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RESEARCH ARTICLE

Glyphosate impairs learning in *Aedes aegypti* mosquito larvae at field-realistic doses

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ABSTRACT

Glyphosate is the most widely used herbicide in the world. Over the past few years, the number of studies revealing deleterious effects of glyphosate on non-target species has been increasing. Here, we studied the impact of glyphosate at field-realistic doses on learning in mosquito larvae (*Aedes aegypti*). Larvae of *A. aegypti* live in small bodies of water and perform a stereotyped escape response when a moving object projects its shadow on the water surface. Repeated presentations of an innocuous visual stimulus induce a decrease in response due to habituation, a non-associative form of learning. In this study, different groups of larvae were reared in water containing different concentrations of glyphosate that are commonly found in the field (50 µg l^{-1}, 100 µg l^{-1}, 210 µg l^{-1} and 2 mg l^{-1}). Larvae reared in a glyphosate solution of 2 mg l^{-1} (application dose) could complete their development. However, glyphosate at a concentration of 100 µg l^{-1} impaired habituation. A dose-dependent deleterious effect on learning ability was observed. This protocol opens new avenues to further studies aimed at understanding how glyphosate affects non-target organisms, such as insects. Habituation in mosquito larvae could serve as a parameter for testing the impact of pollutants in the water.

KEY WORDS: Habituation, Ecotoxicology, Pesticide, Herbicide, Fresh-water, Behaviour, Bio-indicator

INTRODUCTION

Glyphosate is the most widespread herbicide in the world, with about 80,000–85,000 tonnes being used in a year (Grube et al., 2011). Glyphosate, N-(phosphonomethyl)-glycine, is a systemic total foliar herbicide – a non-selective herbicide absorbed by the leaves – and it has a generalised action. Unlike other non-selective herbicides, glyphosate is thought to target a certain biosynthetic pathway that is found only in plants and some microorganisms. This fact is expected to minimise the toxicity to non-targeted organisms, such as animals.

Only a few experiments have been conducted in the field to study the final destination of glyphosate in the environment. These studies show that glyphosate and, in particular, the major degradation product of glyphosate, aminomethylphosphonic acid (AMPA), can persist in the soil for several weeks or months (Laitinen et al., 2006; Mamy et al., 2008). Run-off waters can therefore transport glyphosate to aquatic environments, while the wind can transport it to other terrestrial locations.

Ninety-five percent of herbicides have an impact on organisms outside their expected targets (Miller, 2004), potentially affecting not only numerous plant species but also animals. During the past decade, research conducted on several species of invertebrates and vertebrates reported different levels of toxicity, causing carcinogeny, teratogeny and even lethality (Relyea and Jones, 2009; Paganelli et al., 2010; for reviews, see Relyea and Hoverman, 2006; Kaur-Gill et al., 2018; Mesnage et al., 2015).

In insects, for instance, neurotoxic effects have been observed in honey bees fed with low concentrations of glyphosate, similar to those found in the environment (Herbert et al., 2014; Balbuena et al., 2015). These authors reported deleterious effects, such as a decline of learning and navigation abilities in exposed bees (see Discussion). In the mosquitoes *Aedes aegypti* and *Aedes albopictus*, glyphosate exposure alters life history traits such as larval development time and sex ratio (Bara et al., 2014), as well as the expression of genes conferring resistance to insecticides (Riaz et al., 2009). The generation of mosquito phenotypes resistant to pesticides results from several mechanisms associated with behavioural, physiological and metabolic changes (Scott, 1990). The exposure of mosquitoes to sub-lethal doses of herbicides can reduce the effectiveness of insecticides by affecting diverse physiological targets (Riaz et al., 2009). Exposure to this herbicide therefore has the potential to influence the vectorial capacity and survival of mosquitoes, leading, for example, to resistant phenotypes (Bourguet et al., 2004; Rivero et al., 2010).

Mosquitoes are holometabolous insects, whose preimaginal instars develop in freshwater environments, both natural and artificial (e.g. above ground pools, basins, storm drains, abandoned tyres), being exposed to environmental pollutants, including pesticides such as glyphosate. In the absence of danger, larvae remain on the surface of the water to breathe atmospheric air. When mosquito larvae detect a potentially dangerous moving object over the water surface, they dive, performing a stereotypical escape response. This escape response can cause significant energy expenditure. Indeed, it has been reported that excessive diving would increase larval mortality (Tuno et al., 2004), also affecting growth to the adult stage (Timmermann and Briegel, 1993; Lucas and Romoser, 2001; Roberts, 2018). Performing escape behaviour less frequently would contribute to preserving oxygen and energy reserves, which is crucial for the animal to reach adulthood (Rodríguez-Prieto et al., 2006). In effect, repeated exposure to a non-harmful visual stimulus, results in the larva progressively reducing its escape response. The stimulus is not perceived as a danger anymore, i.e. the larva is habituated to the stimulus (Baglan et al., 2017).

Aedes aegypti (Linnaeus in Hasselquist 1762) is the most cosmopolitan insect vector, transmitting a number of arboviruses responsible for human diseases, such as dengue and yellow fever (Jansen and Beebe, 2010), which according to the WHO cause 100,000 and 30,000 deaths every year, respectively (http://www.who.int/denguecontrol/arbo-viral/en/). More recently, *A. aegypti* was responsible for the outbreak of Zika virus in Brazil. In the

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Received 25 June 2018; Accepted 13 August 2018

present work, we studied the impact of field-realistic doses of glyphosate on learning in *A. aegypti* larvae. The selected doses correspond to concentrations that can be found in treated areas (Byer et al., 2008; Struger et al., 2008). A negative impact of glyphosate on learning would indicate neurotoxic effects, such as those observed in bees in the terrestrial environment. We used a bioassay based on habituation that we had developed previously and which allows the study of learning under fully controlled conditions (Baglan et al., 2017)

MATERIALS AND METHODS

Animals

We used fourth-instar larvae of *A. aegypti* Bora strain. Mosquitoes were reared in a climate room at 25°C with a 12 h:12 h light:dark cycle. Larvae were fed with shrimp food (JBL Novo Prawn, Neuhofen, Germany; www.jbl.de). Larva were obtained from eggs produced by Laboratoire de Lutte contre les Insectes Nuisibles (MIVEGEC-IRD, Montpellier, France). Eggs were placed in small plastic containers with dechlorinated tap water. Under these conditions, larvae reached their 4th instar 5 days after hatching and moulted into pupae 5 days later; i.e. it took around 10 days from hatching to pupal moult. In order to avoid taking any pharate pupa, larvae at a maximum of 8 days old were used in our experiments.

Stimulus

Larvae were presented with a visual habituation stimulus (H) over the course of 15 trials. This stimulus consisted of a black cardboard 3 cm square that was presented horizontally 11 cm above each Petri dish under a spotlight (Fig. 1). We used a servomotor associated with a control board (Arduino Uno; http://www.arduino.cc) to automatically deliver the habituation stimulus at an angular speed of 315.8 deg s^{-1} (for details, see Baglan et al., 2017).

Habituation protocol

Experiments were performed in a different room at 25°C, in a setup allowing us to train five larvae simultaneously. Each larva was individually placed into a Petri dish (3 cm in diameter and 1.2 cm height) filled with dechlorinated tap water. Five Petri dishes were placed on a table, each under a spot light. In this way, the shadows of the stimuli projected on each Petri dish. The Petri dishes were visually isolated from each other by opaque walls (30 cm in height and width). To avoid any disruption caused by movements or vibrations, each Petri dish was placed on an insulating foam block.

To reduce the stress due to context changing from the rearing container to the Petri dish, we left the larvae to familiarise with the new environment for 30 min before starting the experiments. The familiarisation time was determined by preliminary observations, which showed that the larvae stopped performing spontaneous escape responses after 15 min.

Sub-lethal effect of glyphosate

The purpose of this experiment was to test the impact of glyphosate on cognitive abilities of mosquito larvae. For this purpose, we reared five groups of mosquito larvae. Four groups corresponded to four different concentrations of glyphosate and the remaining group was reared without glyphosate. Rearing solutions were prepared from a stock solution of 2 mg l^{-1} (recommended concentrations for field application by manufacturers vary from 2 mg l^{-1} to 3 mg l^{-1}), produced with solid glyphosate (96% purity; Sigma-Aldrich) dissolved in distilled water. Stock solution was renewed every 2 weeks, to avoid any degradation effect and to avoid any possible

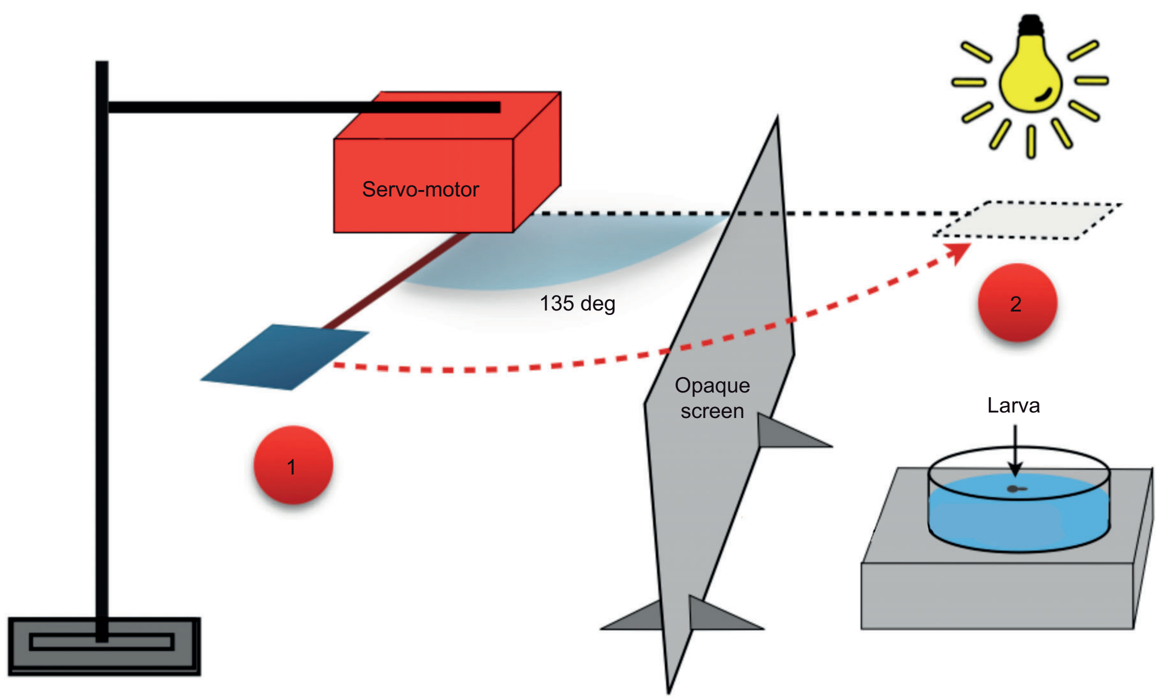

Fig. 1. Stimulation apparatus. A single larvae was placed into a Petri dish. A servomotor delivered the visual stimulus (H) moving it from position 1 to position 2. While in position 2 the visual stimulus H covered the light source. Modified from Baglan et al. (2017).

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concentration error in derived solutions. From the stock solution, four derived solutions were prepared, with the following concentrations: 50 µg l^{-1}, 100 µg l^{-1}, 210 µg l^{-1} and 2 mg l^{-1}. Larvae were exposed to one of these solutions of glyphosate for their entire lives, until the experiments were performed. These four different concentrations of glyphosate are similar to those found in the field (Byer et al., 2008; Struger et al., 2008). These concentrations have also been used in tests on several aquatic animals: amphibians such as *Lithobates sylvaticus* and *Lithobates clamitans* (Edge et al., 2013; Lanctôt et al., 2014) and the mosquitoes *A. aegypti* and *A. albopictus* (Bara et al., 2014).

The no-glyphosate (NG) group and the four treated groups were trained following the habituation protocol described above, which allowed evaluation of learning performance in the course of training. We compared the learning abilities of larvae reared with sub-lethal doses of glyphosate to larvae reared without glyphosate. For each concentration, 20 larvae were assayed.

Potential lethal effects of glyphosate

To test for any lethal effect of the applied doses, we compared mortality in larvae reared with 2 mg l^{-1} glyphosate (the highest dose used in this work) to mortality in larvae reared without glyphosate. No significant differences were observed (see Table S1).

Statistical analysis

All statistical analyses were done using R software, version 3.1.3. To analyse changes in response level during habituation trials, we used a generalised linear mixed model (GLMM, lme4 package) with a binomial structure (logit-link). This method of statistical analysis has been used in recent studies to analyse categorical behavioural responses associated with learning in insects (Bos et al., 2014; Perez et al., 2015; Baglan et al., 2017). To compare the performances between different groups, we used the 'lsmeans' function. The differences between non-paired samples (lethal effects of glyphosate) were tested with a χ^2 test (Fathala et al., 2010).

Dose effect: model Weibull 1

An equation that describes the response of amplitude variation with an effector agent (glyphosate) is known as a dose-dependent model (Murado et al., 2002). A basic condition for the development of a dose-dependent model is visualised by a Sigmoid profile (Scholze et al., 2000). Models of Weibull, log-logistic and lognormal are dose-dependent models already used in many ecotoxicology studies (Boedeker et al., 1993; Christensen and Nyholmt, 1984; Moore and Caux, 1997). Among the different models, Weibull model 1 is the dose-dependent model that best fits our data (R^2=0.81). It describes the evolution of the impact of glyphosate on learning based on the concentration of glyphosate. This model allows us to determine the curve inflection point (Ritz, 2010). The inflection point corresponds to the concentration at which 50% of negative effects of glyphosate on learning can be observed.

Using the least-square method, the sigmoidal dose-response model that best suits our data is the Weibull model 1 (R^2=0). The Weibull model 1 is an asymmetrical model whose equation is: $W_1=c+(d-c)\exp\{\exp[b(\log x-\log e)]\}$. The parameter b reflects the slope of the dose-response curve and the parameter e is the dose at which the inflection point of the dose-response curve is situated. The parameters c and d correspond to the lower and upper limit of the sigmoid curve.

Ethics

All the animals assayed in this work were treated according to ethical rules and regulations. Glyphosate waste was discarded following the corresponding security policies.

RESULTS

Sublethal effects of glyphosate

This experiment allowed us to highlight the impact of glyphosate on learning abilities in mosquito larvae. Escape response levels significantly decreased over the course of the habituation phase in all five groups: NG, 50 µg l^{-1}, 100 µg l^{-1}, 210 µg l^{-1} and 2 mg l^{-1} (GLMMs; in all cases, P<0.01). For each concentration, 20 larvae were assayed. The dynamics of acquisition was different across groups as revealed by the curves in the habituation phase (Fig. 2). The three groups with the higher concentrations of glyphosate: 100 µg l^{-1}, 210 µg l^{-1} and 2 mg l^{-1} (lsmeans, P<0.01) yielded significantly slower acquisition than the groups NG and 50 µg l^{-1}. The NG and 50 µg l^{-1} groups did not significantly differ from each

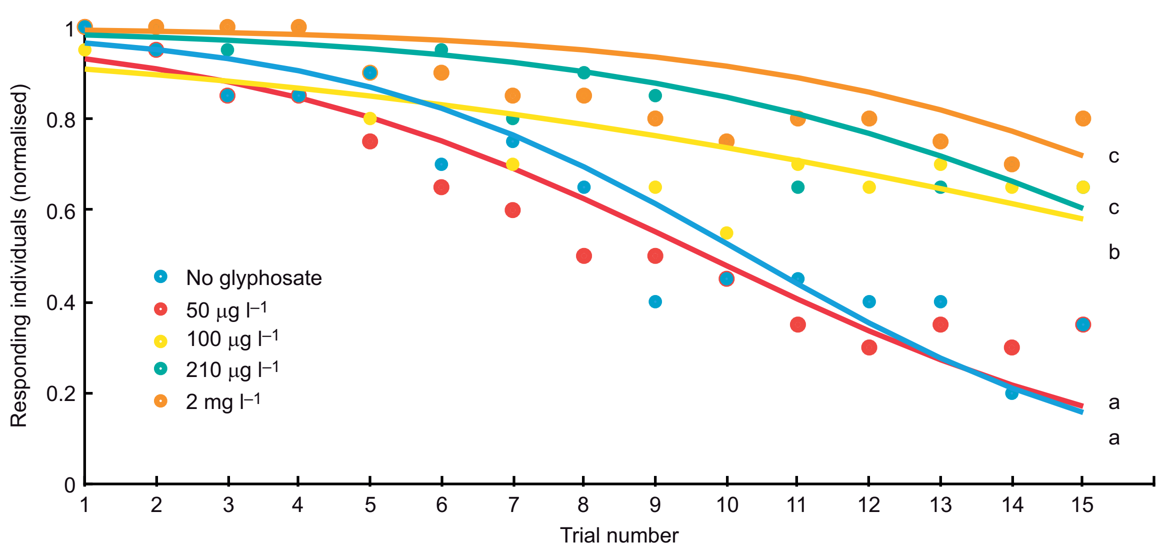

Fig. 2. Escape responses during habituation in increasing concentrations of glyphosate. Each concentration of glyphosate is represented with a different colour. Curves were obtained by GLMMs. Different letters indicate significant differences (P<0.05). For each concentration, N=20 larvae were assayed.

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other. The 100 μg l^{-1} group showed significantly faster acquisition than the other two groups with higher glyphosate concentrations: 210 μg l^{-1} and 2 mg l^{-1} (lsmeans, P=0.0104 and P=0.0313, respectively). Finally, the acquisition curves did not differ between the two groups with the highest concentrations, 210 μg l^{-1} and 2 mg l^{-1} (lsmeans, P=0.9970).

Dose effect assessed using the Weibull 1 model

These results show that the higher the glyphosate concentration, the slower the acquisition. In order to describe our results in the frame of a dose-dependent effect, we determined the number of necessary trials to reach 50% of the initial response level for the five different conditions (Fig. 3). This function, detailed in the Materials and Methods, is defined with the four following parameters: b=−14.385933; c=9.998682; d=17.779996; e=0.082903.

DISCUSSION

Learning performance of mosquito larvae was impaired by glyphosate at concentrations that can be found in the environment. Remarkably, larvae reared with a concentration of 100 μg l^{-1} glyphosate (half the concentration that is mostly found in the environment) showed much lower intensity of learning than the no glyphosate (NG) control. Furthermore, larvae reared in water containing 210 μg l^{-1} glyphosate showed a lower response level than that seen in mosquitoes reared with 100 μg l^{-1}, and even more so when compared with larvae reared without glyphosate. These observations highlight the deleterious effect of glyphosate on learning in mosquito larvae. Beyond 210 μg l^{-1}, the impact of glyphosate on learning seems to have reached an asymptote. Indeed, learning intensity in the 2 mg l^{-1} group was not different from the group reared in 210 μ l^{-1}. This type of curve is characteristic of a dose-dependent system with saturation. In the case of this study, this concentration was 0.083 μg l^{-1}, which is a lower dose than those found in the environment. Performing eco-toxicology studies based on this concentration would be extremely enlightening because the exposure of mosquito larvae to such a concentration may have impacts on life history traits, on the fitness and on dispersal capabilities (See Bara et al., 2014).

The decrease in learning performance evinced by our results was not due to any damage on the receptors or to effector fatigue (Baglan et al., 2017). In fact, larvae responded to the visual stimulus even at the highest concentration of glyphosate over the course of the whole habituation phase. These observations led us to hypothesise that glyphosate impacts the central nervous system of the mosquito larvae. To provide further evidence of the effects of glyphosate on learning, additional neurophysiological studies are necessary.

Several studies have shown that exposure to glyphosate had an impact on the inhibition of acetylcholinesterase in the nervous system and on muscles in aquatic organisms (Cattaneo et al., 2011; Glusczak et al., 2006; Lajmanovich et al., 2011; Menendez-Helman et al., 2012; Modesto and Martinez, 2010; Salbego et al., 2010; Sandrini et al., 2013). Those observations suggest that glyphosate impairs cognitive abilities of mosquito larvae by inhibiting acetylcholinesterase. This inhibition would alter the quantity of neurotransmitters released at synapses and disrupt the habituation abilities of mosquitoes.

This study is among the few that show the impact of glyphosate at field-realistic doses on the cognitive system of a non-target species. Recent studies showed that glyphosate disrupts cognitive abilities in bees (Balbuena et al., 2015; Herbert et al., 2014). Those studies showed that bees fed with glyphosate, at doses similar to those found in the environment, take longer to find their hives (Balbuena et al., 2015) and that glyphosate affected the learning ability of bees and could have a negative long-term impact on bee colonies (Herbert et al., 2014). Those findings highlighted the role of bees as bio-indicators to evaluate the impact of glyphosate spread in terrestrial environments. Indeed, any changes in behaviour can be taken as an indirect proof for the physiological impact of pollutants, such as glyphosate and pesticides. While bee performance was impaired at doses of glyphosate corresponding to cumulative amounts after several foraging flights, learning abilities in mosquito larvae were impaired at 100 μg l^{-1}, almost half the usual residual concentration found in superficial water (Byer et al., 2008; Struger et al., 2008). Complementary experiments are necessary to find out which physiological paths underlie the observed behavioural modifications.

Our work opens the way to future ecotoxicological studies using mosquito larvae as a bio-indicator to evaluate the impact of glyphosate on aquatic environments. Further research is urgently required to accurately evaluate the impact of glyphosate on the biology of mosquitoes, which are one of the major human disease

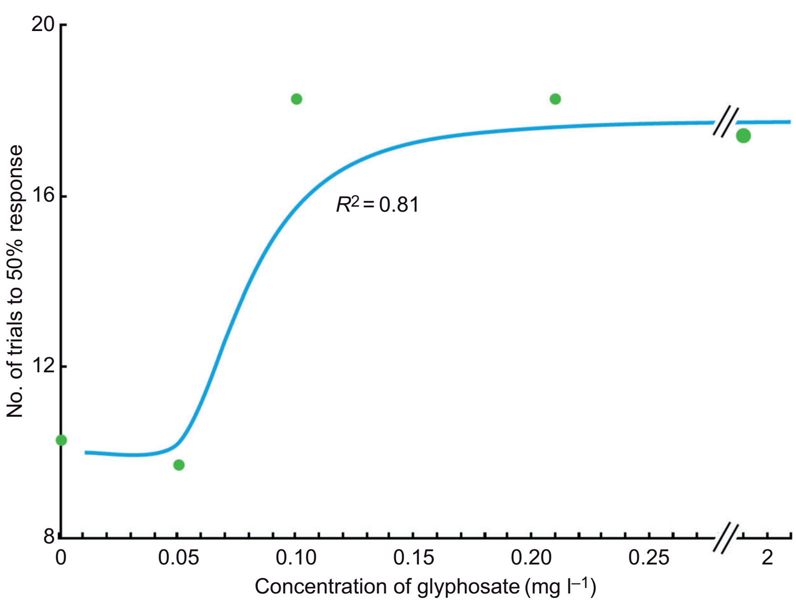

Fig. 3. Learning according to glyphosate concentration. Response levels for each concentration correspond to a Weibull I dose-dependent model (see Materials and Methods for further details). For each group, learning intensity is measured as the number of trials necessary to reach a 50% response. N=20 larvae assessed for all the concentrations.

vectors. In addition, a missing, yet relevant, piece of information is the impact of herbicides on the behaviour of mosquito larvae. A comprehensive understanding the effects of glyphosate on mosquito biology will contribute to a more judicious use of herbicides.

Acknowledgements
We thank our colleagues of the INOV team at IRBI, Tours and two anonymous reviewers for their constructive comments on early versions of the manuscript.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: H.B., C.R.L., F.J.G.; Methodology: F.J.G.; Validation: F.J.G.; Formal analysis: H.B., C.R.L., F.J.G.; Investigation: H.B., F.J.G.; Resources: H.B., C.R.L., F.J.G.; Data curation: H.B., F.J.G.; Writing - original draft: H.B., C.R.L., F.J.G.; Writing - review & editing: H.B., C.R.L., F.J.G.; Visualization: H.B., F.J.G.; Supervision: F.J.G.; Project administration: F.J.G.; Funding acquisition: F.J.G.

Funding
This work was financially supported by Marie Curie Actions FP7-IRSES (grant no. 319015: IBIAL–Integrative Biology of Insects affecting Human Life).

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.187518.supplemental

References
Baglan, H., Lazzari, C. and Guerrieri, F. (2017). Learning in mosquito larvae (*Aedes aegypti*): habituation to a visual danger signal. *J. Ins. Physiol.* **98**, 160-166.

Balbuena, M. S., Tison, L., Hahn, M.-L., Greggers, U., Menzel, R. and Farina, W. M. (2015). Effects of sublethal doses of glyphosate on honeybee navigation. *J. Exp. Biol.* **218**, 2799-2805.

Bara, J. J., Montgomery, A. and Muturi, E. J. (2014). Sublethal effects of atrazine and glyphosate on life history traits of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae). *Parasitol. Res.* **113**, 2879-2886.

Boedeker, W., Drescher, K., Altenburger, R., Faust, M. and Grimme, L. H. (1993). Combined effects of toxicants: the need and soundness of assessment approaches in Ecotoxicology. *Sci. Total Environm.* **134**, 931-939.

Bos, N., Roussel, E., Giurfa, M. and d'Ettorre, P. (2014). Appetitive and aversive olfactory learning induce similar generalization rates in the honey bee. *Anim. Cogn.* **17**, 399-406.

Bourguet, D., Guillemaud, T., Chevillon, C. and Raymond, M. (2004). Fitness costs of insecticide resistance in natural breeding sites of the mosquito *Culex pipiens*. *Evolution* **58**, 128-135.

Byer, J. D., Struger, J., Klawunn, P., Todd, A. and Sverko, E. (2008). Low cost monitoring of glyphosate in surface waters using the elisa method: an evaluation. *Environm. Sci. Technol.* **42**, 6052-6057.

Cattaneo, R., Clasen, B., Loro, V. L., de Menezes, C. C., Pretto, A., Baldisserotto, B., Santi, A. and De Ávila, L. A. (2011). Toxicological responses of *Cyprinus carpio* exposed to a commercial formulation containing glyphosate. *Bull. Environm. Contam. Tox.* **87**, 597-602.

Christensen, E. R. and Nyholmt, N. (1984). Ecotoxicological assays with algae: weibull dose-response curves. *Envir. Sci. Tech. Lib.* **18**, 713-718.

Edge, C. B., Gahl, M. K., Thompson, D. G. and Houlahan, J. E. (2013). Laboratory and field exposure of two species of juvenile amphibians to a glyphosate-based herbicide and *Batrachochytrium dendrobatidis*. *Sci. Total Envir.* **444**, 145-152.

Fathala, Mdel V., Kunert, M. C. and Maldonado, H. (2010). A field model of learning: 2. Long-term memory in the crab *Chasmagnathus granulatus*. *J. Comp. Physiol. A* **196**, 77-84.

Glusczak, L., Dos Santos Miron, D., Crestani, M., Braga Da Fonseca, M., De Araújo Pedron, F., Duarte, M. F. and Vieira, V. L. P. (2006). Effect of glyphosate herbicide on acetylcholinesterase activity and metabolic and hematological parameters in piava (*Leporinus obtusidens*). *Ecotox. Environ. Safe.* **65**, 237-241.

Grube, A., Donaldson, D., Kiely, T. and Wu, L. (2011). Most commonly used conventional pesticide active ingredients in the U.S. agricultural market sector. In *Pesticide Industry Sales and Usage: 2006 and 2007 Market Estimates*, pp. 14-15. Environmental Protection Agency.

Herbert, L. T., Vazquez, D. E., Arenas, A. and Farina, W. M. (2014). Effects of field-realistic doses of glyphosate on honeybee appetitive behaviour. *J. Exp. Biol.* **217**, 3457-3464.

Jansen, C. C. and Beebe, N. W. (2010). The dengue vector *Aedes aegypti*: what comes next. *Microbes Infect.* **12**, 272-279.

Kaur-Gill, J. P., Sethi, N., Mohan, A., Datta, S. and Girdbar, M. (2018). Glyphosate toxicity for animals. *Environ. Chem. Lett.* **16**, 401-426.

Laitinen, P., Siimes, K., Eronen, L., Rämö, S., Welling, L., Oinonen, S., Mattsoff, L. and Ruohonen-Lehto, M. (2006). Fate of the herbicides glyphosate, glufosinate-ammonium, phenmedipham, ethofumesate and metamitron in two finnish arable soils. *Pest Manag. Sci.* **62**, 473-491.

Lajmanovich, R. C., Attademo, A. M., Peltzer, P. M., Junges, C. M. and Cabagna, M. C. (2011). Toxicity of four herbicide formulations with glyphosate on *Rhinella arenarum* (Anura: Bufonidae) Tadpoles: B-esterases and glutathione S-transferase Inhibitors. *Arch. Environ. Contam. Toxicol.* **60**, 681-689.

Lanctôt, C., Robertson, C., Navarro-Martín, L., Edge, C., Melvin, S. D., Houlahan, J. and Trudeau, V. L. (2013). Effects of the glyphosate-based herbicide Roundup Weathermax(R) on metamorphosis of wood frogs (*Lithobates sylvaticus*) in natural wetlands. *Aquat. Toxicol.* **140**, 48-57.

Lucas, E. A. and Romoser, W. S. (2001). The energetic costs of diving in *Aedes aegypti* and *Aedes albopictus* pupae. *J. Am. Mosq. Control Assoc.* **17**, 56-60.

Mamy, L., Gabrielle, B. and Barriuso, E. (2008). Measurement and modelling of glyphosate fate Compared with that of herbicides replaced as a result of the introduction of glyphosate resistant oilseed rape. *Pest Manag. Sci.* **64**, 262-275.

Menéndez-Helman, R. J., Ferreyroa, G. V., dos Santos Afonso, M. and Salibian, A. (2012). Glyphosate as an acetylcholinesterase inhibitor in cnesterodon decemmaculatus. *Bull. Environ. Contam. Toxicol.* **88**, 6-9.

Mesnage, R. Defarge, N. Spiroux de Vendômois, J. and Séralini, G. E. (2015). Potential toxic effects of glyphosate and its commercial formulations below regulatory limits. *Food Chem. Toxicol.* **84**, 133-153.

Miller, G. T. (2004). Chapter 9, Water ressources and water pollution. In *Sustaining the Earth: An Integrated Approach*, 7th edn, pp. 211-216. Thomson, CA, USA: Brooks Cole Ed.

Modesto, K. A. and Martinez, C. B. R. (2010). Effects of Roundup transorb on fish: Hematology, antioxidant defenses and acetylcholinesterase activity. *Chemosphere* **81**, 781-787.

Moore, D. R. J. and Caux, P.-Y. (1997). Estimating low toxic effects. *Environ. Toxicol. Chem.* **16**, 794-801.

Murado, M. A., González, M. P. and Vázquez, J. A. (2002). Dose–response relationships: an overview, a generative model and its application to the verification of descriptive models. *Enzyme Microb. Technol.* **31**, 439-455.

Paganelli, A., Gnazzo, V., Acosta, H., López, S. L. and Carrasco, A. E. (2010). Glyphosate-based Herbicides produce teratogenic effects on vertebrates by impairing retinoic acid signaling. *J. Am. Chem. Soc.* **23**, 1586-1595.

Perez, M., Giurfa, M. and D'Ettorre, P. (2015). The scent of mixtures: rules of odour processing in ants. *Sci. Rep.* **5**, 8659.

Relyea, R. A. and Hoverman, J. (2006). Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. *Ecol. Letters* **9**, 1157-1171.

Relyea, R. A. and Jones, D. K. (2009). The toxicity of Roundup Original Max to 13 species of larval amphibians. *Environ. Toxicol. Chem.* **28**, 2004-2008.

Riaz, M. A., Poupardin, R., Reynaud, S., Strode, C., Ranson, H. and David, J. P. (2009). Impact of glyphosate and benzo[a]pyrene on the tolerance of mosquito larvae to chemical insecticides. Role of detoxification genes in response to xenobiotics. *Aquat. Toxicol.* **93**, 61-69.

Ritz, C. (2010). Toward a unified approach to dose-response modeling in ecotoxicology. *Environ. Toxicol. Chem.* **29**, 220-229.

Rivero, A., Vezilier, J., Weill, M., Read, A. F. and Gandon, A. (2010). Insecticide control of vector-borne diseases: when is insecticide resistance a problem? *PLoS Pathog.* **6**, e1001000.

Roberts, D. M. (2018). Predator feeding vibrations encourage mosquito larvae to shorten their development and so become smaller adults. *Ecol. Entomol.*

Rodríguez-Prieto, I., Fernández-Juricic, E. and Martin, J. (2006). Anti-predator behavioral responses of mosquito pupae to aerial predation risk. *J. Insect Behav.* **19**, 373-381.

Salbego, J., Pretto, A., Gioda, C. R., de Menezes, C. C., Lazzari, R., Radünz Neto, J., Baldisserotto, B. and Loro, V. L. (2010). Herbicide formulation with glyphosate affects growth, acetylcholinesterase activity, and metabolic and hematological parameters in piava (*Leporinus obtusidens*). *Arch. Environ. Contam. Tox.* **58**, 740-745.

Sandrini, J. Z., Rola, R. C., Lopes, F. M., Buffon, H. F., Freitas, M. M., Martins, C. M. G. and da Rosa, C. E. (2013). Effects of glyphosate on cholinesterase activity of the mussel *Perna perna* and the fish *Danio rerio* and *Jenynsia multidentata*: in vitro studies. *Aqu. Toxicol.* **130-131**, 171-173.

Scholze, M., Boedeker, M., Faust, M., Backhaus, T., Altenburger, R. and Grimme, L. H. (2000). A general best-fit method for concentration-response curves and the estimation of low-effect concentrations. *Environ. Toxicol. Chem.* **20**, 448-457.

Scott, J. G. (1990). Investigating mechanisms of insecticide resistance: methods, strategies and pitfalls. In *Pesticide Resistance in Arthropods* (ed. R. T. Roush and B. Tabashnik). pp. 39-57. New York, U.S.A: Chapman and Hall.

Struger, J., Thompson, D., Staznik, B., Martin, P., Mcdaniel, T. and Marvin, C. (2008). Occurrence of glyphosate in surface waters of southern ontario. *Bull. Environ. Contam. Toxicol.* **80**, 378-384.

Timmermann, S. E. and Briegel, H. (1993). Water depth and larval density affect development and accumulation of reserves in laboratory populations of mosquitoes. *Bull. Soc. Vector Ecol.* **18**, 174-187.

Tuno, N., Miki, K., Minakawa, N., Githeko, A., Yan, G. and Takagi, M. (2004). Diving ability of *Anopheles gambiae* (Diptera: Culicidae) larvae. *J. Med. Entomol.* **41**, 810-812.