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Exposure to a glyphosate-based herbicide does not alter maternal care and offspring quality in the European earwig.

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Summary

Sublethal exposure to pesticides can affect the reproduction and population dynamics of many non-target organisms, such as insects. Among these pesticides, glyphosate-based herbicides (GBHs) were long thought to have no effect on insects because their active compounds can only affect plants and microorganisms. However, a growing body of evidence suggests that GBH can directly or indirectly alter a wide range of fitness-related traits in insects. In this study, we tested whether exposure to the GBH Roundup® affects maternal care behaviour and juvenile development, locomotion and immunity in the European earwig, an insect commonly found in vineyards and orchards. First, we exposed female earwigs to Roundup at concentrations ten times below, equal to and ten times above the normal application rate (NAR) and then measured the expression of maternal care in terms of egg collection, egg and juvenile care and defense. We also measured maternal self-grooming and locomotor activity, and the development time, weight and size of newly produced juveniles. In a second experiment, we exposed earwig juveniles to a control solution or to Roundup and then measured their locomotion, the expression of genes involved in their development (the juveniles hormone pathway), and their survival after exposure to a fungal pathogen. Overall, our results showed no significant effect of Roundup on any of the parameters measured. This finding suggests that direct exposure to a GBH formulation may not necessarily induce behavioural, physiological and developmental alteration in this species. It also calls for future studies to explore the underlying mechanisms behind this apparent lack of sensitivity. More generally, these results highlight the importance of assessing the impact of pesticide use and the factors driving potential resilience across a wide range of non-target organisms to ensure sustainable agricultural practices.

Keywords: *Forficula auricularia*, Maternal care, Pesticide, Pest control, Larvae

1. Introduction

Glyphosate, or N-(phosphonomethyl) glycine, is the active ingredient in Roundup, one of the world's most widely used herbicides in small and large scale crop production (Benbrook 2016). This molecule typically acts by inhibiting the 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS), an enzyme that enables the biosynthesis of aromatic amino acids in plants, fungi and bacteria (Duke and Powles 2008). Because this enzyme is not present in animals, it was long thought that exposure to glyphosate-based herbicide (GBH) would have no effect on mammals, birds and arthropods. However, a growing body of evidence indicates that this is not necessarily the case (Defarge et al. 2018). In a taxonomically large number of species, exposure to GBH has been shown to alter fitness-related traits such as behaviour, development, and immunity (for reviews see de Brito Rodrigues et al. 2017; Battisti et al. 2021; Talyn et al. 2023). For example, GBH exposure modifies locomotor activity in termites, cockroaches and in rats (Rocha et al. 2019; Kanabar et al. 2021; Ekaye et al. 2022), alters the moulting and metamorphosis process in juveniles of *Spodoptera littoralis*, *Spodoptera frugiperda* and honey bees (El-Sheikh et al. 2016; Vázquez et al. 2018), reduces immunocompetence and immunity in silkworms (Feng et al. 2023), as well as impairs reproduction in the mallard duck (Oliveira et al. 2007) and other birds populations in wetlands (Santillo et al. 1989; Linz et al. 1996). The mechanisms driving these alterations remain unclear and are often attributed to the toxic impact of adjuvants present in GBHs (Defarge et al. 2018), GBH-induced changes in the host microbiota (Vázquez et al. 2018; Cullen et al. 2023), or endocrine disruptor properties of GBH (for reviews see Kalofiri et al., 2021; Levine et al., 2020; Muñoz et al., 2021). They may also involve other physiological parameters of the hosts, as suggested by the age-specific effects of GBH – stronger effects in juveniles than adults - reported in some mammals (Pope and Liu 1997) and insects (Stuijzand et al. 2000; Fogel et al. 2016).

In addition to the host traits detailed above, recent data indicate that GBHs may also alter the expression of social behaviours, such as maternal care, which have major implications for individual fitness and population dynamics (Cummings et al. 2010; Fong-McMaster et al. 2020). For instance, GBH exposure decreases the amount of time mothers spend caring for their offspring in rats (Dechartres et al. 2019) and reduces the expression of maternal defensive and grooming behaviours in rats and mice (Rocha et al. 2019; Ait-Bali et al. 2020). However, these data are mostly from studies in mammals, raising questions about the effect of GBH exposure on the expression of parental care in other animals, including insects. This lack of data on insects is surprising for three main reasons. First, parental care is a taxonomically widespread phenomenon in arthropods (Machado and Trumbo 2018; Meunier et al. 2022), in which it is well documented that even subtle changes in the expression of this behaviour can dramatically affect reproduction and population dynamics, with major consequences for terrestrial ecosystem functioning, biodiversity maintenance, and crop protection (Schowalter et al. 2018). Second, studies suggest that exposure to various pesticides and chemical pollutants can alter insect parental care, opening the possibility of an effect of GBHs on this trait (Gallegos et al. 2016; de Oliveira et al. 2022). Finally, insects are frequently exposed to GBHs in crops and gardens (Mazzia et al. 2015). Studying their effects is, therefore, biologically relevant and timely, as it could enhance our understanding of the maintenance and population dynamics of many species that play crucial role in current agroecosystems.

The European earwig is an ideal species to study the effect of GBH on maternal care, as it is well known for the extensive care that mothers give to their eggs and newly hatched juveniles, and its ubiquity in multiple agricultural areas worldwide (for reviews see Orpet et al. 2019; Meunier 2024; Honorio et al. 2024). Earwig mothers typically care for their eggs during 50 days, during which they regularly groom, clean and move their eggs during development (Koch and Meunier 2014; Thesing et al. 2015). They then care for their juveniles (called

nymphs) for two weeks, providing them with food, protecting them against pathogens and defending them against predators (Lamb 1976; Kölliker 2007). Unlike egg care, post-hatching care is facultative in this species, as newly hatched nymphs quickly become mobile and able to forage independently (Wong and Kölliker 2012). Regardless of its maternal care, the European earwig is widespread in vineyards and orchards, where a wide variety of pesticides and herbicides are frequently used (Malagnoux et al. 2015b). This species is considered a crop auxiliary in pip-fruit orchards, where it feeds on aphids, moths and psyllids without eating the fruit, while it is considered a pest in stone fruit orchards, where it also feeds on leaves and fruit (Dib et al. 2011; Orpet et al. 2019; Alins et al. 2023). Previous studies have shown that exposure of earwig females to pesticides such as deltamethrin reduces the expression of maternal care and increases their investment in future reproduction (Meunier et al. 2020; Mauduit et al. 2021a). Similarly, a recent study showed that direct exposure of males to the GBH Roundup GT Max® altered their locomotor activity, but not their boldness, aggregation level or immunity (Pasquier et al. 2024). In contrast, female exposure to other chemicals such as cadmium, a heavy metal pollutant, and pyriproxyfen, another pesticide, does not alter the expression of maternal care (Merleau et al. 2022; Honorio et al. 2023a, b).

In this study, we conducted two experiments in the European earwig to investigate the effects of (1) direct exposure of females to a GBH formulation on the expression of maternal care and the associated clutch fate, and (2) direct exposure of juveniles to the same GBH formulation on their behaviour, pathogen resistance and expression of genes involved in the development. The GBH used in each experiment was RoundUp GT Max® (hereafter referred to as Roundup; Scotts France SAS), a formulation widely applied for weed control in agricultural systems and commonly used in domestic gardening. In the first experiment, we exposed 239 females to one of four solutions of Roundup and then measured the expression of six classical forms of egg and nymph care, as well as the development time and hatching rate of their eggs, and the size and weight of newly hatched nymphs. In the second experiment, we exposed 282 nymphs to either a control solution or a solution containing the highest concentration of the same glyphosate formulation (detailed above) and then measured their general activity, the expression of genes involved in their development [Juvenile Hormone (JH) pathway] and their survival after infection with the common entomopathogenic fungus *Metarhizium brunneum*. Overall, we predicted that female exposure to GBH would induce changes in their expression of maternal care and the development and quality of their resulting nymphs, and that juvenile exposure to Roundup would alter their behaviour, gene expression of JH production and survival to pathogen infection.

2. Materials and methods

2.1 Animal breeding and overview of experiments 1 and 2

The two experiments involved 239 field-sampled females and 282 of their first generation laboratory-born nymphs. All these individuals belonged to *Forficula auricularia* Linnaeus, 1758, also called *Forficula auricularia* clade A (González-Miguéns et al. 2020). We collected the females in July 2022 in an organic peach orchard near Valence, France (Lat 44.978526, Long 4.926383). We then maintained them under standard laboratory conditions until oviposition, which occurred in December 2022 (Mauduit et al. 2021b; Merleau et al. 2022). The first experiment started 16 days after oviposition. This timing corresponds to the first third of the egg care period and reflects a stage at which care behaviours are already well established (Honorio et al. 2024). On that day, we counted and trimmed the number of eggs produced by each mother to 25 to standardise the amount of care subsequently required by the clutch. We then exposed each mother to one of three doses of glyphosate formulation or a control solution

and subsequently used standard protocols to measure the expression of care and non-care behaviours, as well as the clutch fate (see details below). The second experiment started 9 days after egg hatching, at a stage when nymphs are already active and have initiated foraging activities outside the nest (Honorio et al. 2024). On that day, we isolated 235 nymphs from 56 control clutches (4 ± 2 nymphs per clutch), exposed each of them to either a high dose of glyphosate formulation or a control solution and then used standard protocols to measure their locomotor activity, immune competence and for gene expression, we isolated 47 nymphs from 24 control clutches of the 56 previous clutches (2 ± 1 nymphs per clutch) using the same protocol (see details below).

Throughout the two experiments, females, eggs and nymphs were maintained under standard laboratory conditions (Meunier et al. 2012). From oviposition to hatching, each female and her eggs were kept in small Petri dishes (diameter 5 cm) at a constant 10 °C in the dark. From hatching, each female and her nymphs were maintained in large Petri dishes (diameter 9 cm) under 12:12 light:dark cycle at 20:18 °C, respectively. Each Petri dish was lined with moist sand to maintain high and constant humidity levels. Before oviposition and after egg hatching, each female (and her nymphs) was fed with laboratory prepared diet consisting mainly of a mixture of pollen, cat food and bird seed (details in Kramer et al., 2015). The food was changed once a week. We did not provide food to the mothers between oviposition and egg hatching, as females typically stop their foraging activity during this period (Kölliker 2007).

2.2 Exposures of mothers and nymphs to glyphosate-based herbicide

In the first experiment, we exposed 239 females to one of four doses of the glyphosate formulation. For this exposure, we used RoundUp GT Max® (Scotts France SAS) and followed a standard protocol that mimics earwig exposure to pesticides (including RoundUp GT Max®) through contact with a contaminated surface (Malagnoux et al. 2015a; Meunier et al. 2020; Pasquier et al. 2024). Following Pasquier et al. 2024, we prepared four GBH solutions by diluting RoundUp GT Max in milliQ water at concentrations equal to the Normal Application Rate (NAR) (240 mg/m²; N=60), 10 times lower than NAR (24 mg/m²; N=60), 10 times higher than NAR (2400 mg/m²; N=59), or to a control solution (0 mg/m²; N=60). As an indication, French tree growers use an average of 1,500g/ha (= 150 mg/m²) of Roundup in their crops (ANSES, 2020). We applied 200 µL of each of these solutions evenly to filter papers (VWR 516-0812; diameter 5.5 cm) previously placed in individual Petri dishes and allowed them to dry for a few minutes under a hood. We subsequently placed each female in these Petri dishes and allowed them to walk on the filter paper for four hours at room temperature (Mauduit et al. 2021b; Merleau et al. 2022). Once this delay is over, we returned each mother to her initial Petri dish with her eggs.

In the second experiment, we randomly selected 6 ± 2 nymphs per family and exposed three of them to the formulated GBH solution (RoundUp GT Max®; Scotts France SAS) at 10 times the NAR (2400 mg/m², N=121) and the three others to the control milliQ water solution (N=137). The exposure was done on single individuals. We used the same protocol as described above to prepare the solutions and do the exposure. After exposure, we transferred each nymph to new Petri dishes until their use for subsequent measurements.

2.3 Measurement of pre- and post-hatching maternal care (experiment 1)

We first tested whether the exposure of the 239 mothers to Roundup affected their expression of egg care in terms of egg collection, frequency of maternal contact with the eggs, egg defence and egg retrieval after abandonment (Meunier et al. 2020; Merleau et al. 2022). (1) Egg

collection shows the extent to which mothers actively collect their eggs after the clutch has been experimentally dispersed (Merleau et al. 2022). For this measurement, we randomly scattered the 25 eggs from each female in the Petri dish while the females were isolated for Roundup exposure. At the end of the exposure period, we replaced each female in the centre of her Petri dish and recorded the number of eggs gathered within a 1 cm circle 2h post-exposure. (2) The frequency of contact between mothers and eggs reflects the number of observations during which a mother was in contact with its eggs over 30 min of observation (Boos et al. 2014). Twenty-four hours after exposure of the females, we used a scan sampling procedure by direct observation through the lids of the Petri dishes. We recorded every minute whether the females were touching/cleaning the eggs with their mouthparts (i.e. grooming) and/or with antennae (i.e. antennation). (3) Just after measuring the frequency of the contact between mother and eggs, we measured the level of egg defence, which shows females' willingness to protect their eggs from a simulated predator attack. We gently opened each Petri dish, standardly poked each female on the pronotum with a glass capillary (1 poke / sec) and recorded the number of pokes required until they moved more than 1 body length away from the clutch. High values of egg defence (poke number) show high maternal investment in egg care and vice versa. Finally, (4) the delay of egg retrieval shows the delay after which a female returns to its eggs after being chased away by a simulated predator attack (Thesing et al. 2015). Immediately after the measurement of egg defence, we recorded the number of seconds it took for each female to touch one of her eggs again. We stopped recordings after 600s (10 min). Long delays of egg retrieval indicate low maternal egg care and vice versa. After this last measurement, we maintained each female under the standard conditions described above until egg hatching.

We then tested whether maternal exposure to Roundup before the eggs hatched later affects their expression of care for the nymphs (Meunier et al. 2012; Thesing et al. 2015). Eight days after hatching, we measured two forms of post-hatching maternal care in the 205 females whose eggs had hatched (D0: N=53, D24: N=50, D240: N=52 and D2400: N=50). These forms of care were (1) the frequency of maternal contact with the clutch of nymphs and then, (2) the level of nymph defence against a simulated predator attack. We performed these measurements using the same procedures described above. In brief, we used the scan sampling technique to record whether females actively touched the nymphs with their mouthparts/antennas and then, to record the number of pokes required to move a mother away from her clutch.

2.4 Measurement of non-care behaviours by mothers (experiment 1)

We also used the females exposed to Roundup to measure two behaviours that were not associated with care. The first one was (1) the frequency of self-grooming, an important behaviour allowing insects to remove dirt and pathogens, to apply self-secreted chemicals on the cuticle to enhance protection against desiccation and to mediate communication with conspecifics (Blomquist and Bagnères 2010; Boos et al. 2014; Weiß et al. 2014). We measured the frequency of self-grooming both during egg care and during nymph care. This occurred during the 30 min of scan sampling where we recorded the frequencies of mother-egg and mother-nymph interactions (see above). We defined self-grooming as when a female touched or scratched any part of her body (i.e., antennae, legs, abdomen, cerci) with her mouthparts and/or legs. The second behaviour was (2) the locomotor activity of females in absence of eggs (Merleau et al. 2022). On day 55 after exposure, we gently transferred each female to an empty circular arena (diameter 18 cm) held between two glass plates on an infrared light table. We then video recorded females for 20 min under darkness with infrared light (BASLER BCA 1300, Germany; Media Recorder v4.0, Noldus Information Systems, the Netherlands) and defined its locomotor activity as the total distance walked by the female during this time. This

distance was automatically extracted from the videos using the software EthoVision XT 16 (Noldus Information Technology©, Wageningen, Netherlands).

2.5 Measurement of offspring development and quality (experiment 1)

In addition to maternal behaviours, we also tested the effect of female exposure to GBH on their clutch fate in terms of egg development time, hatching rate, nymph weight at hatching, nymph size and nymph survival rate during the first eight days of family life. (1) We defined egg development time as the number of days between oviposition and hatching. (2) We defined egg hatching rate as the number of newly hatched nymphs divided by the number of eggs present with the female on the day of exposure, i.e. 25 days after oviposition. (3) We measured the mean weight of newly hatched nymphs by weighing a random group of ten nymphs per clutch (or the total number of nymphs available if less than ten) to the nearest 0.01 mg using a microbalance (OHAUS© Discovery DV215CD). (4) As a proxy of body size, we measured the minimum inter-ocular distance of one random 2-days old nymph per family using a binocular scope (Leica M80®) and ImageJ v1.54d software. Finally, we (5) defined nymph survival during the first eight days of family life as the number of nymphs still alive on the eighth day of family life divided by the number of nymphs initially present in the clutch.

2.6 Measurement of nymph locomotion, pathogen resistance and gene expression (experiment 2)

In the second experiment, we tested the effects of nymph exposure to Roundup or control solution on their locomotor activity (2 nymphs per family), pathogen resistance (2 other nymphs per family + the 2 nymphs used for locomotor activity), and gene expression (2 other nymphs per family). We conducted all these measurements one day after Roundup exposure (i.e. 10 days after hatching). (1) We measured locomotor activity using the same protocol as described above for mothers. It was defined as the total distance a nymph walked in an open arena 3.75 cm in diameter for 20 minutes. (2) We measured pathogen resistance as the survival rate of nymphs after exposure to spores of the entomopathogenic fungus *M. brunneum*. *M. brunneum* is a common, natural and lethal pathogen of *F. auricularia* (Kohlmeier et al. 2016). We individually placed each nymph on a filter paper and then covered them with 100µL of either a solution of *M. brunneum* spores (3×10^8 spores/mL, diluted with 70% water and 30% humectant in 0.01% Tween 80 – Mycelia #04-21-5000SPW) or to a control solution (milliQ water). We allowed the nymphs to rest on the filter paper for five seconds and then transferred them to a new Petri dish (diameter 5.5). This Petri dish contained a layer of moist sand and was kept under a 12:12 light:dark cycle at 20:18°C and received the laboratory prepared food that was changed once a week. We then checked each Petri dish daily to record whether the nymphs were dead or alive for the next 21 days. Overall, this provided us with a total of 67 nymphs exposed to both Roundup and *M. brunneum*, 53 nymphs exposed to Roundup only, 59 nymphs exposed to *M. brunneum* only and 56 nymphs exposed to neither Roundup nor *M. brunneum*. (3) We used RT-qPCR to measure the expression of four genes involved in the juvenile hormone pathway in nymphs (Table 2). Juvenile hormone is one of the main hormones involved in metamorphosis regulation in insects (Riddiford 1994). These genes were JHAMT, which catalyses one of the last steps in juvenile hormone synthesis (Riddiford 1994), Kr-h1 which encodes a transcription factor in the juvenile hormone signalling pathway (Shinoda and Itoyama 2003), and JHE (JH esterase) and JHEH (JH epoxyde hydrolase) which encode for enzymes breaking down juvenile hormone (Li et al. 2022). We performed RNA extraction using NucleoSpin® RNA kit (Macherey Nagel, Düren, Germany), according to the manufacturer's instructions. In brief, cells were lysed by mechanical grinding with Tissue Lyser (Qiagen,

Hilden, Germany) and 2 Tungsten beads (Qiagen, Hilden, Germany) in buffer containing β -mercaptoethanol. After filtration of the lysate, nucleic acids were retained on a silica column in a salt-rich medium. The membrane was washed with 70% ethanol, and the DNA digested by rDNase. Finally, the RNA was eluted in 40 μ L of water. The extracted RNA was quantified using the Qubit[®] 2.0 flowmeter (Invitrogen, Eugene, USA) and the Qubit[™] HS RNA Assay kit (Invitrogen, Eugene, USA). Reverse transcription was performed on 750ng of RNA using the QuantiTect kit Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA removal was performed prior to the reverse transcription at 42°C for 2 min in a volume of 14 μ L. The reverse transcription was performed at 42°C for 15 min and 3 min at 95°C. The cDNAs obtained from the earwigs' RNA were stored at -20°C and diluted 1:10 prior to use in qPCR. Real-time PCR was performed in a final volume of 13 μ L using SYBR 1X (i.e. 6.25 μ L MESA Blue qPCR[™] Mastermix Plus for SYBR[®] Assay, (EurogenTech, Seraing, Belgium), primers at 3.125 μ M final (1.2 μ L forward and reverse primer mix) and cDNA (3 μ L, i.e. 11.25ng RNA equivalent per well). Samples (triplicates for housekeeping genes, *Krh1* and *JHAMT* and duplicates for *JHEH* and *JH esterase*) were distributed in a 384-well plate (4309849, Applied Biosystems[™], Waltham, USA) using the EpMotion5070 robot (Eppendorf, Montesson, France). QPCR was performed using the QuantStudio[™] 6Flex machine (Thermo Fisher, Waltham, USA) and associated software. The primers used are shown in Table 1. We used Actin and *Mnf* as housekeeping genes, which are reference genes in the European earwig (Roulin et al. 2014). The relative gene expression quantification was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) with $\Delta Ct = Ct \text{ target gene} - Ct \text{ housekeeping genes}$ and $\Delta\Delta Ct = \Delta Ct \text{ sample} - \Delta Ct \text{ D0}$.

Table 1. Primers pairs used in RT-qPCR

| Primer name | Gene name | GenBank ref (TSA) | Amplified fragment length (bp) | Sequence (5' - 3') |
|------------------------|-----------|-------------------|--------------------------------|-----------------------|
| 2062.Fa.Kr-h1.F4 | Kr-h1 | GAYQ02035509.1 | 165 | CCTGTATGGGTGCGTGTATG |
| 2063.Fa.Kr-h1.R4 | | | | GAGGCCTCACAAATGCTCAG |
| 2022.Fa.JHEH.F2 | JHEH | GAYQ02037617.1 | 204 | ATGCTAAACCGCCACCTAGT |
| 2023.Fa.JHEH.R2 | | | | CCCCGAGTCCAGGTTTAGTT |
| 2240.Fa.JH_esterase.F3 | JHE | GAYQ02037746.1 | 173 | TGCTGCTTTTGGTGGGAATC |
| 2241.Fa.JH_esterase.R3 | | | | TTTCACGTGCTGCTTTCTGG |
| 2056.Fa.JHAMT.F3 | JHAMT | GAYQ02029801.1 | 168 | CAAGGCGATATTGGAGCACC |
| 2057.Fa.JHAMT.R3 | | | | GGGACAATAGGCAAGGAAAGC |
| 2130.Fa.Actin.F6 | actin | GAYQ02043878.1 | 247 | CACCCCGTTTTACTGACGGA |
| 2131.Fa.Actin.R6 | | | | GACCAGCAAGGTCAAGACGA |
| 2126.Fa.mnf.F4 | mnf | GAYQ02036296.1 | 178 | GAAACCCCTCTTTGGCGAC |
| 2127.Fa.mnf.R4 | | | | CCGCTGATAAAGGATGGCAG |

2.7 Statistical analysis

We conducted all statistical analyses using the software R v4.1.1 (<https://www.r-project.org/>) loaded with the packages *car* (Fox and Weisberg 2023), *DHARMA* (Hartig 2020), *emmeans* (Lenth et al. 2023) and *survival* (Therneau 2015). We analysed the egg grooming, the self-grooming, the number of pokes for eggs and juveniles, the delay of maternal return for eggs, the activity of the females, the egg development, the number of newly hatched juveniles, the weight of juveniles at hatching and the size of the juveniles using ten linear models (*lm* function R). We analysed the egg gathering (proportion of females with gathered clutch after 2h) and the ratio of full clutch failure (proportion of clutch failure) using two generalized linear models (*glm* function R) with binomial error distribution and balanced using the *cloglog* function for the ratio of full clutch failure. We analysed the number of contacts from mothers to juveniles using a generalized linear model (*glm* function R) with poisson error distribution. For the analyses of gene expression (JHEH, JHAMT, Krh1 and JH esterase) and for the activity of the juveniles we used five linear mixed models (*lmer* function R). Finally, we analysed the survival of juveniles after pathogen infection using a Cox proportional hazard regression model allowing for censored data to account for juveniles still alive at the end of the observation time. In each of these models, we entered as response variables the type of exposure (GBH alone, *Metarhizium* alone, GBH+*Metarhizium* and control categorical). We checked that all model assumptions were met using the *DHARMA* R package and transformed the response variable where it was required. In particular, we used log+20 transformation for the activity of the females, log+1 transformation for the egg grooming, the self-grooming, the number of pokes for juveniles, and the activity of the juveniles, log transformation for the egg development and the weight of juveniles, log+0.1 transformation for the number of pokes for eggs, and squared transformation for the number of newly hatched juveniles. Statistical values were obtained using Type II ANOVA (*Anova* function in the *car* package in R), where the effect of each variable is corrected by the variance explained by the other variables. Finally, we conducted pairwise comparisons using estimated marginal means of the models with P-values corrected for multiple testing using Tukey methods (*emmeans* package).

3. Results

Experiment 1 - Maternal exposure to Roundup

Maternal exposure to Roundup had no effect on the expression of maternal care and non-care behaviours, as well as no effect on the fate of their clutch. Roundup exposure did not affect the likelihood of egg collection by mothers after dispersal (Fig. 1a; LR $\chi^2_3 = 4.69$, $P = 0.196$), the frequency of egg grooming behaviour (Fig. 1b; $F_{3,235} = 0.38$, $P = 0.765$), the level of egg defence (Fig. 1c; $F_{3,233} = 0.84$, $P = 0.471$), the time taken by mothers to return to their clutch of eggs (Fig. 1d; LR $\chi^2_3 = 2.30$, $P = 0.512$), the frequency of mother-nymph contacts (Fig. 1e; LR $\chi^2_3 = 3.01$, $P = 0.390$) and the level of nymph defence (Fig. 1f; $F_{3,148} = 2.01$, $P = 0.115$). It also had no effect on the frequency of self-grooming during both egg care and nymph care ($F_{3,235} = 0.69$, $P = 0.560$ and $F_{3,153} = 0.53$, $P = 0.665$ respectively), and on the general locomotor activity of the exposed females ($F_{3,74} = 1.35$, $P = 0.264$). Finally, Roundup exposure did not affect egg development (Fig. 2a; $F_{3,200} = 0.24$, $P = 0.867$), the probability of total clutch failure (Fig. 2b; LR $\chi^2_3 = 0.71$, $P = 0.871$), hatching rate (Fig. 2c; $F_{3,196} = 0.77$, $P = 0.512$), fresh weight of newly hatched nymphs (Fig. 2d; $F_{3,195} = 0.84$, $P = 0.472$), the size of these nymphs (Fig. 2e; $F_{3,135} = 0.91$, $P = 0.437$) and the nymph survival during the first eight days of family life (Fig. 2f; LR $\chi^2_3 = 2.63$, $P = 0.452$).

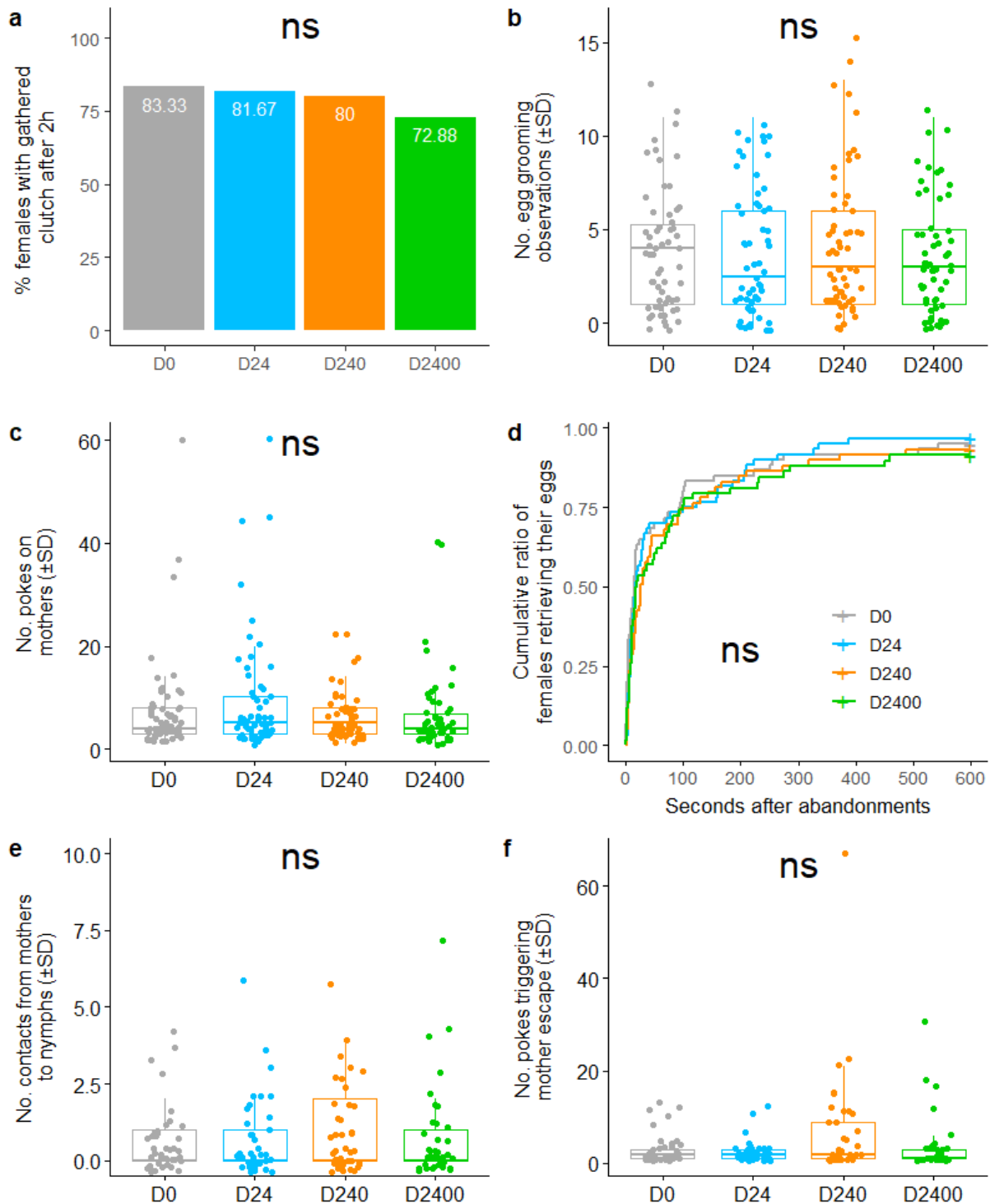


Fig.1 Results of experiment 1 on the effects of maternal exposure to Roundup on the expression of egg care and post-hatching care. (a) Probability that mothers collected their experimentally dispersed eggs. (b) Frequency of mother-egg contacts over 30 min of observation. (c) Level of maternal egg defence against a simulated predator attack. (d) Time spent by females to retrieve their eggs after this simulated predator attack. (e) Frequency of mother-nymphs contacts over 20 min of observation. (f) Level of nymph defence against a simulated predator attack. Boxplots depict median and interquartile ranges, with whiskers extending to 1.5 times the interquartile range and dots representing jittered experimental values. ^{ns} $P > 0.05$.

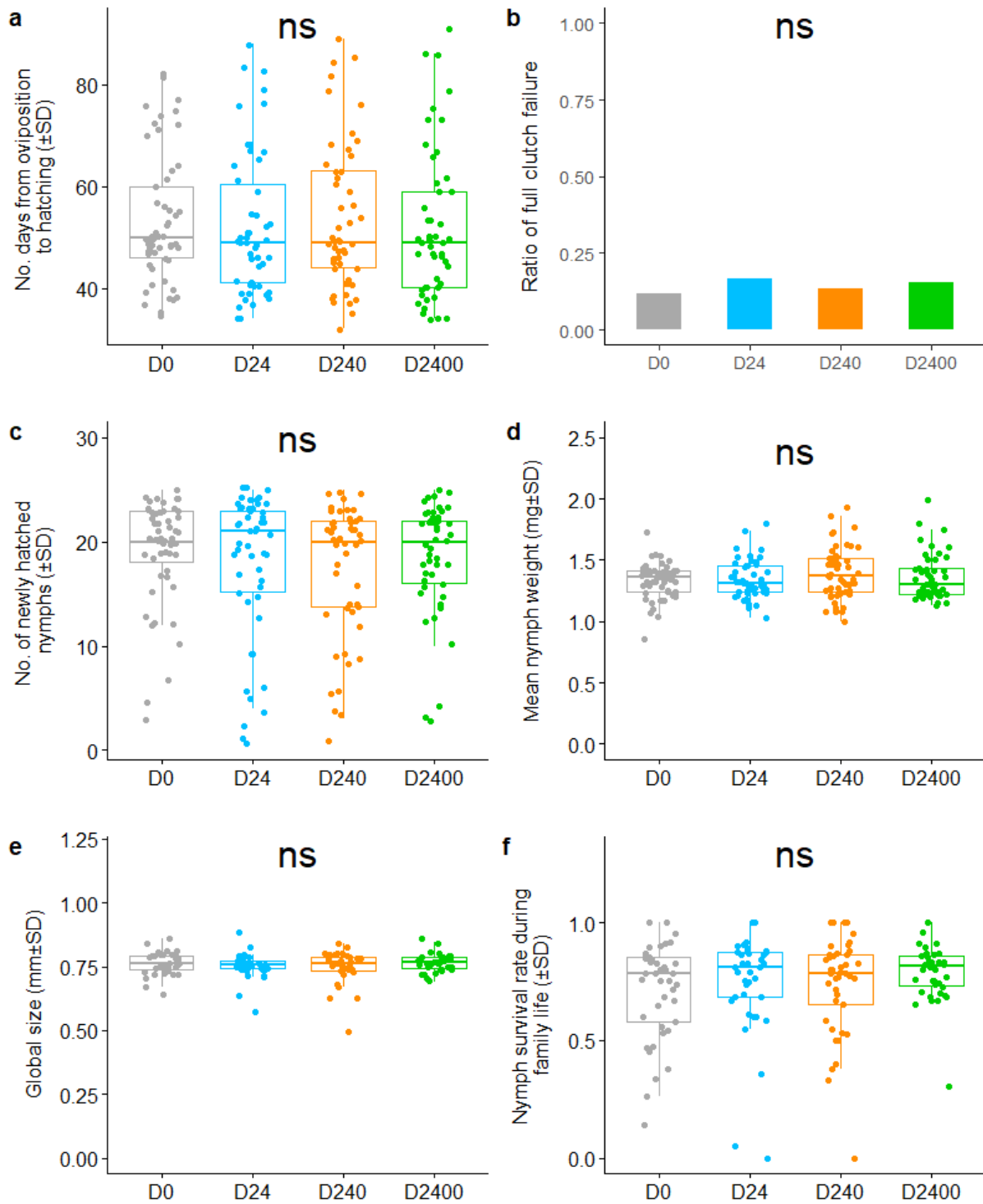


Fig.2 Results of experiment 1 on the effects of maternal exposure to Roundup on clutch fate. (a) Egg development time. (b) Proportion of clutches that completely failed before hatching. (c) Number of nymphs produced at egg hatching. (d) Mean fresh weight of newly hatched nymphs. (e) Size of newly hatched nymphs. (f) Nymph survival during the first eight days of family life. Boxplots depict median and interquartile ranges, with whiskers extending to 1.5 times the interquartile range and dots representing jittered experimental values. ^{ns} $P > 0.05$.

Experiment 2 – Nymph exposure to Roundup

As in the mothers, exposure of nymphs to Roundup had no effect on their locomotor activity (Fig. 3a; LR $\chi^2_1=0.56$, $P=0.455$), their resistance to pathogens (Fig. 3b; LR $\chi^2_1=0.16$, $P=0.691$) and the expression of the four genes of the juvenile hormone pathway (Fig. 4; JHAMT: LR $\chi^2_1=0.01$, $P=0.918$, Krh1: LR $\chi^2_1=0.15$, $P=0.702$, JHEH: LR $\chi^2_1=1.03$, $P=0.306$ and JH esterase: LR $\chi^2_1=0.07$, $P=0.797$). Contact with the fungal pathogen reduced nymph survival rate overall (Fig. 3b; LR $\chi^2_1=12.04$, $P<0.005$), but this effect was independent of Roundup exposure (LR $\chi^2_1=0.02$, $P=0.899$).

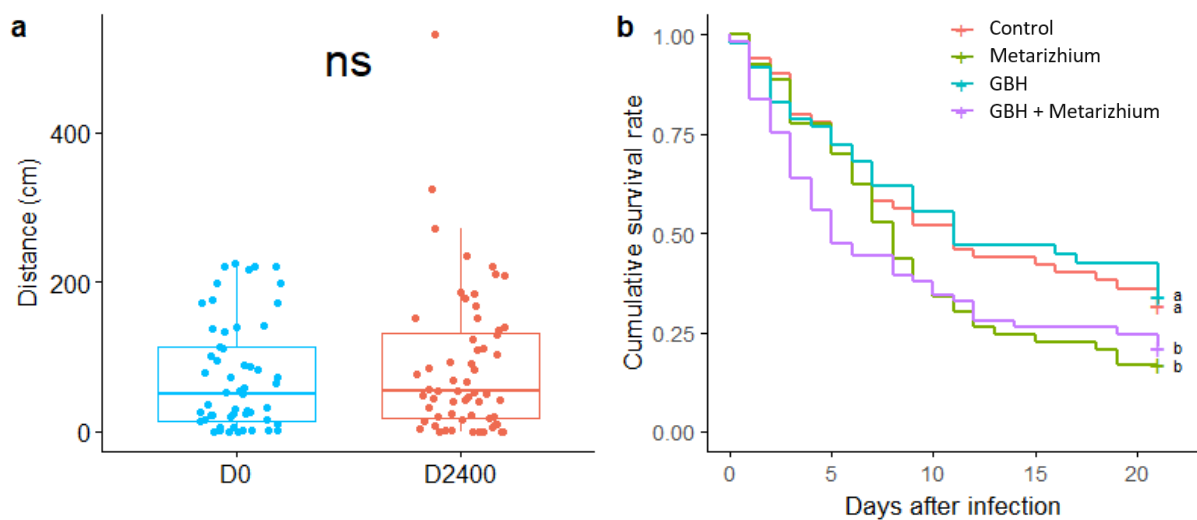


Fig.3 Results of experiment 2 on the effects of nymph exposure to Roundup on locomotion and pathogen resistance. (a) Locomotor activity defined as the distance walked by a nymph during 20 min of observation. (b) Survival rate of the nymphs after exposure to spores of an entomopathogenic fungus. Boxplots depict median and interquartile ranges, with whiskers extending to 1.5 times the interquartile range and dots representing jittered experimental values. ^{ns} $P > 0.05$. Different letters indicate $P < 0.05$.

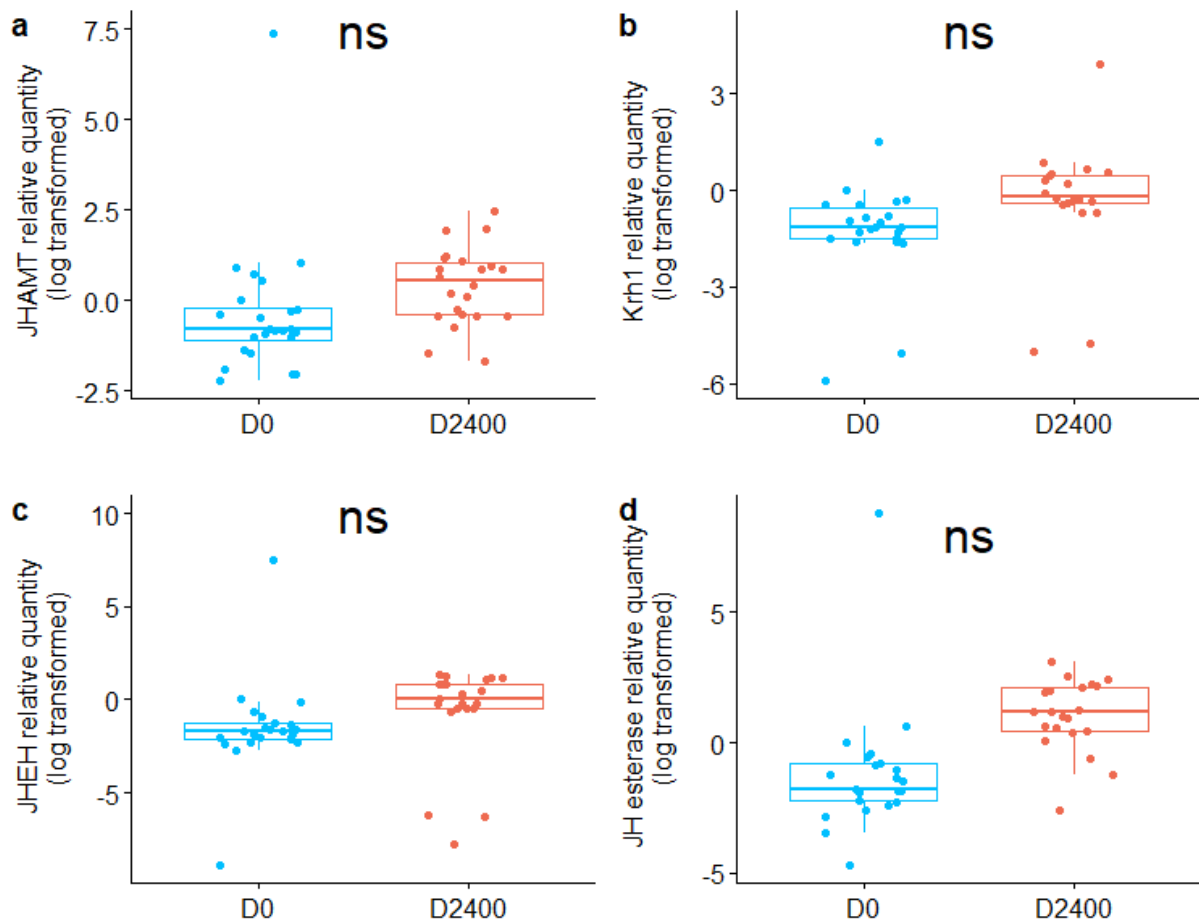


Fig.4 Results of experiment 2 on the effects of nymph exposure to Roundup on gene expression of JH pathway. Relative quantity (log transformed) of genes relative to actin housekeeping gene. (a) JHAMT, (b) Krh1, (c) JHEH, (d) JH esterase. Boxplots depict median and interquartile ranges, with whiskers extending to 1.5 times the interquartile range and dots representing jittered experimental values. ^{ns} P > 0.05.

4. Discussion

A growing body of research suggests that exposure to glyphosate-based herbicides (GBH) can affect the survival and reproduction of non-target animals ranging from mammals to arthropods (Defarge et al. 2018; Pereira et al. 2018; Motta and Moran 2020). Our data on the European earwig do not align with these findings. We found that direct exposure to Roundup, even at doses 10 times higher than those authorized in French orchards, did not alter the behaviour, physiology, gene expression or immune competence of either adult female or juvenile earwigs. Given that exposure to Roundup using the same doses and methods significantly altered the activity of male earwigs (Pasquier et al. 2024), the present results suggest that tolerance and/or resistance of the European earwig to Roundup exposure could be trait, sex and stage specific. A sex-specific effect of GBH would be consistent with previous results showing that exposure to the three pesticides spinosad, acetamiprid, and chlorpyrifos-ethyl altered the predatory behaviour of female but not male European earwigs (Malagnoux et al. 2015a).

Contrary to what has been shown in several rodents (Rocha et al. 2019; Dechartres et al. 2019; Ait-Bali et al. 2020), we found that direct exposure to Roundup does not alter the expression of maternal care in the European earwig. The apparent absence of effect in the European earwig suggests that the genetic, physiological and/or hormonal mechanisms regulating parental care differ between mammals and insects. This could mean that insects, such as the European earwig, might be less susceptible to disruption by exogenous chemical compounds. Unfortunately, our current knowledge of these regulatory mechanisms is very limited in insects (Trumbo, 2019; Trumbo, 2018; Wu et al., 2020), in contrast to mammals (for review Keller et al., 2019). Therefore, future studies are needed to describe their nature and explain whether they could explain this apparent resilience to Roundup. Notwithstanding the mechanisms involved, our results show that the risk of Roundup for the expression of maternal care is limited in the European earwig.

In addition to maternal care, we detected no effect of Roundup on the juveniles in terms of general activity, expression of JH pathway genes and survival after a fungal infection. These findings contrast with results showing that GBHs exposure alters larvae development, behaviour and immunity in various species, including honeybees, amphipods, mosquitoes, damselflies, and lacewings (Lajmanovich et al. 2003; Janssens and Stoks 2017; Baglan et al. 2018; Vazquez et al. 2020; Feng et al. 2022; Gauthier et al. 2023; Du et al. 2024). However, they are in line with previous results in earwigs showing that direct exposure to a chemical pollutant does not alter nymph survival and weight change during development (for review see Honorio et al. 2023b). Interestingly, the fact that Roundup exposure does not alter the expression of JH pathway genes in juveniles suggest that it may lack endocrine-disrupting effects in insects, contrary to the effect commonly observed in mammals (for reviews see Kalofiri et al., 2021; Levine et al., 2020; Muñoz et al., 2021). However, additional support in other insect species is required to confirm this hypothesis.

Overall, there could be three main explanations for the apparent lack of effect of direct exposure to Roundup on earwig females and juveniles. First, our exposure methods could be ineffective. However, this is unlikely to explain our results. The use of identical methods and dose ranges allowed us to detect an effect of roundup exposure on male earwig activity (Pasquier et al. 2024). Similarly, a previous study using the same methods and doses found effects of Roundup in wolf spiders (Lacava et al. 2021). Finally, our methods have proven effective in detecting the effects of various pesticides, such as deltamethrin, spinosad, acetamiprid, and chlorpyrifos-ethyl, on earwigs (Mauduit et al. 2021b; Meunier et al. 2020; Malagnoux et al. 2015a). Second, the European earwig may not be sensitive to Roundup because Roundup acts on parameters that are not involved in the regulation of its physiology, behaviour or immunity. For example, glyphosate's mode of action makes it possible to alter the host microbiota (Vázquez et al. 2018), where even subtle changes are increasingly known to have profound effects on many host functions, including metabolism, development, cognition, nutrition and immunity (Engel et al. 2016; Liberti and Engel 2020; Girard et al. 2023). However, recent data show that the gut microbiota of the European earwig may not be an important driver of its biological functions (Van Meyel et al. 2021; Cheutin et al. 2024), which would render this effect of Roundup inoperative in this species. A third hypothesis is that the European earwig is not sensitive to Roundup because it possesses efficient detoxification mechanisms that act before Roundup can have an effect. In line with this hypothesis, the expression of detoxification enzymes is generally higher in earwigs exposed to several pesticides compared to non-exposed ones (Fricaux et al. 2023), and this species has acquired resistance to insecticides like chlorpyrifos (Le Navenant et al. 2019). However, whether and how this detoxification applies to Roundup remains to be further investigated. Overall, the fact that we did not detect an effect of Roundup on the traits measured in earwig females and

juveniles calls for future studies to explore the physiological mechanisms at play in this species and to conduct longitudinal studies to monitor the apparent lack of effects of Roundup over several generations (e.g., Arreguin-Rebolledo et al., 2023; Kubsad et al., 2019; Le Du-Carrée et al., 2021; Milesi et al., 2021). Future research could also explore the effects of different GBH formulations, as variation in adjuvant composition may lead to differing impacts on behaviour, development, and immunity (Defarge et al. 2018).

In conclusion, the data presented in this study show no effect of direct exposure to Roundup GT Max®, a formulated glyphosate solution, on females and juveniles of the European earwig, even at high concentrations. This apparent lack of effect can be surprising, as this GBH is known to alter life history traits ranging from behaviour, to development and immunity in a broad range of animals (for reviews see de Brito Rodrigues et al. 2017; Battisti et al. 2021; Talyn et al. 2023). However, it is consistent with recent studies suggesting that the European earwig (particularly females) is only poorly affected by direct exposure to various pesticides and chemical pollutants (Malagnoux et al. 2015a; Le Navenant et al. 2019; Merleau et al. 2022; Honorio et al. 2023a, b) (but see Mauduit et al. 2021b). These results highlight the variable sensitivity of animal species to chemical contaminants and open the way for future work to explore the underlying mechanisms that explain this variation and its implications for the evolution of these species in contaminated areas, as well as their conservation and population dynamics in and around agrosystems.

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COMPETING INTERESTS

The authors have no relevant financial or non-financial interests to disclose.

DATA AVAILABILITY

Data can be provided on request if necessary by contacting us.

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