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# GLYPHOSATE AND PARAQUAT HERBICIDES' ACUTE EXPOSURE AND CHRONIC EFFECTS ON YELLOW MEALWORMS, *TENEBRIO MOLITOR*

*P. PAEJAROEN[1], C. PHAENARK[1], S. CHUNCHOB[1], W. SAWANGPROH[1*]*

*[1] Division of Conservation Biology, School of Interdisciplinary Studies, Mahidol University (Kanchanaburi Campus), 199 Moo 9 Lumsum, Sai Yok District, Kanchanaburi 71150, Thailand*

** Corresponding author: weerachon.saw@mahidol.ac.th*

COLEOPTERA
INSECTS
NON-TARGET ORGANISMS
CHRONIC EFFECTS
TOXICOLOGY

ABSTRACT. – Glyphosate and paraquat are broad-spectrum and non-selective herbicides used for weed control worldwide. Their potential impacts on alterations in non-target organisms are known. In this study, we reported the effects of glyphosate and paraquat on the mortality and growth response of *Tenebrio molitor* larvae after 48-hr exposure to glyphosate and paraquat. The results indicated that the $LC_{50}$ value for paraquat ($LC_{50}$ = 0.31 mg/cm$^2$) was lower than for glyphosate ($LC_{50}$ = 1.77 mg/cm$^2$), indicating that paraquat was more acutely toxic to *T. molitor*. At evaluated concentrations, the third- and fourth-instar larvae exposed to glyphosate showed less impact on body length and weight compared to the control in the post-exposure development of surviving larvae. Most pupae developed from surviving larvae of glyphosate exposure showed incomplete emergence of adults from their cocoons, with most dying during ecdysis. Some emerging adults survived, but their moults broke into pieces. In contrast, following exposure to paraquat, surviving larvae showed more shrinkage of body length and weight loss at higher doses than at lower doses. Our results regarding a non-target organism suggest that both glyphosate and paraquat should be used with caution.

## INTRODUCTION

Modern agricultural practices rely on the use of herbicides to control weeds in crop production (Dennis *et al*. 2018). These chemicals can in turn harm non-target organisms (Imfeld & Vuilleumier 2012). Herbicides such as glyphosate and paraquat are widely used weed killers around the globe. Glyphosate is commercially known for agricultural use under the trade name Roundup, and paraquat is commercially known as Gramoxone (Grichar *et al*. 2000). Both herbicides are broad-spectrum and nonselective for controlling weeds such as deep-rooted perennial species of annual and biannual species of grasses, sedges, and broad-leaved weeds in agricultural, industrial, and domestic settings (Alberdi *et al*. 1996, Yu *et al*. 2007). When dissolved in water, paraquat is highly toxic to several aquatic organisms, and especially animals compared to glyphosate (Edwards 1977, Gabryelak & Klekot 1985, Tortorelli *et al*. 1990). In Thailand, paraquat has been banned for production, import, export, and possession since 1st June 2020.

Despite growing concern about the effects of using herbicides in agroecosystems, intense agrochemical use has grown globally as a result of the need to supply food for a growing population (Marcato *et al*. 2017). According to numerous ecotoxicological studies, herbicides can cause changes in non-target organisms such as soil microbes, pollinating insects, small animals, and aquatic organisms (Grisolia 2005). Herbicides such as glyphosate are causative agents of reproductive abnormalities in both wildlife and humans (Mensah *et al*. 2015) and induce developmental and reproductive abnormalities in earthworms in laboratory conditions (Correia & Moreira 2010). A toxic fast-acting herbicide such as paraquat is an inducer of the male hypofertility parasitoid wasp *Anisopteromalus calandrae* (Lacoume *et al*. 2009). To our knowledge, the adverse effects of herbicides on terrestrial organisms, particularly insects, are relatively sparse and limited.

Insects are recognized to be the most crucial elements of biodiversity at a global scale in terms of their ecosystem services and ecosystem disservices (Eggleton 2020). Eggleton (2020) pointed out that some marked declines in insect species, especially in flying insects in the northern temperate region, came from overuse of pesticides. A study of pesticide practices on insect biodiversity in peach orchards suggested that the magnitude of the adverse effect might be greater for herbicide application than for insecticide application per se (Sonoda *et al*. 2011).

In this study, the mealworm beetle (*Tenebrio molitor*) was chosen as the test insect because it is commercially available and is found worldwide (Ghaly & Alkoaik 2009), is very easy to reproduce in captivity, is a frequently used model species in scientific studies (Vijver *et al*. 2003, Armitage & Siva-Jothy 2005), is an important pest of stored grains in farm silos (Rantala *et al*. 2003, Patterson 2016), and is one of the most preferred food items for many farmland bird species (Castilla *et al*. 2008), and therefore, the negative impacts of farming practices on mealworm populations might affect the survival and successful reproduction of birds as a whole (Savory 1989, Hart *et al*. 2006). The current study aims to determine the $LC_{50}$ values of glyphosate and paraquat-based herbicides for acute toxicity in the mealworm, *Tenebrio molitor* L. (Coleoptera, Tenebrionidae). Subsequently, the development of surviving larvae after herbicide exposure was evaluated for chronic effects.

## MATERIALS AND METHODS

*Rearing technique of test insects*: *Tenebrio molitor* is holometabolous with four distinct life stages, egg, larva, pupa, and adult (Patterson 2016). Mealworms or the larval form of *T. molitor* L. were purchased from a vendor at Thonburi Market in Thawee Watthana District, Bangkok, Thailand. The larvae were collectively reared in a cubic plastic chamber (14 × 16 × 23 cm), with two holes in the top of the chamber for aeration. The floor of the chamber contained wheat bran to provide a burrowing substrate and food source for the larvae. The chamber was cleaned, and wheat bran was renewed once a week to avoid bacterial and fungus accumulation. Once a larva pupated, the pupa was reared separately in a cylindrical plastic chamber (17 cm in length) with wheat bran as bedding material. Adults that emerged from pupae were sexed under a dissecting microscope. A male was paired with a randomly chosen female in a new container for mating. The rearing of *T. molitor* was conducted in an insectary at ambient room temperature at Mahidol University (Kanchanaburi). The mean temperature within the rearing room is 28 ± 2° C.

*Preparation of herbicide solution*: Based on our preliminary data, we prepared different concentrations of glyphosate and paraquat for bioassay using the dilution method from their commercial formulations: glyphosate-isopropyl ammonium (48 % w/v soluble liquid) and paraquat dichloride (27.6 % w/v soluble liquid). Five different concentrations of glyphosate (*i.e*., 1.00, 2.00, 4.00, 8.00, and 16.00 g/L) and paraquat (*i.e*., 0.75, 1.50, 3.00, 6.00, and 12.00 g/L) were prepared by diluting with distilled water.

*Toxicity testing*: The effects of glyphosate and paraquat were tested against third- and fourth-instar larvae of *T. molitor*. We treated the third- and fourth-instar larvae at the same stage because it is difficult to distinguish between them at this stage of development (9-10 days) and body length (0.5-0.6 cm) (Park *et al*. 2014). Bioassays were performed using impregnated filter paper. Impregnation with herbicides was done by spraying a Whatman No. 1, 90-mm diameter paper with 5 ml of the desired herbicide concentration without runoff for the treatment condition, or with 5 ml of distilled water for the control condition. Then, the filter papers were dried at room temperature for 60 minutes before use. The active ingredient of herbicide (g/L) per area of filter paper with a 90 mm diameter was calculated (% w/v = g of solute per 100 mL of solution). Therefore, the final concentrations of the active ingredient of the herbicides on the filter paper were 0.08, 0.16, 0.31, 0.63, and 1.26 mg/cm$^2$ for glyphosate and 0.06, 0.12, 0.24, 0.47, and 0.94 mg/cm$^2$ for paraquat, respectively. **Acute exposure**: To determine maximum exposure concentrations relevant to the survival of *T. molitor*, their larvae were placed directly onto the impregnated filter paper in a new plastic chamber without food and observed at 48 hours of exposure for mortality. Larvae that did not move any appendages after 30 seconds of gentle mechanical stimulation were considered dead. A group of 10 larvae per treatment per replicate were randomly distributed to 10 treatment concentrations and two control conditions of filter papers (12 experiments × 3 replicates × 10 larvae = 360 larvae). Later, the acute 48-h $LC_{50}$ concentrations of glyphosate and paraquat were determined. **Chronic effect**: This trial evaluated the post-exposure development of *T. molitor* larvae that survived after the acute exposure test to evaluate the capacity of recovery after herbicidal exposure. Each surviving larva was cultured in a separate chamber and observed for post-treatment effects on the growth increment, *i.e*., body length (mm) and body weight (mg), and pupal ecdysis. The body length of the larvae was measured using an Olympus stereo microscope with a micrometer. The body weight of larvae was measured using a Sartorius digital weighing machine with four digits of a decimal number. The growth increment is calculated from the body length and weight on days 3, 6, 9, and 12 and is deducted from the body length and weight on day 0 (before herbicide exposure).

The acute exposure and chronic effect of larvae were performed at room temperature using a photoperiod of 12:12 h (light:dark).

*Statistical analyses*: Lethal concentration ($LC_{50}$) values (the concentrations that kill 50 % of the test insects during the observation period) were estimated using Probit analysis (Finney 1952). A one-way ANOVA test was determined if the growth increase in body length and weight of the larvae is different between the control and all treatments on days 3, 6, 9, and 12 after 48-h exposure. Differences between the treatments were determined by Tukey's HSD test at $p < 0.05$.

*Ethics statements:* The study was approved by the Mahidol University-Institute Animal Care and Use Committee, Protocol No. F01-64-002.

## RESULTS

### *Acute exposure of* **T. molitor** *to glyphosate and paraquat*

The result of the Probit modelling gave an estimated $LC_{50}$ value of 1.77 mg/cm$^2$ for glyphosate, whereas the $LC_{50}$ value was 0.31 mg/cm$^2$ for paraquat (Table I).

### *Chronic effects of glyphosate on the growth of* **T. molitor**

The larval body length and weight increased continuously from day 3 to day 12 in both the control groups and the treatment groups (Fig. 1A, B). The exposure of third- and fourth-instar larvae to evaluated concentrations of glyphosate showed clearly adverse chronic effects on body length increase on day 3 for the treatment of 0.63 mg/cm$^2$ and on day 6 for all treatments. However, no adverse effects on body length increase were seen on days 9 and 12 (Fig. 1A). No adverse effects on body weight increase were seen on day 3 for all treatments. However, adverse effects on body weight increase were seen on day 6 for all treatments, and on days 9 and 12 for the treatment of 0.63 mg/cm$^2$. On day 12, the statistical analysis showed no significant difference in body weight increase in all treatments compared to the control (Fig. 1B), except the treatment of 1.26 mg/cm$^2$, which had a positive effect on body weight increase in *T. molitor* larvae (Fig. 1B). Observation of ecdysis from the majority of pupal cocoons developed from larvae exposed to different concentrations of glyphosate showed incomplete emergence of adults. Most emerging adults died in their single piece of cocoon, while some emerging adults survived but showed moults broken apart into pieces. In contrast, the

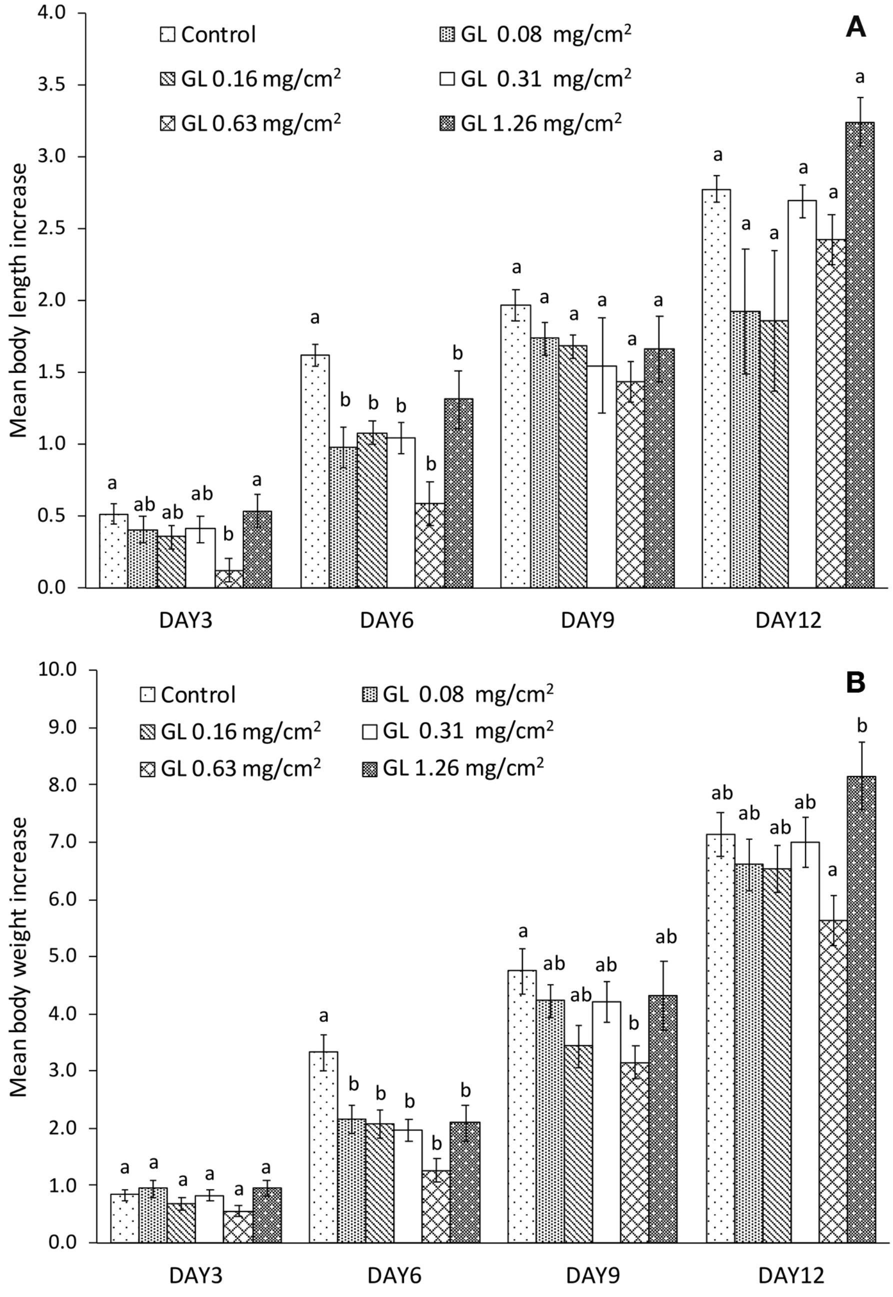


Fig. 1. – The impact of glyphosate on (**A**) body length increase in mm and (**B**) body weight increase in mg of third- and fourth-instar larvae of *T. molitor*. The body length and body weight are displayed as means ± 1 SE. The same letters above bars indicate no significant difference among treatments within the same day (*Post-hoc* Tukey's HSD test at the significant level of 0.05 %).

pupae from the control emerged successfully from their cocoons into adults.

### *Chronic effects of paraquat on the growth of* T. molitor

Larval body length and body weight increased continuously from day 3 to day 12 in both the control and the treatment groups, but those treated with evaluated concentrations of paraquat showed a slower increase in body length and body weight (when exposed to higher doses) (Fig. 2). After 48-h exposure to paraquat, the larvae reduced the increment of body length compared to the control in the treatments of 0.24, 0.47, and 0.94 mg/cm$^2$ on day 3, of 0.12, 0.24, 0.47, and 0.94 mg/cm$^2$ on day 6, of 0.24, 0.47, and 0.94 mg/cm$^2$ on day 9, and of 0.24, 0.47, and 0.94 mg/cm$^2$ on day 12, respectively (Fig. 2A). Similarly, the larvae reduced the increment of body weight compared to the control in the treatment of 0.24, 0.47, and 0.94 mg/cm$^2$ on day 3, of 0.06, 0.12, 0.24, 0.47, and 0.94 mg/cm$^2$ on day 6, of 0.06, 0.12, 0.24, 0.47, and 0.94 mg/cm$^2$ on day 9, of 0.06, 0.12, 0.24, 0.47, and 0.94 mg/cm$^2$ on day 12, respectively (Fig. 2B). The majority of surviving larvae exposed to paraquat showed a delay in pupation and finally died. Some pupae underwent the pupal stage but showed incomplete emergence of deformed adults.

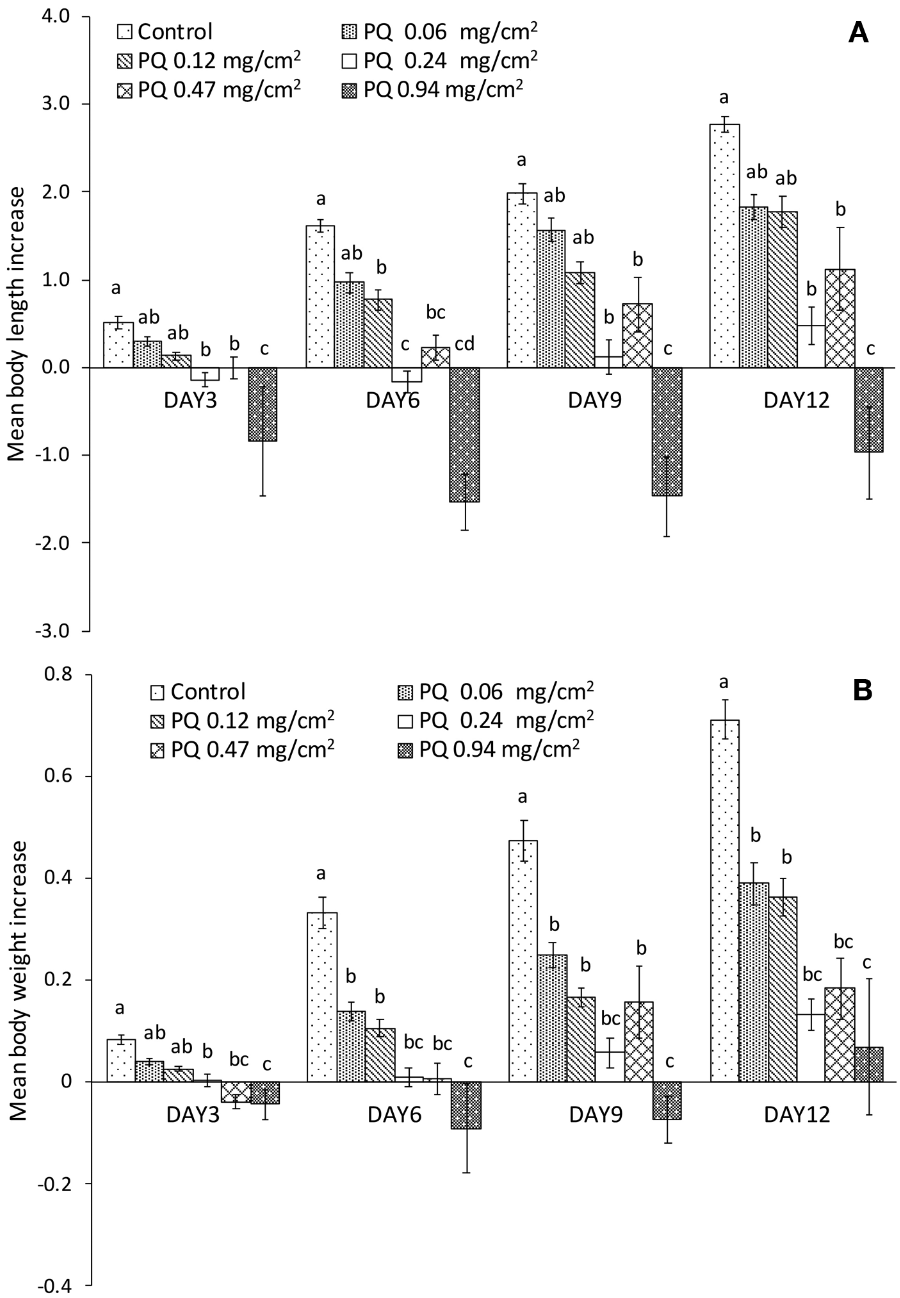


Fig. 2. – The impact of paraquat on (**A**) body length increase in mm and (**B**) body weight increase in mg of third- and fourth-instar larvae of *T. molitor*. The body length and body weight are displayed by means ± 1 SE. The same letters above/ below bars indicate no significant difference among treatments within the same day (Post-hoc Tukey's HSD test at the significant level of 0.05 %).

## DISCUSSION

### *Acute exposure of glyphosate and paraquat to* T. molitor

In this study, mortality data showed that paraquat was more toxic to larvae ($LC_{50}$ = 0.31 mg/cm$^2$) through dermal exposure than glyphosate ($LC_{50}$ = 1.77 mg/cm$^2$) (Table I). Similar results were found in studies of the comparative acute toxicity of paraquat and glyphosate in the crustaceans *Daphnia magna* and *D. spinulata* (Alberdi *et al.* 1996), the African catfish *Clarias gariepinus* (Ayanda *et al.* 2015), and the climbing perch *Anabas testudineus* (Sawasdee *et al.* 2016). The $LC_{50}$ of glyphosate for *T. molitor* ($LC_{50}$ = 1.77 mg/cm$^2$) was more than five times higher than that of paraquat ($LC_{50}$ = 0.31 mg/cm$^2$) in this study. This suggests that glyphosate is less toxic to *T. molitor*. This result was in accordance with previous studies on the effects of commercial glyphosate on terrestrial insects, which demonstrated that glyphosate at low concentrations through direct contact was considered harmless to two aphelinid parasitoids (Teran *et al.* 1993) and the water hyacinth weevil *Neochetina eichhorniae* (Ding *et al.* 1998).

In contrast to glyphosate formulations, commercial paraquat products contain dichloride salt in the forms of ionic and nonionic or cationic surfactants (Smith & Foy 1967). The acute toxicity mechanism of paraquat is described as a non-specific inducer of apoptosis or programmed cell death that occurred in many multicellular organisms such as fishes (Eisler 1990), Collembola (Choi *et al.* 2008), birds (Bauer 1985), gastropods (Bacchetta *et al.* 2002), and mice (Dial & Dial 1987) and as oxidative stress and mitochondrial dysfunction in fruit fly *Drosophila melanogaster* (Hosamani & Muralidhara 2013).

In this study, we did not know for certain about surfactants in an aqueous mixture of commercial glyphosate and paraquat formulations that are often protected as proprietary information by the manufacturer (Chen *et al.* 2004). It would be very interesting to carry out a study to compare the effects of herbicides with and without surfactants on *T. molitor* in the future.

### *Chronic effects of glyphosate and paraquat on* T. molitor

Desneux *et al.* (2007) described chronic effects as physiological and/or behavioral impacts on individuals that survive exposure to a toxic substance at low concentrations. The chronic effects of herbicides on organisms may vary depending on the types of chemicals, stages of development, and test species. We noticed that the majority of surviving larvae exposed to glyphosate and paraquat showed delayed pupation and died, and the transformation rate of surviving larvae into pupae was low, especially for those exposed to paraquat. Compared to glyphosate, the negative effects of paraquat on larval growth (*i.e.*, body length and weight) and certain developmental stages of *T. molitor* were higher in our study. Our findings were consistent with previous research that glyphosate is less toxic and safe for a wide range of living organisms, such as Borggaard & Gimsing (2008), Valavanidis (2018), and Meftaul *et al.* (2020).

Table I. – 48-h mortality from glyphosate and paraquat applied to third- and fourth-instar larvae of *T. molitor* at five concentrations. The percentage (%) of total dead larvae was calculated from the total number of treated animals.

| Chemical | Concentration (mg/cm$^2$) | 48-h Mortality (%) |
|---|---|---|
| Glyphosate | Control | 0 |
| | 0.08 | 0 |
| | 0.16 | 0 |
| | 0.31 | 0 |
| | 0.63 | 20 |
| | 1.26 | 30 |
| Paraquat | Control | 0 |
| | 0.06 | 3 |
| | 0.12 | 10 |
| | 0.24 | 37 |
| | 0.47 | 87 |
| | 0.94 | 90 |

The chronic effects of glyphosate could delay the larval and adult stages of the fruit fly *Drosophila* (Strilbytska *et al.* 2022) and the larval development of honeybees (*Apis mellifera*) (Vázquez *et al.* 2018, Farina *et al.* 2019), reduce the ability of the aphid *Metopolophium dirhodum* to reproduce (Saska *et al.* 2016), and reduce the body weight of honeybees (*Apis mellifera*) (Vázquez *et al.* 2018, Farina *et al.* 2019). The chronic effects of paraquat could result in observable malformation and growth reduction in *Xenopus laevis* embryos (Osano *et al.* 2002), growth retardation in *Rana pipiens* tadpoles and *Oreochromis niloticus* fingerlings (Dial & Bauer 1984, Babatunde 1997), a decrease in life span, oviposition capability, and ovipositor thrusting frequency of the parasitoid *Diaeretiella rapae* (Xu *et al.* 2013), and a decrease in the climbing ability and longevity of adult *Drosophila melanogaster* when exposed to a combination of atrazine and paraquat (Lovejoy & Fiumera 2019). Although the final points considered by the cited authors do not coincide with those evaluated by us, they allow us to infer that chronic effects related to species-specific life history traits must be carefully considered in order to assess herbicide risks to non-target organisms in the future.

In some cases, herbicides might not show a measurable impact at certain stages of organism development in comparison with other stages. For example, Affeld *et al.* (2003) tested pure glyphosate and glyphosate plus polydimethylsiloxane against pupae of the broom twig miner (*Leucoptera spartifoliella*) and found that both treatments showed no significant impact on the emergence of adults from cocoons when applied at the dose recommended in the field. Similarly, Stecca *et al*. (2016) tested different glyphosate-based pesticides against pupae (inside host eggs) of the parasitoid *Telenomus remus* (Hymenoptera), and all tested glyphosate-based products did not reduce adult emergence or parasitism capacity. However, when tested against *T. remus* adults, glyphosate-based products reduced parasitism two days after adult emergence. We believe the cocoons may act as an effective protector against glyphosate during pupation. However, this explanation for such effects and validation of our hypothesis still needs more investigation.

To summarize, all glyphosate and paraquat tested toxic to *T. molitor* larvae. However, glyphosate is less toxic or safer to *T. molitor* larvae and pupae than paraquat in terms of acute exposure and chronic effects. Glyphosate is considered harmless to *T. molitor* larvae, whereas paraquat is highly harmful to *T. molitor*. Therefore, research on the effect of these herbicides on non-target organisms should be continued.

Conflict of interest – The authors declare that there are no conflicts of interest.

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