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Thirteen-week toxicity study in rats exposed to acetamiprid, glyphosate, tebuconazole and their mixture from prenatal life

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ABSTRACT

Background: The SPRINT project is an EU-funded initiative aimed at promoting a transition toward more sustainable plant protection. Growing evidence shows that combined pesticide exposures might have additive or synergistic toxic effects.
Objective(s): We conducted an *in vivo* rodent study on three widely used pesticides—glyphosate, tebuconazole, and acetamiprid—and their mixture, focusing on prenatal exposure at doses considered safe, to better understand long-term health risks.
Methods: Three pesticides (glyphosate, tebuconazole, acetamiprid) were administered in drinking water to male and female Sprague-Dawley rats, starting from prenatal life until 13 weeks after weaning. The main toxicological endpoints were evaluated, including hematological, biochemical, endocrine development parameters, and histopathological analysis.
Findings: In males a statistically significant increased trend in aspartate aminotransferase was observed in tebuconazole, acetamiprid and mixtures, while in females a trend of increased aspartate aminotransferase was observed in glyphosate and mixture. Other increases in lesions were observed in the cardiocirculatory system and genital system.
Conclusions: In our present study an important design characteristic was the prenatal exposure. We observed adverse effects at doses currently considered "safe", namely equal or lower than the No Observed Adverse Effect Level in rodents and other animal models for glyphosate, acetamiprid, tebuconazole and their mixture. Based on our results, a mixture approach indicated additivity in toxicity and no signs of synergism nor antagonism were observed.

1. Background

The SPRINT project (on Sustainable Plant Protection Transition), funded under the EC program H2020 and launched in September 2020 to contribute, both at regional and European level, to accelerate the adoption of innovative transition pathways towards more sustainable plant protection in the context of a global health approach (https://sprint-h2020.eu/). A variety of pesticides, used to eliminate, repel or control unwanted organisms, include insecticides (for insects), herbicides (for weeds) and fungicides (for fungi) (Hassaan and El Nemr,

Abbreviations: ADI, Acceptable Daily Intake; CMCRC, Cesare Maltoni Cancer Research Center; AST, Aspartate Aminotransferase; EU, European Union; GBH, Glyphosate-Based Herbicide; IARC, International Agency for Research on Cancer; INHAND, International Harmonization of Nomenclature and Diagnostic Criteria; NOAEL, No Observed Adverse Effect Level; NTP, National Toxicology Program; RI, Cesare Maltoni Cancer Research Center, Ramazzini Institute; SD, Sprague Dawley; SPRINT, Sustainable Plant Protection Transition.

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2020). Modern conventional agriculture is highly dependent on the massive use of pesticides, that are commonly applicated in a way that results in mixtures of different pesticides in the same crop due to multiple pesticide applications usually involved throughout the year in a single field, leading to complex pesticide mixtures in the agricultural environment (Weisner et al., 2021; Abdelrahman et al., 2024); moreover farmers are sometimes utilizing pesticides in excess of the recommended dosage, which has been associated with serious health hazards in humans and negative impacts on various ecosystems, environment and wildlife (Mark et al., 2024; Kosnik et al., 2022). A new regulation on the sustainable use of pesticides has been recently adopted by the European Commission in the context of the European Green Deal, aimed to achieve a 50% reduction in the use of chemical pesticides by 2030 and to adopt new approaches to preserve biodiversity and the food value chain (Farm to Fork Strategy) (Abrantes et al., 2024). Furthermore, in 2019, the EFSA (European Food Safety Authority) also published new guidelines describing the harmonized application of risk assessment methods for combined exposure to multiple chemicals, emphasizing the need for a new approach to pesticide risk assessment (More et al., 2019). Despite the limited number of studies addressing combined exposures, emerging evidence suggests that mixtures can exert synergistic or additive effects, leading to more pronounced toxicity (Cattaneo et al., 2023; Shahid et al., 2025).

The entire population is exposed to pesticides, and this exposure has been shown to be associated with an increased risk of various diseases such as cancers, chronic diseases, neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, reproductive and developmental disorders, etc. (Alaoui et al., 2024). In the SPRINT project pesticide residues have been detected in different types of environmental samples, such as outdoor air, indoor dust, surface water and sediment samples at 10 study sites, which covered major European crops and conventional and organic farming systems (Silva et al., 2023; Navarro et al., 2023; Navarro et al., 2024; Alaoui et al., 2024).

The SPRINT project prioritized through a tiered approach, based on EFSA guideline (More et al., 2019), the pesticides and mixtures of greatest concern, using available data and *in silico* modelling to assess the top 20 pesticides of concern, next studying *in vitro* models for the top 10 pesticides of concern and finally then studying *in vivo* only the top 3 pesticides of concern.

In this framework the Cesare Maltoni Cancer Research Center (CMCRC) of the Ramazzini Institute (RI) performed an *in vivo* study aimed to investigate the effects of 3 prioritized pesticides and their mixture identified in the SPRINT project. A relevant aspect of the *in vivo* experiment was prenatal exposure, following the principle known as "Developmental Origins of Health and Disease" (DOHaD), according to which harmful exposures occurring early in life, during tissue and organ development, can increase the risk of disease later in life (The Consortium For Children's Environmental et al., 2025; Haugen et al., 2015).

2. Methods

The Cesare Maltoni Cancer Research Center (CMCRC) of the Ramazzini Institute (RI) performed a study aimed to investigate the effects of 3 pesticides and their mixture that have been identified as the 3 highest ranking substance of concern for *in vivo* testing by the SPRINT project. These prioritized pesticides belong to the main groups of pesticides used in agriculture, namely a herbicide (glyphosate), a fungicide (tebuconazole) and an insecticide (acetamiprid). In addition to the 3 pesticides individually dosed, the mixture of the 3 compounds is studied. Doses selected to be tested *in vivo* correspond to levels currently considered safe in humans (or without adverse effects in animals), i.e. between Acceptable Daily Intake (ADI) and No Observed Adverse Effect Level (NOAEL).

2.1. Test substances

The three test substances administered to female and male Sprague Dawley (SD) rats in drinking water were the following:

- Glyphosate, CAS 38641-94-0 [N-(phosphonomethyl)glycine], purity 99%, Sigma-Aldrich, Milan, Italy].
- Tebuconazole, CAS 107534-96-3 [Ethanol, a-[2-(4-chlorophenyl) ethyl]-a-(1,1- dimethylethyl)-1H-1,2,4-triazole], purity 99%, ThermoFisher Scientific, United Kingdom.
- Acetamiprid, CAS 135410-20-7 [[(E)-N-[(6-Chloro-3-pyridyl) methyl]-N-cyano-N-methylacetamidine], purity 99%, ThermoFisher Scientific, United Kingdom.

Solutions of these three test substances were prepared by the addition of an appropriate volume of tap drinking water, to obtain the desired concentration of each pesticide, in line with analyses of the solutions stability and concentrations of the test substances, described in more details below.

2.2. Animals and experimental design

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 CMCRC of the RI, Bentivoglio, Italy. The study protocol was evaluated by the Ethical Committee of the RI and approved and formally authorized by the *ad hoc* commission of the Italian Ministry of Health (ministerial approval n. 1060/2023-PR). The RI animal breeding facility was the supplier of SD rats. Female breeder (F0) SD rats (104) were placed individually in polycarbonate cages (42x26x18cm; 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 the female was housed separately during gestation and delivery. Newborns were housed with their dams until weaning. Weaned offspring were co-housed, by sex and treatment group, 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). The animals drank fresh tap water from glass bottles *ad libitum* and bottles were changed daily. Tap drinking water was tested for possible glyphosate, acetamiprid, and tebuconazole contamination in compliance with Directive 2020/2184. All the methods used comply with standard guidelines and were performed in certified (ISO, Accredia) laboratories in Italy. The pelleted feed was tested for possible glyphosate, acetamiprid, and tebuconazole contamination in compliance with Commission Regulation (EU) n. 396/2005. All the methods used comply with standard guidelines and were performed in certified (ISO, Accredia) laboratories in Italy. The cages were placed on racks at room temperature of 22°C ± 3°C and 50% ± 20% of relative humidity. Daily checks on temperature and humidity were performed. The light was artificial, and a light/dark cycle of 12 h was maintained.

Analyses of chemical characteristics (pH, ashes, dry weight, and specific weight) and possible glyphosate (<0.01 mg/kg), tebuconazole (<0.01 mg/kg), and acetamiprid (<0.005 mg/kg) or other chemicals contaminations (metals, aflatoxin, polychlorinated biphenyls, organophosphorus and organochlorine pesticides) of the bedding were performed. All the methods used comply with standard guidelines and were

performed in certified (ISO, Accredia) laboratories in Italy.

The F0 female breeders received the treatment through drinking water from gestational day (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 until 16 weeks of age. After weaning at postnatal day (PND) 21, F1 animals were randomly selected to continue the exposure in order to have no more than 2 males and 2 females per litter per group. Each F1 experimental group was composed of 12 males and 12 females, belonging to the same treatment group of their exposed dams. The total number of animals was 312 (156 males and 156 females). The numerosity selection of F1 rats and respectively parents were assessed based on sample size analysis evaluation required for animal welfare to ensure compliance with Italian Legislative Decree 26/2014, that implements Directive 2010/63/EU on the protection of animals used for scientific purposes.

Each of the 3 selected test substances and the mixture of the 3 were administered in drinking water, respectively, at doses equivalent to Acceptable Daily Intake (ADI), ADIx10 and ADIx100, which is equivalent to the No Adverse Effect Level (NOAEL) in laboratory animals, based on mean water consumption (40 ml) and a mean body weight (400 g). Final concentrations were therefore the following: glyphosate doses- 0.5 mg/kg bw/day (corresponding to 5 mg/L), 5 mg/kg bw/day (corresponding to 50 mg/L), 50 mg/kg bw/day (corresponding to 500 mg/L); tebuconazole doses- 0.03 mk/kg bw/day (corresponding to 0.30 mg/L), 0.3 mk/kg bw/day (corresponding to 3 mg/L), 3 mk/kg bw/day (corresponding to 30 mg/L); acetamiprid doses- 0.025 mk/kg bw/day (corresponding to 0.25 mg/L), 0.25 mk/kg bw/day (corresponding to 2.5 mg/L), 2.5 mk/kg bw/day (corresponding to 25 mg/L). The same doses were also used in the 3 Mixtures. One control group was administered drinking water only. ADI and NOAEL values were derived from the EU pesticide database at the beginning of the studies (EFSA et al., 2024).[2]

2.3. In vivo endpoints

Body weight, water and feed consumption: body weight, water and feed consumption were monitored prior to dosing, 24 h after the first administration of test substances (GD 7), and then every three days (GD 9, 12, 15, 18 and 21) during pregnancy of dams. During lactation, body weight, water and feed consumption were recorded in dams on Lactational Day (LD) 1, 4, 7, 10, 14, 17, and at weaning of their pups; pups body weight by sex and litter were recorded on PND 1, 4, 7, 10, 14, 17, and at weaning. Individual F1 body weight was recorded, the first day after weaning, and then every week. All animals were weighed at sacrifice.

Presumed fetal mortality was evaluated during the 24 h following delivery when all the death pups found death were subjected to the evaluation of the lung floatation.

2.4. Parameters relating to sexual development

Vaginal opening (VO) was determined by daily inspection of all female pups starting after weaning (PND 21). The body weight of each female was recorded on the day that this was observed. Evaluation of first estrus was done by daily vaginal swab for 14 days beginning from the day after the vaginal opening, in all females. Estral cycle pattern was monitored in all females from 13 weeks of age for 3 consecutive weeks and at terminal sacrifice. Balano-preputial separation (BPS) was determined by daily inspection of males beginning on PND 40. The body weight of each male was recorded on the day that this was observed.

[2] It should be noted that in September 2024, thus after the conclusion of the *in vivo* phase of the study, the EU Commission lowered the ADI of acetamiprid from 0.025 mg/kg bw/day to 0.005 mg/kg bw/day.

2.5. Other toxicological endpoints

Urine samples: after 13 weeks of post-weaning treatment, F1 animals were located individually in metabolic cages for around 16 h, starting from afternoon, prior to sacrifice. During this time, the animals had free access to test solutions administered in drinking water and feed. The day after, in the morning, samples of individual urine were collected for the urinalysis. Aliquot of urine was destinated to IDEXX Laboratory for the urinalysis for the following parameters: appearance, urine volume, specific gravity, pH, total protein, glucose, ketone bodies, urobilinogen, bilirubin, and occult blood.

Blood samples: at the end of treatment each surviving animal (from F1 and F0) was anesthetized by mixture CO_2/O_2 (70% and 30% respectively) inhalation and the blood was withdrawn from the cava vein. When the sample was drawn, the animal was sacrificed by an overdose of the anesthetic CO_2/O_2 mixture (no recovery procedure); death is taken to be complete when the circulation stops permanently (cardiac arrest).

Blood from F1animals was analyzed for the following parameters:

- *Hormonal analysis:* measurement of thyroxine (T4), triiodothyronine (T3), thyroid stimulating hormone (TSH) were performed in duplicate on serum samples collected from each animal at necropsy and stored frozen until the analysis. Enzyme Linked Immunosorbent Assay (ELISA) was performing following the instruction provided by the manufacturer (CUSABIO).
- *Biochemical evaluation (on serum sample):* parameters evaluated were sodium (Na), potassium (K), chloride (Cl), glucose (GLU), inorganic phosphate (PHOS), calcium (Ca), globulins (GLOB), total cholesterol (CHOL), triglycerides (TRIG), blood urea nitrogen (BUN), creatinine (CREA), total protein (TP), albumin (ALB), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALKP), total bilirubin (TBIL), aspartate aminotransferase (AST), Osmolality (OsmCalc).
- *Hematological evaluation:* parameters evaluated were hematocrit (HCT), hemoglobin (HGB), erythrocyte (RBC), reticulocyte (RET and RET%), total and differential leukocyte count (BASO- basophil count; EO- eosinophil count; LYMPH- lymphocyte count; MONO-monocyte count; NEUT- neutrophil count; WBC- total number of white blood cells), platelets (PLT), platelets distribution width (PDW), mean platelet volume (MPV), mean erythrocyte volume in total sample (MCV), mean hemoglobin volume per red blood cell count (MCH), mean hemoglobin concentration of erythrocytes (MCHC), calculated distribution width of erythrocytes-coefficient of variation (RDW-CV), calculated distribution width of erythrocytes-standard deviation (RDW-SD), plateletcrit value (PCT).

2.6. Necropsy and histopathology

The *in vivo* phase of the investigation ended at around 16 weeks of age when all surviving animals were euthanized 13 weeks after weaning. All animals underwent a complete necropsy including an initial physical examination of the external surfaces and all orifices followed by an internal examination of tissues and organs *in situ.* Histopathology was performed on the following organs and tissues of all animals: skin and subcutaneous tissue, mammary gland (4 sites: axillary and inguinal, right and left), brain with cerebellum and medulla/pons, pituitary gland, salivary glands, Harderian glands, tongue, esophagus, thyroid and parathyroid, thymus and mediastinal lymph nodes, trachea, lungs, heart, liver (2 lobes), spleen, pancreas, kidneys, adrenal glands, stomach (forestomach and glandular stomach), small intestine, large intestine (with the Peyer's patches), bladder, uterus (including cervix), ovaries, vagina, testes and epididymis, seminal vesicles and coagulating glands, prostate, subcutaneous lymph nodes, mesenteric lymph nodes, sternum, cranium and all gross lesions and other tissues when anomalies were present. All organs and tissues were fixed in alcohol 70%, except bone

parts that were fixed in buffered formalin (10% neutral formaldehyde in water). After fixation, samples were trimmed, processed, embedded in paraffin wax, sectioned to a thickness of 4–5 μm and then processed in alcohol-xylene series and stained with hematoxylin and eosin for microscopic evaluation. Complete histopathological evaluations were performed by the pathologists on all organs and tissues from all F1 animals. Histopathology evaluation was performed by at least two pathologists, and all lesions were peer-reviewed. The pathological evaluation was performed according to the procedures of the International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) (INHAND) and The NTP Non-neoplastic Lesion Atlas (Nonneoplastic Lesion Atlas).

2.7. Statistical methods

Statistical analyses were performed by comparing each treated group to control or considering one substance at a time for trend tests (glyphosate vs control, tebuconazole vs control, acetamiprid vs control, mixture vs control). Results were considered statistically significant when the p-value was < 0.05. Prior to statistical analysis, extreme values were graphically identified and were examined by CMCRC personnel. All statistical analyses conducted on the entire litter were corrected to account for any litter effect. A multilevel mixed-effects linear regression was applied to the data that were found to be biologically relevant and statistically significant, treating the litter as a random effect. Litter size, hematology, clinical chemistry and hormonal analysis data, that typically have skewed distributions, were analyzed using the nonparametric Jonckheere–Terpstra test for trend and each dose was compared to the control with the nonparametric multiple comparison methods of Dunn. Organ and body weight measurements, water and food consumption, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett.

Analyses of age (in days) and body weight at occurrence of VO or BPS were performed using contrasts within a one-way ANOVA followed by Dunnett's post-hoc test, to test for treatment effect, as data resulted homogeneous using Levene's test (to test the distribution assumption). For these endpoints related to the time-to-event (BPS and VO), which are usually influenced by body weight and litter, a multilevel mixed-effects linear regression model was also used.

Cycle length in days was defined from the first day of oestrus in one sequence of contiguous days to the first day of oestrus in the following sequence of stages. Statistical analyses were conducted on proportions of animals with abnormal cycles, extended oestrus, extended diestrus, and excessive proestrus. Two-sided p-value for the Fisher's exact test was used to evaluate the pairwise differences in proportions and Cochran-Armitage method was used for dose-related trends within different exposures.

The incidence of histopathological lesions compared with the control was assessed by Fisher's exact test. The Cochran-Armitage trend test was performed to test dose-related trends within different exposures by treatment (glyphosate vs control, tebuconazole vs control, acetamiprid vs control, mixture vs control). All reported p-values are one-sided, to visualize the increase in pathological lesions with increasing dose, following the basic hypothesis of the toxicity bioassay study, as reported in OECD guidelines (OECD, 2014).

Statistical analyses were performed using the German Cancer Research Center software (https://biostatistics.dkfz.de/) and Stata 18 software (StataCorp 2023. Stata Statistical Software: Release 18. College Station, TX: StataCorp LLC).

3. Results

3.1. Gestational and perinatal parameters

Fertility index (number of pregnant females which delivered pup/total copulation positive), gestational index (number of females that delivered at least one live pup/total females having pups or evidence of pregnancy) and gestational lengths (ranging from 21.6 ± 0.7 to 22.0 ± 0.4 days) by group were generally homogeneous among groups (Appendix A).

Throughout the gestation period, body weight, food and water consumption of pregnant dams were not affected by the treatment (Appendix B).

Throughout the lactation period, body weight, food and water consumption of pregnant dams or litter were not affected by the treatment. Only sporadic fluctuations were observed during the lactation period (Appendix C).

Number of pups deaths during lactation (males and females) is reported in Tables 1A, 1B, 1C, 1D and Appendix D considering days 1–4 and 5-end of lactation. Some statistically significant differences were detected in tebuconazole treated group dose 0.3 and 0.03 mg/kg bw/day, with an increased litter mortality.

3.2. Post-weaning parameters

No statistically significant difference in water and food consumption was observed after weaning. Sporadic differences in body weight were detected in the first week of post-weaning observation; these differences were not considered to be associated with treatment, but only with biological changes naturally occurring in the first few days after weaning (Appendix E).

3.3. Sexual maturation index

A statistically significant anticipation of day of occurrence of sexual maturation in treated females was detected in different treated groups (Fig. 1 and Table 2; the effect of the treatment remained statistically significant even when analyzed together with body weight in a regression model. No significant differences between weights at VO were observed. However, the effects were not clearly dose-related and in the mixture were observed only at the lowest dose. No differences in the onset of first estrous was observed. All animals have their first estrous between day 1 and 5 after VO; no differences in percentage of animals with abnormal cycles were detected among treated groups and control.

A statistical significant anticipation of day of occurrence of sexual maturation in males was detected only in some groups without a clear dose response attributed to the treatment and no effects were observed in the mixture (Fig. 2 and Table 3); the effect of treatments remained statistically significant even when analyzed together with body weight in a regression model, without a clear biological response attributable to the treatments. No significant difference between weights at BPS was observed.

3.4. Clinical Pathology

A statistically significant positive trend was observed in the increase

Table 1A
Number of pups deaths during lactation in glyphosate treated groups and control.

	Control	Glyphosate 0.5 mg/kg bw	Glyphosate 5 mg/kg bw	Glyphosate 50 mg/kg bw
Survival				
Total dead 1–4	0	1 (M)	2 (1 M + 1F)	1 (F)
Total dead 5–21	0	0	0	0
Total dead	0	1 (M)	2 (1 M + 1F)	1 (F)
Total (live + dead) at birth	86	101	101	101

Table 1B
Number of pups deaths during lactation in tebuconazole treated groups and control.

	Control	Tebuconazole 0.03 mg/kg bw	Tebuconazole 0.3 mg/kg bw	Tebuconazole 3 mg/kg bw
Survival				
Total dead 1–4	0	9 *0.005 #0.003 (3 M + 6F)	6 *0.030 #0.028 (2 M + 4F)	1 (M)
Total dead 5–21	0	5 (4 M + 1F)	0	0
Total dead	0	14 *0.000 #0.000 (7 M + 7F)	6 *0.030 #0.010 (2 M + 4F)	1 (M)
Total (live + dead) at birth	86	107	97	94

* p-value for Fisher's exact test (two-sided), considering all pups at birth (dead + live).
# p-value for mixed-effects model in which the litter is treated as a random effect.

Table 1C
Number of pups deaths during lactation in acetamiprid treated groups and control.

	Control	Acetamiprid 0.025 mg/kg bw	Acetamiprid 0.25 mg/kg bw	Acetamiprid 2.5 mg/kg bw
Survival				
Total dead 1–4	0	2 (2F)	0	4 (2 M + 2F)
Total dead 5–21	0	0	4 (2 M + 2F)	1 (F)
Total dead	0	2 (2F)	4 (2 M + 2F)	5 (2 M + 3F)
Total (live + dead) at birth	86	102	104	128

Table 1D
Number of pups deaths during lactation in mixture treated groups and control.

	Control	Mixture ADI	Mixture ADIx10	Mixture ADIx100
Survival				
Total dead 1–4	0	0	2 (2F)	0
Total dead 5–21	0	2 (2F)	1 (1F)	0
Total dead	0	2 (2F)	3 (3F)	0
Total (live + dead) at birth	86	117	126	99

of aspartate aminotransferase (AST) blood concentration for tebuconazole, acetamiprid, and mixtures in females and for glyphosate and mixtures in males (Table 4). In addition, several statistically significant individual differences per group compared to the control were detected using Dunn's test and are reported in Table 4 (Figs. 3 and 4).

There were only sporadic changes in hematological, urinalysis, hormonal parameters and organ weight measures that were not related to treatment. A complete summary of results is reported in Appendix F-I.

3.5. Histopathology

Pregnant and non-pregnant dams (F0) were histopathologically evaluated only at higher doses and control. Biological relevant increase (3/8) in aorta mineralization was detected in female which developed pups, even if not statistically significant (Table 6), in acetamiprid group.

All other data on female which developed pups are summarized in Appendix J. No lesions of biological interest were detected in females that did not give birth (data on non-pregnant female –F0- were not reported).

No differences in survival were observed in F1 generation at the end of treatment.

Histopathology of F1 rats by organ site revealed few slight increases in incidences of pathological lesions in males (Tables 7–10). In particular, increase in trend was observed in acetamiprid and glyphosate treated group even if with a borderline significance) for lesion related to the circulatory system (Tables 7 and 9); in the acetamiprid intermediate dose one animal bearing circulatory system lesion died spontaneously before terminal sacrifice at 12 weeks of age. In the tebuconazole an increase (25%) of lesions related to the exocrine pancreas was observed in high dose group (Table 8).

Considering all together the 3 dosed for each treatment an increase in incidence of genital system lesions was observed in males meanwhile no cases were detected in the control groups. The lesions considered were inflammation, fibrosis, tubular degeneration and mineralization from mild to severe degrees. The incidence of animals bearing these lesions for each treatment was 0/36 (control), 5/36 (glyphosate), 2/36 (tebuconazole), 4/36 (acetamiprid) and 3/36 (mixture).

Notably in males in the glyphosate low dose a case of leukemia was observed, at histopathological examination, at the end of the treatment (Appendix K). In female no statistically significant increase in incidence of lesions per organ site was observed. Moreover, in the tebuconazole intermediate dose a case of leukemia was observed at the end of the treatment (Appendix L). No cases of leukemia were observed in control animals.

4. Discussion

In the present *in vivo* experiment, we have investigated the toxicity of 3 different pesticides and a mixture of the 3, administered to male and female SD rats. The 3 pesticides involved in the study are part of the 3 main functional categories of plant protection products used in agriculture, namely herbicides (glyphosate), fungicides (tebuconazole) and insecticides (acetamiprid) (Mark et al., 2024; FAO, 2024; US EPA Ingredients Used in Pesticide Products; eurostat). In addition to the 3 pesticides individually dosed, the mixture of the 3 pesticides was studied in order to expose the animals to a scenario closer to realistic human exposure (Silva et al., 2021). The 3 selected pesticides and a mixture of them were administered with drinking water to SD rats, starting during prenatal life (GD 6) util 13 weeks after weaning. The study was designed following the principle of DTT 2023 (Roberts, 2023) and the previous NTP 2011 guidelines. The experimental protocol included 13 groups of 12 SD rats per sex, originated from 8 female breeders. Each of the 3 selected test substances and the mixture of the 3 substances were administered in drinking water, respectively, at doses of ADI, ADIx10 and ADIx100. One control group was administered drinking water only.

During the study, data were collected from different *in vivo* endpoints at different stages, gestation, lactation, and post-weaning, observing a general normal trend at all stages. In particular, no difference was detected among treatment and control groups for fertility and gestational indices, dams body weight, or water and feed consumption. Marginal fluctuation in water consumption at the start of the lactation period was detected, but no differences were related to feed consumption or body weight in the same period, therefore considered normal for the peculiar moment.

There was a statistically significant decrease in litter size in tebuconazole low and intermediate doses. In 4 litters (on a total of 7) belonging to low dose tebuconazole group, pups' death was recorded from PND1 and 4, and in 2 litters from PND5 to PND21. In 2 litters (on a total of 7) pups' death was observed also in intermediate dose of tebuconazole through PND1 and PND4. Tebuconazole, a triazole fungicide,

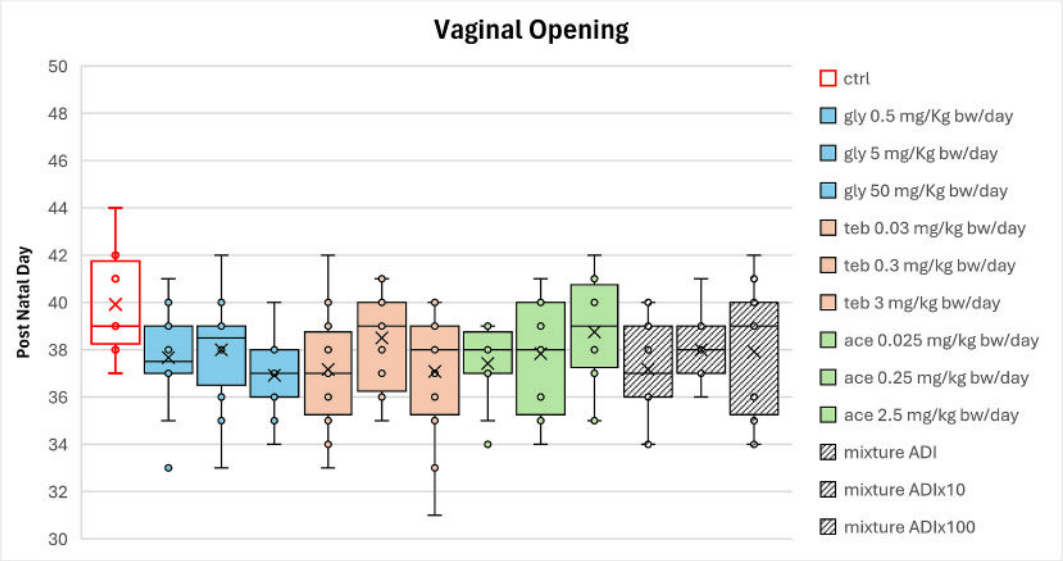

Fig. 1. Post Natal Day when VO was observed.

Table 2
Age and body weight (mean ± standard deviation) at vaginal opening (VO) of female rats in the 13-week prenatal study.[a]

Treatment	Dose (mg/kg bw/day)	PND at VO [a]				Body weight at VO		
		Mean		St. Dev.	p-value	Mean		St. Dev.
Control	–	39.92	±	2.06		127.00	±	13.72
Glyphosate	0.5	37.67	±	2.19	*0.029	118.00	±	12.15
Glyphosate	5	38.00	±	2.37	#0.012	129.92	±	15.26
Glyphosate	50	36.92	±	1.56	*0.003 #0.000	128.17	±	15.16
Tebuconazole	0.03	37.17	±	2.52	*0.020 #0.036	123.25	±	15.75
Tebuconazole	0.3	38.50	±	2.07	#0.041	130.50	±	12.96
Tebuconazole	3	37.08	±	2.84	*0.016 #0.058	119.00	±	16.01
Acetamiprid	0.025	37.42	±	1.56	*0.015 #0.003	115.17	±	12.92
Acetamiprid	0.25	37.83	±	2.33	*0.049 #0.015	124.75	±	11.72
Acetamiprid	2.5	38.75	±	2.03		120.83	±	8.25
Mixture	ADI	37.17	±	2.12	*0.008 #0.017	117.92	±	12.92
Mixture	ADIx10	38.00	±	1.35		117.92	±	11.65
Mixture	ADIx100	37.92	±	2.78		118.00	±	11.43

* Significantly different from the control group by Dunnett's test.
# Multilevel mixed-effects linear regression including doses, the body weight at the day of VO as covariates and litter as random effect.
[a] : Post Natal Day at Vaginal Opening.

has been reported to be related to reproductive, developmental and embryonic toxicity (Ma et al., 2021); in particular the findings observed in our study is consistent with what has been observed in *in vivo* studies by Taxvig et al., where *in utero* exposure to tebuconazole increased perinatal death of pups (Taxvig et al., 2007).

Throughout the postweaning phase, rats had mean body weights, water and feed consumption similar between control and treated groups.

No biologically relevant alterations in organ weights in either treated males or females compared with the control were observed.

Some statistically significances were observed in age at VO in females, compare to control, but none of these are considered of biological interest considering the relative high age at VO in the control group, also compared to previous data from our historical controls (Manservisi et al., 2019). Moreover, no statistically significant differences were observed in the first estrous or in the estrous cycle.

AST concentration increased in all treated groups in both males and females compared to the control groups. In males a statistically significant increased trend in AST was observed in tebuconazole, acetamiprid and mixtures, while in females the increased trend was observed in glyphosate and mixture; significances were confirmed and extended also with Dunn test in 10/12 groups in males (increased concentration from a minimum of 8% to a maximum of 24%) and in 8/12 groups in females (increased concentration from a minimum of 5% to a maximum of 36%). Several pesticides could impact the biochemical assessment in rodents, in particular when the exposure starts prenatally (Ndonwi et al., 2019). A study in rats on different pesticides revealed an increase in AST concentration compared to the control group, suggesting a liver injury, in line with our results (Ndonwi et al., 2019). Ku et al. (2021) also studied how tebuconazole accumulated mainly in the liver, causing damage at the histopathological level (Ku et al., 2021) and induced oxidative stress in the liver of adult rats, altering the concentration of some hematological parameters (Berrouague et al., 2019).

Fluctuations in serum TSH, T3, and T4 concentrations were found across all groups, with no clear dose response. It is therefore difficult to

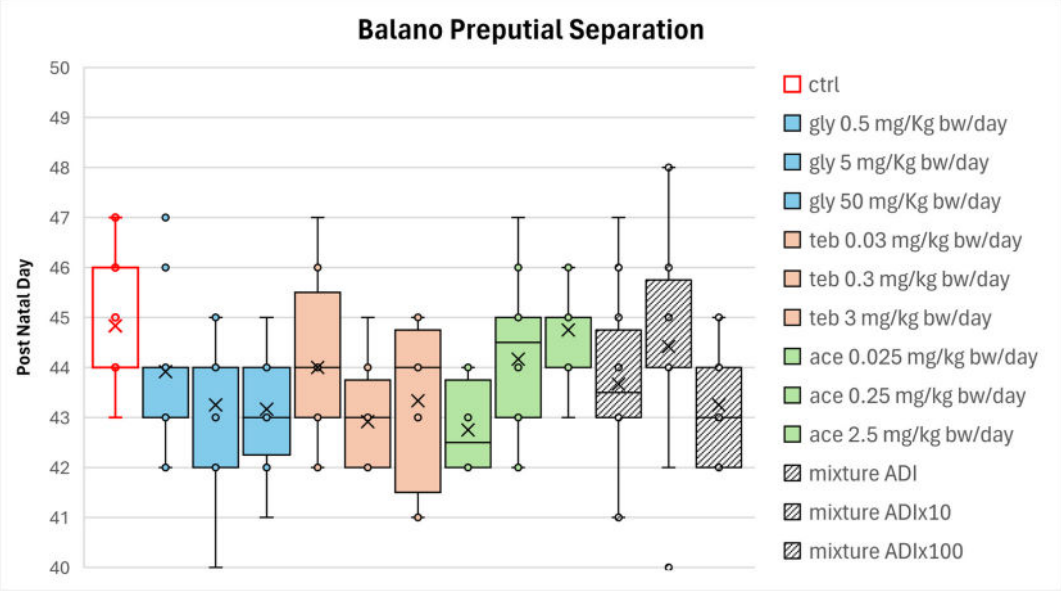

Fig. 2. Post Natal Day when BPS was observed.

Table 3
Age and body weight (mean ± standard deviation) at balano-preputial separation (BPS) of male rats in the 13-week prenatal study.[a]

Treatment	Dose (mg/kg bw/day)	PND at BPS [a]				Body weight at BPS		
		Mean		St. Dev.	p-value	Mean		St. Dev.
Control	–	44.83	±	1.34		182.17	±	13.70
Glyphosate	0.5	43.92	±	1.44		179.50	±	14.59
Glyphosate	5	43.25	±	1.48	*0.017 # 0.006	181.33	±	8.85
Glyphosate	50	43.17	±	1.11	*0.012 #0.004	187.42	±	25.00
Tebuconazole	0.03	44.00	±	1.59		182.42	±	16.32
Tebuconazole	0.3	42.92	±	0.99	*0.004 #0.003	185.25	±	15.92
Tebuconazole	3	43.33	±	1.56	*0.031 #0.031	181.08	±	12.07
Acetamiprid	0.025	42.75	±	0.87	*0.000 #0.002	181.50	±	12.74
Acetamiprid	0.25	44.17	±	1.58		190.50	±	14.08
Acetamiprid	2.5	44.75	±	0.87		185.08	±	13.91
Mixture	ADI	43.67	±	1.77		180.50	±	12.21
Mixture	ADIx10	44.42	±	2.23		183.25	±	13.38
Mixture	ADIx100	43.25	±	1.14		170.67	±	12.84

* Significantly different from the control group by Dunnett's test.
# Multilevel mixed-effects linear regression including doses, the body weight at the day of BPS as covariates and litter as random effect.
[a] : Post Natal Day at Balano-Preputial Separation

attribute precise causality of these alterations to the treatments. Studies in rats with gestational exposure inherent in the assessment of thyroid hormones are limited to a relatively small group of chemicals, as reported in a recent review, suggesting that alterations can be varied and not always concordant (Forner-Piquer et al., 2023). In addition, no significant changes were detected in the histopathological evaluation of the thyroid gland (Appendix K-L).

At histopathological level, considering the low doses tested and the relative short duration of the treatment, a few interesting numbers of non-neoplastic lesions were observed, particularly in males. Interesting also in dams, euthanized after weaning of their pups (10 weeks of treatments) some lesions were recorded at the highest doses analyzed.

In males the target organs were related to the circulatory system (for glyphosate and acetamiprid), to the alimentary system (tebuconazole) and to the genital system (all treatments).

In the male animals exposed to tebuconazole, a significantly increased trend in incidence of exocrine pancreas lesions was observed. In a recent study, low-dose exposure to pesticide mixtures, also containing glyphosate and tebuconazole, over a similar period of time as in the present study, caused hemorrhage, degeneration, and inflammation in the pancreas and mesenteric tissue of male SD rats (Sevim et al., 2024). This is in line with our findings suggesting a real effect of, at least, tebuconazole, on the exocrine pancreas. Tebuconazole currently accounts for 25% of all global fungicide sales (Carnib et al., 2025). Azoles are used in both medicine and agriculture and act by inhibiting lanosterol 14α-demethylase (CYP51), enzyme of the cytochrome P450 complex and blocking the ergosterol biosynthesis pathway. Ergosterol is a key component of the cell membrane of mycetes, where it performs the same functions as cholesterol does in animal cells (Choy et al., 2023).

The circulatory system in the presented study seems to be a very peculiar target system for different treatments. In fact, in males exposed to glyphosate and acetamiprid a significantly increased trend in incidence of heart, pericardium and ascending aorta lesions was observed. Additionally, the same inflammatory lesion in the ascending aorta was

Table 4
Aspartate aminotransferase (AST) concentration (mean ± standard deviation) of male rats in the 13-week prenatal study.[a]

Treatment		Animals		Parameter			Animals		Parameter		
Compound	Dose (mg/kg bw/day)	Sex	N.	AST(U/L)			Sex	N.	AST(U/L)		
				Mean ± St.Dev.		p-value			Mean ± St.Dev.		p-value
Control	–	M	12	96.33 6.83	±		F	12	89.83 9.42	±	
Glyphosate	0.5	M	12	119.92 31.45	±	*0.0002 +0.0010	F	12	98.58 14.36	±	*0.0113
Glyphosate	5	M	12	112.50 11.04	±	*0.0003 +0.0210	F	12	97.42 14.44	±	
Glyphosate	50	M	12	104.08 8.10	±		F	12	99.08 8.86	±	*0.0056 #0.0252 +0.0500
Tebuconazole	0.03	M	12	105.00 15.17	±		F	12	101.33 11.93	±	*0.0020
Tebuconazole	0.3	M	12	106.08 13.05	±	*0.0234 +*0.0550*	F	12	121.92 96.26	±	
Tebuconazole	3	M	12	113.67 12.35	±	*0.0002 #0.0005 +0.0010	F	12	95.17 10.74	±	
Acetamiprid	0.025	M	12	110.42 9.03	±	*0.0007 +0.0020	F	12	107.75 16.16	±	*0.0002 +0.0001
Acetamiprid	0.25	M	11	110.55 14.36	±	*0.0031 +0.0020	F	12	100.75 9.72	±	*0.0015 +0.0160
Acetamiprid	2.5	M	12	112.36 14.24	±[b]	*0.0006 #0.0030 +0.0010	F	12	94.33 5.19	±	
Mixture	ADI	M	11	111.09 12.64	±	*0.0014 +0.0010	F	12	98.33 8.71	±	*0.0116 +0.0220
Mixture	ADIx10	M	12	115.83 11.19	±	*0.0000 +0.0001	F	12	101.17 7.64	±	*0.0007 +0.0010
Mixture	ADIx100	M	12	112.17 13.73	±	*0.0009 #0.0017 +0.0001	F	12	96.50 7.82	±	*0.0170 #0.0188

Significantly different with Jonckheere–Terpstra test for trend considering 1 compound and the control group.
+ Multilevel mixed-effects linear regression including litter as random effect.
*Significantly different from the control group with Dunn's test.
[a] AST = aspartate aminotransferase.
[b] Statistical analysis performed on 11 values of 12.

recorded in 3 dams out of 8 (37.5%) belonging to the acetamiprid high dose group. A recent study by Abdelrahman et al. (2024) showed increased micronuclei and 8-hydroxyguanosine formation and increased all comet parameters with acetamiprid treatments, underlying genotoxic effects on the myocardium; moreover a general dysregulation of genes related to oxidative stress was observed by the authors (Abdelrahman et al., 2024). In humans, the association between *in utero* exposure to neonicotinoid pesticides and heart disease, the most common birth defects, remains poorly explored (Qu et al., 2024). A dose-related increase in vascular inflammation affecting the mesenteric vessels (the only target organ observed microscopically by the authors) was observed in a recent study on male SD rats exposed to a mixture of pesticides containing also glyphosate (Sevim et al., 2024).

All treatments affected the testes and testes adnexa in different ways and degrees, ranging from mild to severe; the mainly observed lesions were inflammation, mineralization fibrosis, tubular degeneration, and spermatocele. None were observed in the control group. For all the treatments the highest dose group has increased incidence compared to control. Considering the low-dose regimen and the young age of the animals (~16 weeks), all lesions, despite statistical significance, can be considered of interest. Degenerative lesions of the testes, a rare finding in this strain at this age, based also on previous study conducted by the CMCRC (Panzacchi et al., 2024), were observed only in treated groups, whereas no degenerative lesions were observed in the control groups. The mammalian testis represents a privileged immune organ, with a dual aspect to be considered in testicular defense: protection of self-antigens from harmful immune responses and control of external microbial pathogens with the blood-testis barrier (Zhao et al., 2014). Tubular degeneration and its sequel, tubular atrophy, are common manifestations of toxicologic injury to the testis, moreover the others lesions observed, as the mineralization, are typical of age rats (Creasy et al., 2012); similarly inflammation of the testes is an uncommon situation, due to the immunologically protected nature of the seminiferous tubules (Creasy et al., 2012). These findings are supported by several authors, connecting all 3 pesticides with alteration of reproductive organs in male rats and blood-testis barrier integrity disruption (Ben Saad et al., 2020; Ma et al., 2021; Zuščíková et al., 2023; El-Hak et al., 2022; Liu et al., 2022; Dai et al., 2016).

Two cases of leukemia, not causing death, were observed each in one female from tebuconazole intermediate dose and one male from low

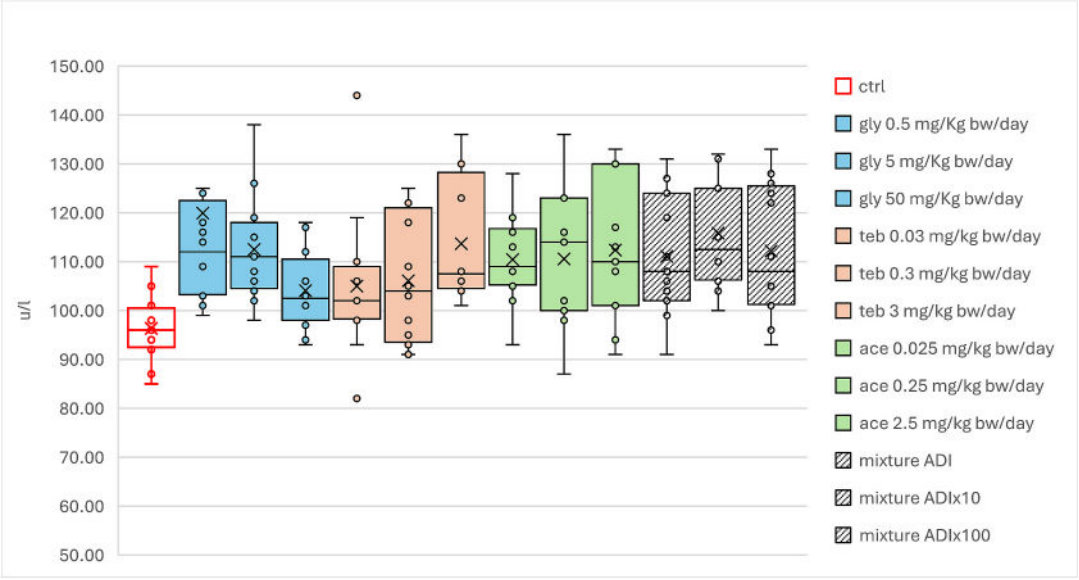

Fig. 3. AST in blood (U/L) in males.

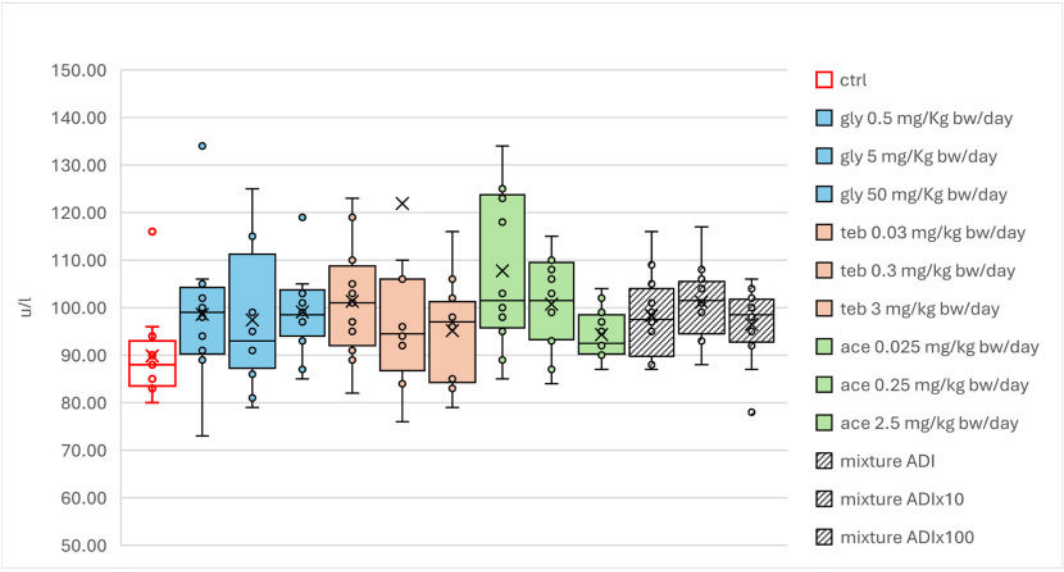

Fig. 4. AST in blood (U/L) in Females.

Table 6
Incidences of select non-neoplastic lesions in dams which developed pups (F0).

Sit		Groups[a]									
	Organ	Control		Glyphosate		Tebuconazole		Acetamiprid		Mixture	
		0 mg/kg		50 mg/kg		3 mg/kg		2.5 mg/kg		ADIx100	
		Tot. 6		Tot. 8		Tot. 7		Tot. 8		Tot. 7	
		N.	%	N.	%	N.	%	N.	%	N.	%
Circulatory system [b]											
	Aorta	0	–	0	–	0	–	3	37.5	0	–

[a] The same animal can bear one or more types of lesions in the same organ, and it is plotted only once.
[b] Aorta lesion: mineralization

dose glyphosate group. These lesions, observed at ~16 weeks of age (terminal sacrifice), were in line with our previous study on glyphosate (Panzacchi et al., 2025) where the first animal died with leukemia was around 20 weeks of age.

Our study has limitations. One limitation is the impossibility of studying all possible mixtures of pesticides in such *in vivo* experiment, so

Table 7
Incidences of select non-neoplastic lesions in male rats (F1) for glyphosate treated groups and control (12 animals per group).

Site		Groups[a]								
	Organ	Control			Glyphosate		Glyphosate		Glyphosate	
		0 mg/kg			0.5 mg/kg		5 mg/kg		50 mg/kg	
		N.	%	p-value*	N.	%	N.	%	N.	%
Alimentary system [b]										
	Salivary gland	0	–		0	–	0	–	0	–
	Liver	0	–		1	8.3	0	–	0	–
	Exocrine pancreas	1	8.3		2	16.7	0	–	1	8.3
Circulatory system [c]										
	Heart, pericardium, aorta	0	–	0.0585 (0.0631)	0	–	0	–	2	16.7
Genital System [d]										
	Testes and genital annex	0	–		1	8.3	2	16.7	2	16.7

* One-sided p-values for the Cochran-Armitage trend test (litter as group size).
[a] The same animal can bear one or more types of lesions in the same organ, and it is plotted only once.
[b] Liver lesions: steatosis, inflammation, fibrosis bile ducts hyperplasia and basophilic focus. Salivary gland and Pancreas exocrine lesions: atrophy with fibrosis or inflammation and acinar hyperplasia.
[c] Heart, pericardium and aorta lesions: inflammation mild or moderate, fibrosis and mineralization
[d] Testes lesions: inflammation, fibrosis, tubular degeneration, mineralization

Table 8
Incidences of select non-neoplastic lesions in male rats (F1) for tebuconazole treated groups and control (12 animals per group).

Site		Groups[a]							
	Organ	Control		Tebuconazole		Tebuconazole		Tebuconazole	
		0 mg/kg		0.03 mg/kg		0.3 mg/kg		3 mg/kg	
		N.	%	N.	%	N.	%	N.	%
Alimentary system [b]									
	Salivary gland	0	–	0	–	0	–	0	–
	Liver	0	–	1	8.3	0	–	1	8.3
	Exocrine pancreas	1	8.3	1	8.3	0	–	3	25.0
Circulatory system [c]									
	Heart, pericardium, aorta	0	–	0	–	0	–	0	–
Genital System [d]									
	Testes and genital annex	0	–	0	–	1	8.3	1	8.3

*One-sided p-values for the exact Cochran-Armitage trend test.
[a] The same animal can bear one or more types of lesions in the same organ, and it is plotted only once.
[b] Liver lesions: steatosis, inflammation, fibrosis bile ducts hyperplasia and basophilic focus. Salivary gland and Pancreas exocrine lesions: atrophy with fibrosis or inflammation and acinar hyperplasia.
[c] Heart, pericardium and aorta lesions: inflammation mild or moderate, fibrosis and mineralization
[d] Testes lesions: inflammation, fibrosis, tubular degeneration, mineralization

we cannot comment on mixtures of pesticides in general; therefore, we tried to mimic as closely as possible the human exposure observed in the SPRINT project with a tiered approach. Considering the results that have emerged, another limitation of this project is the lack of epididymal sperm count and other hormone analyses that could be explored in the future. It would be also appropriate to perform further molecular tests to investigate the observed lesions, and the possible mechanisms of action relate to single pesticides and mixtures.

5. Conclusion

In the present study we observed adverse effects at doses currently considered “safe”, namely equal or lower than the ADI or the NOAEL in rodents and other animal models for glyphosate, acetamiprid, tebuconazole and their mixture. It is well known that the study of low-dose regimens is a key element in assessing real-world exposure, which is not always easy to evaluate. Despite the lack of dose–response data for certain endpoints in the case of mixtures, it has nevertheless been possible to compare the individual chemical components of a complex mixture—tested in parallel with the mixture itself—thereby filling a gap in the scientific literature. Based on our results from a component-based mixture approach, no signs of synergism nor antagonism were observed at the low dose regimes applied. Synergy involves a mechanism of supra-linear potentiation or enhancement, which often leads to a superior and amplified result. In this experiment, the mixture does not appear capable of amplifying the result obtained with the individual pesticides. This is consistent with previous observations in the literature and compatible with additive toxicity (Elcombe et al., 2022). Sex-specific effects were observed in male and female rats which calls for more sex-differentiated research in animal testing and need confirmation in humans. Although the sample sizes were small, it is worth noting that the low doses tested nonetheless revealed certain changes associated with liver toxicity and, in particular, with effects on the endocrine system, where the classic dose–response relationships do not always apply.

In our study an important design characteristic was prenatal exposure. Starting with the evidence provided by Prof. Maltoni in its

Table 9
Incidences of select non-neoplastic lesions in male rats (F1) for acetamiprid treated groups and control (12 animals per group).

Site	Groups[a]								
Organ	Control 0 mg/kg			Acetamiprid 0.025 mg/kg		Acetamiprid 0.25 mg/kg		Acetamiprid 2.5 mg/kg	
	N.	%	p-value*	N.	%	N.		%	%
Alimentary system [b]									
Salivary gland	0	–		0	–	2	16.7	1	8.3
Liver	0	–		2	16.7	2	16.7	0	–
Exocrine pancreas	1	8.3		2	16.7	0	–	0	–
Circulatory system [c]									
Heart, pericardium, aorta	0	–	0.0459 (0.0630)	1	8.3	2	16.7	3	25.0
Genital System [d]									
Testes and genital annex	0	–		2	16.7	0	–	2	16.7

*One-sided p-values for the Cochran-Armitage trend test (litter as group size).
[a] The same animal can bear one or more types of lesions in the same organ, and it is plotted only once.
[b] Liver lesions: steatosis, inflammation, fibrosis bile ducts hyperplasia and basophilic focus. Salivary gland and Pancreas exocrine lesions: atrophy with fibrosis or inflammation and acinar hyperplasia.
[c] Heart, pericardium and aorta lesions: inflammation mild or moderate, fibrosis and mineralization
[d] Testes lesions: inflammation, fibrosis, tubular degeneration, mineralization

Table 10
Incidences of select non-neoplastic lesions in male rats (F1) for mixture treated groups and control (12 animals per group).

Site	Groups[a]							
Organ	Control 0 mg/kg		Mixture ADI		Mixture ADIx10		Mixture ADIx100	
	N.	%	N.	%	N.	%	N.	%
Alimentary system [b]								
Salivary gland	0	–	0	–	1	8.3	0	–
Liver	0	–	0	–	1	8.3	1	8.3
Exocrine pancreas	1	8.3	0	–	0	–	0	–
Circulatory system [c]								
Heart, pericardium, aorta	0	–	0	–	1	8.3	1	8.3
Genital System [d]								
Testes and genital annex	0	–	1	8.3	0	–	2	16.7

[a] The same animal can bear one or more types of lesions in the same organ, and it is plotted only once.
[b] Liver lesions: steatosis, inflammation, fibrosis bile ducts hyperplasia and basophilic focus. Salivary gland and Pancreas exocrine lesions: atrophy with fibrosis or inflammation and acinar hyperplasia.
[c] Heart, pericardium and aorta lesions: inflammation mild or moderate, fibrosis and mineralization
[d] Testes lesions: inflammation, fibrosis, tubular degeneration, mineralization

carcinogenicity studies on early exposures to vinyl chloride, benzene and ethyl alcohol from the 1970s (Maltoni et al., 1981; Soffritti et al., 2008; Maltoni et al., 1989), until its most recent adoption by the US National Toxicology Program (Toxicology and carcinogenesis studies, 2020) prenatal design for cancer assays in SD rats are now widely considered a predictive and reliable model for humans. Indeed, it has been clearly shown that chemical exposures even at low doses during prenatal and developmental phases of life can elicit a number of detrimental effects, including not only cancer but also other effects such as endocrine disorders (Toledano et al., 2024). This concept, called the Developmental Origins of Health and Disease, or DOHaD, is now widely accepted and represents a cornerstone of public health prevention strategies for a variety of diseases including obesity, type 2 diabetes, insulin resistance, asthma, cardiovascular diseases, behavioral disorders, neurodegenerative diseases, reproductive disorders and cancer (Heindel et al., 2015). The extended use of prenatal studies have been also recommended for regulatory purposes in order to protect children from the effect of early exposures to chemicals (The Consortium For Children's Environmental H et al., 2025).

6. Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Simona Panzacchi: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Daria Sgargi:** Formal analysis, Data curation. **Eva Tibaldi:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Francesca Truzzi:** Writing – review & editing. **Luana De Angelis:** Investigation. **Laura Falcioni:** Investigation. **Rita Giovannini:** Investigation. **Martina Iuliani:** Investigation. **Marco Manservigi:** Investigation. **Isabella Manzoli:** Investigation. **Ilaria Menghetti:** Investigation. **Roberta Noferini:** Investigation. **Valentina Strollo:** Investigation. **Paul T.J. Scheepers:** Writing – review & editing, Conceptualization. **Daniele Mandrioli:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Ethics approval and consent to participate

Animal study underwent ethics approval.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2026.110319.

Data availability

Data will be made available on request.

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