

Research Paper

Stronger effects of Roundup than its active ingredient glyphosate in damselfly larvae

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ABSTRACT

Pesticides are causing strong decreases in aquatic biodiversity at concentrations assumed safe by legislation. One reason for the failing risk assessment may be strong differences in the toxicity of the active ingredient of pesticides and their commercial formulations. Sublethal effects, especially those on behaviour, have been largely ignored in this context, yet can be equally important as lethal effects at the population and ecosystem levels. Here, we compared the toxicity of the herbicide Roundup and its active ingredient glyphosate on survival, but also on ecologically relevant sublethal traits (life history, behaviour and physiology) in damselfly larvae. Roundup was more toxic than glyphosate with negative effects on survival, behaviour and most of the physiological traits being present at lower concentrations (food intake, escape swimming speed) or even only present (survival, sugar and total energy content and muscle mass) following Roundup exposure. This confirms the toxicity of the surfactant POEA. Notably, also glyphosate was not harmless: a realistic concentration of 2 mg/l resulted in reduced growth rate, escape swimming speed and fat content. Our results therefore indicate that the toxicity of Roundup cannot be fully attributed to its surfactant, thereby suggesting that also the new generation of glyphosate-based herbicides with other mixtures of surfactants likely will have adverse effects on non-target aquatic organisms. Ecotoxicological studies comparing the toxicity of active ingredients and their commercial formulations typically ignore behaviour while the here observed differential effects on behaviour likely will negatively impact damselfly populations. Our data highlight that risk assessment of pesticides ignoring sublethal effects may contribute to the negative effects of pesticides on aquatic biodiversity.

1. Introduction

One of the main drivers of the current biodiversity crisis is the increasing use of pesticides (Williams et al., 2004). Besides negatively impacting populations of targeted pest species, pesticides indeed have been shown to also reduce fitness of non-target species (Relyea, 2005). An alarming recent finding is that current legislation in Europe fails to protect aquatic biodiversity with up to 42% of taxa disappearing at pesticide concentrations assumed to be safe (Beketov et al., 2013). One explanation is that the difference in toxicity between the active component and the commercial form of a pesticide may considerably increase the impact of pesticides in natural systems (e.g. Beggel et al., 2010; Kroon et al., 2015; Mansano et al., 2016). The increased toxicity of commercial products is thought to result from the toxicity of the assumed inert ingredients that act as solvents, emulsifiers, surfactants and preservatives, and/or from their synergistic interactions with the active component (Cox and Surgan, 2007).

One pesticide group that has attracted considerable attention in the

context of higher toxicity of its commercial formulations and their risk to aquatic biodiversity are glyphosate-based herbicides (e.g. Relyea 2005; Glusczak et al., 2007). This is worldwide the most commonly used herbicide group and its use has increased with a factor of more than 12 in the last 20 years from about 67 million kg in 1995–826 million kg in 2014 (Benbrook, 2016). In natural surface waters concentrations between 0.1–5.2 mg/l of the active ingredient have been detected (Edwards et al., 1980; Thompson et al., 2004) and worst-case exposure scenarios predict concentrations up to 7.6 mg/l (Solomon and Thompson 2003; Wagner et al., 2013).

Due to its specific mode of action, glyphosate specifically targets plants and not animals. Indeed, glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphatase synthase, involved in the synthesis pathway of aromatic amino acids, which is only present in plants and microorganisms (Carlisle and Trevors 1988;). For that reason, glyphosate-based herbicides have been advocated as animal-friendly chemicals for control of plant pests in nature reserves (Monteiro et al., 2005; Tsui and Chu, 2008; Mazzolari et al., 2011). Despite this, lethal effects

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of glyphosate-based herbicides on aquatic animals have been shown (e.g. crustaceans LC_{5048h} 35.3–147 mg/l for glyphosate and 1.77–5.39 mg/l for Roundup (Tsui and Chu 2003); tadpoles tested at a concentration of 3.8 mg/l of Roundup almost all died within three weeks of exposure (Relyea, 2005); tadpoles LC_{5096h} 1.80–4.22 mg/l (Moore et al., 2012)). Glyphosate-based herbicides contain a surfactant which facilitates the penetration of the cuticle layer (Giesy et al., 2000; Solomon and Thompson 2003). While glyphosate can be toxic to non-target species as well, evidence suggests that the added surfactants are more toxic, even though these surfactants are typically considered inert ingredients (Tsui and Chu, 2003; Moore et al., 2012). The often added nonionic surfactants such as polyoxyethylene tallow amines (POEA) have been shown to disrupt the respiratory surfaces of aquatic organisms (Lindgren et al., 1996) what might be the reason for their lethal effects.

There is increasing appreciation that besides lethal effects, also sublethal effects may contribute considerably to the negative impact of pesticides in natural populations (Hanazato 2001; Dell'Omo, 2002). Sublethal effects may directly reduce population growth rates for example through reductions in body mass increase, but also indirectly by making exposed organisms more vulnerable to natural enemies. Sublethal effects of both glyphosate and glyphosate-based herbicides on life history, behaviour and physiology have been documented. For example, fish exposed for 96 h to 0.2 mg/l Roundup suffered increased oxidative damage (Gluszcak et al., 2007), and frogs exposed for 21 days to 0.1 mg/l Roundup had a lower growth rate (Paetow et al., 2012). The larval amphibian stage seems to be less sensitive, since Miko et al. (2017) documented that tadpole behaviours were often only affected at 6.5 mg/l of a glyphosate-based herbicide. In general, invertebrates might be less sensitive than vertebrate species. For example, in damselfly larvae, concentrations from 1.5 (Roundup) to 2 (glyphosate) mg/l reduced growth, increased the (foraging) activity and resulted in higher expression levels of the stress protein Hsp70 (Janssens and Stoks 2012, 2013), while lower concentrations had no effect. Hansen and Roslev (2016) even showed that the swimming behaviour of *Daphnia magna* was only affected starting at 14.1 mg/l glyphosate. Although several studies investigated the sublethal effects of glyphosate or glyphosate-based herbicides, very few studies directly compared such effects between glyphosate and its commercial formulations (but see e.g. Howe et al., 2004 [growth and development]; Peixoto 2005 [mitochondrial energetics]; Webster et al., 2014 [reproduction]). Moreover, no studies directly compared effects on behaviour, this despite the importance of impaired behaviour for fitness and the population dynamics of species (Pyle and Ford, 2017). In general, studies that included behaviour when comparing the effects of the active component and the commercial form of a pesticide are rare (but see Beggel et al., 2010). Whether the toxicity of glyphosate-based herbicides for non-target organisms is caused by the active ingredient itself or by the added products is important, since with the removal of the patent protection of glyphosate in 2000, many new glyphosate-based herbicides have been developed with other combinations of surfactants (Howe et al., 2004).

In this study we compared the toxicity of glyphosate and its commercial formulation Roundup® both with regard to lethal and sublethal effects on a non-target aquatic species. Roundup® is the most known commercial formulation of glyphosate and contains POEA as surfactant. Specifically, we studied ecologically relevant sublethal effects on life history (growth), behaviour (foraging behaviour and escape swimming) and physiology (energy storage, muscle mass, activity of acetylcholinesterase). As study organisms we used damselfly larvae, aquatic insects that occupy an important position in aquatic food webs, being both predators of small invertebrates and prey for large invertebrates and fish (Corbet, 1999). Pesticide concentrations negatively affecting the damselfly larvae are therefore likely to have a much wider impact, since also the rest of the aquatic community may be indirectly affected. Moreover, as adult damselflies are terrestrial, pesticide effects on the aquatic larvae may affect the terrestrial communities (Stoks & Cordoba-

Aguilar 2012). As study species we chose *Coenagrion pulchellum* that is listed as endangered on the red list of threatened species in Flanders (De Knijf et al., 2006). As many other Red List species, *C. pulchellum* typically is restricted to nature reserves where pesticide use is not allowed in Flanders (Vlaamse Gewest, 2013). However, an exception was made for glyphosate and glyphosate-based herbicides to fight invasive plant species (Vlaamse Gewest, 2013), making it especially relevant to study and compare effects of glyphosate and this glyphosate-based herbicide on this regionally endangered damselfly.

2. Methods

2.1. Collection and housing

Fifteen mated females of the damselfly *C. pulchellum* were collected in a nature reserve in Limburg (Flanders). Females were brought to the laboratory for egg laying and their hatchlings were placed individually in 200 ml cups filled with aerated tap water and kept under standard conditions of light (14:10 L:D), food (*ad libitum* *Artemia* nauplii seven days a week) and temperature (21 °C). When the larvae moulted into the final instar, they were used in the experiment. The offspring of each female was randomly divided over the different treatments.

2.2. Experimental setup

In order to compare the toxicity of Roundup with its active compound glyphosate on growth, behaviour and physiology, we exposed individual larvae to one of five treatments: control, glyphosate 1 mg/l, glyphosate 2 mg/l, Roundup containing 1 mg/l of the active compound glyphosate and Roundup containing 2 mg/l of the active compound. We took into account the concentration of the active compound in the commercial formulation, which was in our case 450 g/l, to obtain equal concentrations of glyphosate in the glyphosate treatments and the Roundup treatments. For Roundup the manufacturer recommends a maximum application rate of 6.4 ml/m², resulting in a water concentration expected in aquatic habitats when spraying for terrestrial and aquatic weeds up to 3.7 mg/l of the active ingredient (thereby following the reasoning used in Relyea, 2005). The nominal concentrations used in our experiment are a factor two to four lower than this worst-case scenario. Moreover, Solomon and Thompson, (2003) estimated that the glyphosate concentrations in the surface water where the pesticide is used can exceed 3 mg/l, whereas Wagner et al. (2013) predicted worst case scenarios up to 7.6 mg/l. Actual concentrations reported in nature can vary between 0.1–5.2 mg/l of the active ingredient in surface waters (Edwards et al., 1980; Thompson et al., 2004). This indicates that although the in our study applied glyphosate concentrations are rather high, they are likely to be encountered in water bodies when glyphosate based herbicides are sprayed.

At the start of the exposure, the larvae were individually transferred to glass jars containing 50 ml of the exposure medium. The exposure period lasted for seven days and the medium was refreshed every 24 h (static renewal experiment). Since glyphosate and Roundup are water soluble no solvent was used, hence they were dissolved in synthetic pond water (for the composition, see Janssens and Stoks, 2013). Also the control larvae were kept in this synthetic pond water. We started 40 larvae per pesticide treatment (total of 200 larvae). The actual glyphosate concentration directly after refreshing the medium in the samples was measured at the University of Gent (Belgium) using liquid chromatography-tandem mass spectrometry (LC-MS/MS) from a mixed sample of ten experimental vials per condition. For the nominal concentration of 1 mg/l the measured concentrations were 0.61 mg/l for glyphosate and 0.59 mg/l for Roundup; for the nominal concentration of 2 mg/l the measured concentrations were 1.15 mg/l for glyphosate and 1.19 mg/l for Roundup. The glyphosate concentration in the control vials was below the detection limit of 0.3 µg/l.

2.3. Response variables

We daily checked survival during the 7 day exposure period. To quantify growth rate, the larvae were weighed individually to the nearest 0.01 mg at the start and at the end of this period. Growth rate was calculated as $[\ln_{\text{finalmass}} - \ln_{\text{initialmass}}]/7$ days. We investigated two ecologically relevant behavioural traits: food intake and escape swimming speed. Food intake was studied on day four of the exposure period by counting the number of *Artemia* nauplii the larvae consumed during a 2 h foraging period following the protocol described in Janssens and Stoks (2013). Per day that we quantified food intake we stored two initial samples of *Artemia* nauplii in 70% ethanol to afterwards count the amount of nauplii given to each individual larva. At the end of the 2 h foraging period, we collected the uneaten *Artemia* nauplii per vial and also stored them in ethanol. Afterwards, fixated nauplii were counted at magnification $10 \times$ using a stereomicroscope. The number of nauplii eaten by each individual was calculated as the difference between the mean initial number of a food ration at that feeding day and the number of remaining uneaten nauplii in the vial of that larva.

Escape swimming speed is an important antipredator mechanism determining the chance of damselfly larvae to escape when attacked by dragonfly predators (Stoks and McPeck, 2006; Strobbe et al., 2009). Escape swimming speed was recorded on the final day of the exposure period following the protocols of McPeck et al. (1996) and Stoks and McPeck (2006). We used a high speed camera (Basler pi A 640, 200 Hz) which was connected to a computer using Streampix software. The swimming speed was scored in a separate room without any disturbance and all measurements were done by the same person. All treatments were tested in a random order and all trials were done between 10 h and 14 h. Individual damselfly larvae were transferred to a plastic container (32 cm \times 21 cm, 4 cm high) filled with 1 l of the synthetic pond water. After 10 min of acclimation time, we simulated a predator attack by tapping the larva on its abdomen with a plastic pipette, causing an escape burst swim. Swimming bouts were recorded and analyzed using Image Pro Plus v5. The escape swimming speed (expressed in cm/s) was calculated based on the distance the larvae moved during the first 0.5 s. This is the relevant time interval to escape predation by attacking dragonfly larvae (Dayton et al., 2005).

To quantify the physiological traits, we used two sets of animals. On the first set we quantified energy storage (protein, sugar and fat contents), and on the second set physiological traits related with the swimming speed (muscle mass and acetylcholinesterase activity). For the first set, we started by homogenizing the larvae using a pestle, diluted them 15 times (v/w) in phosphate buffer saline (PBS, 100 mM, pH 7.4) and centrifuged the samples for 5 min (16,100g, 4 °C). The resulting supernatant was used in the physiological assays. Exact sample sizes per response variable are given in the figures.

The fat content was measured following the method described in Janssens and Stoks (2013). We filled a glass tube with 75 μ l of the supernatant and 250 μ l of sulfuric acid (100%). After heating the mixture for 15 min at 200 °C, we added 350 μ l of milli-Q water. We filled the wells of a 96 well microtiter plate with 100 μ l of the sample and measured absorbance at 340 nm. Fat concentrations were calculated using a standard curve of glyceryl tripalmitate. The total sugar content (glucose + glycogen) was quantified following the protocol of Stoks et al. (2006) based on the glucose kit of Sigma Aldrich USA. First, the glycogen was transformed to glucose by mixing mixed 65 μ l milli-Q water, 25 μ l supernatant and 10 μ l amyloglucosidase (1 U/10 μ l) (Sigma A7420) in a 96 well microtiter plate and incubating this mixture at 37 °C for 30 min. In the next step, 200 μ l glucose assay reagent (Sigma G3293) was added and the mixture was incubated at 30 °C for 20 min. Absorbance was measured at 340 nm and the sugar concentration based on a standard curve of known concentrations of glucose and their absorbance. Since the results for glucose and glycogen concentrations were very similar, we only report the total sugar

content. Protein content was quantified following the Bradford method (Bradford, 1976). Total fat, total sugar and protein contents were expressed as μ g/mg wet mass. All analyses were done in duplicate and the mean was used for the statistical analyses. To assess the overall energy content of the larvae we converted the concentration of the different energy storage molecules into energetic equivalents using the enthalpy of combustion (Gnaiger, 1983): 39.5 J/mg for fat, 17.5 J/mg for sugars and 24 J/mg for proteins.

For the second set of animals, we first separated the head from the body. We measured the activity of AChE on the heads using a for insects modified version of the protocol of Ellman (Jensen et al., 1997). This enzyme has an important function in the nervous system by catalysing the breakdown of acetylcholine and higher activity levels have been shown to be positively related to escape swimming speed (Trekels et al., 2012; Bonansea et al., 2016). We homogenized the heads in 250 μ l PBS and after centrifugation we used the supernatant for the analysis. We filled a 96-well microtiter plate with 20 μ l supernatant and then added 100 μ l of a reaction mixture (13.2 ml PBS, 255 μ l 10 mM acetylcholine iodide, 495 μ l 3 mM DTNB). Absorbance at 412 nm was measured every 30 s during 30 min at 30 °C. As an estimate of the AChE activity we used the slope of the linear part of the reaction curve. To quantify muscle mass, we followed the protocol by Swillen et al. (2009). We dried the individual bodies for 24 h at 60 °C and weighed them to the nearest 0.01 mg using an electronic balance. Afterwards, we added 1.5 ml NaOH (0.35 M) to break down all muscle (protein) tissue in the samples, and placed the tubes on a shaker for 24 h. The exoskeletons were dried for another 24 h at 60 °C and weighed. The muscle mass was expressed as a percentage of the total dry mass.

2.4. Statistical analyses

We performed ordered heterogeneity (OH) tests associated with ANOVAs to evaluate the effects of glyphosate and Roundup. This is a more powerful approach than classical ANOVAs, since we had ordered expectations which classical ANOVAs do not take into account (Rice and Gaines, 1994a, 1994b). The OH test extends the classical ANOVA by also incorporating the ordering information of the treatment groups; more specifically it combines the *p*-value derived from the classical ANOVA with a Spearman's rank correlation between the observed and expected ranks of the groups, to obtain a *p*-value for the ordered test. For each pollutant (glyphosate or Roundup) we had the a priori ordered prediction that the pesticide effects would be stronger at the higher than at the lower concentration. Since it was not possible to make a priori ordered expectations across all five treatment levels (for example, whether 1 mg/l Roundup would be more or less toxic than 2 mg/l glyphosate), we ran separate OH tests for glyphosate and Roundup.

To test for the effects of the pesticide treatment on all response variables we used OH tests associated with ANOVAs with a normal error structure, except for survival (dead vs alive) during the exposure period we used a binomial error structure. When an OH test indicated a significant pesticide effect, we performed a Dunnett post hoc test to investigate which of the two pesticide concentrations differed from the control. Because for models with a binomial error structure no post hoc tests can be performed, we ran separate Fisher's Exact tests to further explore the significant effects glyphosate or Roundup. For food intake and escape swimming speed the mass of the larvae was included in the models as continuous predictors, resulting in OH tests associated with ANCOVAs for these two response variables. The ANOVA assumptions were met without the need of transformations. All tests were done in Statistica v13 (Statsoft Inc, OK, USA).

3. Results

Survival was affected by Roundup exposure ($\chi^2_2 = 7.72$, $p < 0.001$), but not by glyphosate exposure ($\chi^2_2 = 2.05$, $p = 0.18$) with larvae exposed to 2 mg/l Roundup having a lower survival

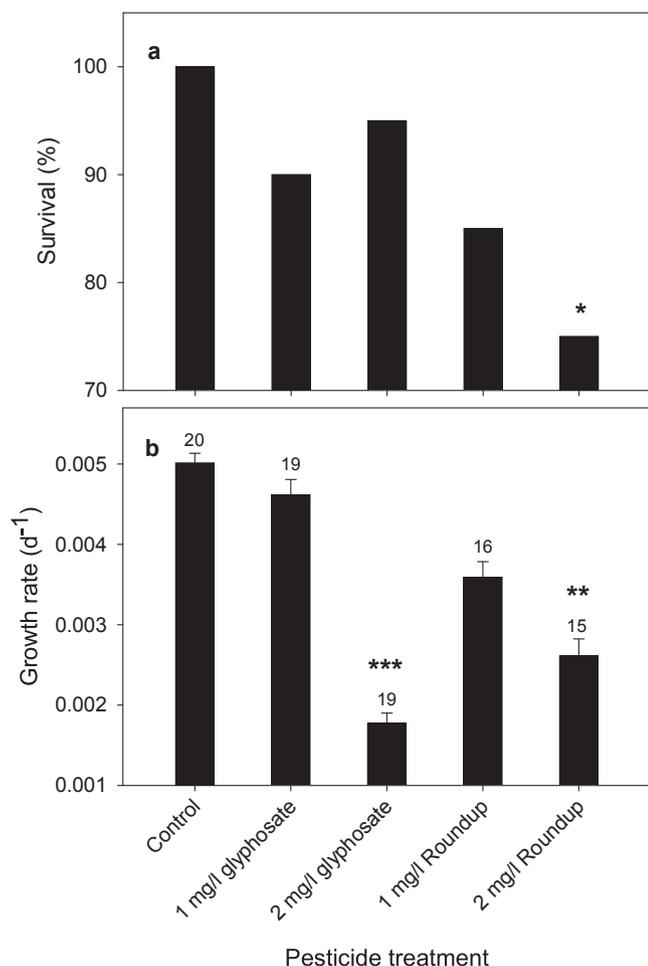


Fig. 1. (a) Percentage survival and (b) mean growth rate (with 1 SE) during the pesticide exposure period of *Coenagrion pulchellum* larvae. Significant differences from the control based on Dunnett post hoc tests, performed when the Ordered Heterogeneity test indicated a significant pesticide effect, are coded with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Numbers above the bars represent the number of replicates.

(Fig. 1a). Both Roundup ($F_{2,49} = 14.27$, $p < 0.001$) and glyphosate ($F_{2,54} = 2.05$, $p = 0.025$) reduced growth rate; more specifically at the highest concentration (Fig. 1b).

Both behavioural traits were affected by Roundup (food intake: $F_{2,48} = 36.33$, $p < 0.001$; escape swimming speed: $F_{2,48} = 20.22$, $p < 0.001$; Fig. 2) and glyphosate (food intake: $F_{2,53} = 5.71$, $p < 0.001$; escape swimming speed: $F_{2,53} = 2.23$, $p = 0.020$). Larvae exposed to both Roundup concentrations had a higher foraging activity and a lower escape swimming speed, while for glyphosate-exposed larvae this was only true at the highest concentration (Fig. 2). Heavier animals swam faster (Roundup: $F_{1,48} = 7.20$, $p = 0.010$; glyphosate: $F_{1,54} = 6.43$, $p = 0.014$), but did not have a higher food intake (Roundup: $F_{1,48} = 0.012$, $p = 0.91$; glyphosate: $F_{1,54} = 2.83$, $p = 0.10$).

Exposure to both Roundup and glyphosate resulted in a lower fat content (Roundup: $F_{2,48} = 26.79$, $p < 0.001$; glyphosate: $F_{2,54} = 23.52$, $p < 0.001$). Additionally, Roundup significantly reduced ($F_{2,48} = 6.68$, $p = 0.044$) and glyphosate tended to reduce ($F_{2,54} = 8.45$, $p = 0.09$) the sugar content. None of the pesticides affected the protein content (both $p > 0.12$; Fig. 3c). Fat contents were lower in all pesticide treatments, while the sugar content was only lower in larvae exposed to 2 mg/l Roundup (Fig. 3a-b). Integrating all energy storage molecules, the available energy was lower when exposed to Roundup ($F_{2,48} = 13.08$; $p < 0.001$), and tended to be lower following glyphosate exposure ($F_{2,54} = 0.83$; $p = 0.09$; Fig. 3d).

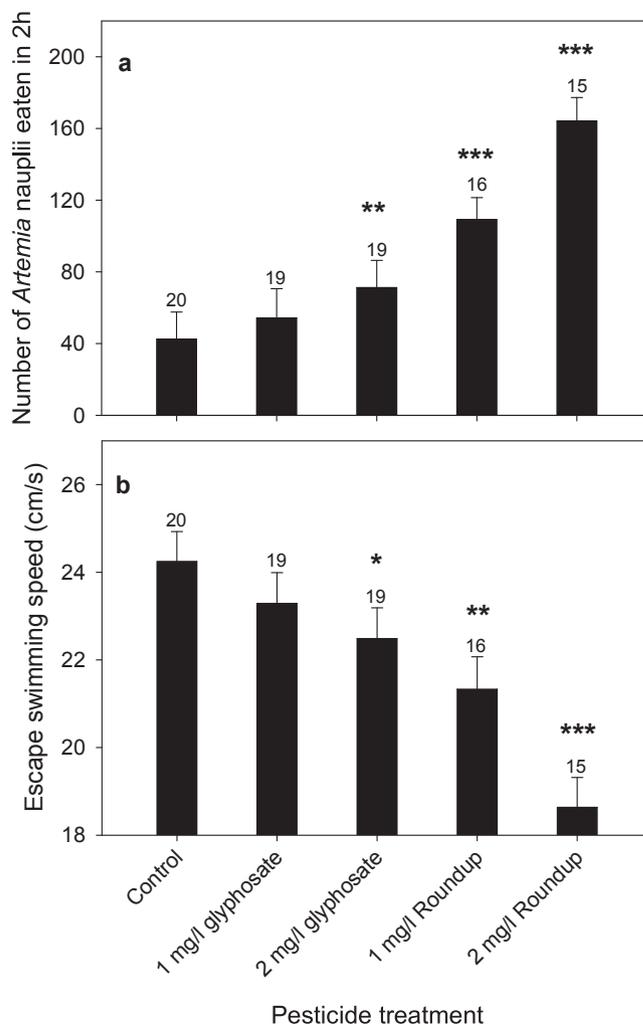


Fig. 2. Mean (with 1 SE) (a) food intake and (b) escape swimming speed as a function of pesticide exposure of *Coenagrion pulchellum* larvae. Significant differences from the control based on Dunnett post hoc tests, performed when the Ordered Heterogeneity test indicated a significant pesticide effect, are coded with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Numbers above the bars represent the number of replicates.

Both Roundup concentrations reduced the muscle mass ($F_{2,48} = 26.74$, $p < 0.001$), while glyphosate had no effect ($F_{2,54} = 0.49$, $p = 0.20$; Fig. 4a). The acetylcholinesterase activity increased in the presence of the pesticide (Roundup: $F_{2,48} = 4.59$, $p = 0.044$; glyphosate: $F_{2,54} = 3.23$, $p = 0.010$), except in animals exposed to 1 mg/l glyphosate (Fig. 4b).

4. Discussion

Pesticide exposure resulted both in increased mortality and in sublethal effects on life history, behaviour and physiology. More importantly, although both glyphosate and Roundup caused adverse effects, there were strong differences in toxicity with the active compound glyphosate being less harmful than an equivalent level of the commercial formulation Roundup. We will first discuss in general the pesticide effects and afterwards zoom in on the differences between glyphosate and Roundup.

4.1. Pesticide effects

The high Roundup concentration caused a significant reduction in survival of the damselfly larvae during the exposure period. Importantly, the concentrations used in this experiment are realistic

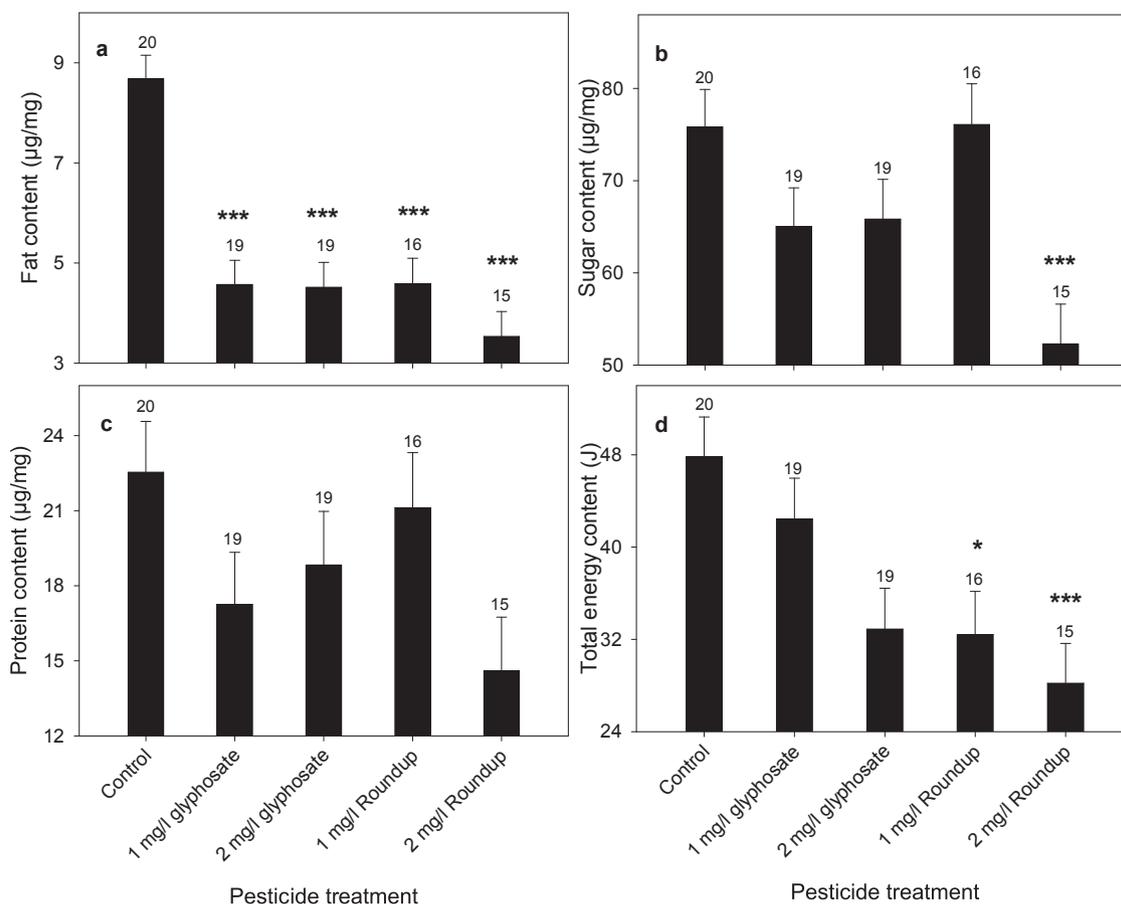


Fig. 3. Mean (with 1 SE) levels of energy storage molecules: (a) fat content, (b) sugar content, (c) protein content and (d) total energy content as a function of pesticide exposure of *Coenagrion pulchellum* larvae. Significant differences from the control based on Dunnett post hoc tests, performed when the Ordered Heterogeneity test indicated a significant pesticide effect, are coded with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Numbers above the bars represent the number of replicates.

concentrations in freshwater ecosystems (Thompson et al., 2004), indicating that in nature this endangered species in Flanders (De Knijff et al., 2006) is at risk of getting locally extinct with the current pesticide use. Lethal effects of ecologically relevant Roundup concentrations have also been reported for other non-target aquatic taxa (e.g. tadpoles: Relyea, 2005; Moore et al., 2012).

A nominal concentration of 2 mg/l glyphosate and Roundup reduced the larval growth rate. A decrease in growth rate following glyphosate exposure has been described before in several species (e.g. crayfish: Frontera et al., 2011; frogs: Paetow et al., 2012) including damselfly larvae (Janssens and Stoks, 2013). As the food intake increased when exposed to glyphosate and Roundup (as in Janssens and Stoks, 2012), behaviour cannot explain the growth reduction. Instead, energy was likely allocated to other processes away from growth; for example, to the upregulation of the expression of stress proteins (Janssens and Stoks, 2013). This may also explain the observed reductions in energy storage and muscle mass.

The pesticide-exposed animals had a lower escape swimming speed than the control larvae. This is expected to result in an increased vulnerability to predation as damselfly larvae swim away to escape and avoid being captured during an attack (Stoks and McPeck, 2006; Strobbe et al., 2009). The pesticide-induced lower swimming speed can be explained by the associated reduction in muscle mass and energy content. Although many studies reported lower escape performance under pesticide exposure (e.g. endosulfan: Trekels et al., 2012; chlorpyrifos: Dinh Van et al., 2014), studies on glyphosate-based herbicides including Roundup did not observe such a lower escape performance (e.g. tadpoles: Wood and Welch, 2015; salamanders: Levis et al., 2016).

Previous studies with a shorter exposure time (24 h – 96 h) found

an inhibition of acetylcholinesterase activity when exposed to glyphosate-based herbicides (e.g. Gluszcak et al., 2007). Instead, AChE activity was higher in the damselfly larvae that were exposed for 7 days to the pesticide in the current experiment. Given that chronic (26 d) exposure at similar glyphosate concentrations (1–6 mg/l) in mussels also did not result in an inhibition of AChE (Iummato et al., 2013), the inhibition of AChE may be transient and depend upon the exposure duration.

4.2. Comparison between glyphosate and Roundup

Our results consistently indicate that at similar levels of the active compound, Roundup was more toxic than glyphosate with negative effects on survival, behaviour and most of the physiological traits being present at lower concentrations (food intake, escape swimming speed) or even only present (survival, sugar and energy content and muscle mass) following Roundup exposure. This extends the observations in other aquatic animals of a higher sensitivity towards Roundup than to glyphosate in terms of survival (e.g. tadpoles: Howe et al., 2004; Moore et al., 2012; waterfleas: Tsui and Chu, 2003). This pattern has been attributed to the toxicity of the surfactant POEA that is added to Roundup in order to increase the absorption of the active ingredient in plants (Giesy et al., 2000). Especially in aquatic animals the contribution of the surfactant POEA to the total Roundup toxicity can be very high (e.g. Tsui and Chu, 2003).

So far, no other studies compared the toxicity of glyphosate and Roundup in terms of behaviour. We here identified stronger effects of Roundup than glyphosate on the escape swimming speed (decrease) and foraging activity (increase). This is expected to have important fitness effects in terms of an increased vulnerability to predation.

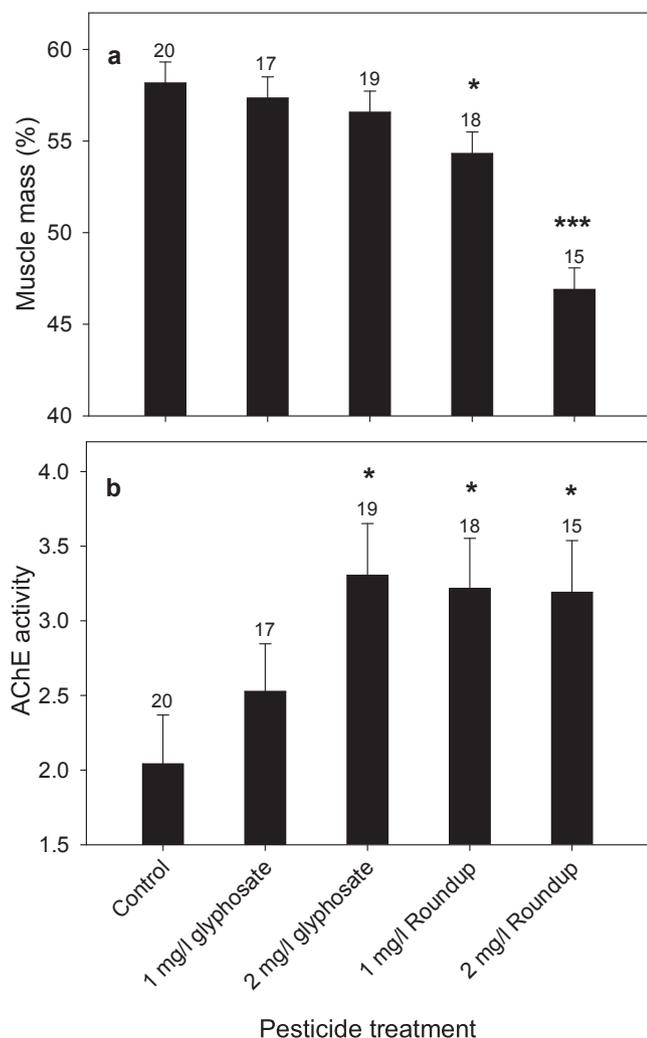


Fig. 4. Mean (with 1 SE) (a) muscle mass and (b) AChE activity as a function of pesticide exposure of *Coenagrion pulchellum* larvae. Significant differences from the control based on Dunnett post hoc tests, performed when the Ordered Heterogeneity test indicated a significant pesticide effect, are coded with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Numbers above the bars represent the number of replicates.

Indeed, in damselfly larvae a higher activity will increase the encounter rates with predators (Stoks et al., 2005), and a lower escape speed will increase the chance of being caught when attacked (Strobbe et al., 2009). Studies only focusing on lethal endpoints may therefore considerably underestimate the impact of Roundup in damselfly larvae and other prey organisms.

The stronger effects of Roundup on behaviour can be explained physiologically. Indeed, only Roundup caused a decrease in total energy content and muscle mass. Also in the only other study directly comparing energy storage between glyphosate and glyphosate-based herbicides, only the mixture of glyphosate and POEA reduced the protein content in the muscles and the total glycogen content of crayfish (Frontera et al., 2011).

4.3. Conclusions and implications for risk assessment

We compared the toxicity of Roundup and its active compound glyphosate for the damselfly *C. pulchellum*. This species is endangered in Flanders where it typically occurs in nature reserves where the use of pesticides is forbidden. However, for glyphosate-based herbicides an exception was made to control invasive plant species such as the black cherry (Vlaamse Gewest, 2013). Our results convincingly show that applying glyphosate and especially its commercial formulations in

nature reserves may be detrimental for the locally present endangered damselflies. The here observed combination of direct lethal and sublethal fitness reductions are expected to strongly negatively impact damselfly populations and likely aquatic biodiversity.

Although several studies have demonstrated that Roundup is more toxic than glyphosate in terms of mortality for non-target species (e.g. Howe et al., 2004; Moore et al., 2012; Tsui and Chu, 2003), much less information is available about sublethal effects at environmentally realistic concentrations (but see Howe et al., 2004). We could for the first time show that not only for several physiological traits (energy content and muscle mass) but also for two ecologically relevant behaviours (food intake and escape swimming) Roundup was the most toxic compound, thereby critically extending our understanding of the impact of Roundup versus glyphosate on non-target organisms. Ecotoxicological studies comparing the toxicity of active ingredients and their commercial formulations typically ignore behavioural effects (Cox and Sorgan, 2007; Kroon et al., 2015; Mansano et al., 2016; but see Beggel et al., 2010). Yet, behavioural effects may not only be more sensitive and detectable at lower concentrations, they may also be highly ecologically relevant and shape population fitness and as such may play an important role when extrapolating laboratory studies to field settings where natural enemies occur (Dell’Omo, 2002; Relyea and Hoverman, 2006).

Notably, glyphosate was not harmless either (in contrast to Howe et al., 2004): at a realistic concentration of 2 mg/l it reduced the growth rate, the escape swimming speed and the fat storage. As larval growth rate will determine the mass at emergence, which is strongly linked with reproduction, hence fitness (Stoks and Cordoba-Aguilar, 2012), this most likely will negatively impact the population dynamics. Also the reduction in escape performance can have severe implications as it will increase mortality by predation (Stoks and McPeck, 2006; Strobbe et al., 2009).

By demonstrating a negative impact of both the commercial product Roundup and the active compound glyphosate on fitness-related traits, our study matches a recent study on fish demonstrating that both Roundup and glyphosate disturb growth and reproduction (Webster et al., 2014). Together with this study, our results indicate that the toxicity of Roundup cannot be fully attributed to the surfactant POEA. This is important as it indicates that also the new generation of glyphosate-based herbicides likely will have adverse effects on non-target aquatic organisms (in contrast with the predictions made by Howe et al., 2004).

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