Limited effects of a glyphosate-based herbicide on the behaviour and immunity of males from six populations of the European earwig

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LIMITED EFFECTS OF A GLYPHOSATE-BASED HERBICIDE ON THE BEHAVIOUR AND IMMUNITY OF MALES FROM SIX POPULATIONS OF THE EUROPEAN EARWIG

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Abstract

The use of herbicides on crops often results in unintentional, low-dose exposure of non-target organisms, such as insects. While these exposures are increasingly known to alter the survival and physiology of insects, it remains unclear whether these effects can vary between populations and modify other fitness-related traits, such as behaviour and immunity. Here, we addressed these questions by testing the effects of sublethal exposure to a glyphosate-based herbicide (GBH) on the behaviour and immunity of European earwig males from six natural populations. We exposed each male to a dose of a common GBH (Roundup©) that was either recommended for crops, five times lower than that recommended for crops, or to a control solution. Twenty-four hours later, we measured the activity, boldness, and aggregation of each male. We then exposed them to an entomopathogenic fungus, monitored their survival for 6 weeks, and measured the immune response of the survivors. We found a condition-dependent effect of GBH exposure on male activity. Exposure to low doses induced a positive association between activity and weight, which was not observed in the high-dose and control groups. However, GBH had no effect on any of the other measured traits. All these results were consistent across the six populations tested, although we did find population-specific differences in almost all measurements on males. Further research is now needed to better understand the dose-response to GBH on male activity and its biological impact, as well as to evaluate the effectiveness of detoxification processes in this species. Overall, these results emphasise the importance of investigating the effects of herbicides on insects to expand our general understanding of the use and potential risks of plant protection products in integrated pest management programs.

Key words: *Forficula auricularia*, pesticide, pest control, non-linear response, dose response

INTRODUCTION

The use of crop protection products (i.e. pesticides) is an important factor in ecosystem dysfunction and biodiversity loss (Ndakidemi et al. 2016; Emmerson et al. 2016). In particular, the spraying of these chemical substances on and off crops can affect not only the target plants and animals but also a wide range of non-target organisms, including insects (Desneux et al. 2007). This inadvertent exposure to crop protection products, even at low and sublethal doses, can alter a wide diversity of parameters in the biology of non-target insects, including their physiology, immunity, behavior, and reproduction (Pereira et al. 2018; Strilbytska et al. 2022; Ekaye et al. 2022; Defarge et al. 2023). As a result, the widespread use of these chemical substances around the world is expected to have major impacts on the ecology, fitness and population dynamics of many insect species and, given the many ecosystem services they provide, on the functioning of most ecosystems (Schowalter et al. 2018). Improving our current understanding of the nature, strength and sources of variation in these effects on non-target insects is therefore timely and crucial, particularly for widely used products.

Glyphosate, also known as N-(phosphonomethyl) glycine, is an active compound that is used in more than 140 countries worldwide (Benbrook 2016). Glyphosate is generally used in the form of glyphosate-based herbicides (GBHs), which are mainly composed of glyphosate, water, salt and co-formulants (FAO 2000). Glyphosate typically inhibits the 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS), an essential enzyme of the metabolic pathway responsible for the biosynthesis of aromatic amino acids in plants, bacteria, and fungi (Duke and Powles 2008). As animals do not have EPSPS, GBHs was long thought to be safe for them. However, a growing body of research shows that this claim is inaccurate, often due to the toxic effects of the co-formulants in GBHs (Defarge et al. 2018). For example, direct exposure to GBH decreases locomotor ability in termites (Ekaye et al. 2022) and cockroaches (Kanabar et al. 2021). Direct exposure to GBH may also alter physiological traits including immunocompetence and immunity, as reported in silkworms (Feng et al. 2023). Broader effects of direct and indirect exposure to GBH have also been reported at the level of whole arthropod communities, for example in crops, where the presence of GBH in the environment reduces the density of predators such as ants, spiders and beetles due to altered foraging behaviour (Pereira et al. 2018). Overall, the mechanisms by which GBH alters these traits are

still unclear, but recent studies suggest that it could be through altering their gut microbiota (Motta and Moran 2020; Smith et al. 2021).

While the negative effects of glyphosate and GBH on the health and fitness of insects are increasingly documented in the literature, it remains surprisingly unclear whether the nature and strength of these effects can be population specific. This is a critical gap of knowledge. Any living organism typically has to adapt to the many constraints of its environment, which are defined by natural parameters such as temperature, humidity, and food availability, and anthropogenic parameters such as urbanisation, light pollution, and exposure to chemical pollutants (Fenoglio et al. 2021; Serrão et al. 2022; Theodorou 2022). Because the combination of these constraints is unique to each population, the resulting physiological, reproductive, immunological and/or behavioural adaptations can lead to population-specific sensitivity, resistance or tolerance to crop protection products, even over very small geographical scales (Serrão et al. 2022). In addition to having profound implications for our understanding of the effects of these chemical substances on non-target insect population dynamics in different areas, such population-specific effects may be the source of conflicting conclusions in the literature, and lead to incorrect decisions about the potential risks of plant protection products in integrated pest management programs. However, research is still lacking on whether the effects of GBH exposure can be population specific.

In this study, we addressed this lack of knowledge by examining the effects of exposure to a common GBH on behaviour, immunity and immune competence (i.e., survival to a pathogen infection) in males from six natural populations of the European earwig *Forficula auricularia.* This gregarious insect is a natural enemy of aphids, moths and psyllids, making it an effective pest control agent in pip-fruit orchards where it does not eat the fruit (Dib et al. 2011; Alins et al. 2023). Conversely, in stone fruit orchards, both adults and juveniles often cause damage by feeding on the fruit and are therefore considered pests (Orpet et al. 2019). Earwigs are particularly abundant in orchards and vineyards (Orpet et al. 2019) - two agroecosystems typically treated with numerous chemical substances, including GBH (Mazzia et al. 2015). Over the last few decades, the impact of pesticide exposure on the biology and population dynamics of this species has been the subject of much research, with contrasting results. The first group of studies show that exposure to sublethal doses of pesticides impairs the expression of key earwig behaviours, such as maternal care toward

eggs and juveniles after exposure to deltamethrin (Meunier et al. 2020; Mauduit et al. 2021) and male predator activity and mobility after exposure to spinosad, acetamiprid or chlorpyrifos-ethyl (Malagnoux et al. 2015a). Conversely, another study shows that exposure to pesticides such as deltamethrin could boost earwig reproduction by increasing the likelihood of females producing a second clutch, the number of eggs produced and the hatching rate of these eggs (Mauduit et al. 2021). Finally, the last group of studies shows that exposure to chemicals like pyriproxyfen or cadmium has no apparent effect on earwig females (Merleau et al. 2022; Honorio et al. 2023a, b). As earwig females exhibit population-specific traits in reproduction, physiology, development, survival and behaviour (Ratz et al. 2016; Tourneur and Meunier 2020), it was hypothesised that local adaptation could explain at least some of the large variation in the effects of chemical exposure reported in these studies. However, whether these population-specific traits are indeed linked to population-specific resistance/susceptibility to crop protection products such as herbicides (and GBH, in particular), and more generally whether they do apply to male earwigs, remained unexplored.

We field-sampled 345 earwig males in a random selection of six natural populations in the Touraine region of France (Fig. 1) and then exposed them to one of three solutions of a glyphosate formulation commonly used in domestic gardens (RoundUp GT Max®, Scotts France SAS). Twenty-four hours later, we measured the effects of GBH exposure on three important behaviours in males, which are their general activity, boldness, and aggregation levels. We then measured the effect of GBH exposure on their immunocompetence and immune system (as reported in other insects such as *Galleria mellonella* and *Anopheles gambiae* but with glyphosate alone; (Smith et al. 2021)) by exposing them to spores of the common entomopathogenic fungus *Metarhizium brunneum* and then testing their survival rate and, when alive, their number of circulating hemocytes. We predicted that exposure to GBH would impair males' activity, boldness, aggregation behaviour, immunocompetence and immunity and that the presence and intensity of these effects would vary between populations.

MATERIALS AND METHODS

Animal sampling and breeding

The experiment started with a total of 332 *Forficula auricularia* males field sampled in July 2022 using wooden traps and then maintained during the next three months in the laboratory following a standard protocol (Mauduit et al. 2021; Merleau et al. 2022). The sampling took place at six sites spaced 10 to 50 km apart (Figure 1). These sites are likely to represent distinct populations, as individuals disperse over a maximum distance of a few tens of metres (Moerkens et al. 2010). These sites were in St-Epain (Lat 47.1575169, Long 0.6059085, n = 49 males), Pont-de-Ruan (Lat 47.2494905, Long 0.5753960, n = 59), St-Branchs (Lat 47.2541374, Long 0.7305669, n = 57), La Chapelle-sur-Loire (Lat 47.2417789, Long 0.17674036, n = 59), Lignières-de-Touraine (Lat 47.3047120, Long 0.4395792, n = 54), and Cinais (Lat 47.1606970, Long 0.1763663, n = 54). They were all orchards, except for Cinais, which was on the edge of a forest. Just after field sampling, we placed the males from each population with a similar number of females (sampled from the same population at the same time) in plastic containers (2 to 4 containers per population) lined with moistened sand and containing egg cardboard as a shelter for the individuals. For the following three months, each terrarium was maintained under a 12:12 light:dark cycle at 20:18°C and received an *ad libitum* amount of laboratory-made food, consisting mainly of a mixture of pollen, cat food and bird seed (details in Kramer, Thesing, et Meunier 2015). This food was changed once a week. This three-month period enabled us to standardise the immediate environment of the males before the experiment to possibly highlight any differences between the populations that reflect their environment of origin, rather than a plastic response to their immediate environment. In October 2022 (i.e., three months later), we isolated each male to conduct the measurements detailed below. We transferred them individually to Petri dishes (diameter 5.5 cm) lined with moist sand and maintained under the same conditions as before, with a change of food once a week. We isolated the females at the same time but used them in another experiment not presented here. Three days after isolation, we weighed each male to the nearest 0.01 mg using a microbalance (Discovery DV215CD, OHAUS).

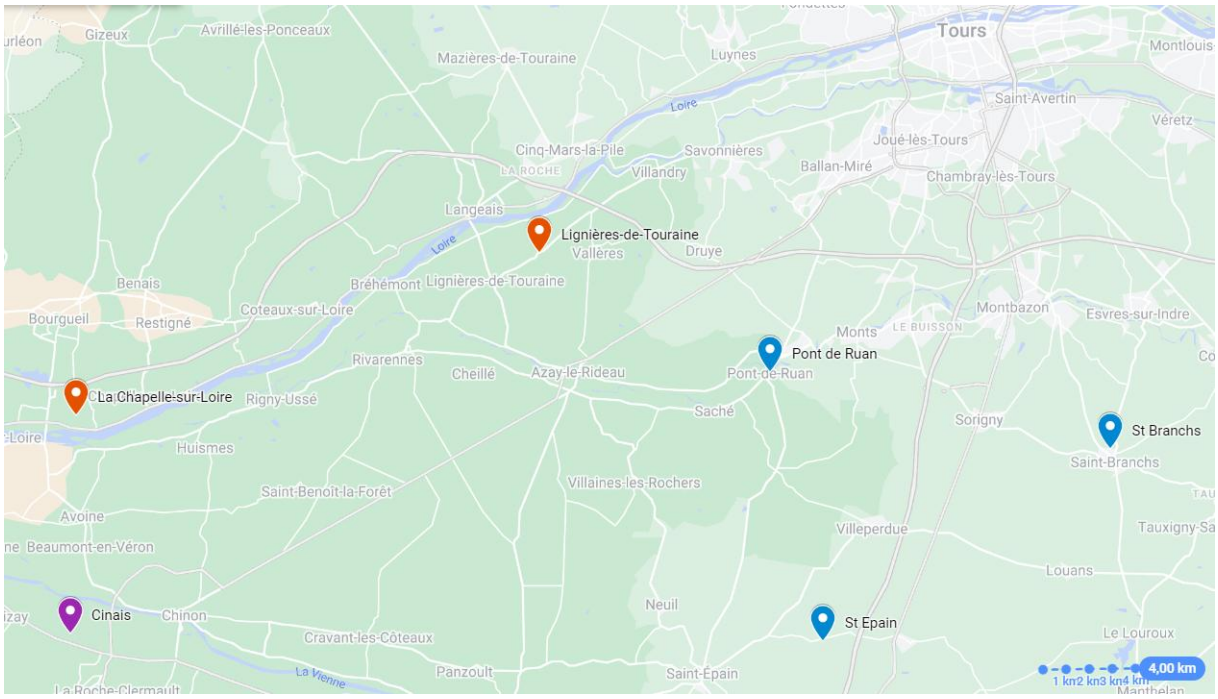

Fig.1 Map of the six tested populations of the European earwig. All these populations were in the Touraine region, France. The population in Cinais (purple = non-cultivated crop) was a non-cultivated field. The populations of St-Branchs, Pont-de-Ruan and St-Epain (blue = organic crop) were organic orchards, while La Chapelle-Sur-Loire and Lignières-de-Touraine (orange = conventional crop) were conventional orchards. Map made with My Maps, Google.

Glyphosate-based herbicide exposure

Two to three weeks after isolation, we exposed each male to residues a glyphosate formulation (RoundUp GT Max®, Scotts France SAS; #GTX800C; Marketing Authorisation No.: 2120018(EAJ); purchased in 2019) commonly used for domestic gardens at a concentration of either 240 mg/m^2 (the recommended dose), 48 mg/m^2 (five times less than the recommended concentration) or 0 mg/m^2 (control). Note that in France, tree growers use on average 1,500g/ha, which corresponds to 150mg/m² of GBH (ANSES, 2020). We followed a standard protocol that mimics earwig exposure to pesticides in orchards through contact with a contaminated surface (Malagnoux et al. 2015a; Meunier et al. 2020). We first prepared three glyphosate solutions by diluting RoundUp GT Max in milliQ water at concentrations of 2.86mg/mL, 0.57mg/mL, or water only (respectively). We then applied 200µL of each of these solutions evenly to single filter papers (VWR 516-0812) covering the bottom of new Petri dishes (diameter 5.5 cm) and allowed filter papers to dry for a few minutes under a hood. We

subsequently placed one male in each Petri dish and let it walk on the filter paper for 4 hours at room temperature (Mauduit et al. 2021; Merleau et al. 2022). Overall, this resulted in the exposure of 113, 111 and 108 males in the 240, 48 and 0 mg/m² treatments, respectively, with 16 to 20 males per population per treatment.

Behavioral measurements

Twenty-four hours after exposure, we measured three behaviours using protocols already established in earwigs (Merleau et al. 2022; Van Meyel and Meunier 2022). The first behaviour was their general activity. We placed each male in an empty circular arena (diameter 8 cm) held between two glass plates on an infrared light table. We then video-recorded their behaviour during the subsequent 20 min under daylight (Camera: BASLER BCA 1300, Germany; Media Recorder version 4.0, Noldus Information Systems, Wageningen, The Netherlands). We defined the level of activity as the total distance walked by a male during these 20 min (Merleau et al. 2022). This distance was automatically extracted from our videos using the video-tracking software EthoVision XT 16 (Noldus Information Technology©, Wageningen, Netherlands).

The second behaviour was boldness. We measured this behaviour using the same 20 min videos and the same software. We defined boldness using two terms: (1) whether a male entered or not the central area (diameter 3 cm) of the tested arena at least once during the 20 min videos and if they entered this area, (2) the total time they spent there during the 20 min videos. These measurements of boldness are standard in rodents (Archer 1973; Stratton et al. 2021) and apply to earwigs, as they are highly thigmotactic and typically avoid open areas (Rankin and Palmer 2009).

The third behaviour was the level of aggregation with conspecifics. We placed each male in a Y-shaped 3D-printed arena held between two glass plates and maintained on an infrared light table. This Y-shaped arena (14 x 20 cm and 0.4 cm high) consisted of an introduction chamber connected to a central chamber, which was itself connected to both an aggregation chamber and a non-aggregation chamber. Each chamber was circular (4 cm diameter) and connected via 0.4 cm wide corridors that were large enough to allow earwig movement between chambers (Van Meyel and Meunier 2022). The aggregation chamber was

connected to another chamber containing four conspecific adults (2 males and 2 females), whereas the non-aggregation chamber was connected to an empty chamber. The connections to these last two chambers occurred via 0.15 cm wide corridors that were large enough to allow odour circulation and antennations but small enough to prevent an earwig from moving through them. We started the test by placing each male in the introduction chamber, waiting 1 min for acclimatization, and then video-recording his movement for 20 min under daylight. The position of the aggregation chamber was swapped with the position of the non-aggregation chamber every other test to avoid laterality bias. As a measure of aggregation, we recorded the total time each male spent in the aggregation chamber (i.e. near conspecifics). The time spent on each chamber was extracted from each video using the video-tracking software EthoVision XT 16 (Noldus Information Technology©, Wageningen, Netherlands). Of the 332 tested individuals, 40 individuals spent no time at all in the test chamber (i.e. they were mostly inactive) and were thus excluded from the statistical analyses. Note that this apparent inactivity was independent of the exposure (generalized linear model with binomial error distribution: GBH: Likelihood Ratio $\chi^2_2 = 0.81$, P = 0.668; Population: LR $\chi^2_5 = 9.46$, P = 0.092; Weight: LR $\chi^2_1 = 0.10$, P = 0.754). The three behaviours were tested on the same day in the order in which they are presented in the text. The tests were done in a blind manner regarding the treatment of the males.

Survival and immune response after pathogen exposure

Immediately after measuring the aggregation level, we exposed each of the 332 males to spores of the entomopathogenic fungus *M. brunneum* following a standard protocol (Kohlmeier et al. 2016). *M. brunneum* (formerly *M. anisopliae*) is a natural pathogen of *F. auricularia*, and is lethal to this species at high concentrations (Kohlmeier et al. 2016). We dipped each male for 5 sec into a 2-mL tube previously filled with 500 µL of a conidiospores solution of *M. brunneum* diluted in 0.05% Tween (10^6 spores/mL – which is lower than the 10^7 spores/mL used in Kohlmeier et al. 2016) or water. We did not dip them in a solution of 0.05% Tween alone, as previous studies have shown that Tween has no effect on earwig mortality (Kohlmeier et al. 2016; Coulm and Meunier 2021). After exposure, we transferred them in individual Petri dishes (diameter 5 cm) lined with moist sand. These Petri dishes were

then maintained at 20°C on a 12:12 light:dark cycle for the next 6 weeks, during which time we checked daily whether the males were alive or dead.

As only 71 (27.2%) males died during these six weeks after exposure to the pathogen, we then measured the immune response (number of circulating hemocytes and percentage of living hemocytes) of the 261 surviving males (n = 90, 87 and 84 for 240, 48 and 0 mg/m², respectively). To this end, we first extracted 2µL of hemolymph in each male using a glass capillary and diluted it in 18µL of Leibovitz's L-15 Medium (Sigma L5520). We then diluted this hemolymph solution (1:200) in Leibovitz's L-15 Medium and added 2µL of trypan blue (Sigma-Aldrich 302643). Finally, we deposited 20µL of the resulting solution on a Cellometer slide (Nexcelom SD100) and counted the number of hemocytes using an automatic Cellometer counter (Cellometer Auto T4 Bright Field Cell Counter, Cellometer software). We disentangled living from dead hemocytes using Trypan blue viability. Dead cells have a membrane that is permeable to trypan blue and therefore appear darker in colour.

Statistical analyses

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 general activity, boldness (time in the open area), aggregation, and total number of circulating hemocytes using four linear models (*lm* function R). We analysed boldness (whether a male entered or not the central area) using a generalized linear model (*glm* function R) with binomial error distribution, and the proportion of living hemocytes (entered with the *cbind* function) using another *glm* with binomial error distribution corrected for overdispersion. Finally, we analysed the survival of males after pathogen infection using a Cox proportional hazard regression model allowing for censored data to account for males still alive at the end of the observation time. In each of these models, we entered as response variables the type of exposure (240, 48 and 0 mg/m²; categorical), the population, the fresh weight of each male and the interaction between the three factors. We entered male's fresh weight in the models as it is known to shape the behaviour and physiology of many insects (Jiménez-Pérez and Wang 2004), including earwigs (Forslund 2003). 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 general activity, log+0.1 transformation for boldness (time in the open area), and square root transformation for both aggregation and total hemocyte number. 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. All statistical models were simplified stepwise by removing nonsignificant interaction terms, after which we confirmed the best model selection based on the smallest Akaike Information Criterion (AIC). This led to the removal of interaction terms in all models except the aggregation model (see results). Finally, we conducted pairwise comparisons using estimated marginal means of the models with P-values corrected for multiple testing using Tukey methods (*emmeans* package).

RESULTS

Glyphosate-based herbicide (GBH) exposure influenced only one of the three behaviours tested. In particular, GBH at 48 mg/m^2 induced a positive association between males' general activity and fresh weight (Fig. 2a; slope ± SE = 0.023 ± 0.007; R^2 = 0.16), whereas this association was absent in males exposed at 240 mg/mL (slope ± SE = -0.004 ± 0.006; R^2 = 0.002) and in control males (slope ± SE = -0.003 ± 0.006; R^2 = 0.005 - Interaction between GBH and weight: $F_{2,321}$= 5.31, P= 0.005; Pairwise comparisons of the slopes; 48 vs 240 : t_{321} = 3.02, P = 0.008; 48 vs control: t_{321} = 2.72, P = 0.019; 240 vs control: t_{321} = 0.15, P = 0.988). By contrast, GBH exposure did not affect boldness in terms of the likelihood of visiting the open area (Fig. 2b; LR χ^2_2 = 1.85, P = 0.396) and time spent in the open area (Fig. 2c; $F_{2,132}$ = 0.89, P= 0.412), and aggregation (Fig. 2d; $F_{2,282}$= 0.43, P= 0.653). It also did not affect the mortality rate of males after pathogen infection (Fig. 3c; LR χ^2_2= 0.44, P= 0.802) and both the total number of circulating hemocytes (Fig. 3a; $F_{2,243}$= 0.34, P= 0.715) and the proportion of living hemocytes (Fig. 3b; LR χ^2_2 = 2.78, P = 0.250) in the surviving males.

Regardless of GBH exposure, the population of origin of the males affected five of the seven parameters measured. This was the case for general activity (Fig. 4a; $F_{5,321}$= 10.31, P < 0.001), boldness (Fig. 4b; likelihood to visit the open area: LR χ^2_2 = 25.39, P = 0.0001, Time in the open area: $F_{2,132}$ = 2.70, P= 0.023), the survival rate after pathogen infection (Fig. 5c; LR χ^2_5 = 12.71, P = 0.026) and the percentage of living hemocytes (Fig. 5b; LR χ^2_5 = 11.77, P = 0.038), but not for aggregation behaviour (Fig.4 d; $F_{2,282}$=0.63, P=0.67) and the number of circulating

hemocytes (Fig.5 a; $F_{5,243}$= 2.17, P= 0.058). Interestingly, the outcome of the five population-specific effects varied from one parameter to another. Males from Cinais (non-cultivated crop) were the most active compared to males from the other populations, while males from La Chapelle-sur-Loire (conventional crop) were the least active (Fig. 4a). Males from Cinais were also the boldest, as they were the most likely to frequent the open area and spent the most time there, while males from the other populations showed intermediate values (Fig. 4b & 4c). By contrast, males from La Chapelle-sur-Loire survived better than males from St-Epain (organic crop), while males from both Pont-de-Ruan (organic crop) and La Chapelle-sur-Loire survived better than males from Lignières-de-Touraine (conventional crop) (Fig.5 c). Similarly, males from St-Epain and Pont-de-Ruan had a larger proportion of living hemocytes than males from La Chapelle-sur-Loire (Fig. 5b). Overall, male weight had no overall effect on the parameters measured (all P > 0.05), except for survival to infection, where heavier males survived longer than lighter males (LR χ^2_1 = 7.58, P = 0.006).

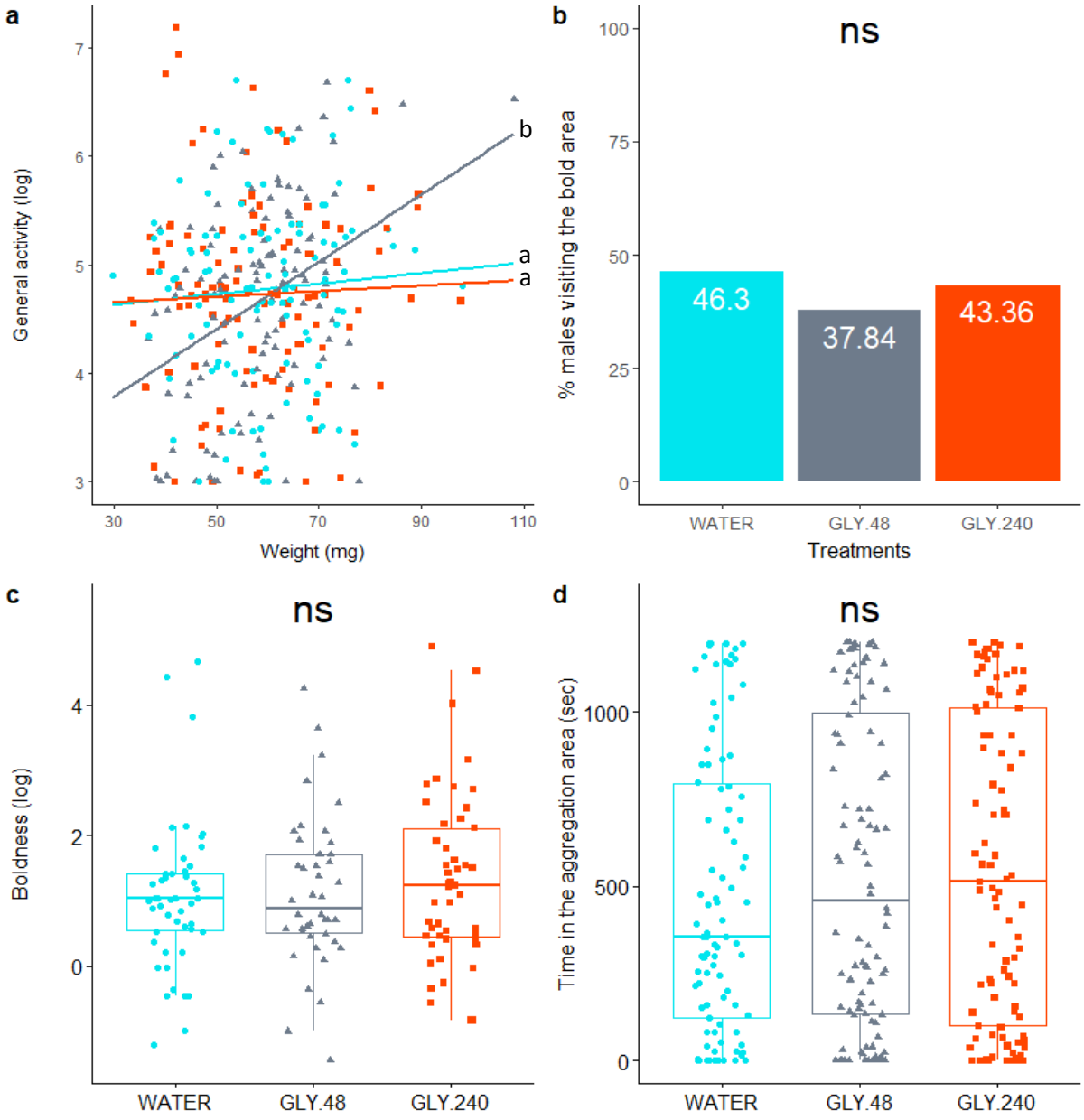

Fig.2 Effects of glyphosate-based herbicide exposure on male behavior. (a) General activity in association to weight, (b) boldness defined as the proportion of males visiting an open area, (c) boldness defined as the time spent by individuals in an open area and (d) aggregation defined as the time spent by individuals close to congeners in separate chambers. Males were exposed to the control solution (WATER, n=108), the GBH solution at 48mg/m^2 (GLY.48, n=111) or the GBH solution at 240mg/m^2 (GLY.240, n=113). Boxplots depict median and interquartile ranges, with whiskers extending to 1.5 times the interquartile range and dots representing jittered experimental values. Ns means non-significant p-value. Different letters indicate statistically significant differences ($P < 0.05$).

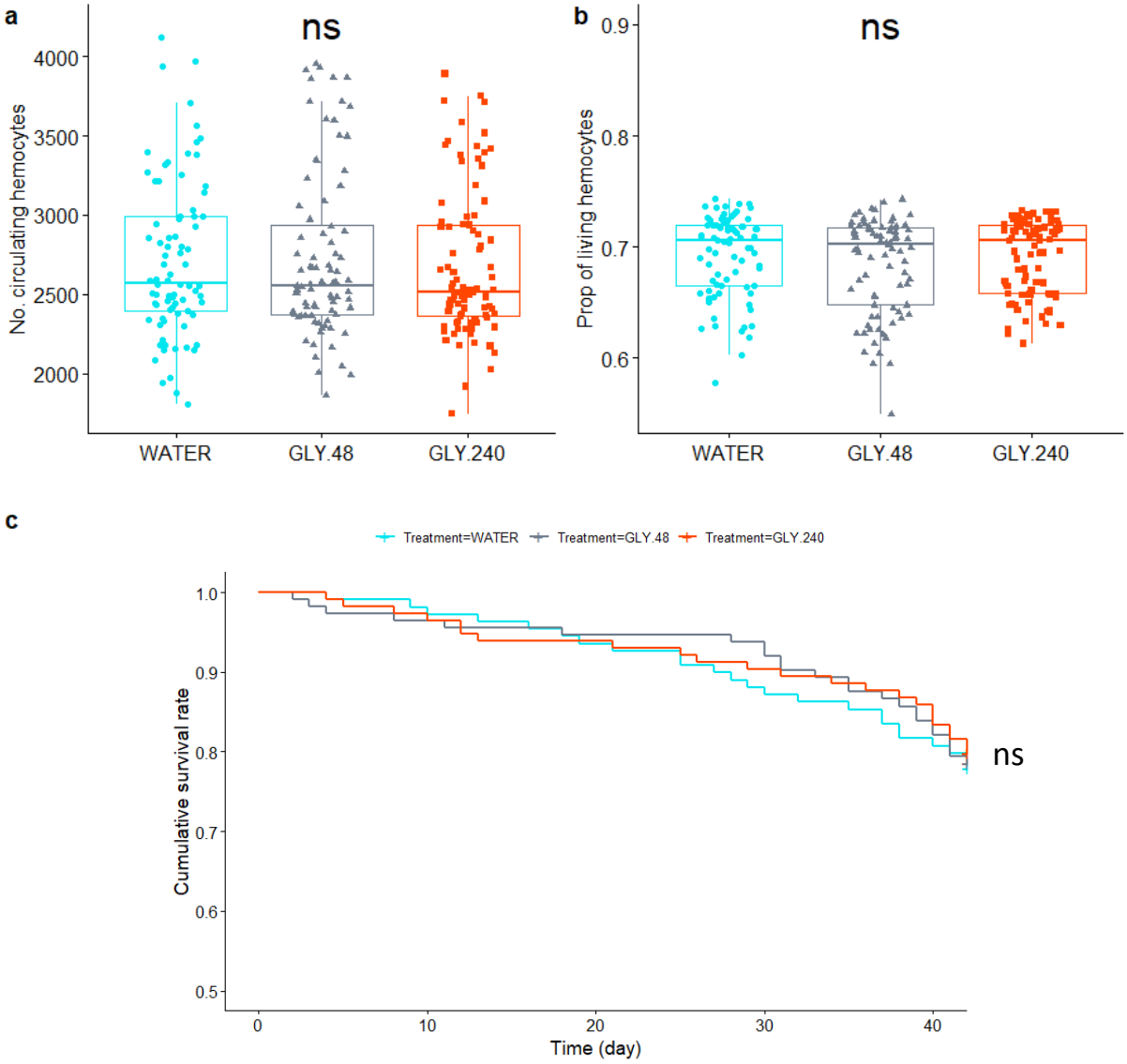

Fig.3 Effects of glyphosate-based herbicide exposure on male immunity and immunocompetence. (a) Number of circulating hemocytes 6 weeks after pathogen exposure, (b) proportion of living hemocytes 6 weeks after pathogen exposure, and (c) male survival rate over 42 days after pathogen exposure. Males were exposed to the control solution (WATER, n=108), the GBH solution at 48mg/m^2 (GLY.48, n=111) or the GBH solution at 240mg/m^2 (GLY.240, n=113). Boxplots depict median and interquartile ranges, with whiskers extending to 1.5 times the interquartile range and dots representing jittered experimental values. Ns means non significant p-value.

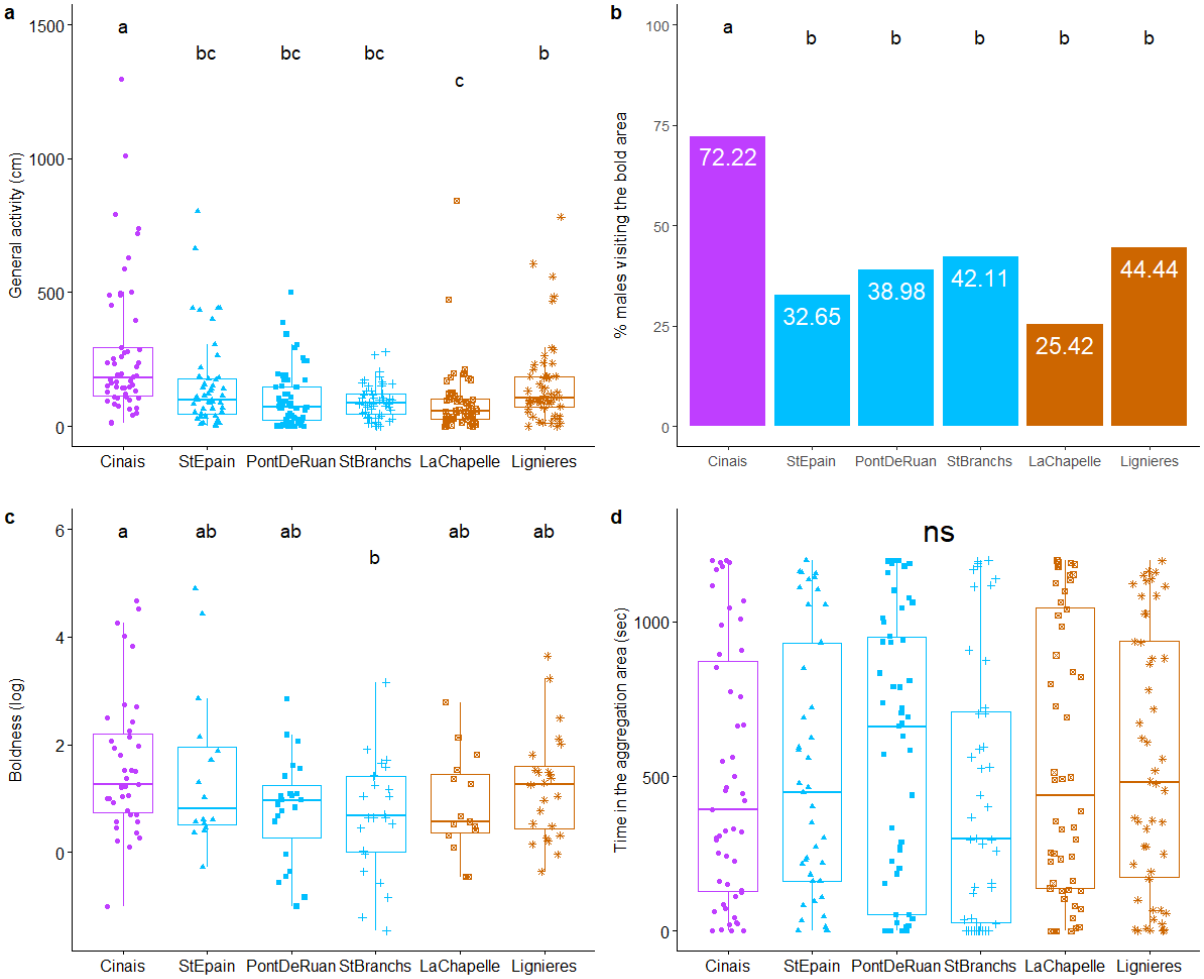

Fig.4 Population effect on male behavior. (a) General activity, (b) proportion of males visiting an open area (c) time spent by males in an open area and (d) time spent by males close to conspecifics. Males come from population of St-Epain n=49, Pont-de-Ruan n=59, St-Branchs n=57 (blue = organic crop), La Chapelle-sur-Loire n=59, Lignières-de-Touraine n=54 (orange = conventional crop), Cinais n=54 (purple = non-cultivated crop). Boxplots depict median and interquartile ranges, with whiskers extending to 1.5 times the interquartile range and dots representing jittered experimental values. Ns means non-significant p-value. Different letters indicate statistically significant differences ($P < 0.05$).

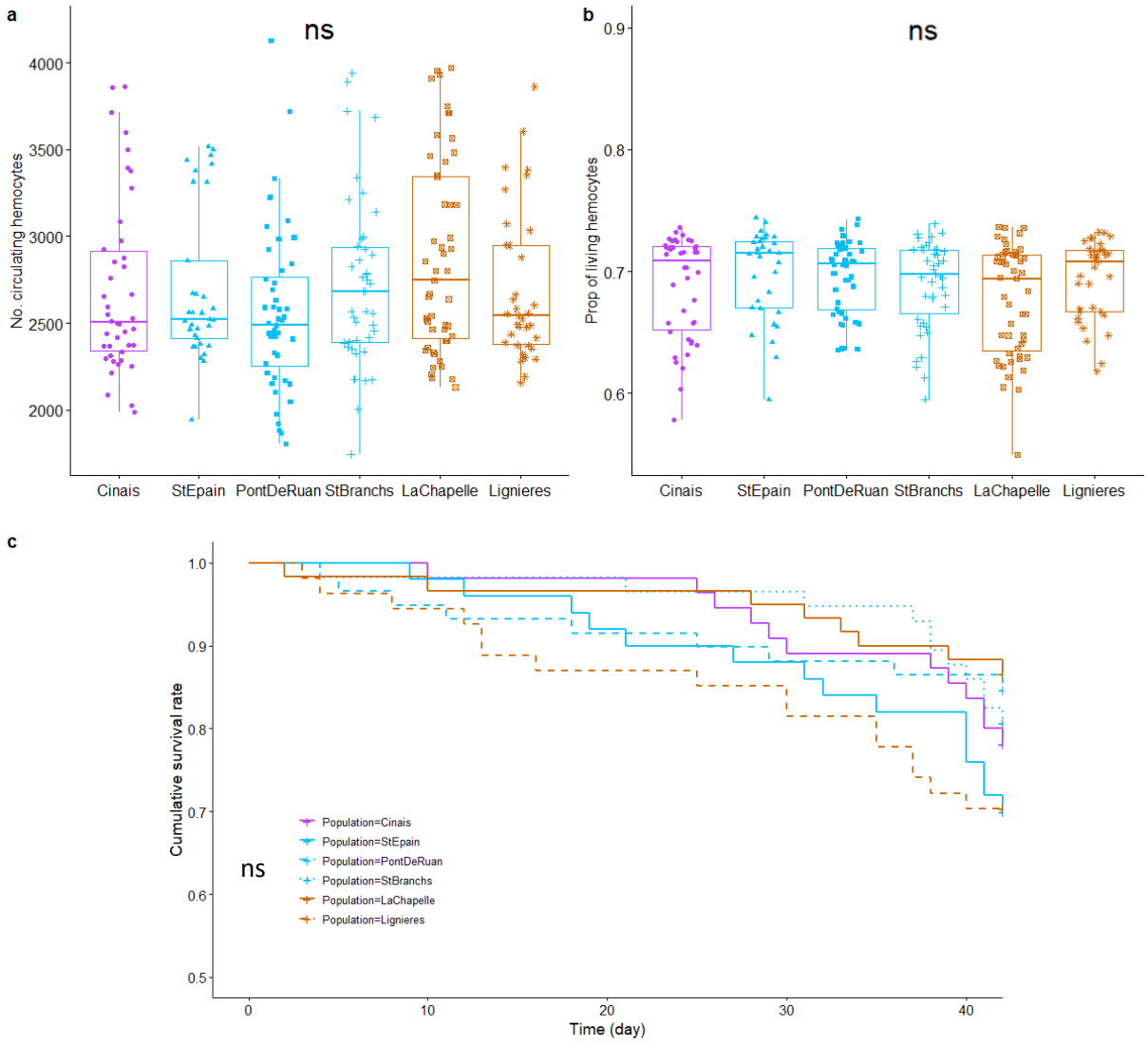

Fig.5 Population effect on male immunity and immunocompetence. (a) Number of circulating hemocytes 6 weeks after pathogen exposure, (b) proportion of living hemocytes 6 weeks after pathogen exposure, and (c) male survival rate over 42 days after pathogen exposure. Males come from population of St-Epain n=49, Pont-de-Ruan n=59, St-Branchs n=57 (blue = organic crop), La Chapelle-sur-Loire n=59, Lignières-de-Touraine n=54 (orange = conventional crop), Cinais n=54 (purple = non-cultivated crop). Boxplots depict median and interquartile ranges, with whiskers extending to 1.5 times the interquartile range and dots representing jittered experimental values. Ns means non-significant p-value.

DISCUSSION

A growing body of research shows that exposure to glyphosate and glyphosate based-herbicide (GBH) can alter many health- and fitness-related traits in insects (Pereira et al. 2018; Motta and Moran 2020; Strilbytska et al. 2022). Our results provide contrasting support for these conclusions in earwigs by showing that exposure to a GBH had a (moderate) effect on only one of the five traits measured in males. In particular, we found a positive association between general activity and weight in males exposed to the lowest dose of GBH, but no association in males exposed to the highest dose of GBH and the control. In contrast, we detected no effect of GBH on any of the other traits we measured, be it boldness, aggregation, immunocompetence and immune investment. The effect of GBH on male activity and the lack of effect on the other traits were consistent across the six populations studied, although we found population-specific differences in male activity, boldness, behaviour, immunocompetence and percentage of live hemocytes.

The (moderate) effect of Roundup© on male activity suggests that *F. auricularia* males may be sensitive to a topical exposure to GBH. Interestingly, the association between male activity and weight occurred after this exposure, which suggests a condition-dependent effect of this herbicide. If good-quality males are better protected from the negative effect of GBH on their activity, we would expect only the low-quality males to have reduced activity and thus an overall positive association between male quality and activity. As adult weight is a proxy for quality in the European earwig (Koch and Meunier 2014; Körner et al. 2017), this would lead to a positive association between male weight and activity – which is what we found. Condition-dependent effects of pesticides are common in insects. For instance, body weight is a predictor of insecticide sensitivity in honeybees (Pamminger 2021), several contact insecticides are more toxic to large compared to small caterpillars in the tomato moth *Diataraxia oleracea* (Way 1954), and adults survive monoterpenes better when they have high compared to low body condition in the mountain pine beetle *Dendroctonus ponderosae* (Reid and Purcell 2011). What was more surprising in the European earwig was that the association between male activity and body weight occurred after exposure to the low dose of GBH and not after exposure to the high dose. This suggests a non-linear dose response, with low doses being more effective than high doses. Although such a non-linear response to GBHs has been reported in rodents, the mechanisms by which low doses may be more

effective than high doses are still unknown (Vandenberg et al. 2012; Pham et al. 2019). Our results may therefore suggest that the existence of a non-linear dose response of GBH may extend to arthropods. However, future studies are needed to confirm the existence of this non-linear response and, if so, to elucidate why low doses of GBH are more toxic to male activity than high doses and the biological implications of such an effect in the European earwig.

Apart from male activity, we did not detect any effect of GBH exposure on male boldness, aggregation, immunocompetence and immune investment. This finding contrasts with the results obtained in other arthropods, such as wolf spiders and beetles, despite using the same exposure protocol (Lacava et al. 2021; Kanabar et al. 2021) and equivalent or even lower doses of GBH (Straw et al. 2021; Kanabar et al. 2021; Strilbytska et al. 2022). These apparent lack of effects were also surprising given that we used a formulation of glyphosate (Roundup©), which is known to trigger more toxic changes in insect physiology and behaviour than the active molecule alone (Straw et al. 2021; Strilbytska et al. 2022). A possible explanation for our findings is that boldness, aggregation, immunocompetence and immune investment (but not on general activity) could be independent of GBH-induced changes in the microbiota of the European earwig. Glyphosate typically alters the shikimate pathway, which is responsible for the biosynthesis of aromatic amino acids in plants, fungi and bacteria. As bacteria are a major component of the insect microbiome, and the microbiome is increasingly known to determine many life history traits of its host (Moran et al. 2019), it has been suggested that the effect of GBH on insects biology is mostly due to changes in their microbiota (Motta and Moran 2020; Smith et al. 2021). However, it is not clear whether changes in the gut microbiota can lead to changes in life-history traits in the European earwig. A previous study indeed showed that experimentally altering the microbiome of female earwigs with antibiotics did not alter any of the 30 physiological, reproductive, behavioural and longevity traits measured over their 300-day lifespan (Van Meyel et al. 2021). Conversely, recent data suggest that the body condition of females is associated with their gut microbial community (Cheutin et al. 2024), possibly explaining why this trait was affected by GBH exposure.

Another potential explanation for the limited effects of GBH is that the European earwig has a high tolerance or resistance to herbicides and pesticides due to highly developed

detoxification mechanisms. In line with this hypothesis, previous studies have shown that the behaviour, physiology, reproduction and survival of *F. auricularia* are poorly affected by several pesticides and chemical pollutants such as spinosad, primicarb, pyriproxyfen and cadmium (Malagnoux et al. 2015b; Merleau et al. 2022; Honorio et al. 2023a). Similarly, chlorpyrifos-ethyl and acetamiprid were less lethal to *F. auricularia* than they were to another earwig species, *F. pubescens* (Malagnoux et al. 2015b). The mechanism of detoxification in insects may be explained by systems for increasing glutathione S-transferase levels, in synergy with greater production of oxidative enzymes such as peroxidases, or greater production of hydrolytic enzymes such as esterases (Coleman et al. 2002; Kliot and Ghanim 2012). In *F. auricularia*, carboxylesterases and acetylcholinesterases appear to be responsible for the detoxification mechanism (Malagnoux et al. 2014; Le Navenant et al. 2019), but further studies are needed to determine their efficiency against GBH.

While our results do not allow us to shed light on a population-specific effect of GBH, they do show that many male traits vary between the six populations studied. This is not the first time that life history differences between populations of the European earwig are reported, but until now they have mainly been found in females from populations separated by hundreds or thousands of kilometres. For example, Tourneur and Meunier (2020) reported physiological differences in females from 19 populations distributed across North America, while Ratz et al. (2016) reported physiological but not behavioural differences between females from three Spanish and French populations. The population-specific effects we found here are unlikely to reflect a plastic response to the sites where males were captured, as we maintained all individuals under standard laboratory conditions for several months prior to the experiments. Instead, they are likely to reflect the local environment in which males have grown up and/or in which their parents (and ancestors) have evolved. These effects are already known in the European earwig, where the conditions in which earwig larvae grow can shape the behaviour, physiology and reproduction of future adults (Thesing et al., 2015; Wong and Kölliker, 2014), and where mothers can 'program' their offspring in anticipation of adverse conditions during development (Raveh et al. 2016). More studies will be needed to investigate which local conditions may give rise to the reported differences and to test whether these differences reflect local adaptations or maladaptations. Among the list of potential parameters that can vary on a small geographical scale, management practices are

unlikely to explain our results (Malagnoux et al. 2015b). This is because we did not find any systematic difference in the measured traits between the three organic orchards (Pont-de-Ruan, St-Branchs and St-Epain) and the two conventional orchards (La Chapelle-sur-Loire and Lignières-de-Touraine). However, it is interesting to note that the population that stood out was often Cinais, the only non-cultivated population. This calls for future work with more non-cultivated populations to determine the direct and indirect effects of agriculture (both organic and non-organic) on these male traits. More generally, a better understanding of the determinants and adaptive value of the reported differences would require a detailed characterisation of the environment of the populations (e.g., levels of anthropogenic pollution, predators and parasites, and microclimate), of the genetic exchange between these populations, as well as the conduct of transplant experiments.

In conclusion, our study shows that exposure to formulated glyphosate, an herbicide known to alter the biology of many insect species (Lacava et al. 2021; Smith et al. 2021; Kanabar et al. 2021), only has limited effects in males of the European earwig. Although these males show population-specific differences in behaviour and immunity, we did not detect population-specific differences in the effect or lack of effect of GBH on these traits. Overall, these results call for future studies to investigate whether these limited effects of GBH are due to the presence of effective detoxification mechanisms and/or the fact that the microbiota (and GBH-induced changes in the microbiota) determine only a few traits in this species. More generally, they stress that studying the diversity of sublethal effects of crop protection products is essential to improve our general knowledge of the use of chemical substances in integrated pest management programs.

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CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation and data collection were performed by LP, JM and CL and statistical analysis were performed by LP and JM. The first draft of the manuscript was written by LP and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

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

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