



A Roundup herbicide causes high mortality and impairs development of *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae)

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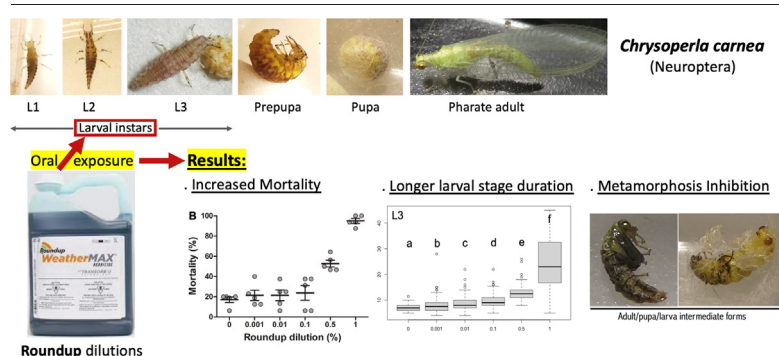
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HIGHLIGHTS

- Herbicides are only cursory tested for effects on non-target arthropods and usually do not include oral exposure
- *C. carnea* larvae were exposed to a Glyphosate herbicide via food at concentrations below the recommended application rate
- Oral exposure resulted in developmental arrest, impaired cocoon formation and high mortality
- The identified hazard is highly relevant for risk assessment and warrants a fresh look at current pesticide testing schemes

GRAPHICAL ABSTRACT



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ABSTRACT

Glyphosate has and is being used extensively in herbicide formulations worldwide. Thus, glyphosate-based herbicides (GBH) substantially add to the environmental load of pesticides and warrant a strict risk assessment. Ecotoxicological testing of herbicides focuses on non-target plants and higher animals while direct effects on arthropods are only cursory tested on the premise of contact exposure. However, oral exposure, as we show in our case, can be highly relevant for systemic pesticides, such as GBH. Specifically, in crop systems including genetically modified crops that are tolerant to GBH, these herbicides and their breakdown products are present both internally and externally of the crop plants and, therefore, are ingested by the crop-associated arthropod fauna.

We tested the effects of oral uptake of the Roundup formulation WeatherMax on larvae of the lacewing *Chrysoperla carnea*, a model organism in ecotoxicity testing programs. Long-term oral exposure of *C. carnea* larvae throughout its juvenile life stages was tested with concentrations ranging from 0.001 to 1 % dilution, thus, lower than the 1.67 % recommended for field applications. Inhibition of metamorphosis was observable at 0.1 % but at a concentration of 0.5 %, GBH significantly impaired cocoon formation and led to massive lethal malformations. At GBH concentration of 1 % half of the individuals remained permanent larvae and no adult hatched alive. The effects observed followed a clear dose-response relationship.

The hazard caused by direct insecticidal action of GHB after oral uptake is highly relevant for the environmental safety and reveals a gap in regulatory risk assessments that should urgently be addressed, specifically in light of the on-going insect decline.

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1. Introduction

Impending re-authorization of glyphosate in Europe is a highly controversial issue. With approx. 800,000 tons a year, glyphosate is the main active ingredient of the most widely used commercial herbicide formulations in the world, with the most well-known formulations being those of the Roundup family (Benbrook, 2016). Glyphosate-based herbicides (GBH) are broad-spectrum (non-selective) herbicides introduced into agriculture half a century ago. However, it was not until the mid 1990s that the use of GBH vastly expanded when cost-effective no-till cropping became possible in conjunction with genetically modified (GM) herbicide-tolerant (HT) crops that could withstand the application of GBHs (Benbrook, 2016). Furthermore, GBHs also became a prime substance of choice for so-called desiccation (chemical acceleration of the ripening process in crops) or 'burn-down' of, for example, grains, soybeans, root crops such as potatoes (Zhao et al., 2020 and references therein). Additionally, it became the most widely used herbicide in non-agricultural environments like private and public areas (e.g. homes, schools, parks) but also along transport routes such as roads and railroad tracks (Antier et al., 2020; Botten et al., 2021; Gandhi et al., 2021). This excessive use of GBH (ca. 825,000 tons in 2014, trend increasing) (Statistica, 2016) led to the exposure of many, if not all, organisms, including humans, in virtually all habitats (Gill et al., 2017).

GBH are systemic herbicides taken up by the plant. In case of GM HT plants, glyphosate will not kill the plant but instead be translocated by xylem and phloem transport processes throughout the plant (Hetherington et al., 1999), especially to apical meristems and roots (Helander et al., 2012). Glyphosate and its degradation products like aminomethylphosphonic acid (AMPA) are persistently present in all plant parts after spraying during the vegetation period and beyond. Fate analyses showed that Glyphosate persists after the vegetation period in plant residues and soil and is present in harvest products (e.g. Bohn and Millstone, 2019; Soares et al., 2021). Consequently, glyphosate and its break-down products have become ubiquitously present in most ecosystems of the world including aquatic habitats (e.g. Hedlin et al., 2020). Thus, all organisms living in these ecosystems will come into contact with GBH and its (break-down) compounds at some point of their life.

The approval of GBHs, roughly 50 years ago, was subject of a regulatory assessment according to the rules in force at the time. Many reviews of regulatory and other impact assessments of GBHs have been published over the past 5 decades (Mohammadi et al., 2021; Richmond, 2018; van Bruggen et al., 2018, 2021). Many are based on usage patterns, i.e. frequencies, timings and concentrations of GBH applications, that pre-date the cultivation of GM HT crops. A still widely cited example summarizing the safety tests at that time with nontarget arthropods is the review by Giesy et al. (2000) wherein the authors evaluated a large body of regulatory data, including those of the manufacturer, to estimate and predict exposure routes and concentrations and calculate risks for a range of terrestrial and aquatic non-target organisms. They concluded that, according to standard ecotoxicity tests recommended for pesticides in those days by organizations like SETAC (e.g. Barrett et al., 1994), GBH pose minimal direct risks for non-target organisms (NTOs) (e.g. van Bruggen et al., 2021) and may only affect arthropods by reducing their food resources and habitats. This safety postulation for non-target arthropods has not changed since then.

Until to date, ecotoxicity tests for pesticides are based chiefly on topical exposure. This is especially true for herbicides for which the focus of tests is on non-target plants while effects on arthropods are only superficially checked by so called glass-plate tests mimicking contact exposure. While contact exposure is an important exposure route for many pesticides, oral exposure is also highly relevant, mainly but not exclusively in the case of GM HT cropping systems that are based on the use of systemic herbicides, such as GBH. Although the exposure route via food has been acknowledged, test methods for oral exposure have to be developed yet (EFSA PPP panel, 2015) and respective estimates for effects of oral exposure to many pesticides are lacking.

Our experiments address this knowledge gap for GBH using larvae of *Chrysoperla carnea* (green lacewings) as test organisms and GBH containing diet as stressor. *C. carnea* larvae are polyphagous foliage-dwelling predators and have received much attention as a potential biological pest control (Vogt et al., 1998, 2001). They also became a model non-target test organism for regulatory ecotoxicity testing schemes for pesticides and GM crops (Barrett et al., 1994; Vogt et al., 1998, 2001; Bigler and Waldburger, 1994). In Spain, *C. carnea* is among the most important beneficial organisms of maize fields (de la Poza et al., 2005; Farinós et al., 2008; Albajes et al., 2009).

2. Methods

Our experiments are based on a series of bioassays exposing *C. carnea* larvae to a GBH via diet. We aimed to identify a potential hazard simulating conservative concentrations of GBH while using a maximum exposure duration as larvae were exposed throughout their entire juvenile period. Effects were monitored from first instar larvae until adult eclosion. We used Roundup WeatherMax, a commercial formulation commonly applied to GM HT crops, so-called 'Roundup-Ready' crops (e.g. used in soybeans and maize). We chose five increasing doses to test for dose-dependent responses. However, all doses remained below the recommended field rate for agricultural use (1.67 %, Defarge et al., 2018). We monitored and recorded *C. carnea* development until the hatching of the adult, death of the individual or the end of the experiment at day 50.

2.1. Insects

C. carnea (Neuroptera: Chrysopidae) occurs in most if not all agroecosystems in the world (New, 1975). While adults feed on nectar and pollen, larvae are fierce, polyphagous predators feeding on many agricultural pests (Albuquerque et al., 1994; Carvalho et al., 1996, 1998). After eclosion from the egg, individuals pass three larval stages. At the end of the third instar, larvae spin a silk cocoon produced by material from their Malpighian tubes. The third instar larva, when enclosed in the cocoon (called prepupa), transforms into a denticious pupa. A pharate adult leaves the cocoon a few hours before the final imaginal ecdysis (Canard et al., 1984; Bortolotti et al., 2005). The typical developmental time from L1 to adult emergence (larval + pupation durations) is 21 days at 24 °C (Khan et al., 2012). All larvae used in the experiments were purchased from Andermatt Biocontrol (Grossdietwil, Switzerland). Only first instar larvae (L1) were used for testing.

2.2. Glyphosate-based herbicide

We used Roundup WeatherMax® as an example GBH formulation. According to the product description, Roundup WeatherMax® contains 540 g/l of the active ingredient glyphosate. As a quality assurance, one batch of the treatment solutions (all treatment groups) was analysed in a DAkkS-accredited laboratory in Switzerland according to DIN ISO 16308. The glyphosate concentration could be verified with a mean difference of 18.3 % (± 10.6) between the theoretical and measured glyphosate concentration (LoD = 25 µg/l; LoQ = 50 µg/l).

Dilutions were prepared fresh for each replicate, with concentrations ranging from 0.001 to 1 % (in detail 0.001, 0.01, 0.1, 0.5 and 1 %) in deionized water (supplemented with 0.5 M sucrose for liquid treatment). All concentrations used were below the recommendations for Roundup for agricultural use which is 1.67 % e.g. for spraying of Roundup-Ready GM crops in Canada.

For a preparation of a 2 % mother solution, we added 1 ml of Roundup WeatherMax to 49 ml of distilled water into a Falcon 50 ml flask. From this mother solution, we mixed 5 ml with 5 ml of deionized water to prepare the 1 % dilution. The 1 % solution was used to prepare the 0.5 % dilution (half solution) and, from a 1/10 dilution series, the 0.01 % and 0.001 % dilutions were prepared. The 0.001 % to 1 % treatment solutions were used to coat

the sterilized eggs of *Ephestia kuehniella* (see [Exposure route and treatments](#)).

In addition, the 2 % mother solution was used for a 1/10 dilution series (1 ml added to 9 ml deionized water) to prepare the 0.2 %, 0.02 %, and 0.002 % Roundup dilutions. Each of these was added to an equal volume of 1 molar-sucrose solution to obtain 0.1 %, 0.01 %, and 0.001 % Roundup solutions containing sugar. These treatment solutions were used to moisten the cotton balls (see [Exposure route and treatments](#)).

All solutions were stored in the dark at 4 °C.

2.3. Exposure route and treatments

Experiments were designed to provide an exposure route via oral uptake. This was achieved by feeding larvae GBH-coated moth eggs and offering them small cotton balls soaked with a solution of the desired GBH concentration mixed into glucose-water as additional source of liquid (Fig. 1).

Sterilized eggs of *E. kuehniella* (Lepidoptera: Pyralidae) moths were used as diet that is known to be a suitable, high-quality food for *C. carnea* larvae and to facilitate good development of adults (e.g. Hilbeck et al., 1998). *E. kuehniella* eggs were purchased from Andermatt Biocontrol AG (Grossdietwil, Switzerland) and mixed with Roundup WeatherMax at the different test concentrations.

The insect diet was prepared as follows: 400 mg of *E. kuehniella* eggs were placed in an Eppendorf tube together with 800 µl of the liquid treatment which was sufficient to cover the egg mass. For the control treatment, the eggs were mixed with 800 µl of deionized water. After adding the liquid, the Eppendorf tube was vortexed for a few seconds. The diet was then stored at 4 °C until use.

2.4. Insect feeding trials

First instar larvae of *C. carnea* (1–2 days old) were transferred into plastic trays for the bioassays (Bio-RT-32-C-D International Inc., Pitman, New Jersey, USA). The trays consisted of 32 square wells of about 4 cm², in each of which one *C. carnea* larva was kept (Fig. 1). One replicate consisted of groups of 16 larvae per treatment, and 5 replicates were carried

out. The trays were closed with a removable, perforated lid allowing for air circulation. A 1–2 cm² piece of blotting paper moistened with 2–3 drops of deionized water was placed in each compartment ensure sufficient humidity. The blotting paper was replaced every day. The treatment diet was placed in the center of the blotting paper piece and consisted of a spatula tip of *E. kuehniella* eggs coated with GHB or the negative control. The diet was renewed every 2 days during the whole larval stage. In addition, each larva received daily 0.1 ml of liquid treatment supplemented with 0.5 M of sucrose solution applied to a cotton ball (organic cotton, Migros, Switzerland; 0.5 cm in diameter). Cotton balls were replaced every four days to prevent fungal growth. After pupation, blotting papers were still moistened with water every day and replaced every four days until the end of the experiments.

The trays were kept in a climate chamber set at a photoperiod of 16 h light (25 ± 1 °C, 50 ± 5 % relative humidity) and 8 h dark (20 ± 1 °C, 65 ± 5 % relative humidity).

Two testing parameters were recorded in all experiments: (1) the survival/mortality rate from neonate larva to either pupal or adult life stage, and (2) the developmental times of the different immature stages (durations of L1, L2, L3, and pupa). The developmental stage could reliably be identified by the remaining exuvium after each molt. Furthermore, we recorded malformations and arrested/partial development, as these became apparent in some individuals during a preliminary trial. While some “light” malformations affected the wings or antennae of surviving adults (and are not reported nor discussed here), others led to immediate death in newly hatched adults: those concerned a visibly non-functional digestive tract or even resulted in adult/larva intermediate forms, outcome of a partial metamorphosis inhibition. All malformations that were lethal were grouped for statistical analyses. A larva that would not react to stimulation by a pencil was considered to be dead, as well as a pupa enclosed in a darkened cocoon.

2.5. Statistical analysis

R (R Core Team, 2021) and, in particular, the packages dunn.test (v1.3.5; Dinno, 2017), fBasics (v3042.89.1; Wuerztz et al., 2020), FSA (v0.8.10; Ogle et al., 2022), ggplot2 (v3.3.5; Wickham, 2016), lawstat

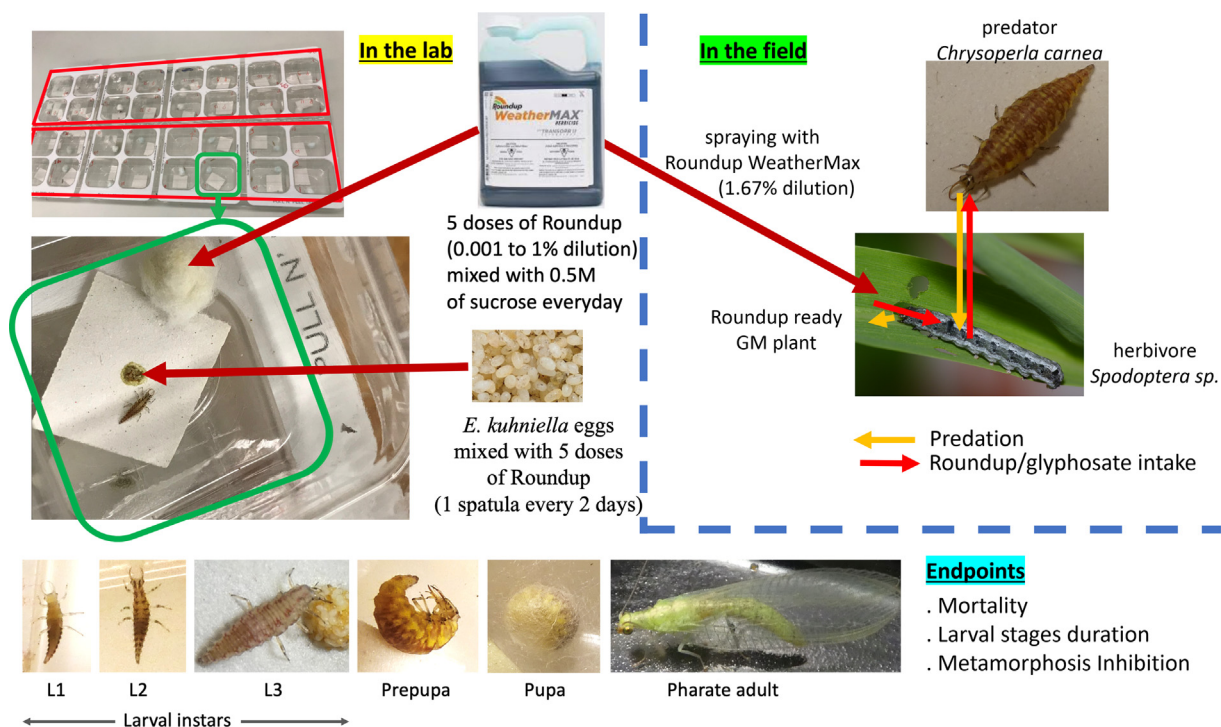


Fig. 1. Experimental setup to simulate exposure of the predator *C. carnea* via consumption of herbivore prey feeding on GM HT crops sprayed with Roundup.

(v3.4; Gastwirth et al., 2020), openxlsx (v4.2.5; Schaubberger and Walker, 2021) and tidyverse (Wickham et al., 2019) were used for statistical analyses. Normality of the data distribution was checked with a Shapiro test for Gaussian distribution followed by Bartlett's equal variance test for homoscedasticity. ANOVA followed by a Tukey test for multiple comparisons was used as a parametric test. In case normality or homoscedasticity were not reached, a Kruskal-Wallis test was carried out followed by Dunn's test with multiple testing correction (p-value adjustment method was Benjamini-Hochberg).

In order to assess the putative dose proportionality of the effects, a model for linear regression was defined and Spearman correlation was calculated. The model was used for prediction and the residues were visualized in a Q-Q plot then checked for normal distribution (Shapiro test) and equal variances (Bartlett test and Levene test).

Data corresponded to the means of a parameter in percentage in each of the five replicates ($n = 16$ insect per group) except for the analysis of developmental times where the five replicates were pooled. Total number of individuals per treatment was 80 at the beginning of the experiment.

3. Results

3.1. Mortality

Increasing exposure to the GBH clearly and significantly caused increasing mortality in *C. carnea* larvae (Fig. 2). Total mortality over all larval stages (first instar to pupa) of *C. carnea* larvae exposed to GBH-treated diets increased significantly and proportionately to the GBH concentration (i.e. dose-dependent). The threshold of statistical significance was 0.5 % GBH dilution ($p < 0.001$) with a mean mortality of $53\% \pm 8$ to compare to $17\% \pm 6$ in the control group. At the highest concentration (1 %), mean larval mortality reached $95\% \pm 5$. Considering that the remaining 5 % were either a permanent larva when reaching the end of the experiment alive (without pupating) or an adult/larva intermediate form that died during eclosion as the result of severe malformations, the factual mortality reached 100 % for the highest treatment group since no healthy adult hatched alive.

Our linear model (Fig. 2) fitted the data well (adjusted $r^2 = 0.89$; $p < 0.001$). The Spearman correlation coefficient (ρ) was $= 0.76$ ($p < 0.001$) indicated a highly significant correlation between GBH concentration and mortality.

3.2. Developmental time

Development times of first instar larvae (L1) only significantly differed for the 0.5 % dose group from all other groups, including the control (Kruskal-Wallis test $p < 0.001$) (Fig. 3, top left panel). However, when reaching the second instar (L2), development times were significantly prolonged at the two highest doses (5.1 ± 1.5 days at 0.5 % and 7.0 ± 2.6 days at 1 %; Kruskal-Wallis test $p < 0.001$; $p < 0.001$ for both groups in post-hoc

multiple comparison tests; Fig. 3, top right panel) when compared to the control group (4.2 ± 0.8 days). Finally, when reaching the third instar (L3), development times of all larvae in all treatment groups were significantly longer than in the control (Fig. 3, bottom left panel). Mean development time was 7 days in the control group (7.0 ± 1.4), but 1–2 days longer in the groups exposed to 0.001 and 0.01 % Roundup (8.4 ± 3.5 , $p < 0.05$ and 8.8 ± 3.2 , $p < 0.001$ respectively) and almost 3 days longer at the 0.1 % dose (9.8 ± 3.3 , $p < 0.001$). Development times almost doubled at 0.5 % and tripled at the 1 % dose (13.0 ± 3.4 and 24.5 ± 10.0 respectively, $p < 0.001$ in both cases). At this dose of 1 %, the dramatic increase was due to the emergence of permanent (L3) larvae (see Section 3.3 Inhibition of metamorphosis). Permanent larvae were larvae that were arrested in the last instar (over 15 days, more than twice the mean duration of the control group) and never managed to reach the pupal stage during the time period of the experiment. While most of these larvae died in this larval stage, a few ones remained alive until the end of the experiment (50 days).

For the pupal stage, no differences in development times were observed, except, again, at the 0.5 % dose level (Kruskal-Wallis test $p < 0.001$). At the 0.5 % dose, development times of pupae were significantly longer than in the controls and other treated groups ($p < 0.01$). However, at the 1 % dose, development time was confounded with other adverse effects (e.g. mortality, malformations) to the extent that only three individuals hatched from their -incomplete- pupa as intermediates forms - see next paragraph - and were moribund.

3.3. Inhibition of metamorphosis and malformation

In the control and at the lowest doses, inhibition of metamorphosis was low and only occurred sporadically during the pupal stage ($< 10\%$) with only very few ($< 3\%$) severely malformed individuals hatching from the cocoon. Adults that exhibited simultaneously partial patterns from larvae and adults were called intermediate forms (see below and Figs. 4 and 5). Around 12 % of the individuals failed to reach the sensitive pupal stage when metamorphosis takes place in the control and lowest GBH dose group (Fig. 5). While the number of individuals failing to complete metamorphosis almost doubled with exposure to 0.01 and 0.1 % GBH dilutions, this only became statistically significant above the 0.5 % concentration ($p < 0.01$ in the Dunn's multiple comparison test following a significant Kruskal-Wallis). In this group (0.5 %), inhibition started already before the pupal stage was reached, around one fourth of the individuals attached to the well wall and started spinning but failed to form a proper cocoon. The proportion of these prepupae that failed to reach metamorphosis was significantly higher at this dose in comparison to the control and to all other groups ($p < 0.01$ in Dunn's multiple comparison test following a significant Kruskal-Wallis test – $p < 0.01$). Another 21 % hatched moribund and severely malformed, usually exhibiting intermediate forms (e.g. adult head and thorax, atrophied wings and larval-shaped abdomen) indicating severe problems with metamorphosis within the cocoon. These intermediate/malformed individuals occurred in all groups, including controls, but

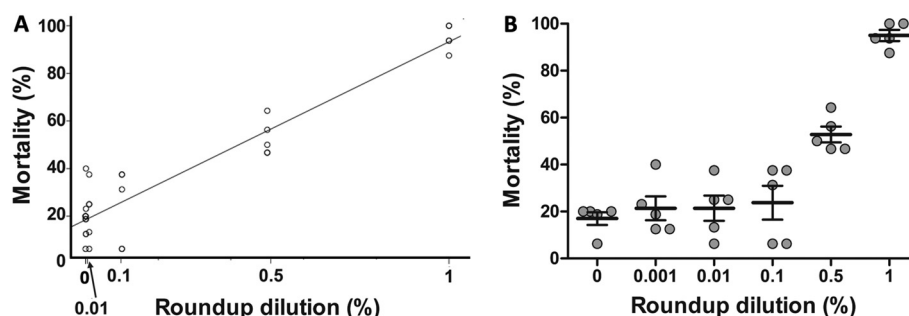


Fig. 2. Mortality in *Chrysoperla carnea* larvae exposed to increased doses of a Roundup herbicide. A. Linear regression of larval mortality as a factor of GBH (Roundup) dilution. Points representing the 0.001 % dilutions are not presented. B. Larval mortality per dose of treatment. Total mortality is shown as mean % in each of the 5 replicates ($N = 16$ individuals per group). The linear model equation is: $y = 75x + 18$ (linear regression adjusted $r^2 = 0.89$; $p < 0.001$). The Spearman correlation coefficient (r) was $= 0.76$ ($p < 0.001$).

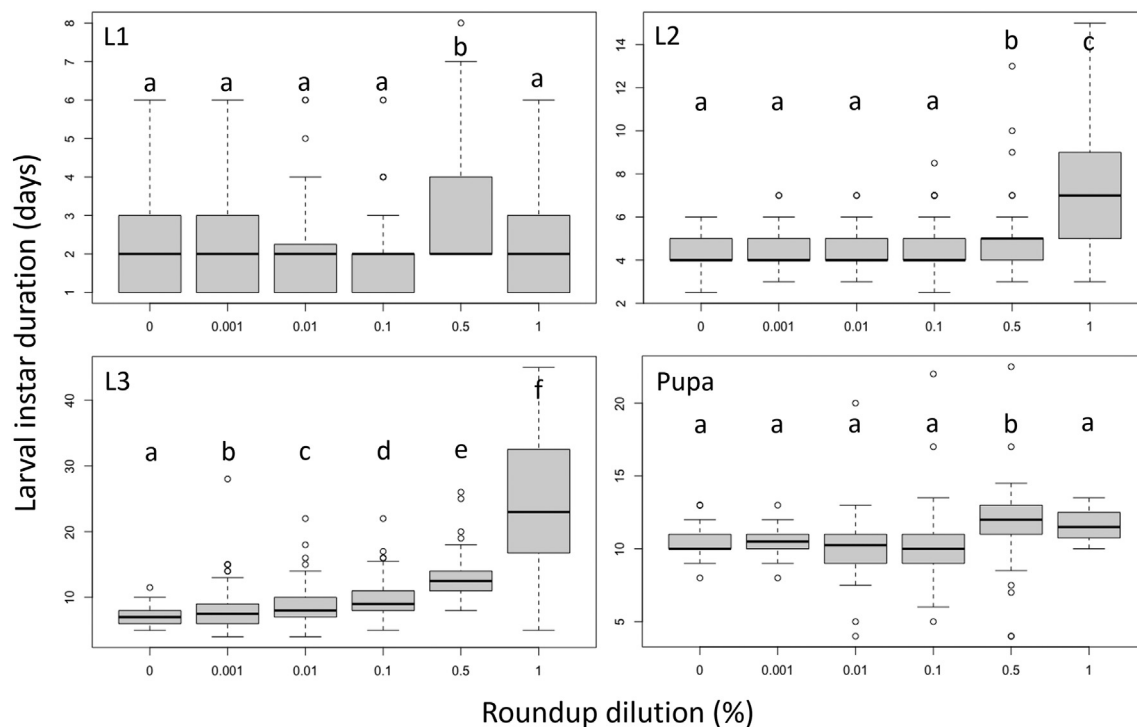


Fig. 3. Developmental time of L1, L2, L3 instars and pupal stage of *C. carnea* larvae exposed to increased doses of a GBH (Roundup). Distribution is shown as geometric mean and interquartile, whiskers represent the 2.5–97.5 percentile.

their proportion was significantly larger at 0.5 % ($p < 0.05$ in the Dunn's multiple comparison test following a significant – $p < 0.05$ – Kruskal-Wallis test). At the highest dose (1 %), metamorphosis inhibition occurred already very early with half of the individuals remaining in the third larval

stage (L3) until death and/or the end of the experiment (50 days). The proportion of permanent larva increased at 0.5 % and became significant at 1 % ($p < 0.001$ in the Dunn's multiple comparison test following a significant – $p < 0.01$ – Kruskal-Wallis test) with half of the individuals reached.

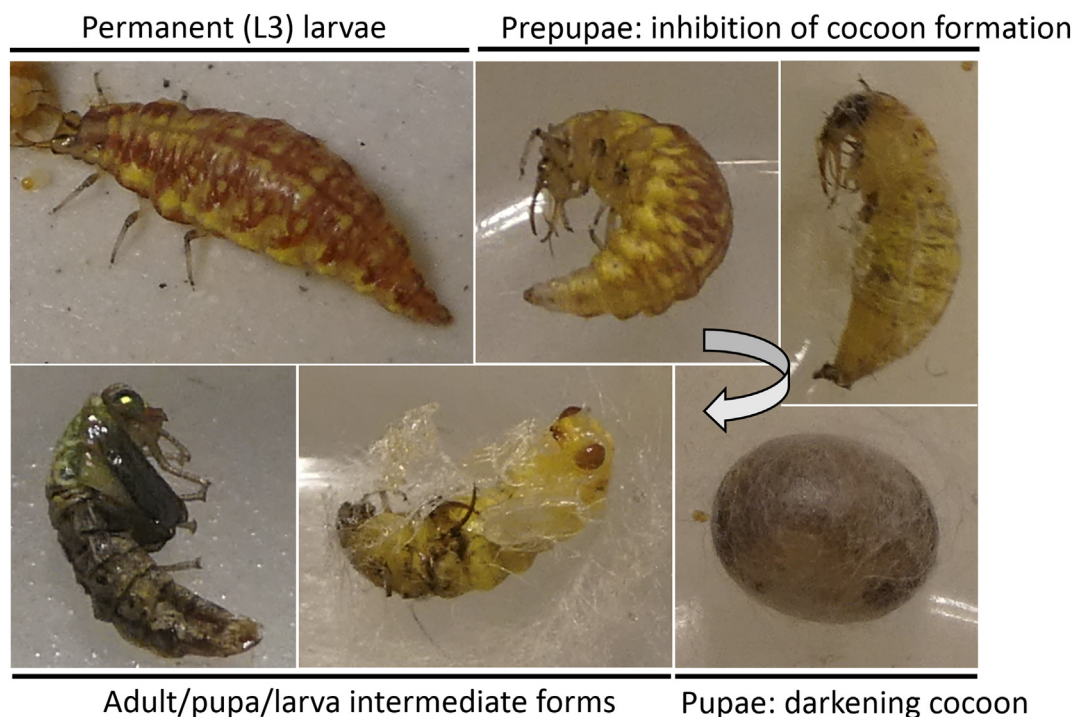


Fig. 4. Arrestment points during metamorphosis of *Chrysoperla carnea* larvae exposed to increased doses of a GBH (Roundup). Chronological order runs clockwise from upper left to upper right, then bottom right to bottom left, as indicated by the arrow.

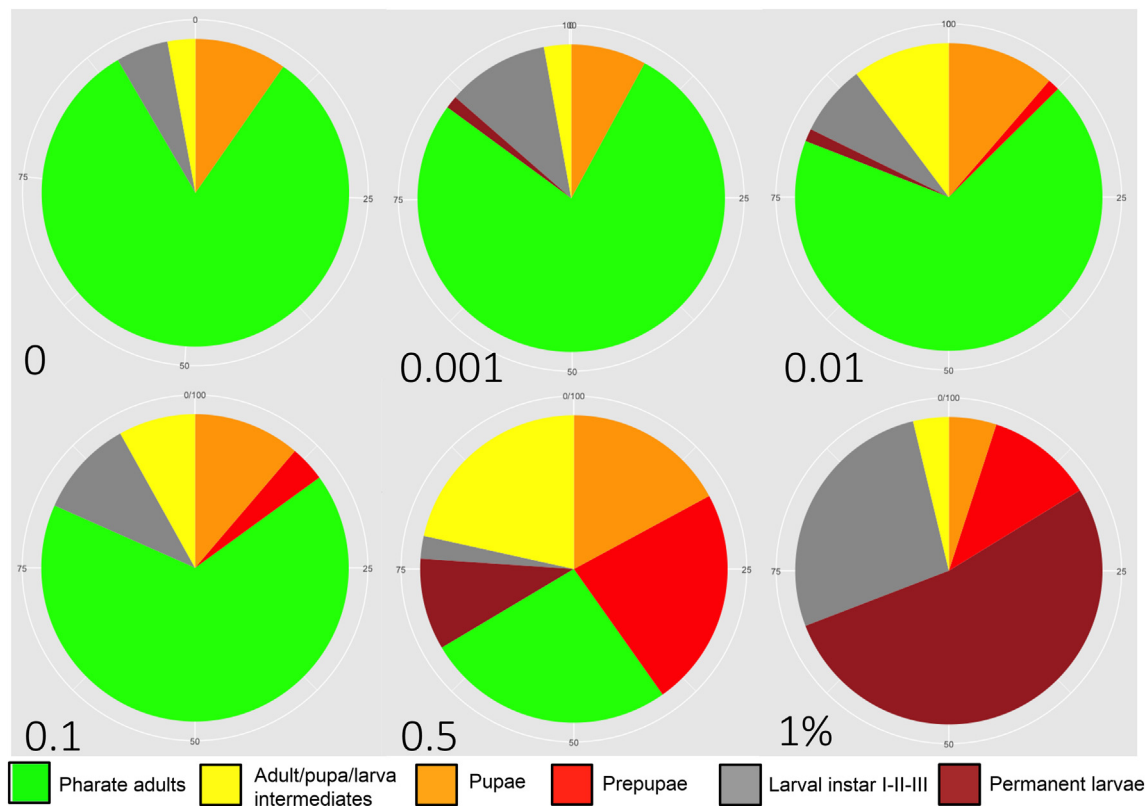


Fig. 5. Proportion of inhibition of metamorphosis in *Chrysoperla carnea* when exposed to increasing doses of a GBH (Roundup) during juvenile development. In green, the mean proportion of healthy pharate adults that successfully completed metamorphosis while the other coloured groups represent the proportion of the various forms of metamorphosis inhibition referred to in Fig. 4 and in the text. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Our experiments revealed a surprisingly high susceptibility of an important biological control agent, the insect predator *C. carnea*, to a GBH when fed with a diet that was sprayed with GBH at different levels that all remained below the agricultural recommended field rates. The chronic exposure of *C. carnea* larvae to the GBH via food (sprayed *E. kuehniella* eggs and treated cotton balls) throughout their juvenile life stage was responsible for massive lethal impairments of its larval development causing prolonged larval stages, inhibition of metamorphosis leading to malformations and mortality. Mortality rates were clearly dose dependent, thus, confirming a valid method of exposure. Metamorphosis inhibition resulting in permanent larvae and adult/larva intermediate forms recalled the findings reported by Bortolotti et al. (2005) when *C. carnea* larvae were exposed to the insecticide fenoxycarb. Fenoxycarb was shown to mimic juvenile hormones, disturbing and inhibiting metamorphosis and, consequently, was banned or declared of limited use in some countries due to these side effects on beneficial insects.

To our knowledge, effects of GBH on *Chrysoperla* larvae have been reported in the literature only once before. Similar to our experiments, Schneider et al. (2009) used GBH sprayed eggs to expose *Chrysoperla* larvae. While experiments of Schneider et al. (2009) were designed to record life table parameters such as longevity and age-stage fecundity, they also noted an approx. 20 % higher mortality in the GBH treatment. Compared to our results, this lower mortality, however, is likely caused by divergent experimental protocols. Schneider et al. (2009) used a different species (*Chrysoperla externa*), a different GBH formulation (Glyfoglex 48®), a single treatment dose and measured acute (48 h exposure compared to chronic exposure in our experiments) toxicity from the 3rd larval stage onward. Despite the fact that Schneider et al. (2009)

used older, and, therefore, presumably less susceptible larvae, they could demonstrate a profound effect of a single exposure on reproduction and the female reproductive tract. Our findings, combined with the ones of Schneider et al. (2009) indicate that sprayed food items ingested by larvae, at a concentration below the recommended field rate, had most severe effects on larvae and adults irrespective of an acute or chronic exposure. In our chronic exposure setting, the concentration at which we began to observe statistically significant effects (longer duration of L3 instar) was the 0.01 % GBH dilution which is >150 times lower than the recommended field rate of 1.67 % (Defarge et al., 2018). We argue that our chronic exposure setting is relevant to estimate field effects not only from sprayed food items but also from exposure to glyphosate via prey feeding on GBH treated (transgenic) plants.

To date, few studies have investigated direct adverse effects of GBHs on terrestrial arthropods and even less experiments considered oral exposure. A still widely cited review has been provided by Giesy and colleagues over two decades ago (Giesy et al., 2000). Effects more recently reported were inhibition of melanisation in Lepidoptera and Diptera (Smith et al., 2021), negative effects in reproduction in Diptera, Hymenoptera and Neuroptera (Muller et al., 2021; Schneider et al., 2009), behavioural changes in spiders after topical exposure (Behrend and Rypstra, 2018; Evans et al., 2010; Haughton et al., 2001). Oral exposure so far has been best studied in honey bees, with exposure mainly during the larval stage. Observed effects range from changes in hypopharyngeal glands (Faita et al., 2018), impairments of the sensory and cognitive abilities such as olfactory learning (Farina et al., 2019), reduced weight and delayed molting (Vázquez et al., 2018) to changes in the gut microbiome of bees (Blot et al., 2019) which can be lethal to larvae (Abraham et al., 2018; Dai et al., 2018; Motta et al., 2020).

Together with the studies above reporting direct, delayed and/or sublethal effects of GBH formulations on arthropod species, our data raises a number of additional serious concerns. These need to be urgently addressed both in further studies as well in the way GBHs are assessed for their effects on biodiversity.

Firstly, the severe negative, and in their magnitude unexpected, effects of our study should be checked by other working groups and include a wider taxonomic range of indicator organisms and other GBH formulations. In this context, we stress the point that chronic, oral exposure should be at the core of experiments as data on this exposure route, which is highly relevant under field conditions, are largely missing. Given the long time and the quantity in which GBHs have been used worldwide, this lack of data is surprising. In addition to data informing about the hazard, the fate of GBHs, including its co-formulants, should be studied, which is a task outside of the scope of this publication. However, such analyses should not be purely based on theoretical assumptions but in fact experimentally testing trophic interactions and food-web effects.

Secondly, our data raise questions on how GBH are assessed in general in the context of their approval as a pesticide. At least two separate regulations apply to formulated pesticides, one for declared active ingredients of pesticides (here, glyphosate) and the other for adjuvants in pesticide formulations (here Roundup Weathermax). In context of GM HT crops, a third separate GMO regulation applies that categorically excludes the impact of herbicides and their potential adverse effects of prey-mediated exposure routes on beneficial arthropods (Hilbeck et al., 2020; Hedlin et al., 2020).

GBHs are old herbicides that received first regulatory approval in Europe almost half a century ago (mid 1970s). In those days, broad spectrum herbicides like Roundup were a minor niche product, hardly used in significant amounts in arable fields and never on a growing crop. cursory assessments of their ecotoxicological profile exclusively through brief external contact exposure (see above) were deemed sufficient then. But even then, thresholds for what classified as 'harmful' were quite lenient. For example, Hassan et al. (1988) reported that of the 19 different test organisms, for 4 species the tested GBH was considered 'slightly harmful' which entailed that >50 % but <80 % died with one species even exhibiting what was called 'moderately harmful' effects, entailing up to 99 % mortality. It seems puzzling that mortality rates of up to 99 % for one species and up to 80 % for another three species led the researchers of those days to conclude that 'Roundup (glyphosate) ... was harmless to all relevant organisms except *Bembidion* and *T. pyri*' (Hassan et al., 1988) also cited in Giesy et al. (2000). *C. carnea* was among those that exhibited 50–79 % mortality when exposed to dried GBH residues (Hassan et al., 1988; Bigler and Waldburger, 1994). No field or semi-field tests were carried out with *C. carnea* and glyphosate (Bigler and Waldburger, 1994; Hassan et al., 1988).

Clearly, the safety of GBHs, if not also of many other herbicides, need to be revisited taking into account the oral uptake of active compounds as well as of co-formulants (Gandhi et al., 2021). This is especially true for herbicides with a systemic mode of action such as GBHs. Although this has been acknowledged by regulatory authorities such as the European Food Safety Authority (EFSA PPP panel, 2015) no standard tests have been developed so far and included into the test protocols relevant for regulatory decisions. Presently, it is not possible to distinguish if the adverse effects we observed are triggered by the active ingredient, the co-formulant (a petroleum distillate with the commercial name Transorb2), a contaminant (for example, this formulation contains around 500 ppb of arsenic and 10 ppb of lead, Defarge et al., 2018) or, as we suspect, their combination.

While many factors contribute to the contemporary insect decline (e.g. Hallmann et al., 2017), the use of pesticides has been suggested repeatedly as one of the main causes (Brühl et al., 2021; Schulz et al., 2021). In order to better protect biodiversity as a whole and to prevent further insect decline, we call for a rethinking of the ERA for non-target arthropods and find ourselves in line with conclusions from Brühl and Zaller (2019) who stated: "Biodiversity decline..." (could be) "...a consequence of an inappropriate Environmental Risk Assessment of Pesticides". With our research, we provide further evidence in support of this conclusion and similar ones voiced

by others (e.g. Seibold et al., 2019). *C. carnea*, along with many other beneficial arthropods, has been almost eliminated from agricultural fields due to the frequent use of non-selective agrochemicals (Nasreen et al., 2005). But it is more commonly thought of as a direct impact of insecticides, or at best indirect impact of herbicides by eliminating the host plants of insects (see above). It is rarely imagined that herbicides could have a direct adverse impact as we found from a GBH on *C. carnea*. However, the massive use of GBHs has led to the situation that Glyphosate/Roundup now is ubiquitous and can be detected in measurable amounts in essentially all environmental compartments. In agroecosystems, but also natural habitats, GBHs have become a significant contaminant in food webs including those leading to the exposure of predatory insects in arable fields via its prey or other contaminated foods. Also, many predatory insects feed directly on plants as an additional source of liquid (e.g. Eubanks and Styrsky, 2005.). In regions where GM crops are grown, like North and South America, exposure to Roundup, in concentrations exceeding those we used in our experiments via prey are very likely.

5. Conclusion

Our data document for the first time serious direct toxic effects from chronic oral exposure to a GBH on the non-target arthropod *C. carnea*. As direct toxic effects from acute and chronic exposure via food are overlooked in current testing schemes for regulatory herbicide approval, potential similar effects on other arthropod taxa cannot be ruled out. In fact, the ubiquitous use of GBHs in combination with reports of negative effects from GBHs on other NTOs published so far, indicate that direct or indirect toxic effects on non-target arthropods may be more common than expected. The vast expansion in space, time, and frequency of GBH concentrations in the environment over the past decades warrants a fresh look specifically at the long-term exposure to these pesticides in conjunction with the insect decline phenomenon.

CRedit authorship contribution statement

MO and AH conceived of the study. ND and AH designed the experiments. ND carried out the experiments and carried out the statistical analyses. All authors drafted and approved the manuscript.

Data availability

Data will be made available on request.

Declaration of competing interest

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

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