li S, Song Y, et al. Global, Regional, and National Trends in the Burden of Anxiety Disorders from 1992 to 2021: An Age-Period-Cohort Analysis Based on the Global Burden of Disease Study 2021. SSRN. Preprint posted online 2024. doi:10.2139/ssrn.4987862
30. Heindel JJ, Balbus J, Birnbaum L, et al. Developmental origins of health and disease: integrating environmental influences. *Endocrinology*. 2015;156(10):3416-3421.
31. Barker DJP. Developmental origins of adult health and disease. *J Epidemiol Community Health*. 2004;58:114-115.
32. Mesnage R. Use of shotgun metagenomics and metabolomics to evaluate the impact of glyphosate or Roundup MON 52276 on the gut microbiota and serum metabolome of Sprague-Dawley rats. *Env Health Perspect*. 2021;129(17005).
33. Mesnage R, Ibragim M, Mandrioli D, et al. Comparative toxicogenomics of glyphosate and Roundup herbicides by mammalian stem cell-based genotoxicity assays and molecular profiling in Sprague-Dawley rats. *Toxicol Sci*. 2022;28;186(1):83-101. doi:10.1093/toxsci/kfab143.
34. Mesnage R, Panzacchi S, Bourne E, et al. Glyphosate and its formulations Roundup Bioflow and Ranger Pro alter bacterial and fungal community composition in the rat caecum microbiome. *Front Microbiol*. 2022;13(888853). doi:10.3389/fmicb.2022.888853.
35. Panzacchi S, Mandrioli D, Manservigi F, et al. The Ramazzini Institute 13-week study on glyphosate-based herbicides at human-equivalent dose in Sprague Dawley rats: study design and first in-life endpoints evaluation. *Env Health*. 2018;17(1).
36. Doeppner TR. Effects of neural progenitor cells on post-stroke neurological impairment—a detailed and comprehensive analysis of behavioral tests.
37. Maurissen JPJ, Marable BR, Andrus AK, Stebbins KE. Factors affecting grip strength testing. *Neurotoxicol Teratol*. 2003;25(5):543-553. doi:10.1016/S0892-0362(03)00073-4
38. Moser VC. Functional Assays for Neurotoxicity Testing. *Toxicol Pathol*. 2011;39(1):36-45. doi:10.1177/0192623310385255
39. Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *Eur J Pharmacol*. 2003;463(1-3):3-33. doi:10.1016/S0014-2999(03)01272-X
40. Luong TN, Carlisle HJ, Southwell A, Patterson PH. Assessment of Motor Balance and Coordination in Mice using the Balance Beam. *J Vis Exp*. 2011;(49):2376. doi:10.3791/2376
41. Apukhtin KV, A.D. S, M.M. K, et al. Analyses of Rodent Grooming and its Behavioral Microstructure in Modern Neurobiological Studies //. *Russ J Physiol*. 2024;110.-N. 6:889-914. doi:10.31857/S0869813924060022

42. Nozdrenko D, Abramchuk O, Prylutska S, et al. Analysis of Biomechanical Parameters of Muscle Soleus Contraction and Blood Biochemical Parameters in Rat with Chronic Glyphosate Intoxication and Therapeutic Use of C60 Fullerene. *Int J Mol Sci.* 2021;22(9):4977. doi:10.3390/ijms22094977
43. Barbosa A, Zazula MF, Oliveira MCD, et al. Maternal exposure to glyphosate-based herbicide promotes changes in the muscle structure of C57BL/6 mice offspring. *Anat Rec.* 2022;305(11):3307-3316. doi:10.1002/ar.24922
44. Fang YW, Wang C, Lin CY. Association between urinary glyphosate levels and hand grip strength in a representative sample of US adults: NHANES 2013–2014. *Front Public Health.* 2024;12:1352570. doi:10.3389/fpubh.2024.1352570
45. Jauregui-Zunzunegui S, Rodríguez-Artalejo F, Tellez-Plaza M, García-Esquinas E. Glyphosate exposure, muscular health and functional limitations in middle-aged and older adults. *Environ Res.* 2024;251:118547. doi:10.1016/j.envres.2024.118547
46. Yang AM, Chu PL, Wang C, Lin CY. Association between urinary glyphosate levels and serum neurofilament light chain in a representative sample of US adults: NHANES 2013–2014. *J Expo Sci Environ Epidemiol.* 2024;34(2):287-293. doi:10.1038/s41370-023-00594-2
47. Ait Bali YA, Kaikai N eddine, Ba-M'hamed S, Bennis M. Learning and memory impairments associated to acetylcholinesterase inhibition and oxidative stress following glyphosate based-herbicide exposure in mice. *Toxicology.* 2019;415:18-25. doi:10.1016/j.tox.2019.01.010
48. Ait Bali Y, Ba-M'hamed S, Gambarotta G, Sassoè-Pognetto M, Giustetto M, Bennis M. Pre- and postnatal exposure to glyphosate-based herbicide causes behavioral and cognitive impairments in adult mice: evidence of cortical ad hippocampal dysfunction. *Arch Toxicol.* 2020;94(5):1703-1723. doi:10.1007/s00204-020-02677-7
49. Ait Bali Y, Madougou FA, Ba-M'hamed S, Giustetto M, Bennis M. Glyphosate-based herbicide exposure affects cognitive flexibility and social cognition in adult mice. *Neurosci Lett.* 2024;837:137912. doi:10.1016/j.neulet.2024.137912
50. Barnett JA, Josephson JK, Yuzbashian E, et al. Prenatal exposure to dietary levels of glyphosate disrupts metabolic, immune, and behavioral markers across generations in mice. *Sci Total Environ.* 2025;1002:180437. doi:10.1016/j.scitotenv.2025.180437
51. Fuhrmann S, Farnham A, Staudacher P, et al. Exposure to multiple pesticides and neurobehavioral outcomes among smallholder farmers in Uganda. *Environ Int.* 2021;152:106477. doi:10.1016/j.envint.2021.106477
52. Jenkins HM, Meeker JD, Zimmerman E, et al. Gestational glyphosate exposure and early childhood neurodevelopment in a Puerto Rico birth cohort. *Environ Res.* 2024;246:118114. doi:10.1016/j.envres.2024.118114
53. Panzenhagen AC, Cavalcanti A, Stein DJ, et al. Behavioral manifestations in rodent models of autism spectrum disorder: protocol for a systematic review and network meta-analysis. *Syst Rev.* 2022;11(1):150. doi:10.1186/s13643-022-02028-w

Table

Table 1. Summary of glyphosate-based herbicide (GBH) exposure effects on activity and behavior outcomes in the Open Field test. Summary reflects results from mixed model analyses for each class of GBH exposure, with GBH treatment, sex, and treatment x sex interaction as model main effects. (see Supplemental Tables 2 and 3 for a summary of the mixed model ANOVA GBH treatment and sex effects).

	Glyphosate			RoundUp			RangerPro		
Outcome	Trt ¹ Effect	Sex ²	Trt x Sex ³	Trt Effect	Sex	Trt x Sex	Trt Effect	Sex	Trt x Sex
Total distance	---	F > M	---	---	F > M	---	---	F > M	---
Avg Speed	---	F > M	---	---	F > M	---	---	F > M	---
Total Distance Wall/Total	↑	F > M	---	---	F > M	---	↑	---	---
Time in Wall Zone	---	---	---	↑	---	---	↑	---	---
Avg Duration Visits in Wall Zone	↑	M > F	---	↑	M > F	---	↑	M > F	---
Time Freezing in the Wall Zone	---	---	↑ F	↑	---	---	↑	---	---
Time Immobile in the Wall Zone	---	M > F	---	↑	M > F	---	↑	M > F	---
Grooming Behavior									
Syntactic/Total Grooming Events	↓	M > F	↓ M	↓	M > F	↓ M	↓	M > F	↓ M

1. Treatment effect = low, medium, or high dose versus controls ($p \leq 0.05$). '---' indicates no statistically significant effect ($p > 0.05$).
2. Sex effect = Relative difference between sexes, or greater treatment effect in one sex versus the other ($p < 0.05$). F = females, M = males.
3. Treatment x sex interaction = Treatment effect greater in the sex indicated ($p < 0.05$; see text).

Figures

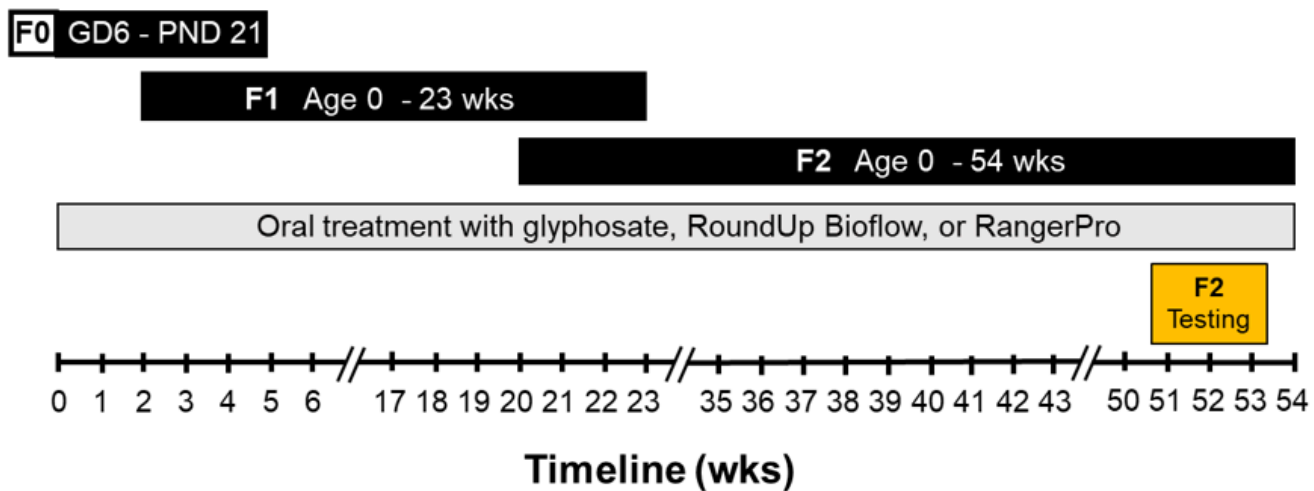


Figure 1

Study design and timeline of multigenerational glyphosate and GBH exposure across F0, F1, and F2 generations, and behavioral testing.

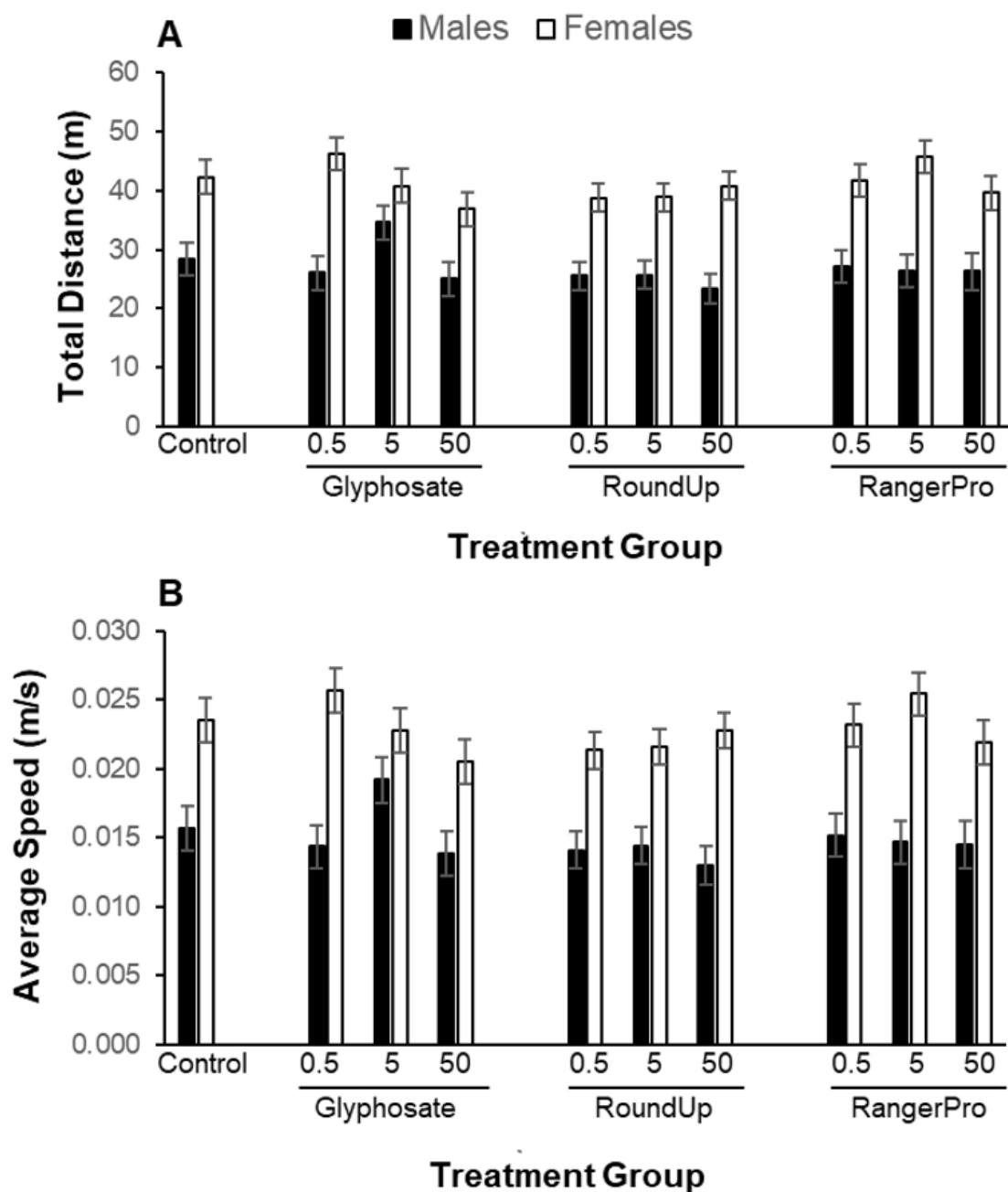


Figure 2

Glyphosate-based herbicide (GBH) exposure did not alter total distance traveled (A) or the average speed of travel (B) in females or males in the open field test. Data are LS means $\pm$ SE from separate mixed model ANOVA analyses for each GBH formulation that included control, 0.5, 5, and 50 mg/kg/d dose levels). N = 11-12 per sex and treatment group. (see Table 1 and Supplemental Table 2 for a summary of the mixed model ANOVA GBH treatment and sex effects).

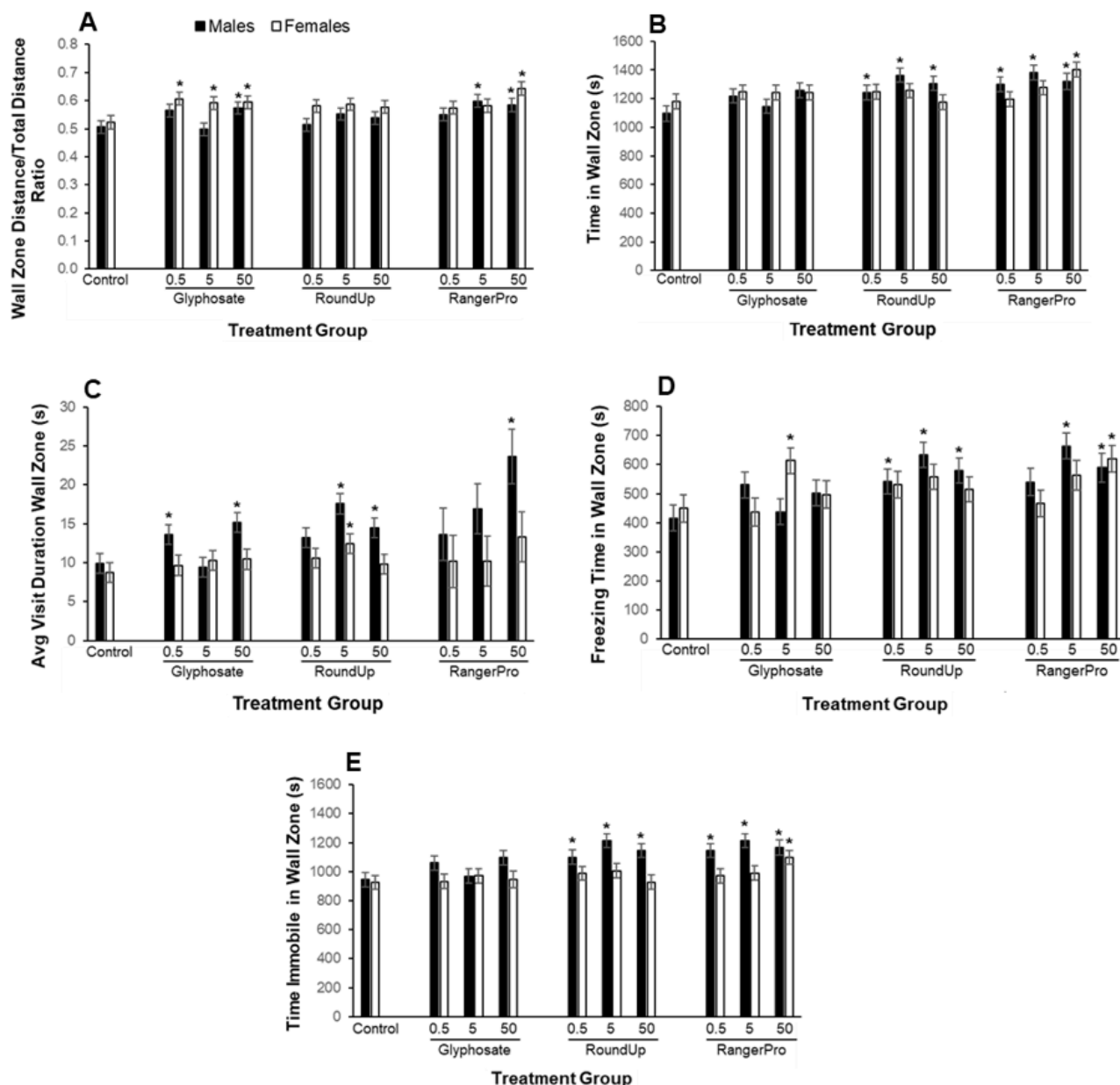


Figure 3

Glyphosate-based herbicide (GBH) exposure altered wall zone exploration behavior in the open field test, consistent with increased anxiety-like behaviors. (A) Ratio of distance traveled in the wall zone to total distance traveled. (B) Time spent in the wall zone. (C) Average duration of visits to the wall zone. (D) Total duration of freezing episodes in the wall zone. (E) Total time spent immobile (not moving) in the wall zone. Data are LS means $\pm$ SE from separate mixed model ANOVA analyses for each GBH formulation that included control, 0.5, 5, and 50 mg/kg/d dose levels). Asterisks (*) indicate significantly different from control, within sex. N = 11-12 per sex and treatment group. (see Table 1 and Supplemental Table 2 for a summary of the mixed model ANOVA GBH treatment and sex effects).

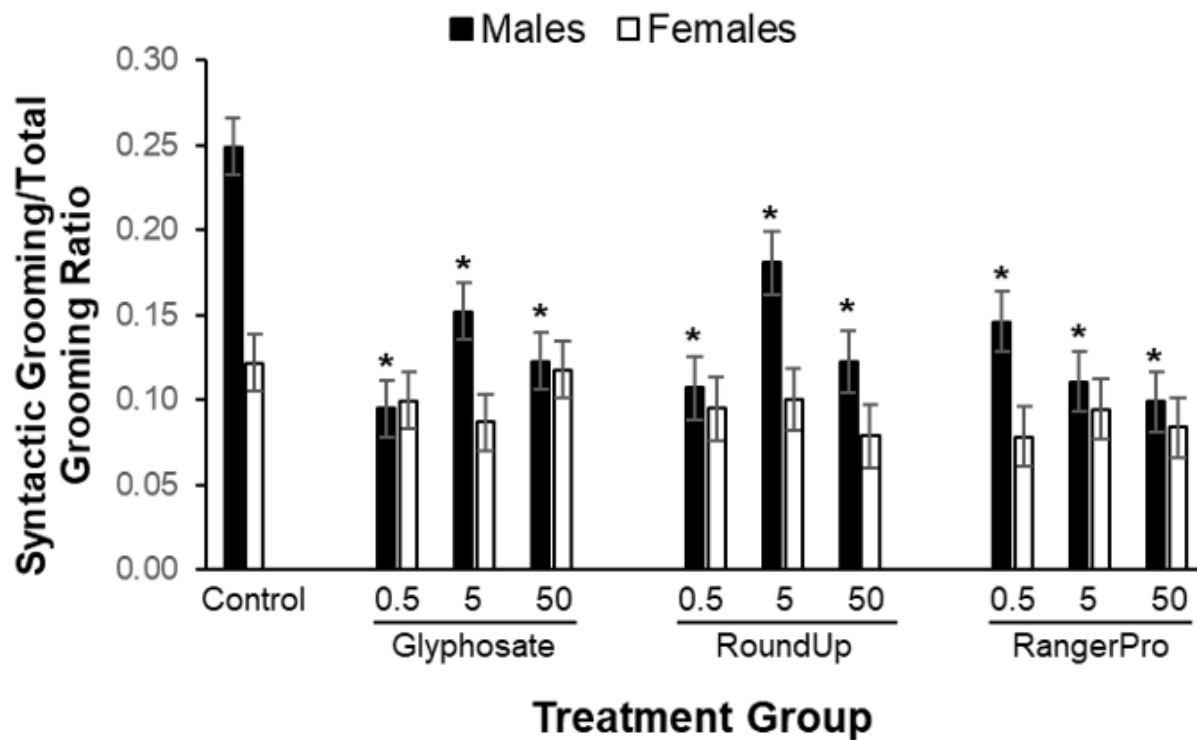


Figure 4

Glyphosate-based herbicide (GBH) exposure reduced the proportion of syntactic grooming episodes in males, consistent with increased anxiety-like behaviors. Data are the ratio of the number of syntactic grooming episodes to total grooming episodes. Syntactic grooming is a highly ordered stereotypical sequence of grooming behaviors occurring in a specific cephalocaudal pattern. Asterisks (*) indicate significantly different from control, within sex. N = 11-12 per sex and treatment group (see Table 1 and Supplemental Table 3 for a summary of the mixed model ANOVA GBH treatment and sex effects).

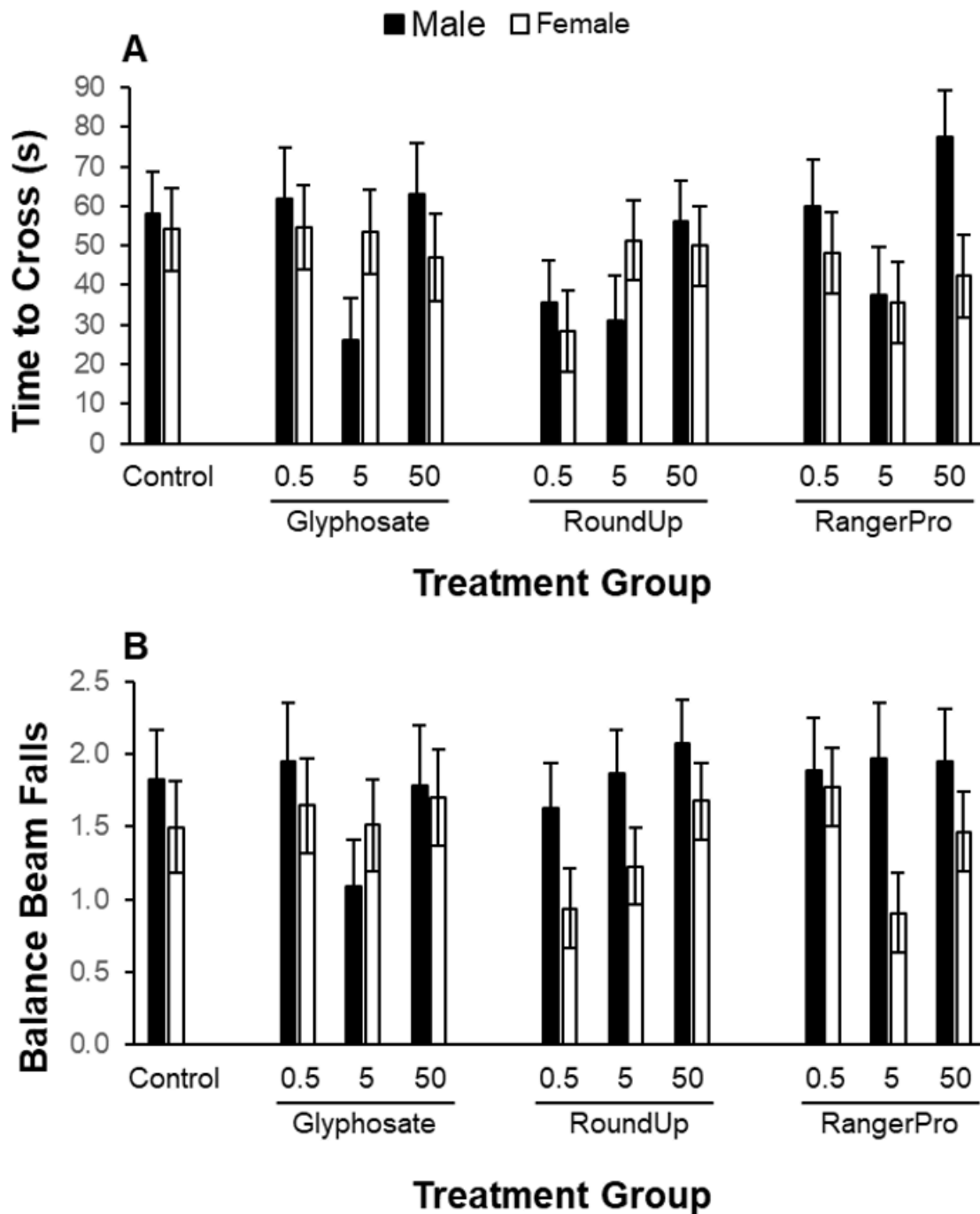


Figure 5

Glyphosate-based herbicide (GBH) exposure did not affect motor coordination on the balance beam test. Neither the time to cross the balance beam (A) or the number balance beam falls (B) were affected by any GBH formulation. There was a significant main effect of sex on the number of falls in the Roundup ($p=0.0147$) and Ranger Pro ($p=0.0319$) groups (though no treatment group $\times$ sex interaction, p 's > 0.49), with males experiencing more falls than females. $N = 11-12$ per sex and treatment group (see Supplemental Table 4 for a summary of the mixed model ANOVA GBH treatment and sex effects).

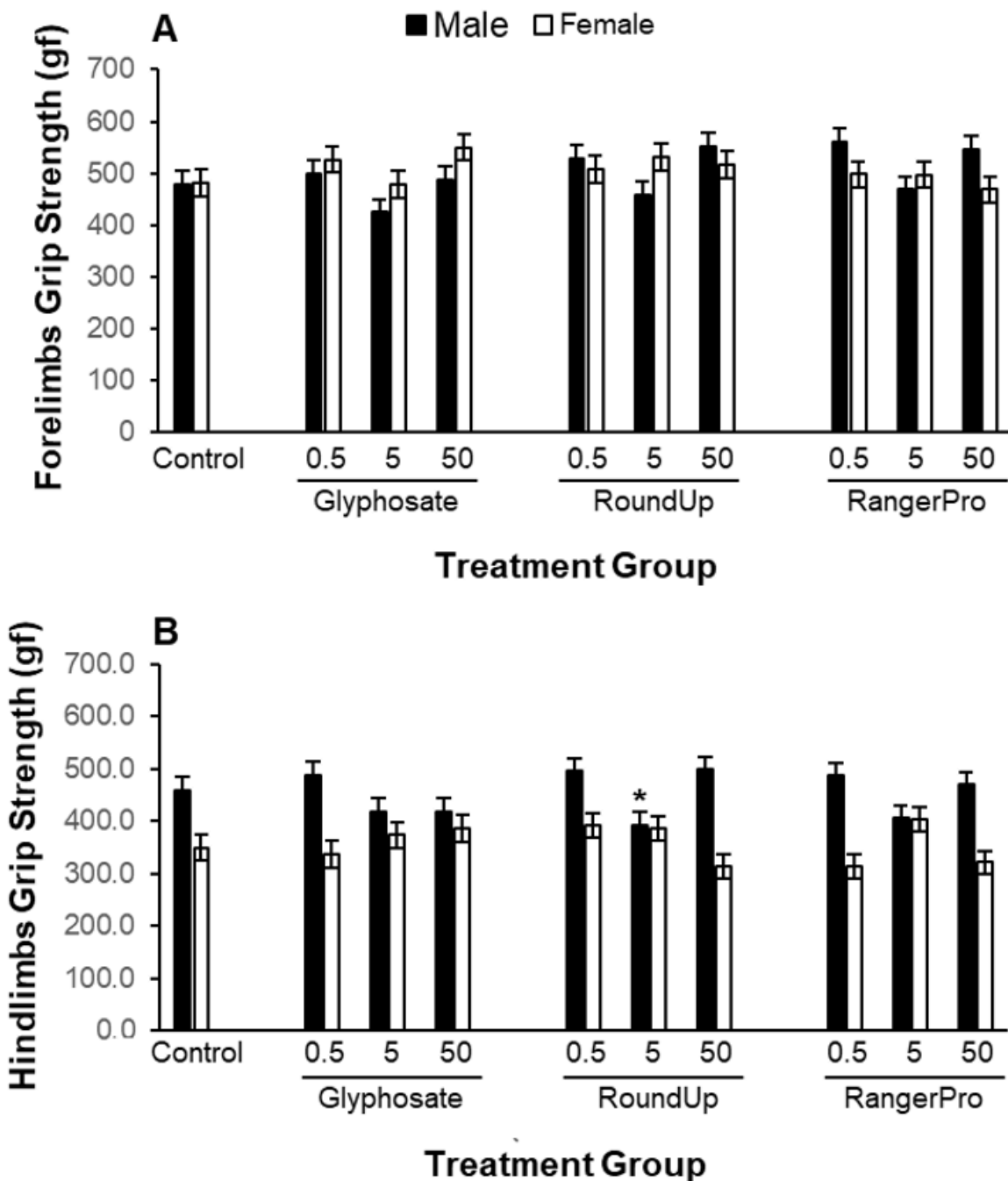


Figure 6

GBH treatment reduced grip strength in male rats, indicating modest deficits in neuromuscular tone.

(A) Forelimb grip strength was affected by glyphosate exposure, based on a glyphosate treatment main effect ($p = 0.0338$). For hindlimb grip strength (B), there were no main effects of any GBH treatment, although there was a highly significant effect of sex on hindlimb grip strength in all three GBH exposure groups (p 's < 0.0001), and a significant treatment group $\times$ sex interaction for the Roundup and Ranger Pro groups (see text). Asterisk (*) indicates significantly different from control, within sex. $N = 12$ per sex and

treatment group (see Supplemental Table 5 for a summary of the mixed model ANOVA GBH treatment and sex effects).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [MenghettietalGlyphosateSupplementalMaterials.docx](#)