Acute and chronic sublethal chemical pollution affects activity, learning and memory in mosquito larvae

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Keywords: habituation, *Aedes aegypti*, paracetamol, glyphosate, atrazine, mixture

Highlights

1. We examined the effect of glyphosate, atrazine, and paracetamol, in the cognitive abilities of *Aedes aegypti* mosquito larvae.

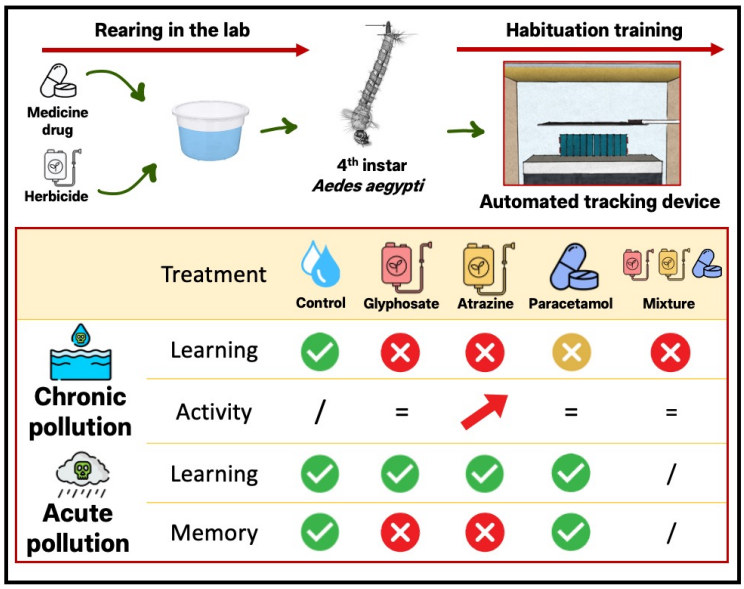
2. We used doses ranging from field-realistic to commercial-recommended concentrations of the pollutants alone and in mixtures.

3. For chronic exposition, spontaneous activity was increased or reduced, and habituation was impaired after pollutant exposure.

4. For acute exposition, memory retention was impaired after pollutant exposure.

5. Using the cognitive abilities of mosquito larvae help to understand the ecological effects of pollutants in vulnerable ecosystems.

Graphical abstract



Abstract

Freshwater ecosystems play a critical role in supporting biodiversity and providing essential environmental services. However, these ecosystems are increasingly threatened by human activities, including habitat loss, pollution, and climate change. Traditional assessment methods focus on water properties, but biomonitoring approaches, particularly those examining behaviour and cognition, provide valuable insights into the ecological effects of pollutants. This study examines the effects of three common pollutants (glyphosate, atrazine, and paracetamol) on the cognitive abilities of *Aedes aegypti* mosquito larvae, a vector for several diseases. We used an automated bioassay to study habituation learning and the effects of the three pollutants alone or in mixtures, at sub-lethal doses ranging from field-realistic to commercial-recommended levels. Our results show that the three compounds modulate individual spontaneous activity, impair habituation and memory retention. These changes may alter the perception or the behavioural response of mosquito larvae to signals of their environment, as the presence of conspecifics or predators, and suggest that other organisms living in freshwater ecosystems may also be affected. Incorporating behavioural and cognitive assessments in ecotoxicological studies provides a more comprehensive understanding of the ecological effects of pollutants which is needed to address economic challenges in fragile ecosystems.

Introduction

Although freshwater ecosystems account for a small proportion of total freshwater (1.3%), which is only 2.7% of the total water on Earth, they provide essential environmental services, support 10% of the world's animal species and are a critical source of biodiversity (Carpenter et al. 2011; Madhav et al. 2020; Zhang et al. 2021). Despite their importance, freshwater ecosystems are the most endangered environments worldwide (Sala et al. 2000; Vári et al. 2022). Threats come from human activities, namely habitat loss and degradation, overexploitation, invasive species, climate change, hydrological alterations and increases in chemical compounds used for industrial, agricultural and domestic purposes (Arthington 2021).

Various methods have been developed to measure changes in these ecosystems. The most common is to take water or sediment samples and measure physical and chemical properties (Bartram et al. 1996; Zhou et al. 2008). In addition, biomonitoring methods have been increasingly explored, ranging from biomarkers at the cellular level to biological indicators or bioindicators at the community level (López-López and Sedeño-Díaz 2015; Zaghloul et al. 2020). These approaches help to address new ecological questions related to ecosystem management and provide tools to target the mechanisms underlying the effects of pollution, to model the toxicity at different intensities and at different temporal and geographical scales (Oertel and Salánki 2003; Previšić et al. 2020; Malhotra et al. 2021). In addition, the multiple sources of toxicity present in the actual environments constantly create unique cocktails, whose effects are difficult to disentangle from individual effects (Hodkinson and Jackson 2005).

At the organism level, studying the effects of toxicity on individual behaviour and cognitive abilities may reveal subtle effects of pollutants that are not accessible to naturalistic observation or standard bioassays (Melvin and Wilson 2013; Bownik and Wlodkowic 2021). In addition, assessing behaviour and cognition in ecotoxicology present other advantages. First, the study of behaviour is more sensitive than community composition or abundance because it integrates physiological processes (Blaxter and Hallers-Tjabbes 1992). Many studies have employed behavioural endpoints to assess the effects of contaminants at sublethal doses which serve as early indicators of

environmental stressors and allow the identification of the mechanisms of action of a toxic response. (Hellou 2011; Hong and Zha 2019). Second, behaviour is linked to individual fitness and is associated with functions such as feeding, anti-predation and reproduction. It is therefore a crucial source of information about the immediate environment, surrounding individuals, and can be used to model population dynamics (Ford et al. 2021). Third, the incorporation of behavioural and cognitive bioassays into ecotoxicological assessment links laboratory studies to more ecologically relevant scenarios (Bertram et al. 2022). Fourth, behavioural adaptations or maladaptations to pollutants provide insight into potential evolutionary changes that may occur within an ecosystem (Jacquin et al. 2020). Finally, despite the lack of standardised methods, behavioural bioassays are easy to perform, non-invasive, inexpensive and have a precise ecological relevance (Bonada et al. 2006).

For example, a recent paper by Li et al. (Li et al. 2019) examined the role of lead at field-realistic concentrations on the behaviour and physiology of the zebrafish *Danio rerio*. First, they assessed the cognitive abilities and physiological alterations of individuals exposed to three concentrations of lead and of a control group. Then, they generated *behavioural fingerprints*. Behavioural fingerprints are the result of a combination of behavioural parameters applied for comprehensively measuring neurotoxic effects (Li et al. 2019). Finally, they measured the expression of mRNA levels and performed histopathological analysis of brain tissue. Within a comprehensive behavioural and physiological analysis, the authors provided a global approach to study subtle effects of lead in behaviour and physiology, as well as ecological interpretations (Li et al. 2019).

In this study, we examined the effects of three pollutants adjusted for acute and chronic toxicity, ranging from doses measured directly in water to spray doses usually recommended for agricultural or gardening use, alone or in combination, on the locomotor, sensory, learning and memory abilities of dengue mosquito larvae. *Aedes aegypti* is the most cosmopolitan disease vector insect, responsible for the transmission of the causative agents of numerous human an animal diseases, including Dengue fever, Chikungunya fever, Zika virus, yellow fever, West Nile virus and Dirofilariasis (Kraemer et al. 2019; Adegoke et al. 2020). Due to the use of insecticides worldwide, a selective pressure has favoured the development of resistance mechanisms in this species, which

are currently under investigation (Rubio-Palis et al. 2023). Exposure of *Aedes aegypti* larvae to atrazine or glyphosate increases the expression of cytochrome P450 monooxygenases (P450s), glutathione-Stransferases (GSTs), and carboxylesterases that can confer larval tolerance to different insecticides (Bara et al. 2014).

The larval stage, which lasts about 4 to 10 days, which is easy to rear and rapidly developing in the laboratory is, in addition, sensitive to pollution (Boyer et al. 2006; Baglan et al. 2018; Black et al. 2021). Mosquito larvae spend most of their time just below the surface of the water. Larvae dive when they perceive potential threat, for example, a moving shadow over the water or a vibration (Clements 1999). If the stimulus is found to be harmless after repeated occurrences, the larvae become habituated to it, namely, they gradually reduce their response to further stimulation (Baglan et al. 2017). The stimulus perceived as dangerous by naïve animals is no longer significant in experienced individuals. We adopted a habituation (a form of non-associative learning) training procedure by Dessart et al. (2023) the basis for a bioassay to evaluate the effects of pollutants on cognitive abilities and behaviour in mosquito larvae (Dessart et al. 2024).

Here, we investigated the effects of two herbicides (atrazine and glyphosate) and a medicinal drug (paracetamol) on habituation in *Aedes aegypti* larvae. Glyphosate is the active ingredient in herbicide formulations and the most heavily used agrochemical in the world (Battaglin et al. 2014; Gill et al. 2018). It can persist in soils for years and is commonly found in aquatic ecosystems (Kanabar et al. 2021). It has been shown to impair collective thermoregulation and learning in bumblebees, and to alter life history traits, nutritional stress, and learning in mosquitoes (Bara et al. 2014; Baglan et al. 2018; Bataillard et al. 2020; Weidenmüller et al. 2022; Nouvian et al. 2023).

Atrazine is another widely used herbicide in the world (Li et al. 2018). It has been banned in the EU since 2004 but remains the most commonly used herbicide in the USA and is often found in water (Bara et al. 2014; Abdulelah et al. 2020).

The effects of atrazine have been studied on the locomotor activity in mammals, amphibians and teleost fishes, honeybees and nematodes (Rohr and McCoy 2010; García-Espiñeira 2018; Araújo et al. 2021), life history traits in mosquitoes (Bara et al. 2014), and spatial learning and memory in mice (Rastegar-Moghaddam et al. 2019). Paracetamol (acetaminophen) is the active ingredient in pharmaceutical products used as analgesics and is the most widely used drug worldwide (Duong et al. 2016; Hider-

Mlynarz et al. 2018; McCrae et al. 2018). Bioassays investigating the effects of paracetamol on behaviour are scarce, but a study in the zebrafish *Danio rerio* showed toxic effects of paracetamol on individual malformations, pigmentation, locomotion, enzyme expression and epigenetics (Nogueira et al. 2019).

In this study, we performed a series of bioassays using each substance alone at different concentrations or in mixtures.

Material and methods

1. Animals

Mosquito eggs (*Aedes aegypti* Bora strain) were obtained from MIVEGEC-IRD (Montpellier, France). Eggs were kept dry or placed in 750 ml polypropylene containers with either 500 ml dechlorinated tap water or 500 ml mixture. Larvae were fed *ad libitum* with shrimp food (JBL Novo Prawn, Neuhofen, Germany) and the containers were maintained at 25°C ± 2°C, under a 12h:12h light/dark cycle (lights on at 8:00 am). Only fourth instar mosquito larvae were trained in the experiments. All animals were reared and manipulated in accordance with European Union ethical guidelines.

2. Experimental procedure

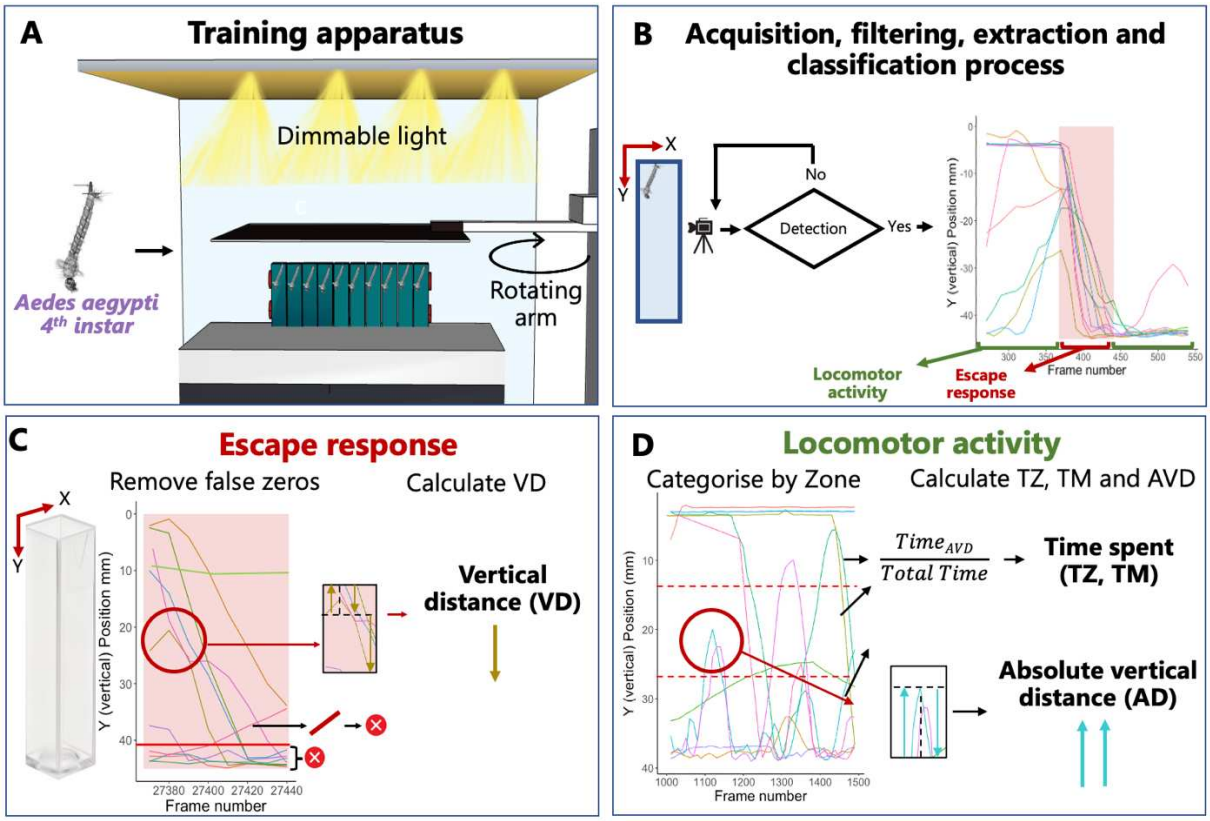


Figure 1: Schematic of the experimental protocol. A) *Aedes aegypti* larvae were reared in the laboratory and trained at the fourth larval stage using our training apparatus. B) Experiments were video-recorded and individual trajectory was extracted. C) We analysed the stimulation response that corresponded to the individual trajectory during the aversive stimulus, using Vertical distance (VD). This quantitative variable was calculated as the relative sum of the distance travelled in the vertical direction toward the bottom of the cuvette. In addition, two filters were applied to exclude individuals located at the bottom of the cuvette during the first frames of the aversive stimulus and the individuals travelling upwards during the stimulus. D) We analysed the spontaneous locomotor activity using 3 variables. Time spent per zone (TZ) was a proportion of time spend in one of the 3 zones delimited. Time spent moving (TM) was a proportion of time where the Absolute vertical distance was above a threshold of 1mm/sec. Absolute vertical distance (AD) was quantitative and calculated as the absolute sum of the distance travelled in the vertical direction.

All experiments were performed in the same experimental room at 25°C ± 0.5°C during the afternoon (i.e., from 12.00 to 19.00 h). The experimental apparatus was the same used in two previous studies and consisted of a platform for isolating individual mosquito larvae, different types of stimuli, and a system for recording and analysing larval behaviour (Dessart et al. 2023, 2024). It included individual containers (i.e., 1.5 ml spectrophotometer plastic cuvettes) filled with treated water (see acute toxicity section below) or dechlorinated tap water (Figure 1A). Fourth instar larvae were placed in 10 horizontally aligned cuvettes and confronted with the appearance of an aversive stimulus consisting of either a black cardboard square (16 cm side) attached to a

servomotor, or 4 mechanical vibrators, all operated by an Arduino Uno board remotely controlled by a computer. The cardboard represented a potential flying predator visually perceived by the larvae, while the vibrator represented the motion of potential fishes transmitted through the water column. The cuvettes were illuminated by two LED panels and the light intensity was fixed at 900 $\mu W.cm^{-2}$ ± 100 $\mu W.cm^{-2}$ (International Light Technology radiometer). All experiments were video-recorded, and the videos were subsequently analysed (Figure 1B, data analysis section).

After 30 min of familiarisation, 10 trials were performed, i.e., 10 passages of the visual stimulation, with a 2 min inter-trial interval. For the acute toxicity (see acute toxicity section), a new trial was performed 3 hours after the 10^{th} trial to test the memory persistence of mosquito larvae. After training, we applied a 3-second mechanical stimulation using the vibrators to check that the larvae were still responsive to a stimulus. Individuals that did not respond to the mechanical stimulus were excluded from the analysis (7 individuals). We also removed individuals that remained motionless during the training (10 individuals), transformed into pupae during the training (1 individual), and when tracking failed to extract individual coordinates (1 individual). A total of 765 individuals were kept for the analyses.

3. Chemical treatment

Two types of toxicity were represented in this study. Chronic toxicity represents the effect of exposure at low concentrations over the development from egg to 4^{th}- instar, whereas acute toxicity was achieved by a single exposure to higher concentrations of chemicals (Environmental Protection Agency 1994). We simulated chronic toxicity by placing eggs in 750 ml polypropylene containers with either 500 ml of water purified using a Millipore Milli-Q lab water system or 500 ml of treated water, until the fourth larval stage. To represent acute toxicity, larvae were reared in 500 ml purified water, and they were subsequently trained in treated water or purified water (during 30 min familiarisation + training period). Three chemicals were considered for both toxicity simulations: glyphosate, atrazine and paracetamol. Glyphosate solutions were prepared by dissolving glyphosate (PESTANAL analytical standard, purity ≥98%, Sigma Aldrich, USA), atrazine (PESTANAL analytical standard, purity ≥98%, Sigma Aldrich, USA) and paracetamol (BioXtra, purity ≥98%, Sigma Aldrich, USA) in Millipore Milli-Q lab water. We

used the pure chemical rather than a formulation to isolate any possible effect due to these substances being in a mixture. Indeed, formulations with several compounds have been shown to be more toxic than the chemical alone (Nagy et al. 2020).

4. Chronic toxicity

To investigate the chronic toxicity of the two herbicides (glyphosate and atrazine) and the medicine drug (paracetamol), mosquito larvae were reared in treated water. For glyphosate, we selected two concentrations related to field measurements: 100 µg/L and 200 µg/L (Struger et al. 2008; Byer et al. 2008; Riaz et al. 2009) and a high concentration was prepared at 2mg/L which corresponds to usual doses applicated in other works (Bara et al. 2014; Balbuena et al. 2015; Baglan et al. 2018; Nouvian et al. 2023). For atrazine, three concentrations were selected: 200 µg/L and 500 µg/L and 2mg/L, consistently with several publications (Dewey 1986; Bara et al. 2014; Johnson 2019; Abdulelah et al. 2020; Adedara et al. 2021). For paracetamol, although field concentrations reported by some studies were relatively low (e.g. (Gracia-Lor et al. 2012): median 44.8 µg/L, min–max values 1.13 – 201 µg/L), we chose to test concentrations of 1 mg/L, 10 mg/L and 100 mg/L in line with previous bioassays in mammals (Adamson et al. 1991; El Menyiy et al. 2018), the zebrafish *Danio rerio* (Nogueira et al. 2019) and on the development of the fly *Calliphora vicina* (O'Brien and Turner 2004).

A control treatment was paired with each set of conditions. A first control (N°1) was associated with atrazine 200 µg/L and 500 µg/L, and a second control (N°2) was associated with glyphosate 100 µg/L and 200 µg/L, and with paracetamol 1 mg/L and 10 mg/L. This first experiment was called "field doses" because it represented low concentrations associated with field measurements. A new control (N°3) was associated with atrazine 2 mg/L and another one (N°4) with glyphosate 2 mg/L and paracetamol 100 mg/L. This second experiment was called "spray doses" because it represented high concentrations associated with field applications.

In a second set of experiments, we compared the effect of chronic toxicity of chemicals alone or in mixtures alongside the control treatment. The first mixture consisted of concentrations measured directly in water, in our region. A paracetamol concentration of 6 µg/L corresponded to the maximum measured in the Loire River basin

between 2018 and 2019 (Ledieu et al. 2021); the concentrations of 0.126 µg/L for atrazine, and 2.3 µg/L for glyphosate corresponded to the highest concentrations found at monitored sites in the Indre et Loire area between 2019 and 2021 (www.naiades.eaufrance.fr). These concentrations were communicated by the Institute of Organic and Analytical Chemistry (ICOA UMR 7311 CNRS – *Université d'Orléans*).

The second mixture consisted of 200 µg/L of atrazine and 1mg/L of paracetamol, two treatments that did not affect individual learning (see Results section below). A control (N°5) was associated with the chemicals alone, and an additional control (N°6) was associated with the two mixtures, in this set of experiments called "field realistic doses" *sensu* Herbert et al. (Herbert et al. 2014).

5. Acute toxicity

To assess acute toxicity, we reared larvae in purified water and trained them in treated water. We chose the highest concentrations similar to those used in the chronic toxicity tests (glyphosate and atrazine: 2 mg/L, paracetamol: 100 mg/L). We analysed the effect of these treatments on individual learning, and we also investigated whether these treatments would affect individual memory retention by applying a new trial 2 h after the training session. This retention time was chosen based on our previous work showing that larvae could retain information for up to 2 h after visual learning (Dessart et al. 2023). A last control (N°7) was associated with these experiments.

6. Data analysis

For each experiment of 10 individuals, two sets of videos were recorded. First, we recorded the familiarisation during 30 min and compared global spontaneous activity between groups by taking the last 5 min of the familiarisation. Then, we recorded the whole duration of the training period. The videos were analysed similarly as in Dessart et al. (2024). Briefly, each individual trajectory was extracted using a tracking software and larval identity and detection rate were verified (Figure 1B); Supplementary Table T1, Supplementary Table T2). Individual vertical positions were smoothed and collapsed to reduce the data number, they were classified into two categories and analysed separately (Figure 1). First, the escape response lasted for the 3 seconds period during which the visual stimulus appeared above the individuals (Figure 1C). These data were

filtered within each trial response to remove false zeros (i.e., when the larvae were already at the bottom of the cuvette at the start of a trial, see [34]). After this step, we defined the vertical distance (VD) as the response variable corresponding to the escape response. This variable was 0 if the individual did not move during a stimulus period, and increased as the individual moved away from the stimulus (i.e., dived downwards, Figure 1C). Second, locomotor activity represented individual spontaneous activity during the familiarisation and the inter-trial interval period (Figure 1D). These data were outside the stimulus period and were therefore not filtered. We analysed locomotor activity using the absolute distance (AD), which measured individual displacement irrespective of the direction of movement (upward or downward). This variable was more consistent in representing the average speed and number of diving events of an individual across the ITIs. Finally, we also divided the cuvette in three equal zones (Figure 1D) and calculated the time spent per zone (TZ) and the time spent moving (TM).

Statistical analyses

7. Learning performance

We modelled the learning performance using the Generalised Additive Model to provide a visual estimate of the behavioural response across trials during the training period. We defined models of increasing complexity and compared the best smoothing function using the GCV-UBRE criterion from the *mgcv* package (Wood 2017). Similar to our previous work, the best smoothing function was the P-spline and was applied to all smoothing curves (Dessart et al. 2024).

For each treatment, we compared the distance travelled during a stimulation on the first and last trials. If the difference was not statistically significant, we concluded that the individuals had not habituated. To make these comparisons, we applied a linear mixed effects model to compare the response at the 1st trial versus the 10th trial. The control treatments were analysed similarly to verify that the individuals reared and trained in clear water were able to habituate. We chose VD as the response variable, trial as a fixed factor and the individual identity as a random factor. We checked variance homogeneity and the distribution of the residuals using the *simulateResiduals* function from the *DHARma* package (Hartig F 2022). For the assessment of acute toxicity, we

applied the same model but including the response at the Test phase. For these models, we evaluated pairwise comparisons using the *emmeans* package with Tukey correction (Lenth 2021).

8. Spontaneous activity

We compared individual spontaneous activity during familiarisation, considering the last 5 min of each treatment. We also compared the 9 inter-trial intervals (ITI) between the treatments. First, the absolute distance (AD) travelled by individuals was averaged to calculate the individual average speed (mm/sec) (Figure 1D). We divided the cuvette into three equal zones (top, middle, bottom, Figure 1D), and counted the time spent in each zone (%). We also confronted AD with a threshold of 1mm/sec and classified our data into the variable time spent moving (Figure 1D). Furthermore, we counted the number of diving events by creating a function that counted each time an individual crossed 1/3 and 2/3 of the cuvette length (i.e. 120 pixels) on its way in and out (Figure 1D). For the average speed, the time spent moving and the number of diving events comparison, we applied a one-way ANOVA with respectively AD, time spent moving or the diving events as the response variable and the treatment as factor, followed by Tukey's post hoc test for multiple comparisons.

9. Dataset and code repository

The R code used to analyse the data and the database are available online at: https://github.com/martindessart/Chemical_toxicity

Results

1. Chronic exposition to field doses

Learning

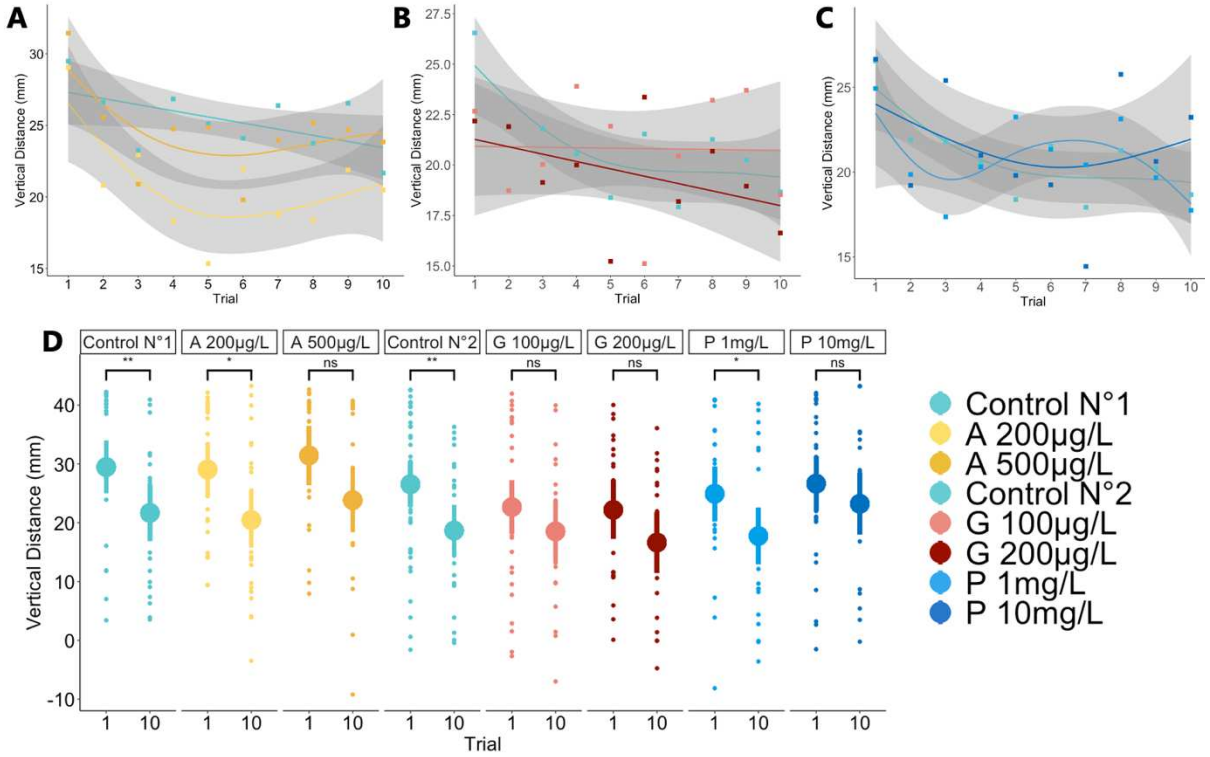


Figure 2: Learning performance for larvae reared at field doses. A) B) C) Habituation curves for larvae reared in control (cyan), atrazine 200 µg/L (light yellow), atrazine 500 µg/L (dark yellow), glyphosate 100 µg/L (pink), glyphosate 200 µg/L (red), paracetamol 1 mg/L (light blue), paracetamol 10 mg/L (dark blue). Vertical distance (in millimetre) corresponds to the distance travelled by one individual during the stimulus period (3 sec), from the 1st to the 10th trial. Smoothing lines indicate the best fitted GAM model. Grey shades indicate 95% confidence interval for the average response. Points indicate mean values. D) For each treatment, vertical distance in millimetre travelled by individuals responding to an aversive stimulus during the 1st to the 10th trial. Points indicate mean values and bars indicate +- 95% confidence intervals. A= Atrazine, G = Glyphosate, P = Paracetamol. NS, not significant; *P<0.05, ***P<0.001.

For control N°1, control N°2, atrazine at 200 µg/L and paracetamol at 1 mg/L, VD was significantly higher in the 1st trial than in the 10th trial: Control N°1: t-ratio = 3.373, df =27, P < 0.01; A 200 µg/L: t-ratio = 2.631, df =28, P = 0.01, Control N°2: t-ratio = 2.801, df =39, P < 0.01; P 1 mg/L: t-ratio = 2.618, df =25, P = 0.01 (Figure 2). On the opposite, the difference between the 1st and the 10th trial was not significant for the other treatments: A 500 µg/L: t-ratio = 1.974, df =23, P = 0.06; G 100 µg/L: t-ratio = 1.059, df =25, P = 0.30; G 200 µg/L: t-ratio = 0.243, df =22, P = 0.81; P 10 mg/L: t-ratio = 1.408, df =22, P = 0.173 (Figure 2).

Activity

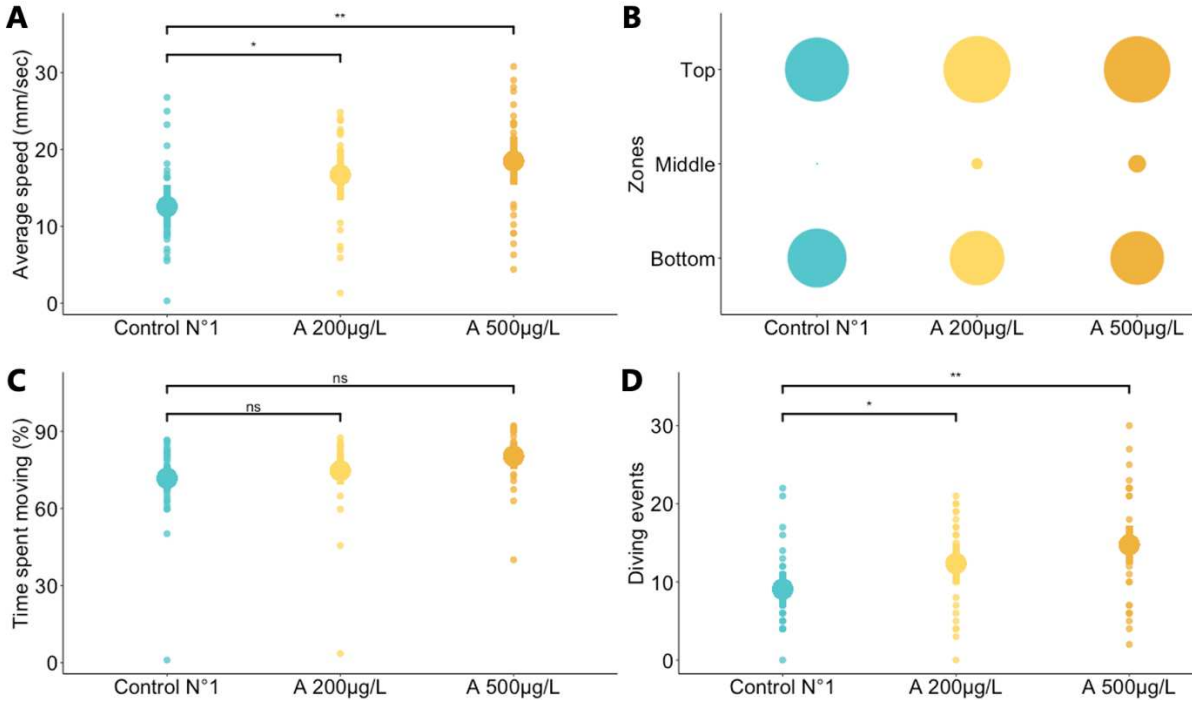


Figure 3: Example of spontaneous locomotor activity for larvae reared in atrazine at field doses during familiarisation. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. A = Atrazine. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant; *P<0.05, **P<0.01.

All the results of spontaneous activity are detailed in supplementary material (Supplementary Table T4). For the treatments at field doses, individuals reared in atrazine were faster and dived more often than control, both during the familiarisation and during the inter-trial intervals (Average speed: Familiarisation: A200 µg/L: t-ratio = -2.427, df =85, P = 0.05; A500 µg/L: t-ratio = -3.593, df =85, P = < 0.01; Inter-trial intervals: A500 µg/L: t-ratio = -2.603, df =90, P = 0.03; Diving events: Familiarisation: A200 µg/L: t-ratio = -1.99, df =85, P = 0.05; A500 µg/L: t-ratio = -3.565, df =85, P = < 0.01; Inter-trial intervals: A500 µg/L: t-ratio = -2.755, df =90, P = 0.01; Figure 3). All other comparisons were not significant (Supplementary Table T4, Supplementary Figure S1, S2, S3, S9, S10).

2. Chronic exposition to spray doses

Learning

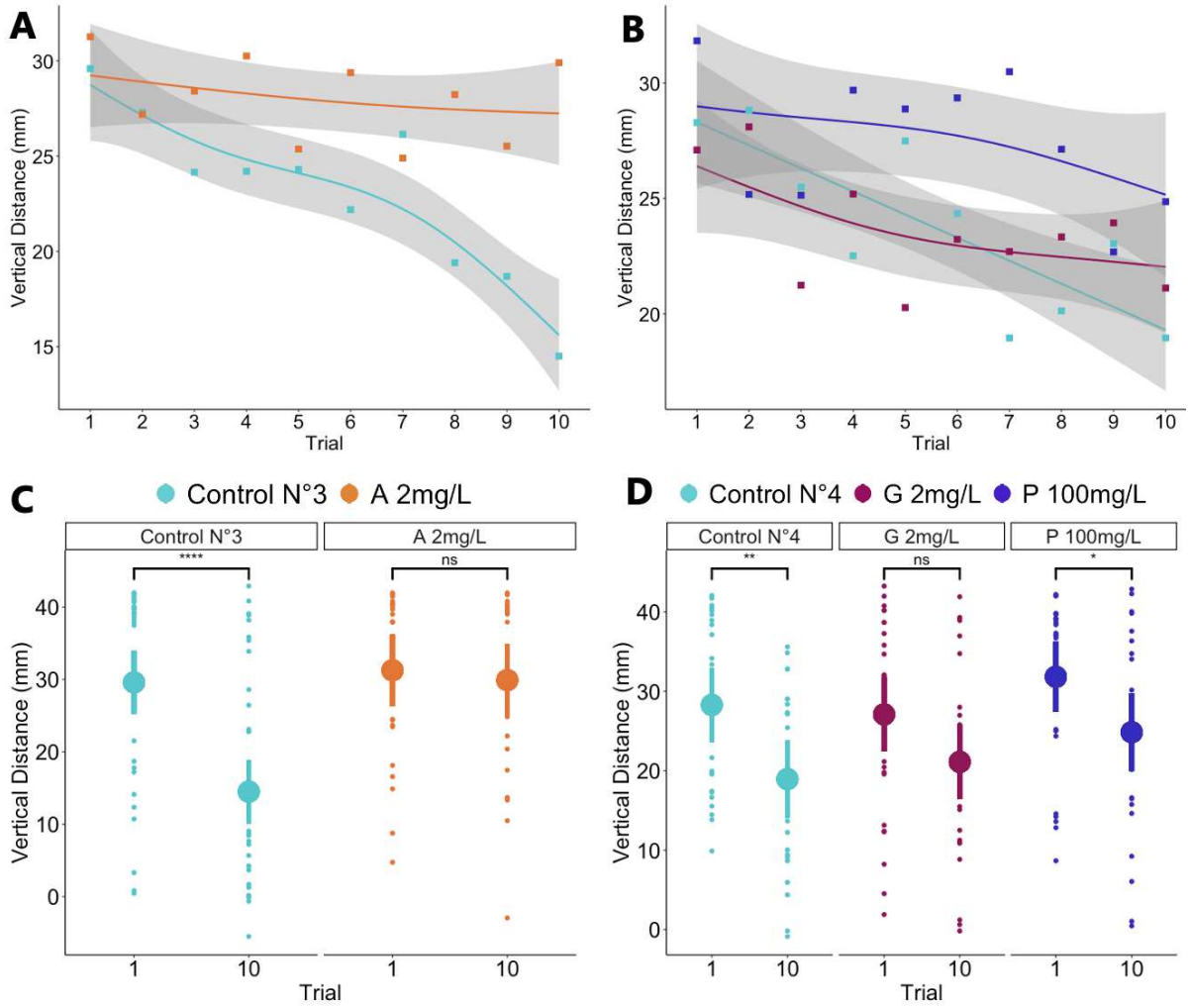


Figure 4: Learning performance for larvae reared at spray doses. A) B) Habituation curves for larvae reared in control (cyan), atrazine 2 mg/L (orange), glyphosate 2 mg/L (purple), paracetamol 100 mg/L (dark blue). Vertical distance (in millimetre) corresponds to the distance travelled by one individual during the stimulus period (3 sec), from the 1st to the 10th trial. Smoothing lines indicate the best fitted GAM model. Grey shades indicate 95% confidence interval for the average response. Points indicate mean values. C D) For each treatment, vertical distance in millimetre travelled by individuals responding to an aversive stimulus during the 1st to the 10th trial. Points indicate mean values and bars indicate +- 95% confidence intervals. A= Atrazine, G = Glyphosate, P = Paracetamol. NS, not significant; *P<0.05, **P<0.01, ****P<0.0001.

For the controls N°3 and N°4 and paracetamol at 100 mg/L, VD was significantly higher in the 1st trial than in the 10th trial: Control N°3: t-ratio = 4.686, df =35, P < 0.0001; Control N°4: t-ratio = 3.063, df = 23, P < 0.01; P: t-ratio = 2.676, df = 25, P = 0.01 (Figure 4). On the opposite, the treatments with atrazine and glyphosate at high dose did not present a significant difference: A 2 mg/L: t-ratio = 0.443, df =27, P = 0.66; G 2 mg/L: t-ratio = 1.761, df = 25, P = 0.09 (Figure 4).

Activity

Larvae reared in glyphosate at 2 mg/L and paracetamol at 100 mg/L dived more than control (Familiarisation: P 100 mg/L: t-ratio = -2.906, df = 83, P = 0.01; Inter-trial intervals: G 2 mg/L: t-ratio = -4.622, df = 83, P < 0.0001; P 100 mg/L: t-ratio = -4.344, df = 83, P < 0.001; Figure 8). Larvae reared in glyphosate were also faster than control during the inter-trial intervals (Average speed G 2 mg/L: t-ratio = -3.033, df = 83, P < 0.01). All other comparisons were not significant (Supplementary Table T4, Supplementary Figure S4, S5, S11, S12).

3. Chronic exposition to field realistic doses

Learning

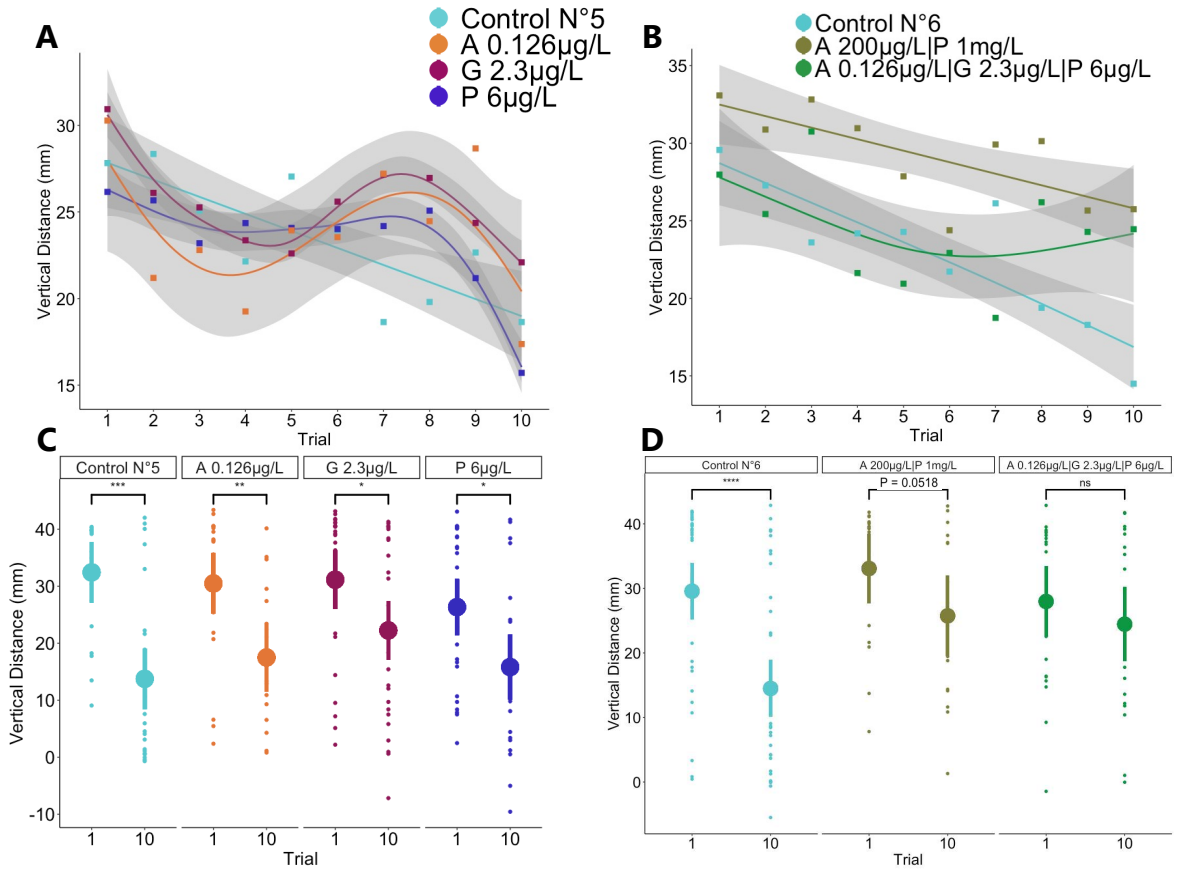


Figure 5: Learning performance for larvae reared at realistic doses. A) B) Habituation curves for larvae reared in control (cyan), atrazine 0.126 µg/L (orange), glyphosate 2.3 µg/L (purple), paracetamol 6 µg/L (dark blue), Mixture N°1 (light green), Mixture N°2 (dark green). Vertical distance (in millimetre) corresponds to the distance travelled by one individual during the stimulus period (3 sec), from the 1st to the 10th trial. Smoothing lines indicate the best fitted GAM model. Grey shades indicate 95% confidence interval for the average response. Points indicate mean values. C) D) For each treatment, vertical distance in millimetre travelled by individuals responding to an aversive stimulus during the 1st to the 10th trial. Points indicate mean values and bars indicate +- 95% confidence intervals. A= Atrazine, G = Glyphosate, P = Paracetamol. NS, not significant; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

For the controls N°5 and N°6 and the chemicals alone, VD was significantly higher in the 1st trial than in the 10th trial: Control N°5: t-ratio = 5.512, df = 24, P < 0.01; A: t-ratio = 4.301, df = 21, P < 0.001; G: t-ratio = 2.205, df = 27, P = 0.04; P: t-ratio = 2.729, df = 26, P = 0.01; Control N°6: t-ratio = 4.686, df = 35, P < 0.001 (Figure 5). However, for the mixture N°1, the difference was not significant: t-ratio = 0.959, df = 22, P = 0.35. Finally, we found a marginal difference for the mixture N°2: t-ratio = 2.058, df = 22, P = 0.0518 (Figure 5).

Activity

Larvae reared in 6 µg/L of paracetamol dived significantly less than control N°5 during inter-trial intervals only (P 6 µg/L: t-ratio = -2.789, df =113, P = 0.03 (Figure 10). Regarding mixtures, larvae reared in the mixture N°2 were faster and dived significantly more than control N°6 during familiarisation and inter-trial intervals (Average speed: Familiarisation: Mixture N°2: t-ratio = -1.988, df =89, P = 0.05; Inter-trial intervals: Mixture N°2: t-ratio = -3.053, df =89, P < 0.01; Diving events: Familiarisation: Mixture N°2: t-ratio = -3.64, df =89, P < 0.01; Inter-trial intervals: Mixture N°2: t-ratio = -3.602, df =89, P < 0.01; Figure 11). All other activity showed no difference between the controls and the treatments (Supplementary Table T4, Supplementary Figure S6, S7, S13, S14).

4. Acute toxicity

Learning and memory

For the four treatments, VD was significantly higher in the 1st trial than in the 10th trial: Control N°7: t-ratio = 2.704, df = 49, P = 0.03; A: t-ratio = 2.801, df = 42, P = 0.02; G: t-ratio = 3.118, df = 53, P < 0.01; P: t-ratio = 4.002, df = 51, P < 0.001 (Figure 6). To evaluate the duration of memory of the larvae tested after 2 hours, we compared the response at the 1st trial to the response at the Test phase. While the difference was significant for Control and paracetamol (Control N°7: t-ratio = 2.906, df = 45, P = 0.01; P: t-ratio = 3.454, df = 52, P < 0.01), it was not the case for atrazine and glyphosate (A: t-ratio = 1.612, df = 41, P = 0.25; G: t-ratio = 0.612, df = 53, P = 0.815, Figure 6).

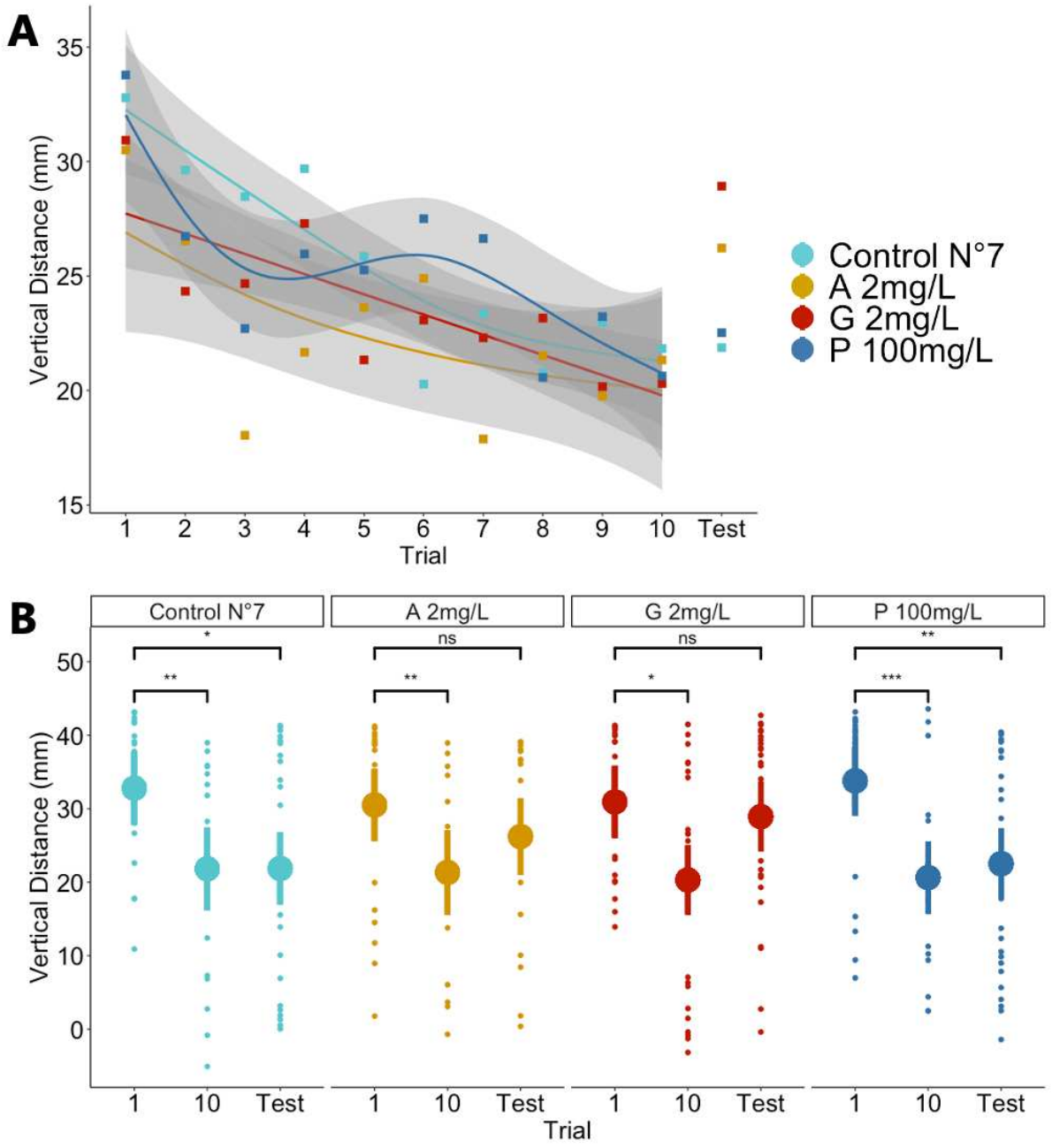


Figure 6: Learning performance for larvae reared at spray doses for acute toxicity. A) Habituation curves for larvae reared in control (cyan), atrazine 2 mg/L (yellow), glyphosate 2 mg/L (red), paracetamol 100 mg/L (blue). Vertical distance (in millimetre) corresponds to the distance travelled by one individual during the stimulus period (3 sec), from the 1st to the 10th trial. Smoothing lines indicate the best fitted GAM model. Grey shades indicate 95% confidence interval for the average response. Points indicate mean values. B) For each treatment, vertical distance in millimetre travelled by individuals responding to an aversive stimulus during the 1st to the 10th trial. Points indicate mean values and bars indicate +- 95% confidence intervals. A= Atrazine, G = Glyphosate, P = Paracetamol. NS, not significant; *P<0.05, **P<0.01, ***P<0.001.

Activity

Larvae trained in paracetamol at 100 mg/L showed reduced time spent moving both during the familiarisation and the inter-trial intervals (Familiarisation: P 100 mg/L: t-ratio = 3.008, df = 114, P = 0.02; Inter-trial intervals: P 100 mg/L: t-ratio = 2.760, df = 112, P = 0.03; Figure 13). All other comparisons showed no difference in spontaneous activity (Supplementary Table T4, Supplementary Figure S8, S15).

Discussion

The development of behavioural tests to assess the effects of pollutants at field realistic concentrations on the cognitive abilities of animals provides an understanding of how pollutants may affect higher neurological functions as well as their environment (Bownik and Wlodkowic 2021). This study investigated how three pollutants at field realistic concentrations affect the activity, learning and memory in mosquito larvae. Our results show that atrazine, glyphosate and paracetamol can reduce or increase individual spontaneous activity, impair habituation and memory retention 2 h after exposure to the pollutants. These changes the animals' ability to perceive and escape from a potential danger or to avoid spending energy escaping from an innocuous object in its natural habitat. In result, it may reduce the individuals' overall fitness. Our results put into evidence behavioural changes due to sub-lethal doses of pollutants in the water. Therefore, mosquito larvae could potentially be used as bioindicators to study the effect of chemicals at sub-lethal doses. Moreover, we observed deleterious effects of glyphosate, atrazine and paracetamol presented in a mixture, at doses in which no effect could be observed due to each substance alone. This means that our bioassay proved to be useful to put into evidence toxic effects of the mixture itself also known as "cocktail effects", which cannot be predicted solely by chemical analyses.

In this study, given that we were interested on sub-lethal hidden effects, we did not perform a survival analysis, as was previously done for glyphosate (Baglan et al. 2018). Although we cannot state that individual survival was not impacted at all, we did not observe any noticeable mortality during the rearing of the larvae. In the study by Baglan et al. (Baglan et al. 2018), the authors used glyphosate concentrations similar to

ours, and no reduction in survival was assessed. In addition, a study by Bara et al. (Bara et al. 2014) examined the effects of atrazine and glyphosate at 5 mg/L on life history traits in *Aedes aegpyti* and *Aedes albopictus* mosquitoes. Atrazine increased the emergence rate and the emergence time of *Aedes aegpyti*, and also skewed the sex-ratio. Another study (Rakotondravelo et al. 2006) assessed the sublethal effects of atrazine on survival, growth and adult emergence of the aquatic midge *Chironomus tentans*, and no effect on these three parameters at 150 □g/L was found. For the cockroach *Nauphoeta cinerea,* (Adedara et al. 2021) no effect on individual survival was found when cockroaches had been administered 1.0 and 0.5 µg g^{-1} of atrazine. Finally, Marcus et al. (Marcus and Fiumera 2016) found a reduction in survival in the fly *Drosophila melanogaster* starting at 2 ppB of atrazine dissolved in the diet. To the best of our knowledge, we did not find any studies on the effect of paracetamol on mosquito survival, and we did not observe any specific mortality in our study.

Atrazine affected our animals by altering their learning ability and by increasing their spontaneous locomotion at 500 □g/L. At 200 □g/L, atrazine slightly increased individual spontaneous activity but not at 2 mg/L. At the latter concentration, however, learning ability was severely impaired, as the average distance travelled during a stimulus was not less than 25 mm, whereas it fell below 20 mm in the control treatments. Furthermore, when looking at the response 2 h after the end of the training phase (i.e., acute toxicity), atrazine impaired the individual's ability to retrieve the information, whereas learning was not impaired. These three independent results provided strong evidence for the neurotoxicity of atrazine in mosquito larvae. As noted by several authors, atrazine disrupts endocrine functions in invertebrates, by increasing oxidative stress and increased the activity of enzymes like P450 known to be involved in insecticide resistance in mosquitoes (Semren et al. 2018). Moreover, atrazine has been shown to alter acetylcholinesterase activity in invertebrate species (Boyer et al. 2006; Vogel 2015).

Besides, atrazine induced changes in locomotion, still with conflicting results. For instance, it decreased locomotor activity in cockroaches, honeybees, nematodes and termites (García-Espiñeira 2018; Ejomah et al. 2020; Araújo et al. 2021; Adedara et al. 2021), while in amphibians it increased activity at low concentrations, and decreased activity at high concentrations (Rohr and McCoy 2010). In our study, we hypothesise that the hyperactivity induced at field concentrations was attenuated by greater toxicity at

higher concentrations. To our knowledge, the only studies of the effects of atrazine on learning and memory have been in mammals. For example, Rastegar-Morghaddam et al. (Rastegar-Moghaddam et al. 2019) found that atrazine ingestion impaired learning and spatial memory in mice and increased the apoptosis of cells located in the hippocampus. Here, we showed for the first time that sub-lethal doses of atrazine impaired learning and memory in an invertebrate species.

Glyphosate also affected learning abilities of mosquito larvae even at the lowest dose: 100 □g/L. In addition, spray doses (5 mg/L) severely impaired individual learning and memory and increased locomotor activity. In acute toxicity tests, we also found a strong effect of glyphosate on memory 2 hours after training. These results confirm the previously demonstrated effects of glyphosate on insect cognition and add to the growing body of work showing negative effect of glyphosate on animals (Gill et al. 2018). Indeed, glyphosate reduced locomotor activity in nematodes, planarians, cockroaches and produced a slight and transient modification in bumblebees (García-Espiñeira 2018; Córdova López et al. 2019; Kanabar et al. 2021; Nouvian et al. 2023). Glyphosate has also been shown to alter spatial learning in honeybees, associative learning in honeybees and bumblebees and memory retention in honeybees (Herbert et al. 2014; Balbuena et al. 2015; Hernández et al. 2021; Nouvian et al. 2023). We hypothesise, in line with the literature, that the alteration primarily affects the central nervous system of mosquito larvae (Gill et al. 2018; Baglan et al. 2018).

Larvae reared at 10 mg/L of paracetamol slightly decreased their behavioural response during the training period, but we observed a clear difference between the 1st and the 10th trials for larvae reared at 1 mg/L and 100 mg/L. In addition, no effect of paracetamol was found for acute toxicity, and only an increase in the number of diving events was observed at the spray doses for the activity assessment. The literature assessing the effects of paracetamol on locomotor and cognitive abilities in animals remains scarce. We found two studies in mice and rats that observed an alteration of learning abilities in the presence of paracetamol. Regarding locomotor activity, Rakotondravelo et al. observed an increase in locomotion in rats, while two studies found a decrease in locomotion in zebrafish and mice (Viberg et al. 2014; Nogueira et al. 2019). Our study cannot determine the exact effects of paracetamol on invertebrate cognition, but we suggest that research into the potential effects and mechanisms of this

drug in the soil, water and organisms around human populations should continue (Hider-Mlynarz et al. 2018).

In our study, we combined the lowest concentrations of atrazine (200 µg/L) and paracetamol (1 mg/L). While these two concentrations alone had no effect, we found a significant increase in locomotion and an alteration in learning abilities in larvae reared with these two compounds in combination. Similarly, we conducted a series of experiments with the three compounds at field realistic concentrations measured directly in water. Alone, the pollutants revealed no effect on learning. However, when pollutants were presented in a mixture, learning was impaired while no effect on locomotion was found. It is important to assess the additive and possible synergistic effects of these compounds in mixtures. Indeed, aquatic organisms are constantly exposed to different types of pollutants and the possible synergistic effect of these pollutants would represent the worst case scenario for these organisms (Siviter et al. 2021). We chose to expose mosquito larvae to pure chemicals in order to access the effect of the combination without the influence of adjuvants, but it is important to point out that commercial herbicide formulation affect organisms not only by their main active ingredient but also by their overall formulation and residual products (Córdova López et al. 2019; Kanabar et al. 2021).

Taken together, these results support the global concern about the lack of knowledge about the impact of agrochemicals combined with pharmaceutical residues on aquatic ecosystems. Mosquitoes are known for their behavioural plasticity and their tolerance to pesticides (Poupardin et al. 2008). Their wide global distribution, tolerance to poor water conditions and their behaviour being affected by chemicals at low concentrations make them appropriate subjects to study the effects of pollutants on aquatic invertebrates. By altering the cognitive abilities of the larval stage, these pollutants increased energy expenditure by means of locomotor activity, which may affect their role as nutrient cycling or as food for predators, and affect the overall role of the food web dynamic in aquatic ecosystems (Kanabar et al. 2021). As mosquitoes are resistant to stressors, these toxicological effects are of concern for other species living in the same environments, and might be transferred by bioaccumulation to higher predators (Corbi et al. 2010).

In a previous paper, we presented an automated experimental approach for evaluating different parameters of the behaviour of mosquito larvae, notably, learning capacity and activity. Here we applied this concept to study the sublethal effects of water pollutants. Although our method is not yet an "ideal biomonitoring tool", as defined by some authors (Bonada et al. 2006), it revealed as an easy-to-use and modulable system for evaluating simultaneously 10 individuals, with high throughput data, making it appropriate for the assessment of risks associated with the presence of pollutants in the aquatic environment.

With this study, we tried to contribute to the field of cognitive ecotoxicology, using a model that is widely used and of great interest for human health. We believe that there is an urgent need to develop, communicate and standardise methods for measuring the impact of pollutants on these vulnerable ecosystems so that they can be used by policy makers to meet the next environmental, social and economic challenges.

Acknowledgements

Financial support for this work was granted by Region Centre-Val de Loire, France APR IR 2020 COMPORTATE. COMPORTATE is supported by Réseau MiDi - Milieux et Diversité, Pole DREAM - French Water Team, Zone Atelier de la Loire – LTER France Loire, Réseau ENTOMOCENTRE, Département d'Indre-et-Loire - Direction de l'Attractivité des Territoires (France).

We thank Elfie Perdereau for her help in handling the chemical products and the dilutions.

Author contributions

Martin Dessart: Conceptualization**,** Data curation, Formal analysis, Investigation, Methodology, Validation, Software, Visualization, Writing – original draft, Writing – review & editing. **Claudio R. Lazzari:** Conceptualization, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. **Fernando J. Guerrieri:**

Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

We declare we have no competing interests.

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Supplementary material

1. Data classification and filtering

Toxicity	Replicate	Treatment	Concentration		ID number		Detection rate	Vertical length (px)	Comment
Chronic - Residual doses	1	Atrazine	200 µg/L		10		0,99	389,5	
	2	Control n°1	/		9		0,89	392	ID#1 dead
	3	Atrazine Control n°1	200 µg/L	/	5	5	0,99	392,6	
	4	Control n°1 Atrazine	/	500 µg/L	5	5	0,98	390	
	5	Atrazine Control n°1	500 µg/L	/	5	5	0,99	391	
	6	Atrazine	500 µg/L		10		0,94	390,7	
	7	Atrazine	500 µg/L	200 µg/L	5	5	0,9	391,9	
	8	Control n°1 Atrazine	/	200 µg/L	5	5	0,95	395,6	
	9	Atrazine Control n°1	200 µg/L	/	4	5	0,96	395,7	ID#2 dead
	10	Control n°1 Atrazine	/	500 µg/L	5	5	0,98	394,1	
	11	Glyphosate	200 µg/L		10		0,93	389,9	
	12	Glyphosate Control n°2	200 µg/L	/	5	5	0,92	393,2	
	13	Glyphosate	100 µg/L		10		0,85	393,5	
	14	Glyphosate Control n°2	100 µg/L	/	5	5	0,91	395,1	
	15	Control n°2 Glyphosate	/	100 µg/L	5	5	0,99	394,8	
	16	Glyphosate Control n°2	100 µg/L	/	5	5	0,89	395,6	
	17	Paracetamol Control n°2	1 mg/L	/	5	5	0,93	393	
	18	Control n°2 Paracetamol	/	1 mg/L	4	6	0,93	389,7	
	19	Paracetamol	10 mg/L	1 mg/L	5	5	0,97	393,5	
	20	Paracetamol	1 mg/L	10 mg/L	5	5	0,98	389,2	
	21	Paracetamol Glyphosate	1 mg/L	200 µg/L	5	5	0,94	396,7	
	22	Glyphosate Paracetamol	200 µg/L	1 mg/L	5	5	0,99	394	
	23	Control n°2	/		10		0,96	390	
	24	Paracetamol	10 mg/L		10		0,96	395,1	
	25	Paracetamol	10 mg/L		10		0,98	391,1	
	26	Glyphosate	100 µg/L	200 µg/L	5	5	0,87	394,2	
Chronic - Spray doses	27	Control n°3	/		10		0,98	309,6	
	28	Control n°3	/		9		0,99	391,7	ID#2 dead
	29	Control n°3	/		2		0,97	389,6	ID#1 Mix2, ID#2 to ID#3 Control, ID#4 dead, ID#5 to ID#10 Mix3
	30	Control n°3	/		10		0,99	389,6	
	31	Atrazine	2 mg/L		10		0,96	390,3	
	32	Atrazine	2 mg/L		10		0,98	390,9	
	33	Atrazine	2 mg/L		10		0,99	391	
	34	Control n°3	/		10		0,99	393,4	
	35	Glyphosate	2 mg/L		10		0,95	391,3	
	36	Glyphosate	2 mg/L		10		0,95	390,8	
	37	Glyphosate	2 mg/L		10		0,91	391,7	
	38	Paracetamol	100 mg/L		10		0,94	384,8	
	39	Paracetamol	100 mg/L		10		0,95	390,3	
	40	Control n°4	/		9		0,96	400,9	ID#8 did not respond to vibration
	41	Control n°4	/		10		0,99	400,4	
	42	Control n°4	/		7		0,95	399,3	ID#2, ID#3 did not respond to vibration, ID#6 did not respond to vibration
	43	Paracetamol	100 mg/L		10		0,88	392,7	

Chronic - Field doses	44	Control n°5	/	10	0.99	395.2	
	45	Control n°5	/	10	0.99	395.2	
	46	Paracetamol	6 µg/L	10	0.89	399.8	
	47	Glyphosate	0.126 µg/L	10	0.94	402	
	48	Control n°5	/	10	0.99	391.3	
	49	Atrazine	2.3 µg/L	9	0.9	399.8	ID#5 did not respond to vibration
	50	Paracetamol	6 µg/L	10	0.98	397	
	51	Glyphosate	0.126 µg/L	10	0.91	398.5	
	52	Atrazine	2.3 µg/L	9	0.95	399.2	ID#10 did not respond to vibration
	53	Paracetamol	6 µg/L	10	0.99	403.9	
	54	Glyphosate	0.126 µg/L	10	0.99	399.2	
	55	Atrazine	2.3 µg/L	9	0.9	400.7	ID#2 did not respond to vibration
	56	Mixture N°1	G: 0.126 µg/L | A:2.3 µg/L | P: 6 µg/L	10	0.98	390.1	
	57	Control n°6	/	10	0.98	309.6	
	58	Mixture N°1	G: 0.126 µg/L | A:2.3 µg/L | P: 6 µg/L	5	0.96	387.5	ID#6 to ID#10 from Glyphosate-only group
	59	Control n°6	/	9	0.99	391.7	ID#2 dead
	60	Mixture N°1	G: 0.126 µg/L | A:2.3 µg/L | P: 6 µg/L	9	0.93	388.4	ID#10 transformed in pupae
	61	Mixture N°2	P:1 mg/L | A:200 µg/L	10	0.99	390.3	
	62	Mixture N°2	P:1 mg/L | A:200 µg/L	10	0.98	394.4	
	63	Mixture N°2 Control n°6	P:1 mg/L | A:200 µg/L /	7 2	0.97	389.6	ID#1 Mix2, ID#2 to ID#3 Control, ID#4 dead, ID#5 to ID#10 Mix3
	64	Control n°6	/	10	0.99	389.6	
	65	Control n°6	/	10	0.99	393.4	
Acute - Spray doses	66	Control n°7	/	9	0.97	402.3	ID#6 dead
	67	Control n°7	/	10	0.9	400.9	
	68	Control n°7	/	10	0.98	405.7	
	69	Glyphosate	2 mg/L	10	0.92	405.8	
	70	Atrazine	2 mg/L	9	0.88	403.3	ID#10 not tracked
	71	Paracetamol	100 mg/L	10	0.94	404.7	
	72	Atrazine	2 mg/L	10	0.96	404.1	
	73	Atrazine	2 mg/L	8	0.93	404.4	ID#5, ID#8 did not respond to vibration
	74	Glyphosate	2 mg/L	10	0.89	406.3	
	75	Paracetamol	100 mg/L	10	0.94	405.6	
	76	Paracetamol	100 mg/L	10	0.96	403.9	
	77	Glyphosate	2 mg/L	10	0.97	405.9	

Supplementary Table T1: Details for each experiment performed. Toxicity represents the type of toxicity studied. Each replicate represents 10 individuals trained during one session. Treatment represents the rearing (for chronic toxicity) or training (for acute toxicity) water treatment. If the cell is divided in two, it means that we tested 5 individuals for one treatment and 5 for another treatment. The concentration column refers to the concentration of chemicals used and ID number correspond to the number of individual for each replicate. Detection rate was calculated as the ratio between the maximum frame number and the actual frame number identified by the tracking software. Vertical length was calculated as the difference between the maximum and the minimum individual position measured on each video by the tracking software.

Chronic pollution

	Field doses								*Spray doses*					
	/	200 µg/L	500 µg/L	/	100 µg/L	200 µg/L	1 mg/L	10 mg/L	/	2 mg/L	/	2 mg/L	100 mg/L	
	Control 1	Atrazine	Atrazine	Control 2	Glyphosate	Glyphosate	Paracetamol	Paracetamol	Control 3	Atrazine	Control 4	Glyphosate	Paracetamol	
Individuals trained	34	**30**	29	44	**30**	**30**	31	30	41	30	26	30	30	
Trials per individuals	340	300	290	440	300	300	310	300	410	300	260	300	300	
Trials filtered by position	258	234	225	367	255	216	241	241	339	248	223	248	240	
% Trials removed	24.1%	22.0%	22.4%	16.6%	15.0%	28.0%	22.3%	19.7%	17.3%	17.3%	14.2%	17.3%	20.0%	
Trials filtered by going up	257	234	224	362	254	214	240	240	337	248	222	244	240	
% Trials removed	0.4%	0.0%	0.4%	1.4%	0.4%	0.9%	0.4%	0.4%	0.6%	0.0%	0.4%	1.6%	0.0%	
Total % Trials removed	24.4%	22.0%	22.8%	17.7%	15.3%	28.7%	22.6%	20.0%	17.8%	17.3%	14.6%	18.7%	20.0%	

Chronic pollution **Acute pollution**

	Realistic doses							*Spray doses*				
	/	2.3 µg/L	0.126 µg/L	6 µg/L	/	A:2.3 µg/L G: 0.126 µg/L P: 6 µg/L	A:200 µg/L P:1 mg/L	/	2 mg/L	2 mg/L	100 mg/L	
	Control 5	Atrazine	Glyphosate	Paracetamol	Control 6	Mix N°1	Mix N°2	Control 7	Atrazine	Glyphosate	Paracetamol	Total
Individuals trained	30	27	30	30	41	25	26	29	27	30	30	740
Trials per individuals	300	270	300	300	410	250	260	319	297	330	330	7516
Trials filtered by position	237	209	228	215	342	203	192	256	217	269	279	5982
% Trials removed	21.0%	22.6%	24.0%	28.3%	16.6%	18.8%	26.2%	19.7%	26.9%	18.5%	15.5%	**20.4%**
Trials filtered by going up	234	205	228	214	340	203	190	255	212	269	279	5945
% Trials removed	1.3%	1.9%	0.0%	0.5%	0.6%	0.0%	1.0%	0.4%	2.3%	0.0%	0.0%	0.6%
Total % Trials removed	22.0%	24.1%	24.0%	28.7%	17.1%	18.8%	26.9%	20.1%	28.6%	18.5%	15.5%	**20.9%**

Supplementary Table T2: Summary of the filtering steps. For each species, 25 to 59 individuals were trained. When the individual's position was close to the bottom, the response to the trial was removed, accounting for a total of 20.5% of trials removed. The trajectory of an individual moving upwards during a stimulation was rare, representing only 0.6% of trials.

Treatment	Control 1	Atrazine	Atrazine	Control 2	Glyphosate	Glyphosate	Paracetamol	Paracetamol	Control 3	Atrazine	Control 4	Glyphosate	Paracetamol
Concentration	/	200 µg/L	500 µg/L	/	100 µg/L	200 µg/L	1 mg/L	10 mg/L	/	2 mg/L	/	2 mg/L	100 mg/L
χ 2	5.313	13.697	11.879	11.231	13.565	7.023	6.286	8	3.028	8	10.421	11.5	8.333
df	9	9	9	9	9	9	9	9	9	9	9	9	9
P =	0.81	0.13	0.22	0.26	0.14	0.63	0.71	0.53	0.96	0.53	0.32	0.24	0.5

Treatment	Control 5	Atrazine	Glyphosate	Paracetamol	Control 6	Mix	Mix	Control 7	Atrazine	Glyphosate	Paracetamol
Concentration	/	2.3 µg/L	0.126 µg/L	6 µg/L	/	G: 0.126 µg/L + A:2.3 µg/L + 6 µg/L	P:1 mg/L + A:200 µg/L	/	2 mg/L	2 mg/L	100 mg/L
χ 2	4.909	9.615	12.421	8.88	4.286	11.936	8.571	6.812	8.306	7.37	10.471
df	9	9	9	9	9	9	9	10	10	10	10
P =	0.84	0.38	0.19	0.45	0.89	0.22	0.48	0.74	0.6	0.67	0.4

Supplementary Table T3: Similar to Dessart et al. (2023, 2024), we tested whether the number of trials deleted by the criterion depended on the trial number, by applying a chi-squared test to the deleted trials as a function of the trial number for each treatment. For all treatments, the deleted trials were not specific to any trial number.

2. Spontaneous activity

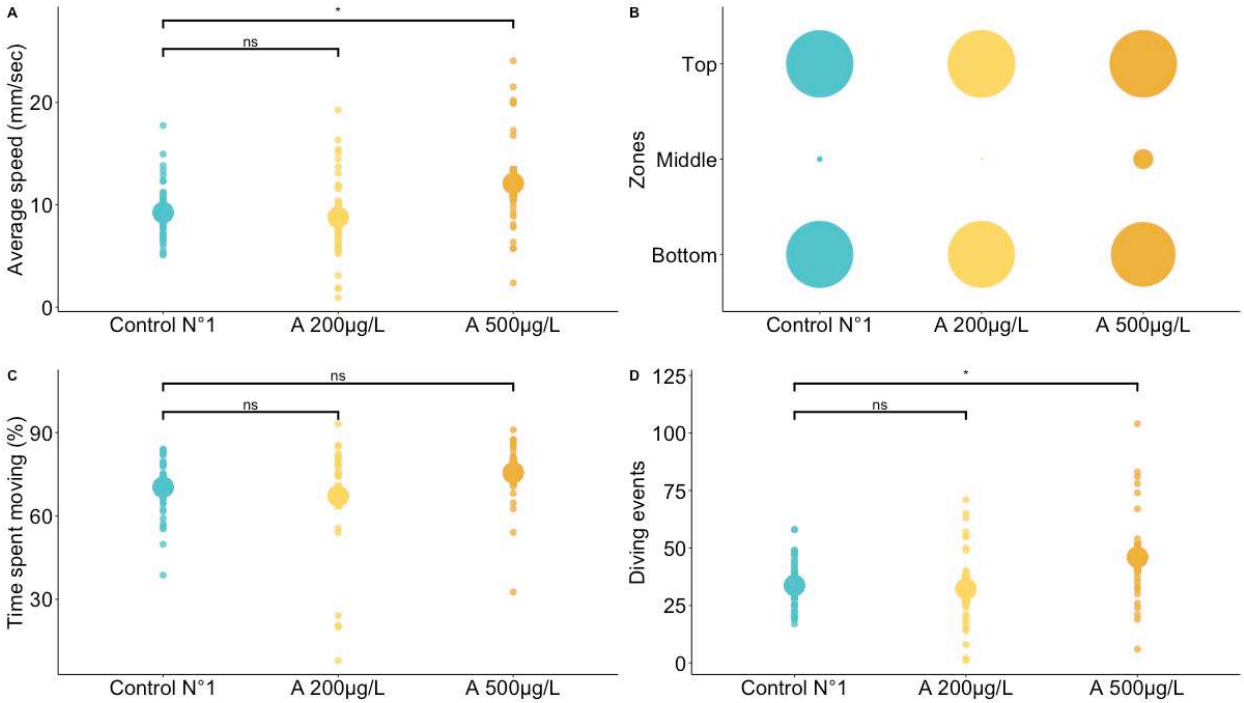
		Treatment	Atrazine	Atrazine	Glyphosate	Glyphosate	Paracetamol	Paracetamol	Atrazine	Glyphosate	Paracetamol
		Concentration	200 µg/L	500 µg/L	100 µg/L	200 µg/L	1 mg/L	10 mg/L	2 mg/L	2 mg/L	100 mg/L
Activity during familiarisation	Average speed	t-ratio	**-2.427**	**-3.593**	-0.165	-0.206	1.524	1.337	1.371	-1.365	-0.351
		df	**85**	**85**	85	85	85	85	50	83	83
		P	**0.05**	**< 0.01**	0.99	0.98	0.28	0.38	0.18	0.36	0.93
	Time spent moving	t-ratio	-0.75	-2.312	-0.709	-0.318	0.94	0.532	1.545	-1.321	0.757
		df	85	85	85	85	85	85	50	83	83
		P	0.73	0.06	0.76	0.95	0.62	0.86	0.13	0.39	0.73
	Diving events	t-ratio	**-1.99**	**-3.565**	-0.217	1.2	0.659	-0.313	1.168	-1.549	**-2.906**
		df	**85**	**85**	85	85	85	85	50	83	**83**
		P	**0.05**	**< 0.01**	0.98	0.46	0.79	0.95	0.25	0.27	**0.01**
Activity during inter-trial intervals	Average speed	t-ratio	0.441	**2.603**	-0.591	0.997	1.043	0.954	1.438	**-3.033**	-2.062
		df	90	**90**	101	101	102	102	69	**83**	83
		P	0.9	**0.03**	0.83	0.58	0.55	0.61	0.15	**< 0.01**	0.1
	Time spent moving	t-ratio	0.832	-1.362	-1.117	-0.237	1.043	0.954	1.357	-1.589	0.219
		df	90	90	101	101	102	102	69	83	83
		P	0.68	0.37	0.51	0.97	0.55	0.61	0.18	0.26	0.97
	Diving events	t-ratio	0.341	**-2.755**	-1.017	1.079	0.958	-0.016	0.237	**-4.622**	**-4.344**
		df	90	**90**	101	101	102	102	69	**83**	**83**
		P	0.94	**0.01**	0.57	0.53	0.61	0.99	0.81	**< 0.0001**	**< 0.001**

		Treatment	Atrazine	Glyphosate	Paracetamol	Mix	Mix	Atrazine	Glyphosate	Paracetamol
		Concentration	2.3 µg/L	0.126 µg/L	6 µg/L	G: 0.126 µg/L + A:2.3 µg/L + 6 µg/L	P:1 mg/L + A:200 µg/L	2 mg/L	2 mg/L	100 mg/L
Activity during familiarisation	Average speed	t-ratio	-1.153	-0.822	0.459	0.037	**-1.988**	1.858	-0.312	1.45
		df	113	113	113	89	**89**	114	114	114
		P	0.66	0.84	0.97	0.99	**0.05**	0.25	0.99	0.47
	Time spent moving	t-ratio	-0.478	-1.48	1.125	-0.67	-0.812	1.623	2.098	**3.008**
		df	113	113	113	89	89	114	114	**114**
		P	0.96	0.45	0.67	0.78	0.7	0.37	0.16	**0.02**
	Diving events	t-ratio	-1.115	-0.776	0.111	-0.346	**-3.64**	1.502	0.606	1.056
		df	113	113	113	89	**89**	114	114	114
		P	0.68	0.87	0.99	0.94	**< 0.01**	0.44	0.93	0.72
Activity during inter-trial intervals	Average speed	t-ratio	-2.547	-0.707	1.166	-0.69	**-3.053**	2.194	2.432	2.185
		df	113	113	113	89	**89**	112	112	112
		P	0.06	0.89	0.65	0.77	**< 0.01**	0.13	0.08	0.13
	Time spent moving	t-ratio	-1.279	-1.032	1.533	-0.52	-2.033	1.716	1.934	**2.76**
		df	113	113	113	89	89	112	112	**112**
		P	0.57	0.73	0.42	0.86	0.11	0.32	0.22	**0.03**
	Diving events	t-ratio	-0.22	1.896	**-2.789**	-0.042	**-3.602**	0.279	1.465	0.526
		df	113	113	**113**	89	**89**	112	112	112
		P	0.99	0.24	**0.03**	0.99	**< 0.01**	0.9	0.46	0.95

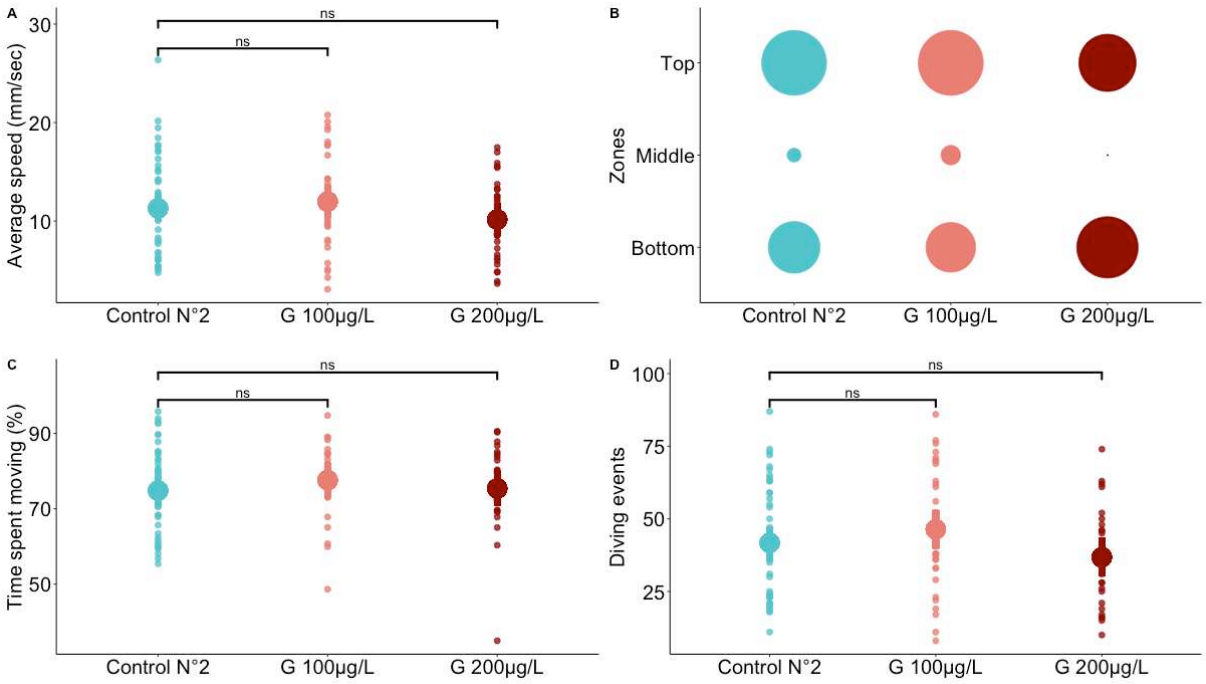
Supplementary Table T4: shows all the comparison between treatment and control.

3. Spontaneous activity during inter-trial intervals

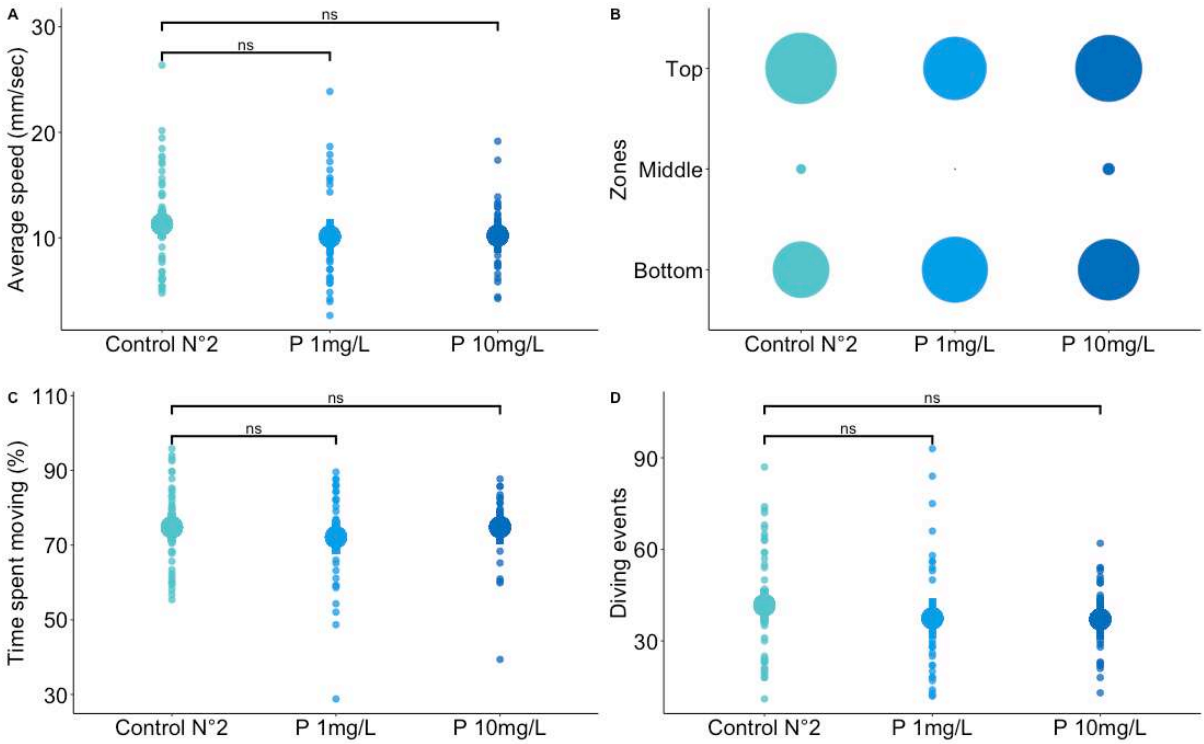
Here are the graphs representing differences during the **inter-trial intervals**.



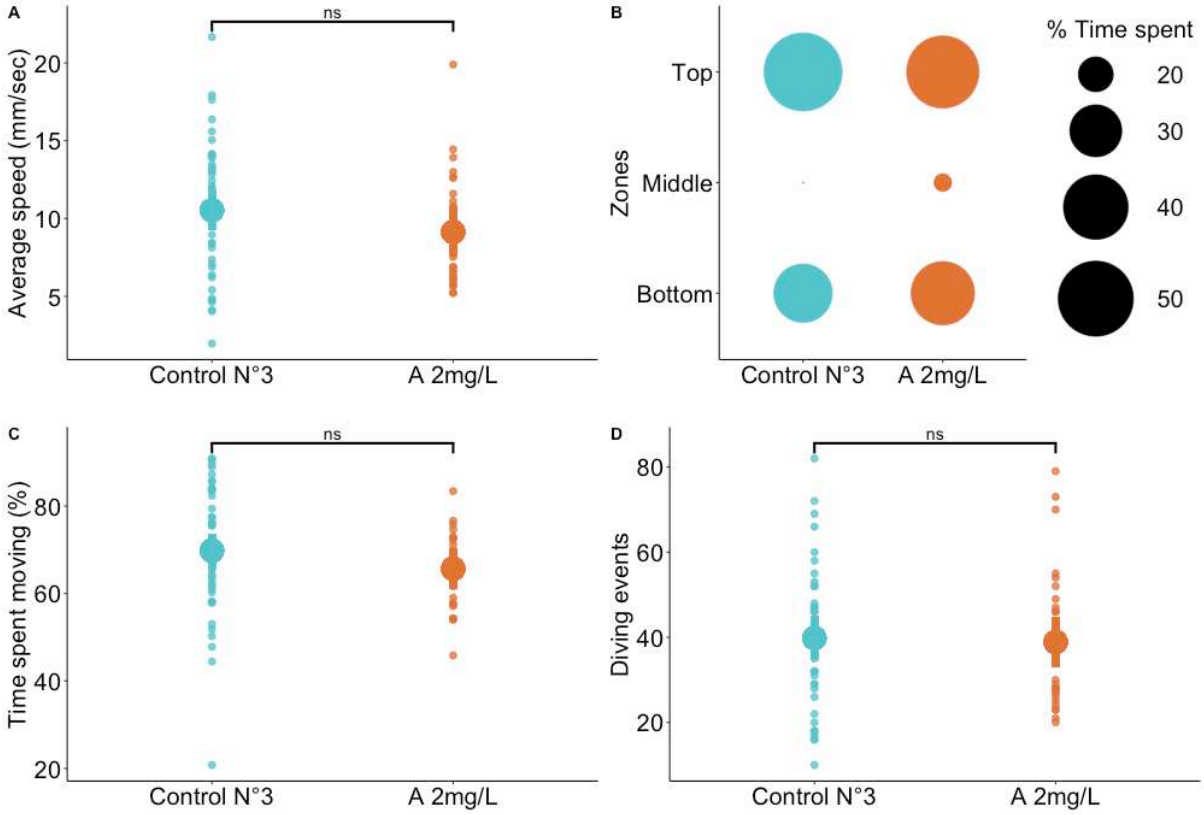
Supplementary Figure S1: Spontaneous locomotor activity for larvae reared in atrazine at field doses during inter-trials intervals. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant; *P<0.05.



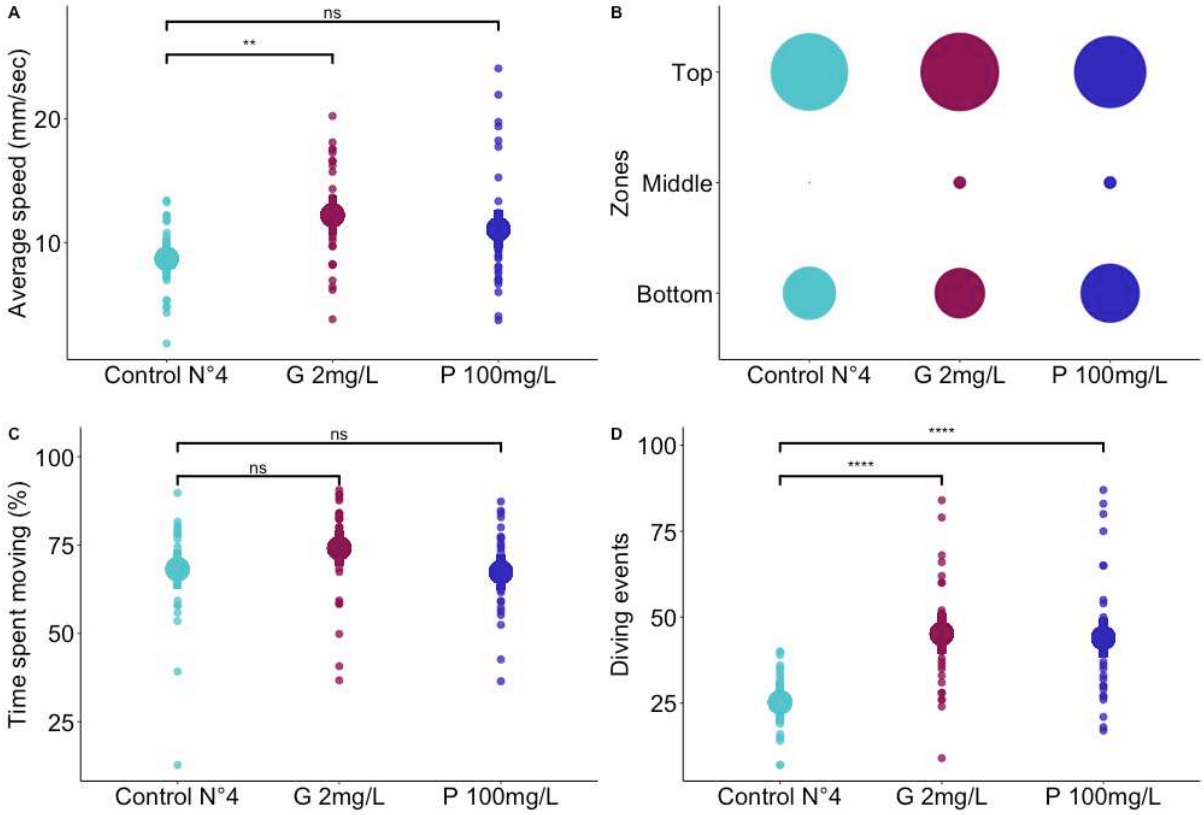
Supplementary Figure S2: Spontaneous locomotor activity for larvae reared in glyphosate at field doses during inter-trials intervals. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant.



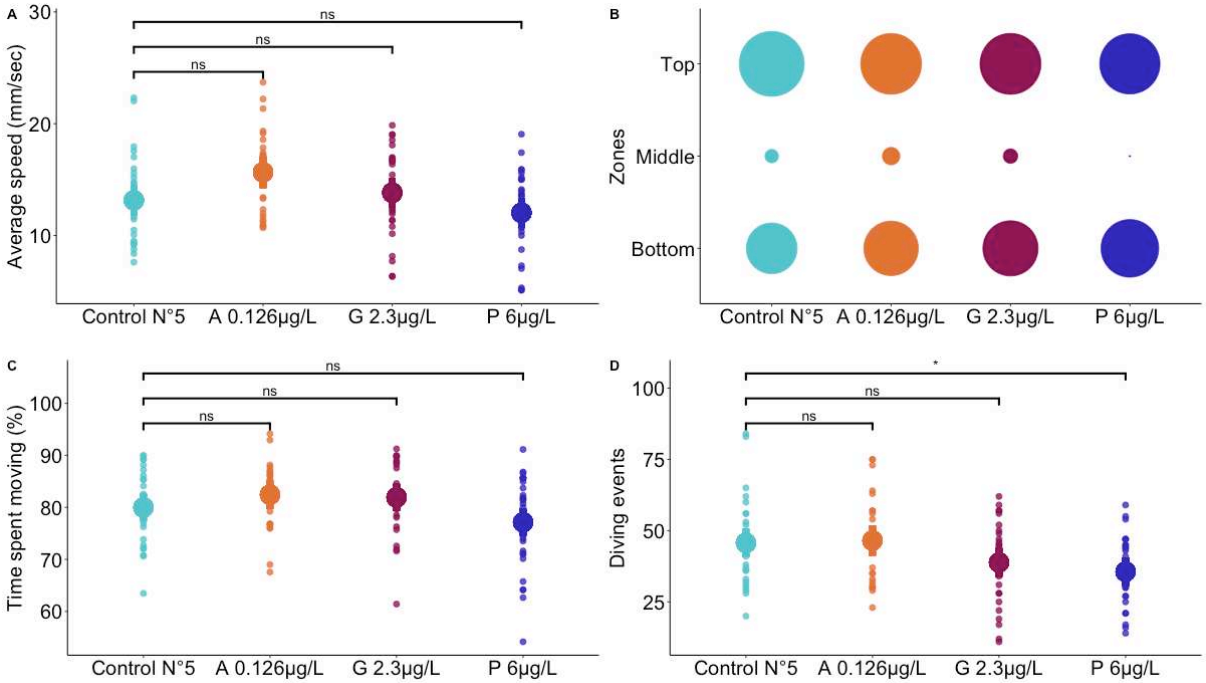
Supplementary Figure S3: Spontaneous locomotor activity for larvae reared in paracetamol at field doses during inter-trials intervals. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant.



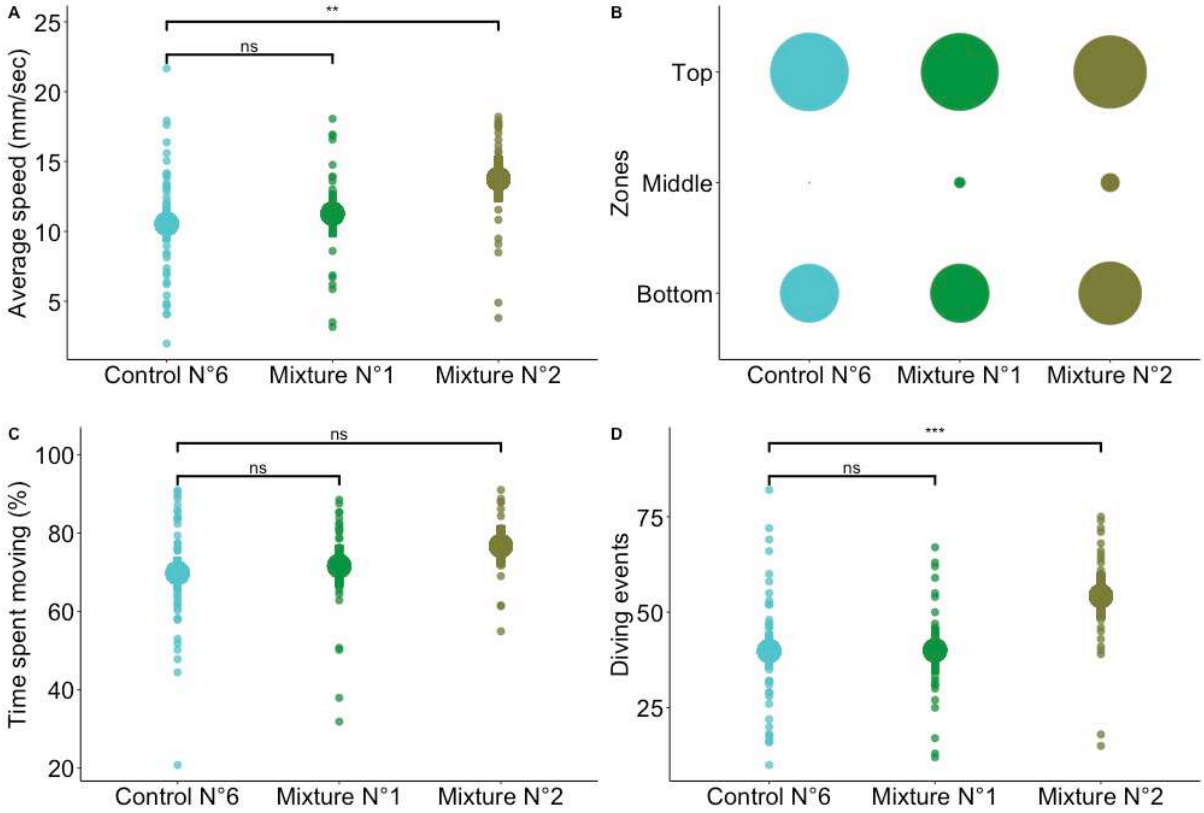
Supplementary Figure S4: Spontaneous locomotor activity for larvae reared in atrazine at spray doses during inter-trials intervals. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. A= Atrazine. NS, not significant.



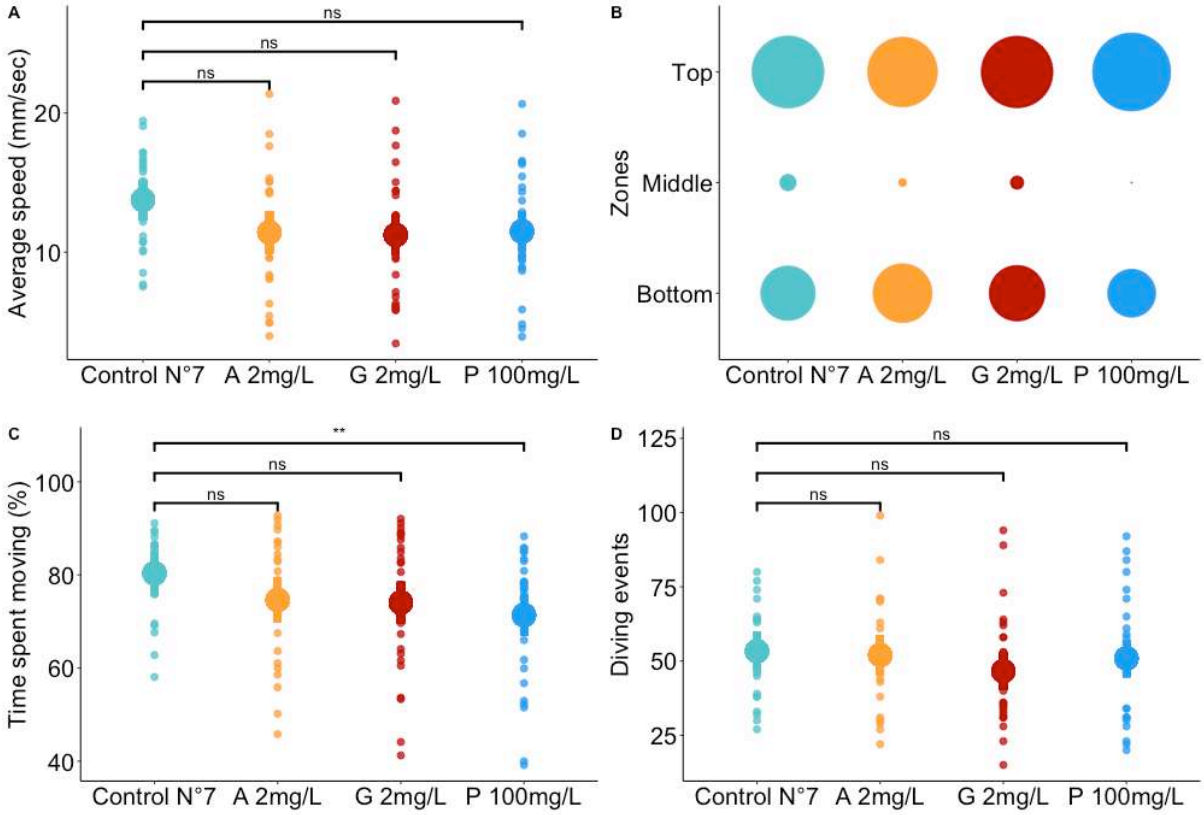
Supplementary Figure S5: Spontaneous locomotor activity for larvae reared in glyphosate and paracetamol at spray doses during inter-trials intervals. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. G = Glyphosate, P = Paracetamol. NS, not significant; **P<0.01, ****P<0.0001.



Supplementary Figure S6: Spontaneous locomotor activity for larvae reared alone at field realistic doses during inter-trials intervals. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. A= Atrazine, G = Glyphosate, P = Paracetamol. NS, not significant; *P<0.05.



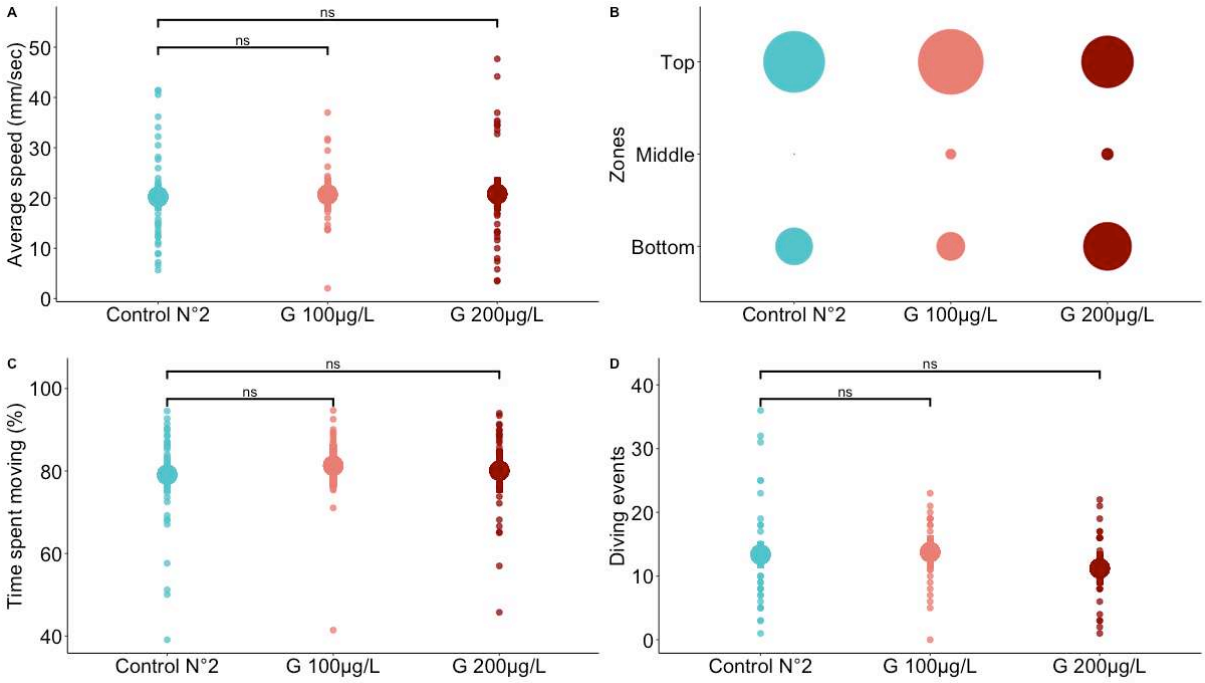
Supplementary Figure S7: Spontaneous locomotor activity for larvae reared in mixture at field realistic doses during inter-trials intervals. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant; **P<0.01, ***P<0.001.



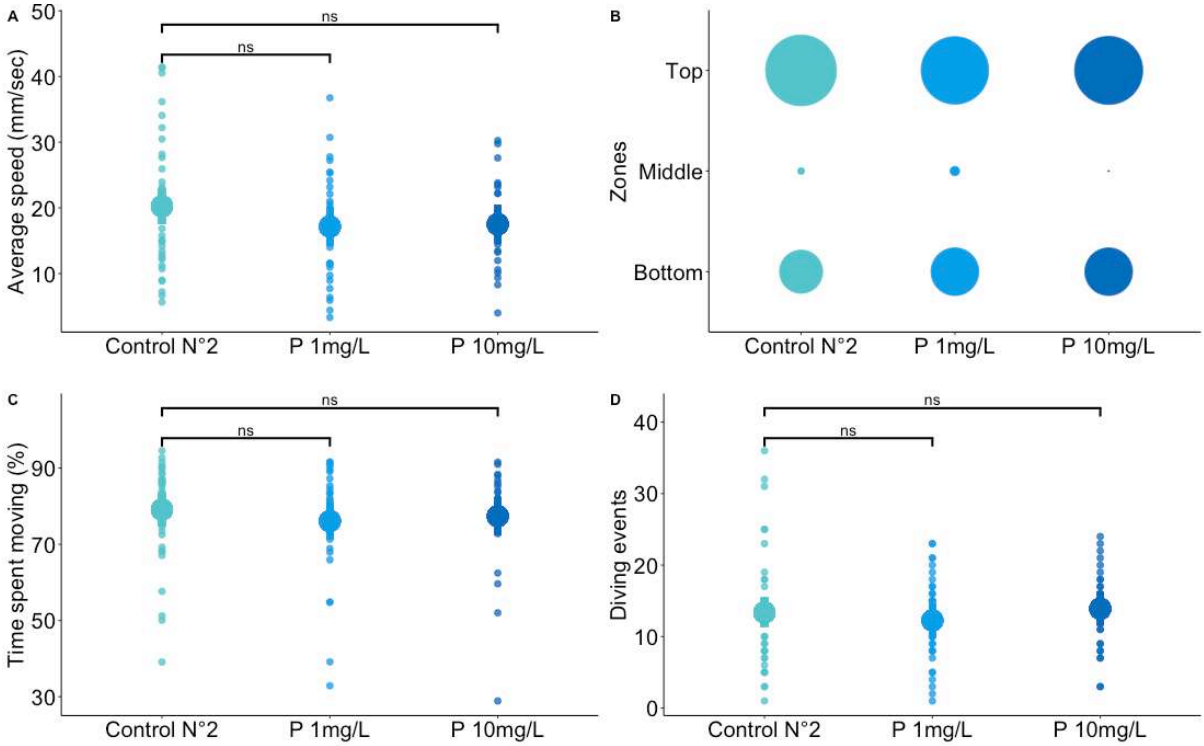
Supplementary Figure S8: Spontaneous locomotor activity for larvae reared at spray doses for acute toxicity during inter-trials intervals. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant; **P<0.01.

4. Spontaneous activity during the familiarisation period

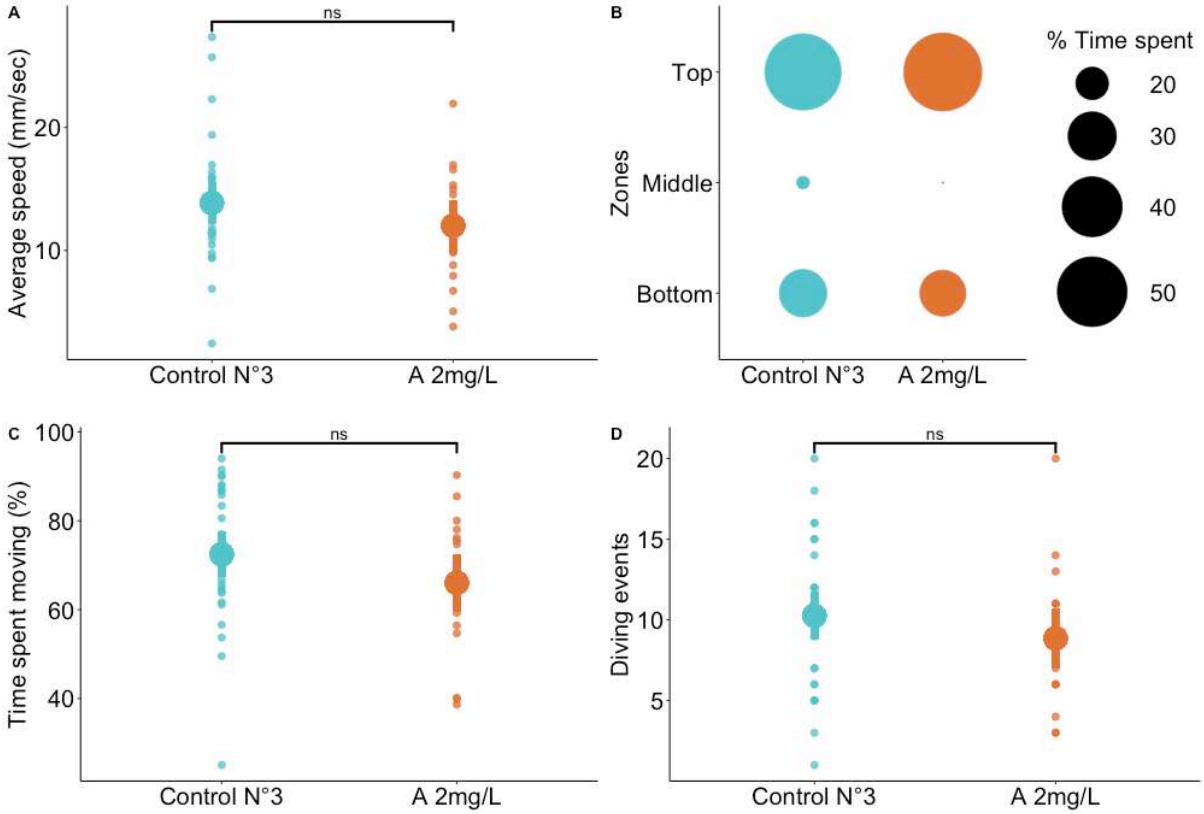
Here are the graphs representing differences during the **familiarisation period**.



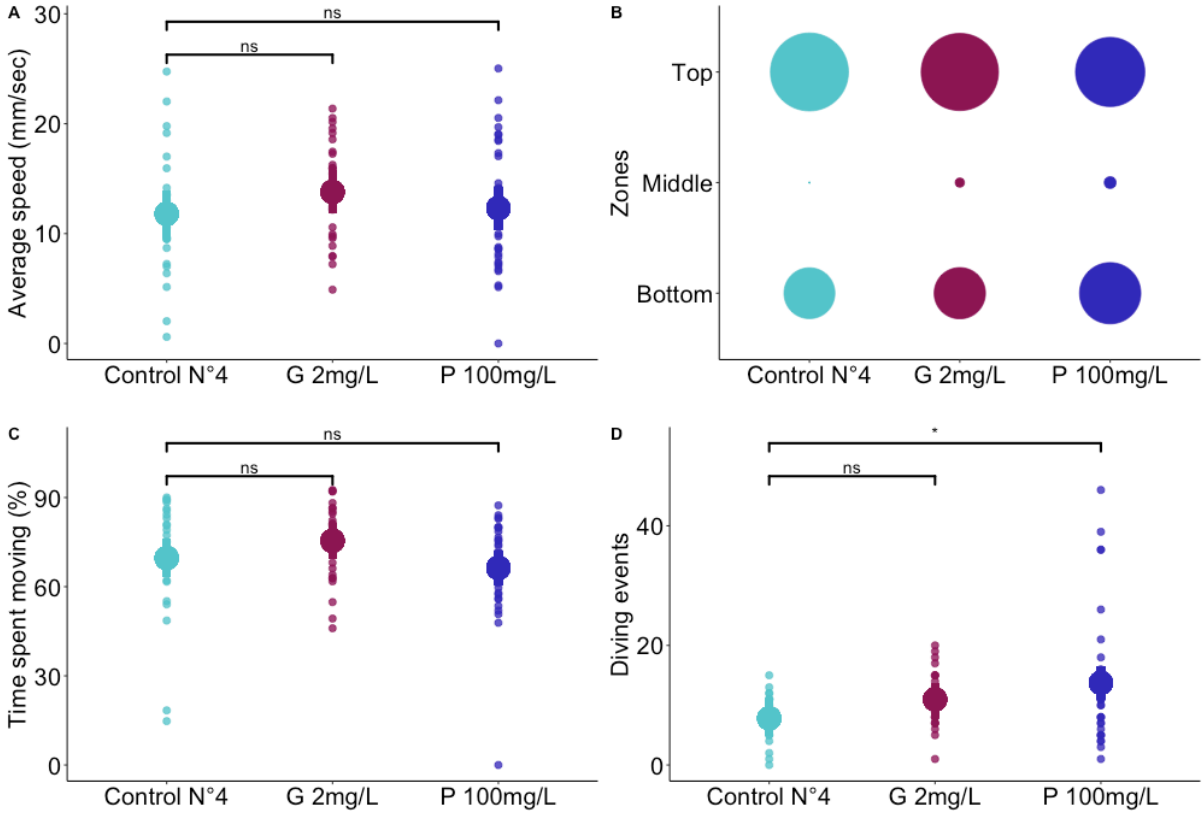
Supplementary Figure S9: Spontaneous locomotor activity for larvae reared in glyphosate at field doses during **familiarisation**. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant.



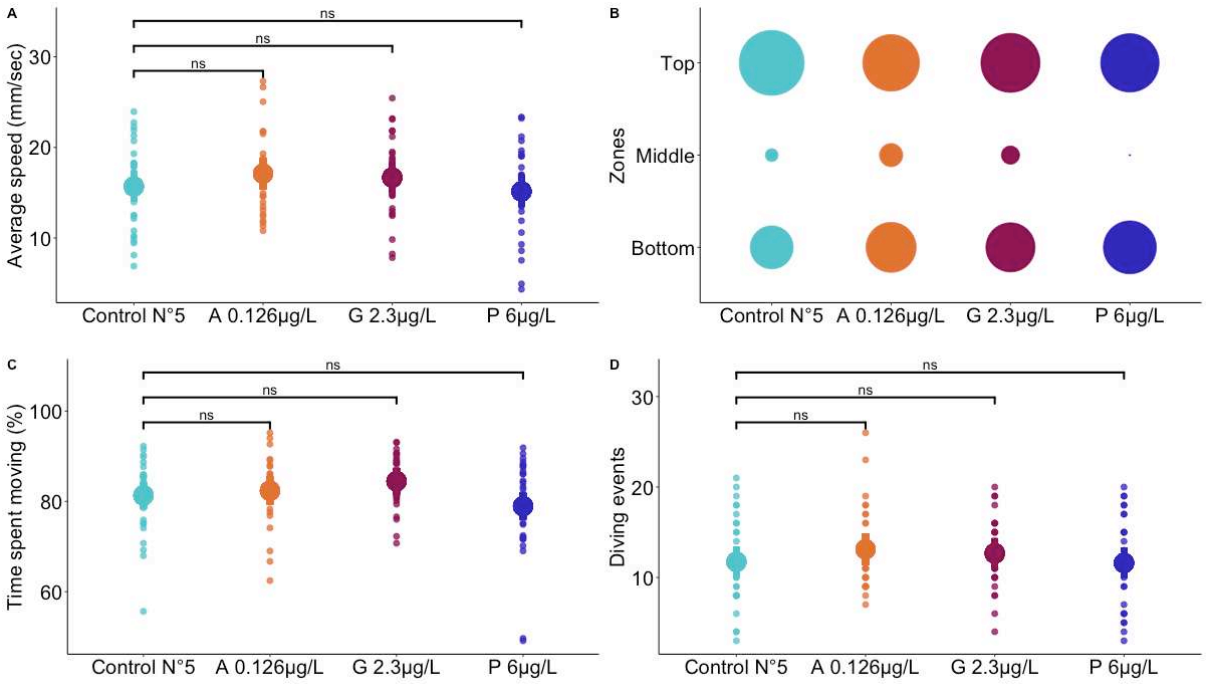
Supplementary Figure S10: Spontaneous locomotor activity for larvae reared in paracetamol at field doses during **familiarisation**. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant.



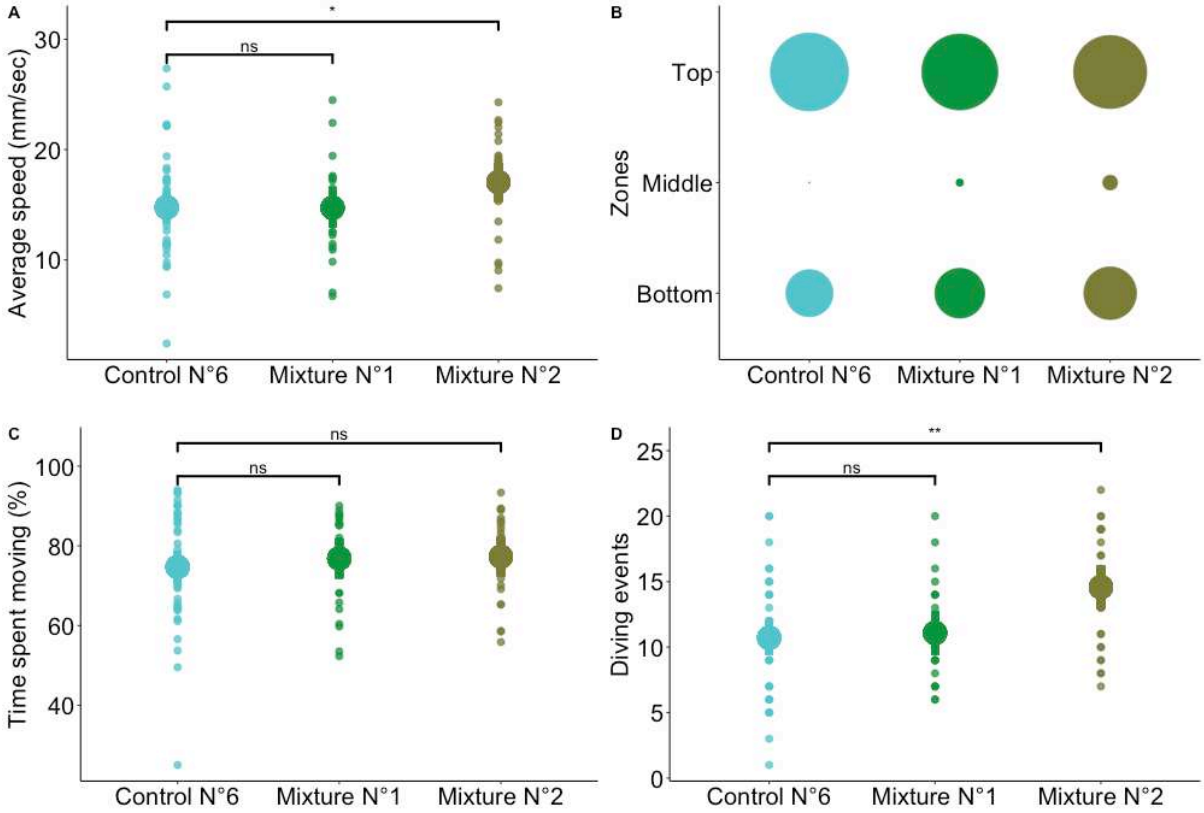
Supplementary Figure S11: Spontaneous locomotor activity for larvae reared in atrazine at spray doses during **familiarisation**. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. A= Atrazine. NS, not significant.



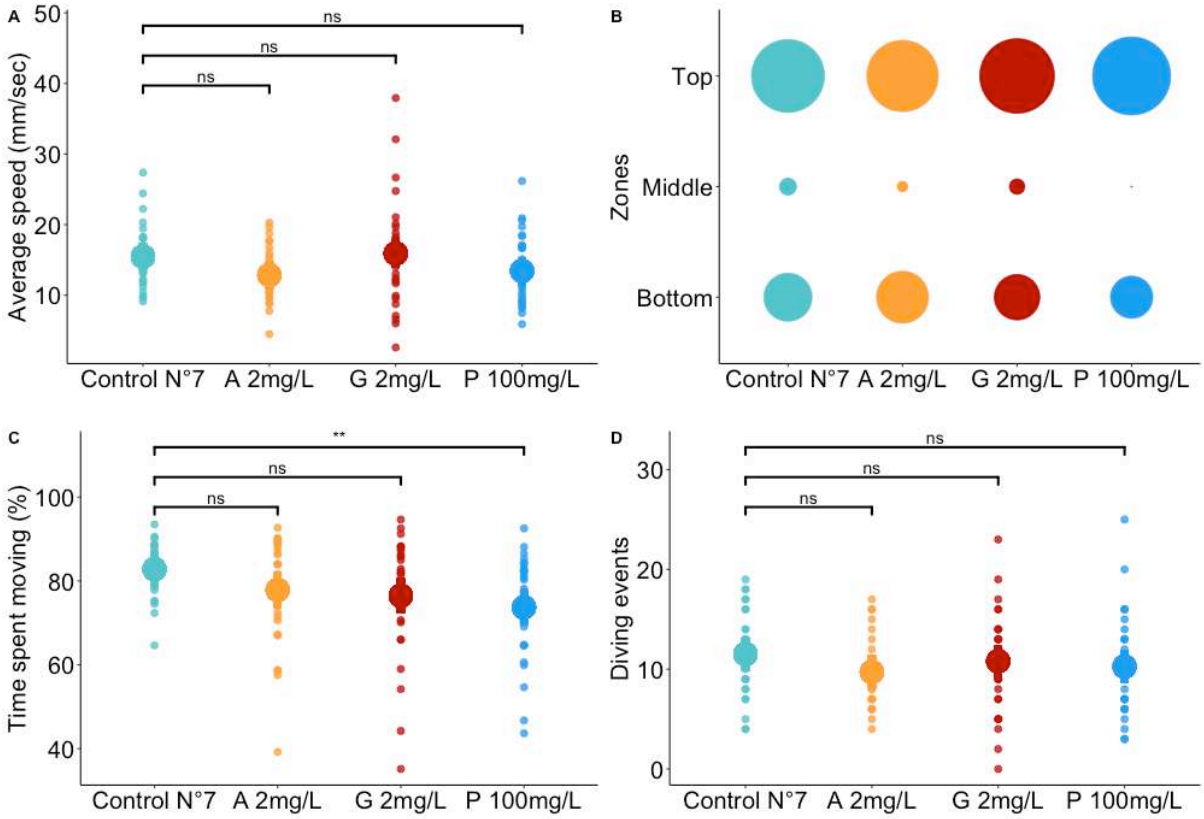
Supplementary Figure S12: Spontaneous locomotor activity for larvae reared in glyphosate and paracetamol at spray doses during **familiarisation**. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. G = Glyphosate, P = Paracetamol. NS, not significant; *P<0.05.



Supplementary Figure S13: Spontaneous locomotor activity for larvae reared alone at field realistic doses during **familiarisation**. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. A= Atrazine, G = Glyphosate, P = Paracetamol. NS, not significant.



Supplementary Figure S14: Spontaneous locomotor activity for larvae reared in mixture at field realistic doses during **familiarisation**. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant; *P<0.05, **P<0.01.

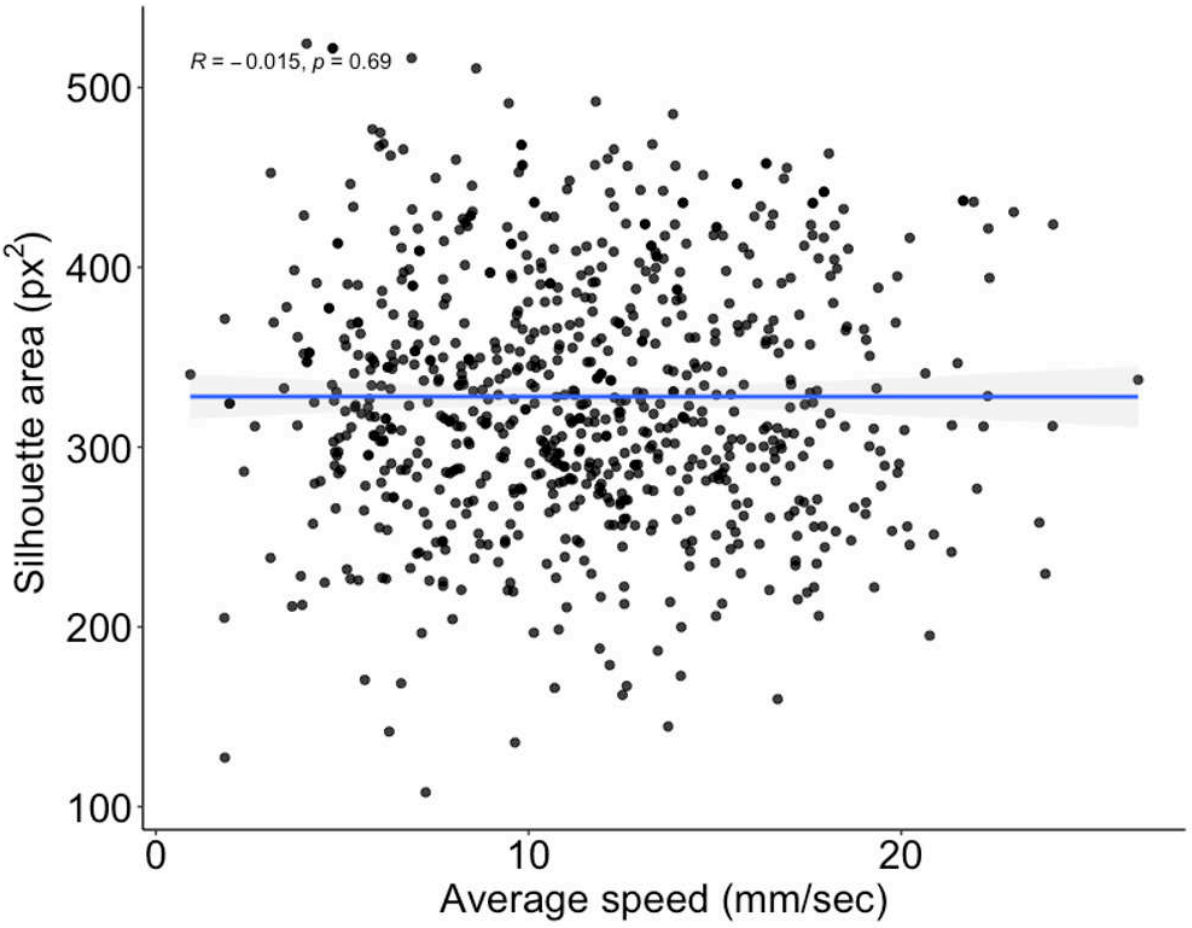


Supplementary Figure S15: Spontaneous locomotor activity for larvae reared at spray doses for acute toxicity during **familiarisation**. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant; **P<0.01.

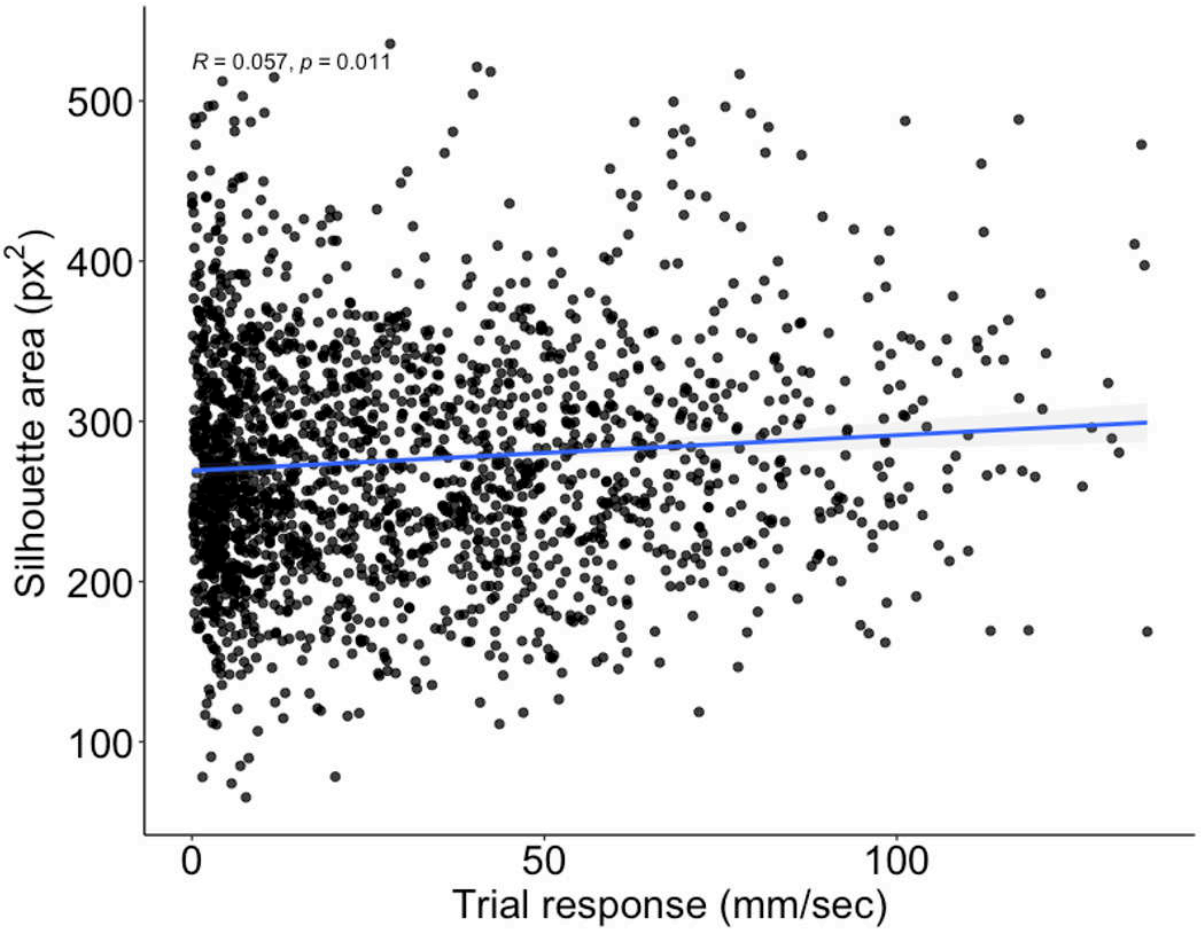
5. Correlations between spontaneous activity and silhouette area

We used the contour of the individual over the videos to check the correlation between the individual surface detected by the tracking software and the response at the first stimulation and spontaneous activity.

We compared the average silhouette area of all individuals retained for this study (n = 740) to their response at the first trial during training (Figure 14) and to their average speed during the training period (Figure 13). While differences in silhouette area could be observed, there were no correlations between the response at the first trial or the average speed, and the silhouette area (R = 0.057, P = 0.011; R = -0.015; P = 0.69, Figure S9 and S10 below).



Supplementary Figure S16: Average silhouette area correlated with individual average speed. Points indicate the mean value for an individual. Blue line corresponds to linear correlation. Grey shades indicate 95% confidence interval for the average speed.



Supplementary Figure S17: Average silhouette area correlated with individual response at the 1st trial. Points indicate the mean value for an individual. Blue line corresponds to linear correlation. Grey shades indicate 95% confidence interval for the average speed.