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

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68 **Running title:** RI Glyphosate Pilot Study

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ABSTRACT

Background

Glyphosate-based herbicides (GBH) are the most widely used pesticides worldwide, and glyphosate is the active ingredient of such herbicides, including the formulation known as Roundup. The massive and increasing use of GBHs results in not only the global burden of occupational exposures, but also increased exposure to the general population. The current pilot study represents the first phase of a long-term investigation of GBHs that we are conducting over the next 5 years. In this paper, we present the study design, the first evaluation of *in vivo* parameters and the determination of glyphosate and its major metabolite aminomethylphosphonic acid (AMPA) in urine.

Methods

We exposed Sprague-Dawley (SD) rats orally via drinking water to a dose of glyphosate equivalent to the United States Acceptable Daily Intake (US ADI) of 1.75 mg/kg bw/day, starting from prenatal life, i.e. gestational day (GD) 6 of their mothers. One cohort was continuously dosed until sexual maturity (6-week cohort) and another cohort was continuously dosed until adulthood (13-week cohort). Here we present data on general toxicity and urinary concentrations of glyphosate and its major metabolite AMPA.

Results

Survival, body weight, food and water consumption of the animals were not affected by the treatment with either glyphosate or Roundup. The concentration of both glyphosate and AMPA detected in the urine of SD rats treated with glyphosate were comparable to that observed in

animals treated with Roundup, with an increase in relation to the duration of treatment. The majority of glyphosate was excreted unchanged. Urinary levels of the parent compound, glyphosate, were around 100-fold higher than the level of its metabolite, AMPA.

Conclusions

Glyphosate concentrations in urine showed that most part of the administered dose was excreted as unchanged parent compound upon glyphosate and Roundup exposure; the adjuvants and the other substances present in Roundup did not seem to exert a major effect on the absorption and excretion of glyphosate. Our results demonstrate that urinary glyphosate is a more relevant marker of exposure than AMPA in the rodent model.

KEYWORDS

Glyphosate, Roundup, 13-week, Sprague-Dawley rat, Glyphosate Based Herbicides, GBH

BACKGROUND

Glyphosate [IUPAC chemical name N-(phosphonomethyl)glycine] is the most widely applied pesticide worldwide and it is an active ingredient of all glyphosate-based herbicides (GBHs), including in the formulation “Roundup” [1, 2]. It is mainly marketed as a broad-spectrum systemic herbicide and crop desiccant [3]. The Asia-Pacific region represents the largest supplier of glyphosate active ingredient worldwide in terms of production. . In 2016, China contributed the largest share in the Asia Pacific, and is likely to remain a dominant market for years to come. The United State trails behind the Asia-Pacific market in the production of GBHs. Latin America, Middle East and Africa are expected to grow in terms of use at a significant rate during 2017-2025 [4]. Production and use of glyphosate have risen dramatically with the introduction in 1996 of genetically modified (GM) glyphosate tolerant crop varieties. In the United States (US) glyphosate is contained in over 750 products, particularly herbicides used for intensive GM crops that have built-in tolerance to glyphosate, but also in other products used in agriculture, forestry, urban, and

133 home applications [5]. In 2015, 89% of corn, 94% of soybeans, and 89% of cotton cropped in the
134 US were genetically modified to be glyphosate-tolerant [6]. Only a few data on the use of
135 individual pesticides are available for certain countries in the European Union (EU), making it
136 difficult to find out how much glyphosate is being used by farmers [7]. However, surveys in
137 individual countries give some indication. Glyphosate is the top ranked herbicide in United
138 Kingdom arable crop production [8]. In Denmark, glyphosate accounts for 35% of all pesticides
139 used in agricultural production [9]. In Germany, it has been estimated that glyphosate is used on 4.3
140 million hectares (39%) of agricultural land each year, with nearly two thirds applied to just 3 crops -
141 oilseed rape, winter wheat and winter barley [10]. The EU has a strict regulation regarding the
142 planting of GM crops (Directive EU 2015/412) [11] and GBHs are mainly applied to cereals for
143 post-harvest desiccation purposes (wheat, rye, triticale, barley and oats), oilseeds (rapeseed,
144 mustard seed and linseed), orchards and vineyards [12].

145 The massive and increasing use of GBHs leads to a global burden of occupational exposures in
146 manufacturing workers and GBH applicators (farmers), as well as increasing exposures in the
147 general population, as demonstrated by environmental contamination from glyphosate residues
148 found in air [13], groundwater [14, 15], drinking-water [16], crops [17, 18], food [19, 20] and
149 animal feed [21]. Microbial biodegradation of glyphosate occurs in soil, aquatic sediment and
150 water. The main pathway of biodegradation of glyphosate appears to be by splitting the C–N bond
151 to produce aminomethylphosphonic acid (AMPA), the major microbial metabolite [22]. In humans,
152 the main exposure routes to glyphosate are inhalation and dermal exposure in the occupational
153 setting and consumption of water and food for the general population [22]. The results of oral
154 studies with [^{14}C] glyphosate in rats, rabbits and goats indicate that absorption from the
155 gastrointestinal tract is incomplete and amounts to up to 30% of the dose [23-25]. The most relevant
156 routes of excretion following oral administration of glyphosate [^{14}C] are feces (70-80%) and urine
157 (20-30%) [26]. In rats, after a single oral administration of [^{14}C] glyphosate, almost all radioactivity
158 was detected in urine and feces, and the radiolabeled detected chemical was present as the

159 unchanged parent compound [27-29]. Elimination through exhaled air was very low. AMPA was
160 the only metabolite detected, accounting for only 0.2–0.3% of the applied dose of [^{14}C] glyphosate
161 [30]. The limited data currently available on glyphosate pharmacokinetics in vertebrates are
162 insufficient to predict transport and fate of glyphosate in different mammalian tissues, organs and
163 fluids in the body, and to determine whether or where bioaccumulation occurs, although animal
164 metabolism studies indicate kidney and liver as target tissues [1].

165 The possible effects of GBHs on human health is the topic of intense public debate, for both its
166 potential carcinogenic and non-carcinogenic effects, including endocrine disruption, neurotoxicity,
167 developmental and reproductive toxicity, which might occur even at doses much lower than the
168 ones considered for risk assessment, in particular during sensitive periods of life (such as fetal
169 development) [5, 12, 31, 32]. Glyphosate, as the pure active substance, and GBHs may not be quite
170 the same from the toxicological standpoint. Glyphosate formulations contain a number of so-called
171 'inert' ingredients or adjuvants to facilitate the uptake by plants, most of which are patented and not
172 publicly known (in many countries the law does not require a full disclosure of pesticide
173 ingredients). GBHs that contain surfactants and adjuvants might act differently than glyphosate
174 alone [33, 34]. In fact, adjuvants might potentiate the toxic effects of glyphosate [35-38].

175 **The Ramazzini Institute 13-week pilot study: aims and experimental design**

176 The present pilot study is the first phase of an integrated long-term project on GBHs that we are
177 conducting during the next 5 years [39]. The initial focus of our pilot study is to assess techniques
178 and methods for glyphosate detection in different matrices (results presented here), then to evaluate
179 target organ toxicity, genotoxicity and endocrine disrupting activities, together with omics and
180 microbiome alterations (not presented here). In our pilot study, we exposed Sprague-Dawley (SD)
181 rats to either glyphosate or Roundup, one of the most popular branded GBHs, with a dosage
182 considered to be "safe", the United States Acceptable Daily Intake (US ADI) of 1.75 mg/kg
183 bw/day, defined as the chronic Reference Dose (cRfD) determined by the US EPA [40]. The design
184 of the pilot study derives from the 13-week cohort protocol of the National Toxicology Program

185 (NTP) guideline Modified One-Generation study (MOG) [39, 41]. It incorporates exposure during
186 the perinatal period (*i.e.*, gestation and lactation) and later for 13 weeks after the pups are weaned,
187 evaluating standard sub-chronic toxicity and functional endpoints (*e.g.*, sperm analysis, vaginal
188 cytology, indices of puberty and sexual differentiation) to investigate possible effects on the
189 reproductive and endocrine systems. In order to provide more information about specific modes of
190 action, we further integrated the 13-week cohort NTP MOG design with transcriptome analyses of
191 potential target tissues and gut microbiome evaluation at different time-points and life stages in
192 both dams and their offspring. The whole-transcriptome analysis can provide important mechanistic
193 information and support the pathological evaluation of target organs and hormone analysis. The gut
194 microbiome evaluation is a novel endpoint representing the potential role of altered balance in the
195 gut microbiota that relate to several health disorders such as metabolic diseases, hepatic, coronary
196 and gastrointestinal diseases (*e.g.*, inflammatory bowel disease) [32]. The experimental plan and the
197 endpoints investigated in the study are presented in Table 1 and Table 2.

198 The protocol of the pilot study commences with exposure from gestation day (GD) 6 (implantation)
199 continuously through pregnancy and lactation. To satisfy the need to consider multiple effects
200 across multiple life stages, at weaning the offspring were assigned to two testing cohorts at *random*,
201 so as to have minimal differences in body weight among groups (standard deviation <10% of the
202 average). The first cohort (6-week cohort) was continuously dosed until full sexual maturity (Post
203 Natal Day-PND 73 ± 2), then sacrificed. The second cohort (13-week cohort) was continuously
204 dosed until adulthood (PND 125 ± 2), then sacrificed. Both cohorts were analyzed for post-natal
205 developmental landmarks, microbiome, target organs toxicity and clinical pathology.

206 The design of the pilot study has been developed by the Ramazzini Institute in collaboration with
207 all Institutions taking part in the overall Glyphosate Study. All of the *in vivo* experimental phases of
208 the study were performed at the Ramazzini Institute, while the other collaborating Institutions have
209 independently assessed different outcomes and endpoints of interest. In this paper, we present the
210 study design, the first evaluation of *in vivo* parameters and the determination of glyphosate and its

211 major metabolite AMPA in urine.

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213 **MATERIALS AND METHODS**

214 **1. Experimental model**

215 The study was conducted following the rules established by the Italian law regulating the use and
216 humane treatment of animals for scientific purposes [Decreto Legislativo (D.Lgs.) N. 26, 2014.
217 Attuazione della direttiva n. 2010/63/UE in materia di protezione degli animali utilizzati a fini
218 scientifici. - G.U. Serie Generale, n. 61 del 14 Marzo 2014]. Before starting, the protocol was
219 examined by the Internal Ethical Committee for approval. The protocol of the experiment was also
220 approved and formally authorized by the *ad hoc* commission of the Italian Ministry of Health
221 (ministerial approval n. 710/2015-PR). The experiment was performed on both male and female SD
222 rats, which belong to the colony used at the Cesare Maltoni Cancer Research Center laboratories of
223 the Ramazzini Institute (CMCRC/RI) for over 40 years. An animal disease screening program
224 enforced by the Italian Health Authority and Research Organization for Animal Health is in place
225 and ongoing on sentinel animals belonging to the RI colony.

226 Female breeders SD rats were placed individually in Polycarbonate cage (42x26x18cm; Tecniplast
227 Buguggiate, Varese, Italy) with a single unrelated male until evidence of copulation was observed.
228 After mating, matched females were housed separately during gestation and delivery. Newborns
229 were housed with their mothers until weaning. Weaned offspring were housed, by sex and treatment
230 group, not more than 3 per each cage. Cages were identified by a card indicating: study protocol
231 code, experimental and pedigree numbers, dosage group. A shallow layer of white fir wood
232 shavings served as bedding (supplier: Giuseppe Bordignon, Treviso, Italy). Analysis of chemical
233 characteristics (pH, ashes, dry weight, specific weight) and possible contamination (metals,
234 aflatoxin, polychlorobiphenyls, organophosphorus and organochlorine pesticides) of the bedding
235 was performed by CONSULAB Laboratories (Treviso, Italy). The cages were placed on racks,
236 inside a single room prepared for the experiment at 22°C ± 3°C temperature and 50 ± 20% relative

237 humidity. Daily checks on temperature and humidity were performed. The light was artificial and a
238 light/dark cycle of 12 hours was maintained.

239 During the experiment SD rats received *ad libitum* the standard "Corticella" pellet feed supplied by
240 Laboratorio Dottori Piccioni Srl (Piccioni Laboratory, Milan, Italy). The constituents of the diet are:
241 ground corn (23%), barley milled (15%), soybean meal extract (20.6%), wheat middling (24%),
242 wheat bran (2%), spray dried whey (2.5%), di-calcium phosphate (2%), calcium carbonate (1.1%),
243 chicken meal (6%), carob bean gum (3%), sodium chloride (0.5%), mixed vitamins (0.3%). Every
244 day, the animals drank fresh municipal tap water from glass bottles *ad libitum*. Both feed and water
245 were periodically analyzed to identify possible chemical or microbiological contaminants or
246 impurities; the analyses are included in the documentation of the experiment. The pelleted feed was
247 tested for possible glyphosate contamination in compliance with Commission Regulation (EU) No
248 293/2013 [maximum residue levels (MRLs) < 1 mg/kg]. Tap drinking water was tested for possible
249 glyphosate contamination in compliance with Directive 2008/105/EC, D.Lgs. 152/2006,
250 Directive2006/118/EC (active substances in pesticides, including their relevant metabolites,
251 degradation and reaction products < 0.1 µg/l).

252 **2.** Active ingredient glyphosate (Pestanal^{TM} analytical standard, CAS number 1071-83-6, purity >
253 99,5%) was supplied from Sigma-Aldrich (Milan, Italy). The commercial formulation Roundup
254 Bioflow (containing 360 g/L of glyphosate acid in the form of 480 g/l isopropylamine salts of
255 glyphosate (41.5%), water (42.5%) and surfactant (16%; chemical name, CAS number and/or
256 exact percentage have been withheld as a trade secret) was supplied from a local agricultural
257 consortium (Consorzio Agrario dell'Emilia, Bologna, Italy). The original containers/bottles of
258 glyphosate and Roundup were stored in its original container and kept in a ventilated storage
259 cabinet at room temperature (22°C ± 3°C) throughout the study. Purity data for each batch of
260 glyphosate and Roundup were provided by the supplier. The opening and the use date of the
261 different batches of test substances were recorded in the raw data. An aliquot of each lot of the
262 test article is maintained in the ventilated storage cabinet, until 5 years from the end of the main

experiment. The solutions of glyphosate and Roundup were prepared by the addition of appropriate volume of tap drinking water.**Experimental plan**

Each of twenty-four virgin female SD rats (17 weeks old, 270-315g) was cohabited outbred with one breeder male rat of the same age and strain. Every day, the females were examined for presence of sperm. Gestational day (GD) 0 was defined as the one in which the sperm was found in vaginal smears. The day on which parturition was completed was designated as lactating day (LD) 0 for the dam and PND 0 for the offspring. Each dam and delivered litter was co-housed in common nesting box during the postpartum period. Following the NTP MOG design, on PND 28, thus 28 days after the last litter was delivered, the offspring were weaned and identified by ear punch according to the Jackson Laboratory system. Sequentially, they were allocated in the same treatment group of their mother in order to have 18 males (8 for the 6-week cohort and 10 for the 13-week cohort) and 18 females (8 for the 6-week cohort and 10 for the 13-week cohort) for each dose group. No more than 2 males and 2 females from the same litter were included in the same cohort/treatment group. Altogether, 108 SD rats (54 males and 54 females) were enrolled in the post-weaning treatment phase. The experimental plan of the pilot study is outlined in Table 1. A summary of the endpoints and relative monitoring time points evaluated in the pilot study, both in dams and in the offspring (6-week and 13-week cohorts) is presented in Table 2.

Two groups of SD rats were treated with either glyphosate or Roundup diluted in tap water administered *ad libitum* and one group received only tap water as control. Roundup was diluted in tap water in order to obtain an equivalent dose of glyphosate of 1.75 mg/kg bw/day. During gestational and lactational periods, embryos and newborns (F1) received the test compounds mainly through their dams (F0). Glyphosate and Roundup water formulations during these periods were freshly prepared on a daily base depending on individual body weight and water consumption of dams as measured at each scheduled time point (see below). After weaning, until the end of the experiment (PND 73 ± 2 or 125 ± 2), the test substances were administered in tap water to F1 animals on the basis of the average body weight and average water consumption *per* sex and *per*

experimental group, as measured at each scheduled time point (see below). Males and females were considered separately because of their difference in weight gain, body weight and water consumption.

At least every week, the exposure doses were recalculated and registered. The actual levels of test compounds that reached the fetus during gestation or that were ingested postnatally by the offspring during the period of lactation were not estimated in the present study.

Animals were monitored during the entire experimental period. The following procedures were performed:

- Health status control: from the start of the experiment, animals were checked three times daily, except on Sundays and non-working days, when they were only checked twice. All observed variations from normal status were recorded.
- Clinical control: status, behavior and clinical observation on the experimental animals were checked before the start of the treatment, and at least every two days until the end of the experiment. Any findings listed below were then recorded: alterations of skin, hair, eyes and mucosa; modification in production of secretions or excretions and in autonomic activity; respiratory symptoms; postural changes or changes in walk; presence of tonic or clonic contractions; unusual stereotypes and behavior.

Dams' body weights were recorded on GD 0, 3, 6 and then daily during gestation until parturition. During lactation, dams' body weights were recorded at LD 1, 4, 7, 10, 13, 16, 19, 21 and 25 (last measurement before weaning). Pups' body weight by sex and litter was determined on PND 1, 4, 7, 10, 13, 16, 19, 21 and 25. After weaning, the body weight was measured twice a week, until PND 73 ± 2, then weekly until PND 125 ± 2 and before terminal sacrifices; the means of individual body weights were calculated for each group and sex.

Dams' feed and water consumption were recorded twice weekly during gestation (GD 0, 3, 6, 9, 12, 15, 18, 21), whereas during lactation were measured at LD 1, 4, 7, 10, 13, 16, 19, 21, 25 and 28.

After weaning the daily water and feed consumption *per* cage were measured twice a week, until PND 73 ± 2, then weekly until PND 125 ± 2; the means of individual consumptions were calculated for each group and sex.

The day before the terminal sacrifices, all the animals were located individually in metabolic cages and starved for around 16 hours. During this time, the animals had free access to water alone or to the programmed test compound solutions. The day after, in the morning, samples of at least 5ml of spontaneous urine from each animal were collected and put in separate labelled tubes. Urine samples for analysis of glyphosate and AMPA excretion were obtained from 3 dams/group and from 10 (5 males + 5 females) rats/group belonging to the 6-week and 13-week cohorts.

3. Glyphosate and aminomethylphosphonic acid (AMPA) detection

Analyses of glyphosate and its metabolite AMPA in drinking water, feed and urine were performed by Neotron Laboratories (Modena, Italy), an officially accredited laboratory by Accredia (Lab. N. 0026) according to European regulation UNI CEI EN ISO/IEC 17025:2005. The specification and results are maintained in the experimental documentation. The analytical method is based on liquid chromatography tandem mass spectrometry (LC-MS/MS) [42-45]. The limit of quantification (LQ) for glyphosate and AMPA corresponded to 0.10 µg/l in water, 50 µg/kg in feed, and 1 µg/kg in urine.

4. Statistical analysis

Summary statistics, means ± standard deviations (sd), were calculated for continuous variables. For body weight, water and feed consumption over time further analyses were performed using multilevel mixed-effect linear regression models, to control for within subject correlation across time; moreover we have considered also the litter effect during the lactation period. Analysis of variance and Dunnett's tests (when applicable) were also performed to compare body weight gain in different periods and consumption of food and water as mean consumption in several periods.

339 All tests were two tailed, with alpha set at 0.05. Statistical analyses were perfomed by using
340 STATA version10 (Stata Corporation, College StationTexas, USA).

341

342 **RESULTS**

343 In dams, during both gestation and lactation, body weight and weight gain were not statistically
344 different among the different groups (Fig.1 A-B). In both female and male offspring, post weaning
345 body weights were homogenous and no statistically significant differences in body weight gain
346 were observed among groups (Fig 1 C-F). All 24 dams and 108 SD rats from the 6-week (48/48)
347 and 13-week (60/60) cohorts survived until sacrifice.

348 Water and feed consumption during gestation and lactation were no different across the groups
349 (Fig. 2 A-B and Fig. 3 A-B). Litter sizes were fully comparable among groups, with mean number
350 of live pups: control group 13.6 (range 10-16); glyphosate group 13.3 (range 11-17); Roundup
351 group 13.9 (range 11-16). Post weaning water and feed consumption were not affected by the
352 treatment (Fig. 2 C-D and Fig. 3 C-D).

353 No unexpected clinical signs or symptoms were observed in the experimental animals during the *in*
354 *vivo* phase. In particular, there was no clinical evidence of alterations in activity or behavior,
355 reflexes, the eye or skin, or the respiratory, gastrointestinal, genito-urinary and cardiovascular
356 systems.

357 The results of glyphosate and AMPA urinary concentrations are reported in Table 3 and Figure 4.
358 The urinary concentration of both glyphosate and AMPA of SD rats treated with 1.75 mg/kg
359 bw/day of glyphosate were comparable to the ones observed in SD rats treated with Roundup dose
360 equivalent to 1.75 mg/kg bw/day, despite limited sample size and the large standard deviations. In
361 the control group, as expected, the glyphosate and AMPA urinary levels were all below or close to
362 the limit of quantitation (0.001 mg/kg). In the treated SD rats, the majority of glyphosate was
363 excreted unchanged (as parent compound), with urinary levels about 100-fold higher than that of its

364 metabolite AMPA. For example, glyphosate and Roundup treated females in the 13-week cohort
365 presented mean urinary levels of glyphosate respectively of 1.354 mg/kg and 1.524 mg/kg, while
366 the AMPA levels were respectively 0.013 mg/kg and 0.021 mg/kg. In glyphosate and Roundup
367 treated SD rats, a time-dependent increase in the mean urinary concentration of glyphosate was
368 observed. In glyphosate and Roundup treated males, an approximate 2-fold increase in the 13-week
369 cohort (animals exposed prenatally until 125±2 days after birth) compared to the 6-week cohort
370 (animals exposed prenatally until 73±2 days after birth) was observed. In glyphosate treated
371 females, the 6-week cohort (animals exposed prenatally until 73±2 days after birth) showed a 2-fold
372 higher value than the dams after weaning (exposed for 49±2 days), while the 13-week cohort
373 (animals exposed prenatally and 125±2 days after birth) showed a 1.5-fold increase compared to the
374 6-week cohort. In the Roundup treatment group, the increase was less steep, but the time-dependent
375 pattern was still evident. In glyphosate and Roundup treated SD rats, the levels of AMPA were
376 comparable at the different time points in both males and females. In these animals, large standard
377 deviations of the values of AMPA concentrations in urine have been observed, in particular for
378 values close to the limit of quantitation as in the control groups.

379 **DISCUSSION**

380 Survival, body weights, food and water consumption of SD rats were not affected by the treatment
381 with glyphosate and Roundup. Clinical changes in the animals were not observed in the various
382 groups. Overall, both glyphosate and Roundup treatments seemed to be well tolerated, which is
383 consistent with previous experiments performed by the US NTP [26].

384 Glyphosate and Roundup exposure led to comparable concentrations of glyphosate and AMPA in
385 urine, indicating that systemic exposure does occur at the selected exposure level of 1.75 mg//kg
386 bw/day, corresponding to the US ADI. The bioavailability of glyphosate in our study is also
387 supported by the evident increase of glyphosate concentration in urine in relation to the length of
388 treatment. The adjuvants and the other substances present in Roundup did not seem to exert a
389 major effect on the absorption and excretion of glyphosate, even though mean values of glyphosate

390 seem to be somewhat higher in the Roundup treated group. The levels in urine were also
391 comparable between the two sexes; however, a consistent inter-individual variability was observed.
392 In rats, glyphosate in urine appears to be the most accurate biomarker of exposure to GBHs. In fact,
393 our results confirm previous evidence that in rodents most of the administered dose of glyphosate
394 (98%) is excreted as unchanged parent compound, whereas the metabolite AMPA in urine is at
395 around 0.2-0.3% of the administered dose [46]. Furthermore, with the level of exposure to
396 glyphosate used in this pilot study, AMPA urinary values of treated animals (0.011-0.027 mg/kg)
397 were already close to the chromatographic LQ (0.001 mg/kg) and this might limit the reliability of
398 the measures. On the other hand, glyphosate concentration in urine of treated animals (0.480-2.280
399 mg/kg) resulted up to 100-fold higher than the AMPA concentration and at least 500-fold higher
400 than the chromatographic LQ (0.001 mg/kg). Therefore, in order to assess exposure to glyphosate in
401 rats, in particular at doses that are equal or lower than the one used in this pilot study (1.75 mg/kg
402 bw/day), glyphosate appears to be the biomarker of choice.

403 The presence of negligible levels of glyphosate (0.003-0.013 mg/kg), close to the chromatographic
404 LQ (0.001 mg/kg), in some of the urine of the control groups might reflect an ubiquitous
405 environmental contamination at ultra-low doses of glyphosate, which is consistent with previous
406 reports from other authors [21]. As the current limit of quantitation of glyphosate in HPLC for
407 pelleted animal feed is 0.050 mg/kg, this represents a technical limiting factor for testing ultra-low
408 doses of glyphosate. As reported by a recent inter laboratory comparative study on the quantitative
409 determination of glyphosate at low levels, caution should be taken when interpreting results if the
410 tested doses of glyphosate are close to the LQ of HPLC [47].

411 It is noteworthy that the commercial formulation used in this study, Roundup Bioflow, was the
412 representative formulated product recently evaluated for the renewal of the approval of glyphosate
413 in EU and considered in the European Food Safety Authority peer review (MON 52276)[48].

414 Our results seem particularly relevant in light of the massive global burden of exposure to
415 glyphosate, as shown by the exponential increase in the last 20 years of the levels of glyphosate and

416 AMPA measured in the urine of the general population in Germany [49] and in the US [50].

417 **CONCLUSION**

418 We performed a pilot study on the health effects of glyphosate and its formulation Roundup
419 administered at currently admitted doses (US ADI = 1.75 mg/kg bw/day) to SD rats. In this paper,
420 we described the study design, the first evaluation of *in vivo* parameters and the determination of
421 glyphosate and its major metabolite AMPA in urine. The treatment with either glyphosate or
422 Roundup seemed to be overall well tolerated, consistently with previous experiments performed by
423 the US NTP [26]. Both glyphosate and Roundup exposure led to comparable urinary concentrations
424 of glyphosate and AMPA with an increasing pattern of glyphosate excreted in urine in relation to
425 the duration of treatment, indicating the systemic bioavailability of the active substance. The
426 adjuvants and the other substances present in Roundup did not seem to exert a major effect on the
427 absorption and excretion of glyphosate. Our results confirm that, in rodents, glyphosate in urine is
428 the much more relevant marker of exposure than AMPA in particular at doses that are equal or
429 lower than the one used in this pilot study (1.75 mg/kg bw/day). The evaluation of different
430 outcomes and endpoints of interest (i.e., pathology of target organs, molecular toxicity,
431 genotoxicity, endocrine disrupting activities, microbiome, developmental toxicity, etc.) is currently
432 ongoing in the different partner laboratories of the project.

433

434 **ABBREVIATIONS:**

435 GBH: Glyphosate-based herbicides; AMPA: aminomethylphosphonic acid; SD: Sprague-Dawley;
436 CMCRC: Cesare Maltoni Cancer Research Center; RI: Ramazzini Institute; US ADI: United States
437 Acceptable Daily Intake; GD: gestational day; LD: lactating day; GM: genetically modified; EU:
438 European Union; NTP: National Toxicology Program; MOG: Modified One-Generation study;
439 PND: Post Natal Day; LC-MS/MS: liquid chromatography tandem mass spectrometry; LQ: Limit
440 of Quantification.

DECLARATIONS:

Ethics approval and consent to participate: N/A

Consent for publication: N/A.

Availability of data and material: all raw data recorded and used during the current study are available from the corresponding author on reasonable request

Competing interests: the authors declare that they have no competing interests

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Authors' contributions: All authors provided substantial contributions to the conception/design of the work, acquisition, analysis or interpretation of the data, revised the manuscript critically, and approved the final version for submission. FM, SP, DM participated in the design of the study, performed the animal experiments and sample collection, and drafted the manuscript. LB and LF performed the animal experiments and sample collection. FB supervised the study, participated in the design of the study and helped to draft the manuscript. MS, GG, GD, RM, AM, SL, JH, JC, MJP, PJL, helped to draft the manuscript. All authors read and approved the final manuscript.

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595

596 **FIGURES TITLES AND LEGENDS**

597 Figure 1. Average body weight: dams during gestation (A), treatment starting at gestation day 6
598 (↓); dams (B), male (C) and female (D) offspring during lactation; male (E) and female (F)

599 offspring after weaning. At week 6 after weaning 8 male and 8 female pups per group were
600 sacrificed.

601

602 Figure 2. Average water consumption: dams during gestation (A), treatment starting at gestation
603 day 6 (↓); dams and litter (B) during lactation; male (C) and female (D) offspring after weaning. At
604 week 6 after weaning 8 male and 8 female pups per group were sacrificed.

605

606 Figure 3. Average feed consumption: dams during gestation (A), treatment starting at gestation day
607 6 (↓); dams and litter (B) during lactation; male (C) and female (D) offspring after weaning. At
608 week 6 after weaning 8 male and 8 female pups per group were sacrificed.

609

610 Figure 4. Average urinary concentrations of glyphosate and AMPA, expressed in mg/kg, collected
611 at terminal sacrifices. Dams glyphosate (A) and AMPA (B) excretion; 6-week cohort male and
612 female offspring; glyphosate (C) and AMPA (D) excretion; 13-week cohort male and female pups
613 Glyphosate (E) and AMPA (F) excretion.

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20 Table 1. Experimental plan

Breeders			Offspring				Treatment [b]			End of the experiment	
Group	Animals		Group	Animals [a]							
					Cohort					Cohort	
	Sex	No.	N.	Sex	6-week (No.)	13-week (No.)	Compound	Dose[c]	Age at start [d]	6-week (PND)	13-week (PND)
I	F	8	I	M	8	10	Control (drinking water)	0	GD 6	70[e]	120[f]
	M	8		F	8	10					
	F+M	16		M+F	16	20					
II	F	8	II	M	8	10	Glyphosate	US ADI	GD 6	70[e]	120[f]
	M	8		F	8	10					
	F+M	16		M+F	16	20					
III	F	8	III	F	8	10	Roundup	US ADI Glyphosate equivalent	GD 6	70[e]	120[f]
	M	8		M	8	10					
	F+M	16		F+M	16	20					
TOTAL	M+F	48		M+F	48	60					

[a] No more than 2 sisters and 2 brothers per litter

[b] Test compounds are administered *ad libitum* in drinking water

[c] Doses are calculated considering the Glyphosate US ADI (1.75 mg/kg bw/day)

[d] Solutions are admistered to dams starting from the 6th day of pregnancy

[e] Animals are treated until the landmarks of sexual development are acquired (PND 73 ± 2).

[f] Animals are treated from embryonic life (GD 6) indirectly from dams milk until PND 28 ± 2, then directly for 90 days after weaning (until PND 125 ± 2)

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22

23 Table 2. Summary of the endpoints and relative monitoring time points evaluated in the study, in dams and offspring (6-week and 13-week cohorts).

Endpoints	Time points	Dams	Offspring 6-week cohort	Offspring 13-week cohort
Gestation length	GD0-delivery	✓	–	–
AGD and body weight in male and female pups	PND 1	–	✓	✓
Litter size	PND 1, 4, 7, 10, 13, 16, 19, 21, 25	–	✓	✓
Live-birth index	PND 1	–	✓	✓
Survival index	PND 4, 7, 10, 13, 16, 19, 21, 25	–	✓	✓
Age and body weight at BPS in male pups	PND 35	–	✓	✓
Age and body weight at VO in female pups	PND 28	–	✓	✓
First estrous in female pups	3 days after VO	–	✓	–
Estrous cycle length and percentage of days in each stage	PND 95 - PND 116	–	–	✓
Estrous cycle prior to necropsy	PND 125 ± 2	–	–	✓
Serum hormone measures	End of lactation (dams), PND 73 ± 2 and PND 125 ± 2	✓	✓	✓
Clinical biochemistry	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Urinalysis	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Glyphosate and AMPA detection in urine	End of lactation (dams), PND 73 ± 2 and PND 125 ± 2	✓	✓	✓
Sperm counts	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Daily Sperm production	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Sperm transit time through the epididymis	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Sperm morphology	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Sperm aneuploidy	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Partial histopathology (reproductive organs, brain, liver, kidney)	End of lactation (dams)	✓	–	–
Complete histopathology	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Organ weight	End of lactation (dams), PND 73 ± 2 and PND 125 ± 2	✓	✓	✓
Micronuclei test (bone marrow)	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Transcriptome on mammary glands	End of lactation (dams), PND 73 ± 2 and PND 125 ± 2	✓	✓	✓
Transcriptome on brain	PND 125 ± 2	–	–	✓
Transcriptome on liver	End of lactation (dams), PND 73 ± 2 and PND 125 ± 2	✓	✓	✓
Transcriptome on kidneys	End of lactation (dams), PND 73 ± 2 and PND 125 ± 2	✓	✓	✓
Microbiome analysis in dams	Before mating, GD 5 (before treatment), GD 13, LD 7, LD 14	✓	–	–
Microbiome analysis in offspring	PND 7, PND 14, PND 31 (before puberty), PND 57 (after puberty), PND 125 ± 2 (adulthood)	–	✓	✓

24 GD: gestation day; LD: lactation day; PND: postnatal day; AGD: anogenital distance; VO: vaginal opening; BPS: balano preputial separation

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27 Table 3. Glyphosate and AMPA concentration in urine

	Treatment	Dams		Offspring (6-week cohort)		Offspring (13-week cohort)	
		Glyphosate (mg/kg)	AMPA (mg/kg)	Glyphosate (mg/kg)	AMPA (mg/kg)	Glyphosate (mg/kg)	AMPA (mg/kg)
Male	Control			0.012 ± 0.010	0.003 ± 0.003	0.011 ± 0.010	0.006 ± 0.004
	Glyphosate	_	_	0.938 ± 0.414	0.014 ± 0.007	1.684 ± 0.768	0.023 ± 0.012
	Roundup			1.174 ± 0.439	0.011 ± 0.005	2.280 ± 1.520	0.027 ± 0.016
Female	Control	0.009 ± 0.001	0.006 ± 0.002	0.013 ± 0.007	0.005 ± 0.001	0.008 ± 0.005	0.003 ± 0.005
	Glyphosate	0.480 ± 0.010	0.024 ± 0.002	0.938 ± 0.377	0.016 ± 0.010	1.354 ± 0.359	0.013 ± 0.006
	Roundup	0.700 ± 0.106	0.024 ± 0.001	0.910 ± 0.383	0.018 ± 0.007	1.524 ± 0.585	0.021 ± 0.007

28 *results are reported as mean ± standard deviations

29

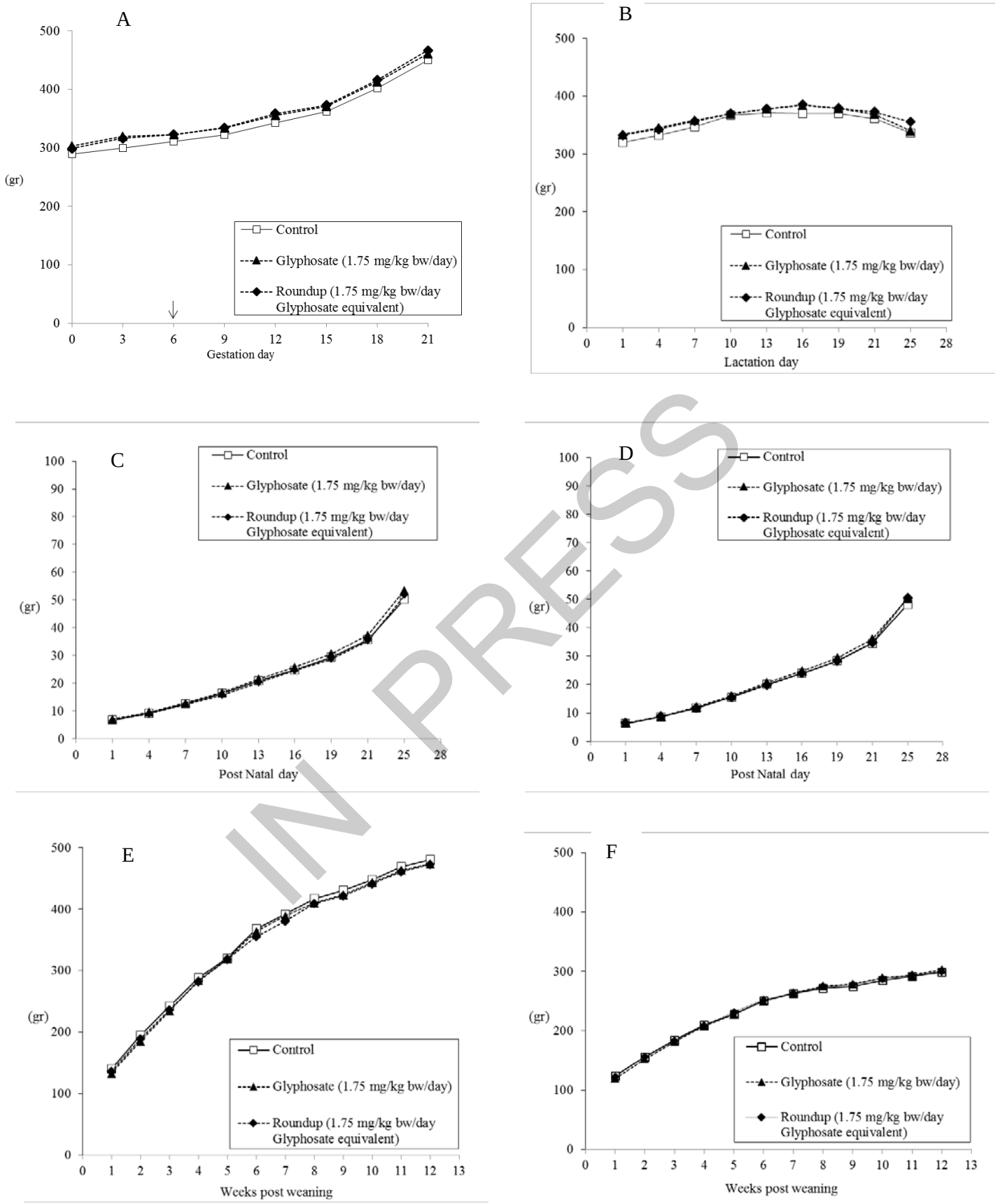

Figure 1.

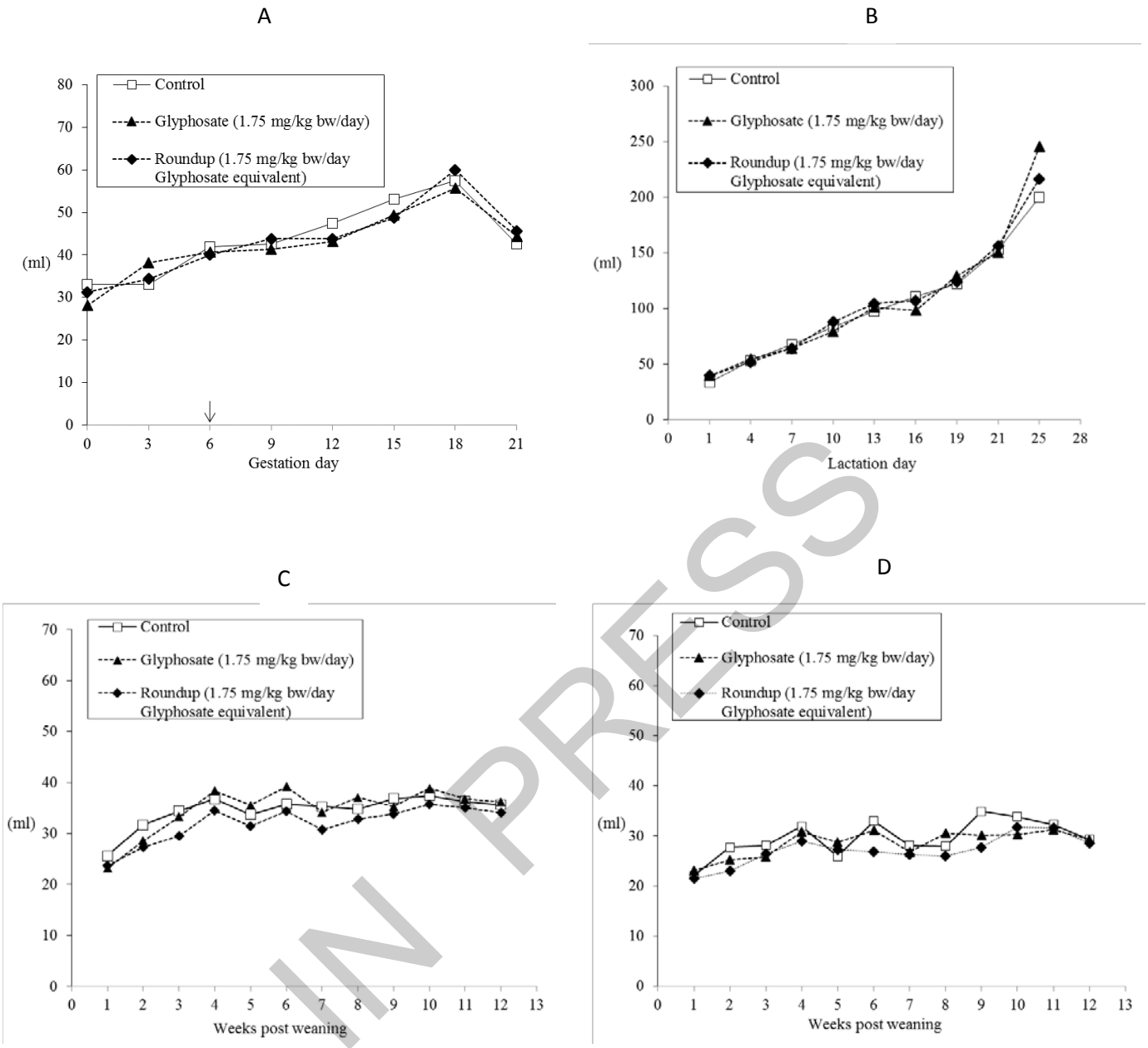

Figure 2

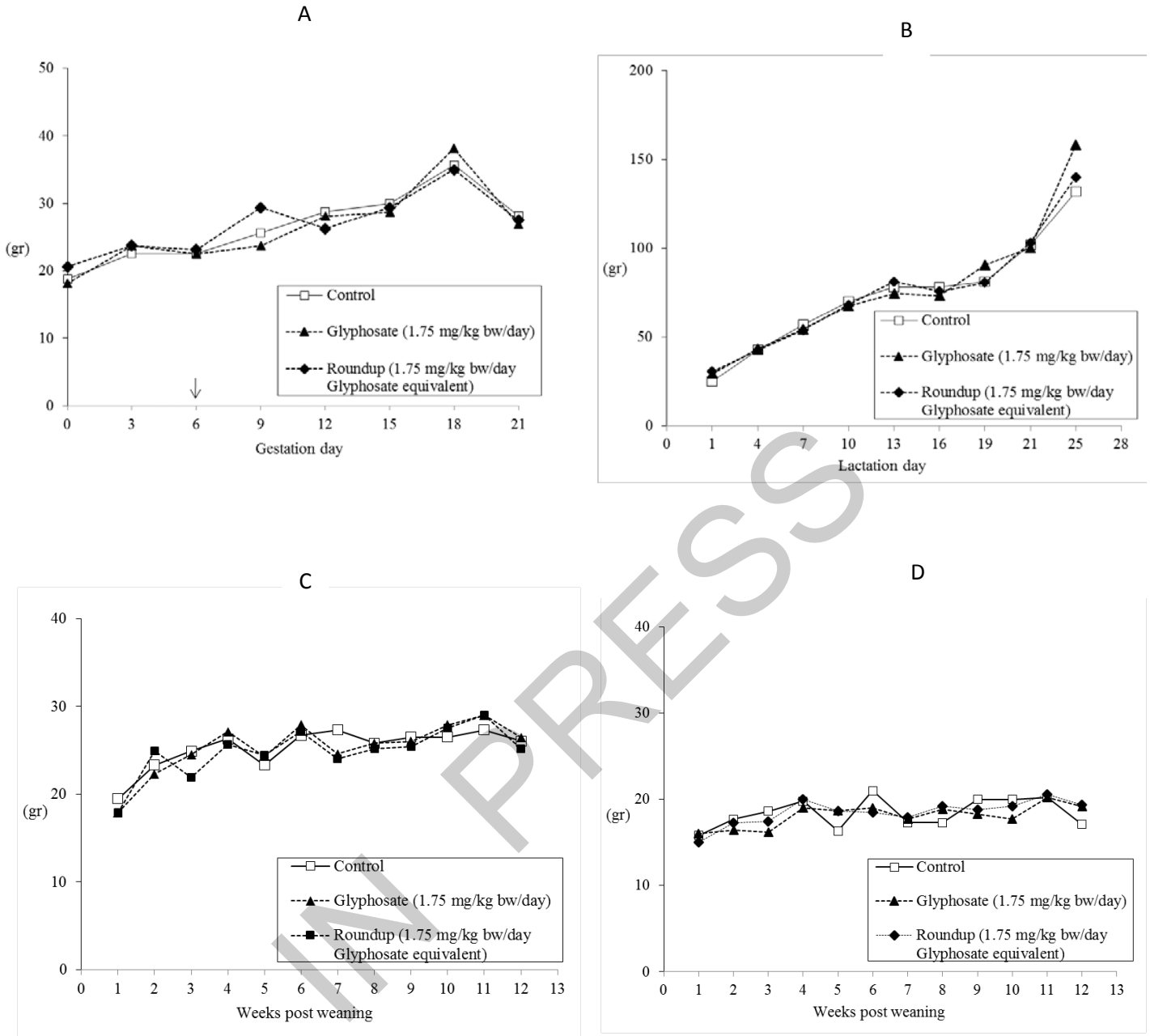

Figure 3

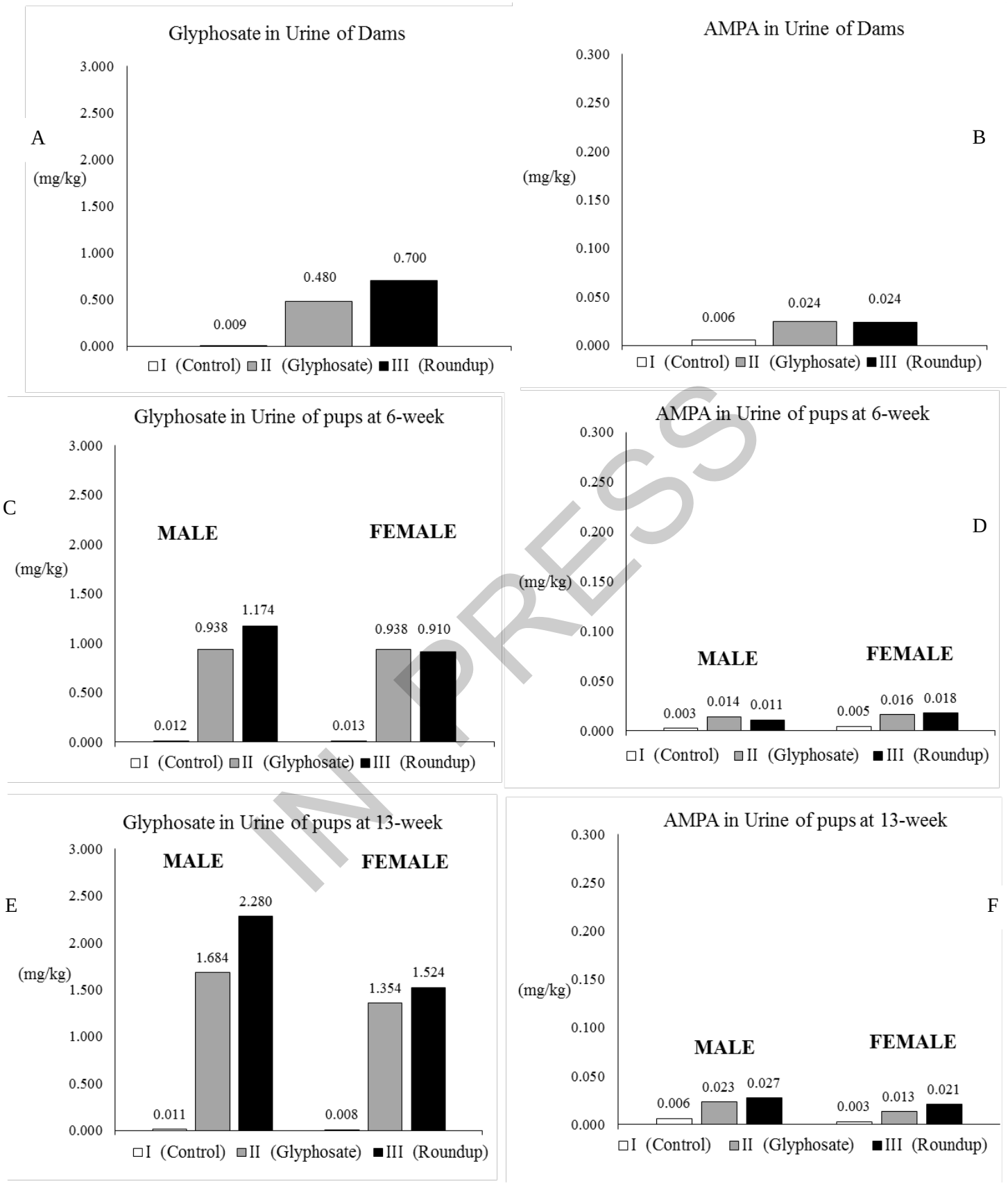

Figure 4