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The surfactant polyethoxylated tallowamine (POEA) reduces lifespan and inhibits fecundity in *Drosophila melanogaster- In vivo* and *in vitro* study

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ABSTRACT

In order to develop an understanding of the role of adjuvants in a popular glyphosate-based herbicide – Roundup® Concentrate Plus (RCP), on non-target organisms, the effects of pure glyphosate [*N*-(phosphonomethyl)-glycine], RCP and a non-ionic surfactant – polyethoxylated tallowamine (POEA) were studied in the fruit fly *Drosophila melanogaster*. Acute exposure to sub-lethal concentrations of RCP (15 μg/mL) and POEA (45 μg/mL) reduced ($p < 0.001$) lifespan of female flies compared to untreated controls or glyphosate (100 μg/mL). Negative geotaxis responses in female flies were reduced ($p < 0.05$) following acute exposure to sub-lethal concentrations of RCP and POEA whereas glyphosate did not significantly affect this response compared to untreated flies. Acute exposure to sub-lethal concentrations of RCP and POEA elevated ($p < 0.05$) protein carbonyl levels while markedly ($p < 0.01$) inhibiting carbonyl reductase activity whereas glyphosate treatment did not significantly affect protein carbonyl levels or carbonyl reductase activity. Fecundity was reduced ($p < 0.05$) following exposure to sub-lethal concentrations of RCP and POEA whereas glyphosate did not affect fecundity. *In vitro* treatment of ovarian stem sheath (OSS) cells with sub-lethal concentrations of RCP and POEA revealed decreased cell viability and enhanced caspase activity indicative of pro-apoptotic processes after 48 h compared to untreated controls. Glyphosate however was non-toxic at the concentration used. The results suggest that RCP and the surfactant POEA are more toxic than pure glyphosate and inhibit fecundity in *Drosophila* by impairing cell viability through enhanced apoptosis.

1. Introduction

Glyphosate (*N*-(phosphonomethyl)-glycine) is the active compound found in over 750 broad-spectrum herbicides widely used across the world for weed control (Myers et al., 2016). Since its first commercialization in 1974, its use has grown to dominate the herbicide market (Duke, 2018). The use of glyphosate-based herbicide (GBH) has increased rapidly with development of genetically modified crops engineered to be resistant to glyphosate (Green, 2009). Glyphosate competitively inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) that subsequently interfere with biosynthetic pathway of the essential aromatic amino acids phenylalanine, tyrosine and tryptophan and other secondary metabolites in plants through the shikimate pathway (Tomlin, 2006). While glyphosate is best known for its direct inhibitory effects on EPSPS in plants, the shikimate enzymatic pathway is also present in most bacteria. Interestingly, every vertebrate (and invertebrate) organism is the host for billions of microorganisms (mostly bacteria), collectively termed the microbiota. Several studies also show that generic GBH exposure can affect gut microbiota in poultry, rats and honey bees leading to dysbiosis (Shehata et al., 2013; Lozano et al., 2018; Motta et al., 2018). Moreover, there has been extensive research on GBH as well as glyphosate as an active ingredient for its properties to produce adverse effects on human health and ecosystems (Williams et al., 2000; Govindarajulu, 2008). Animal exposure to this herbicide can lead to adverse effects, such as endocrine disruption, behavioral disorders and oxidative stress (Larsen et al., 2012; Sandrini et al., 2013; Mottier et al., 2015). The generation of oxidative stress has been linked with exposure to glyphosate and GBH in non-target organisms such as the fresh water oligochaete, fishes, oysters and the fruit fly *Drosophila* (Contardo-Jara et al., 2009; Glusczak et al., 2007; Sinhorin et al., 2014; Mottier et al. 2015; de Aguiar et al., 2016). Generation of protein carbonyls as a product of lipid peroxidation can be one of the consequences of exposure to pesticides and is often measured as a biomarker of oxidative stress (Parvez and

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Raisuddin, 2005; Bednářová et al., 2017). It has been reported that GBH mechanism of toxicity can also act through inhibition of acetylcholinesterase activity in fishes and mussel (Modesto and Martinez, 2010; Sandrini et al., 2013). Inhibition of this enzyme results in neurotransmitter accumulation which subsequently interfere with the cholinergic pathway, which causes muscle hyperstimulation and disturbance in movement. Consequently, behavioral processes can be compromised (Bretaud et al., 2000). It has also been shown that GBH may negatively influence fecundity and fertility in neuropteran species (Schneider et al., 2009).

Herbicide or pesticide formulations always contain mixtures of an active principle with adjuvants/surfactants to enable effective penetration into target tissues. While most of the adjuvants are classified as inert, it is essential to know the differential toxicity of the various compounds of the formulations. The commonly used Roundup® Concentrate Plus herbicide contains 18% glyphosate isopropylamine salt (~143 g/L), 0.73% diquat dibromide and 81.27% other ingredients. The other ingredients in this case are the adjuvants/surfactants which are often confidential proprietary information (Defarge et al., 2018). The most common adjuvants in herbicide formulations are surfactants which are non-ionic (Foy, 1996). Of these, the most widely used are the polyethoxylated tallowamines (POEA), adjuvants used in many Roundup® formulations as MON 0139 or MON 0818 (Monsanto Company – now acquired by Bayer, St Louis, MO, USA). The oxide to tallowamine ratio varies in range from 5:1 to 25:1. As the ratio increases, the solubility of POEA in water increases (Brausch and Smith, 2007). Toxicity of POEA has been documented in amphibians, fishes and aquatic invertebrates, mammals and even in human cells by several studies as exceeding the toxicity of glyphosate (Folmar et al., 1979; Howe et al., 2004; Brausch et al., 2007; Brausch and Smith, 2007; Mesnage et al., 2013; Séralini, 2015; Chłopecka et al., 2017). This body of work demands a more intensive study of the possible toxicological and pharmacological effects of such adjuvants or surfactants on non-target organisms.

In this study we used *Drosophila melanogaster* as a model non-target system since it has been utilized previously as an effective tool in toxicological tests and facilitates scientific research in many areas (Rand, 2010; Peterson and Long, 2018). We investigated the toxicity (72 h LC_{25} and LC_{50}) and effects of acute and chronic exposure of glyphosate as a pure chemical [*N*-(phosphonomethyl)-glycine], Roundup® Concentrate Plus as the herbicide formulation, as well as the surfactant POEA on lifespan, negative geotaxis response, fecundity, oxidative damage to proteins and pro-apoptotic events in *D. melanogaster*.

2. Material and methods

2.1. Chemicals

Glyphosate [*N*-(phosphonomethyl)- glycine] CAS Number: 1071-83-6, MW. 169.07 was obtained from Sigma-Aldrich (now Millipore-Sigma) (St Louis, MO, USA). Roundup® Concentrate Plus-hereafter referred to as RCP (Bayer, St Louis, MO, USA) was obtained locally from Walmart. Polyethoxylated tallow amine (POEA) 15:1 CAS Number 61791-26-2 was obtained from Xi'an Benherb Biotech Co.Ltd., PR China. The surfactant polyoxyethylene sorbitan monolaureate (20 POE) (~10% solution in water) was obtained from Millipore-Sigma, St. Louis, MO, USA).

2.2. Drosophila stocks and husbandry

For this study laboratory cultures of the wild type (w^{1118}) *D. melanogaster* were used. They were maintained on a diet of agar (1%), cornmeal (6.25%), molasses (6.25%) and active dry yeast (Red star, 6.25%) and in a 12 h-light/12 h-dark cycle (≈2000 lux) at 25 °C. The parental generation was regularly flipped on new diets to harvest the F1 generations. Male and females were separated after eclosion and used for testing. Other than longevity, the assays were conducted on 5 day old adults.

2.3. Concentration-mortality response studies

Glyphosate was dissolved in a small volume of dimethyl sulfoxide (DMSO) before diluting to a stock solution with water. Concentration-mortality studies were conducted using six concentrations ranging from 0 to 6000 μg/mL in 5% sucrose solution. RCP was diluted with water as stock and concentration-mortality studies were conducted using nine concentrations ranging from 0 to 10,000 μg/mL in 5% sucrose solution. POEA (15:1) was diluted in water and seven concentrations ranging from 0 to 10,000 μg/mL was prepared in 5% sucrose solution. For establishment of LC_{25} and LC_{50} of the different chemicals, testing was done in three cohorts of 30 male and female flies separately with three independent biological repetitions. The flies were kept in glass vials with cotton plugs and a filter paper at the bottom. Chronic exposure over the testing period was done by pipetting sucrose-solutions with varying concentrations on the filter papers (100 μl) with a frequency of 12 h of replenishment. This ensured both feeding by the flies as well as topical exposure. Mortality was scored every 12 h in a total span of 72 h of testing.

2.4. Lifespan measurements

Lifespan measurements were conducted in three cohorts of ca. 70 females respectively for each treatment group, including untreated controls. Following a two time acute exposure to the sub-lethal (around the determined LC_{25}) concentration of chemical (Glyphosate (100 μg/mL), RCP (15 μg/mL) and POEA (45 μg/mL)) over the period of 24 h, they were kept in polypropylene bottles (8 oz. Genessee Scientific, San Diego, CA, USA) with tissue culture dishes (60 mm Falcon Primeria from Becton Dickinson Labware, Franklin Lakes, NJ, USA) serving as lids and containers for the regular diet (15 mL). The diet dishes were replaced on alternate days after tapping the flies down to the bottom of the bottle. The mortality was recorded daily.

2.5. Rapid iterative negative geotaxis assay

The negative geotaxis of the different treatment groups was assessed using rapid iterative negative geotaxis (RING)-assay (Gargano et al., 2005) at room temperature (25 ± 1 °C). For each iteration a maximum of 30 flies per tube were used for the assay. The flies were loaded into the tubes of a RING apparatus after a 24 h acute exposure period to sub-lethal concentrations of the chemicals (Glyphosate (100 μg/mL), RCP (15 μg/mL) and POEA (45 μg/mL)) and were given an acclimatization period of 5 min. Untreated controls were run in parallel. The apparatus was then rapped sharply against a table to initiate the negative geotaxis response. The climbing movements were recorded as a digital video. Three consecutive trials were done with at least 30 s rest in between for recovery. Testing was done on female flies of each treatment group with a total amount of tested flies of ca. 150 flies for each group (five independent repetitions). The number of flies that passed the half-way mark (5 cm) of the tubes after 5 s was analyzed and the performance was averaged over all trials of each testing group and their respective biological repetitions.

2.6. Fecundity assay

Fecundity was assessed as number of eggs laid daily by individual females during the first 10 days following mating after exposure to the chemical. Each treatment was acute exposure to sub-lethal concentrations of the chemicals viz. Glyphosate (100 μg/mL), RCP (15 μg/mL) and POEA (45 μg/mL) for 24 h. Untreated controls were run in parallel. Reciprocal treatments were as follows: Males treated and females untreated, males untreated and females treated, both males and females

treated. Cohort size was 6 virgin females and 6 males for each technical replicate repeated thrice in three biological repetition (three independent repetitions with three technical replicates in each repeat). Diets were changed daily, and the number of laid eggs were counted daily under a stereo-microscope. The average number of laid eggs per female was assessed and recorded as fecundity. Care was taken to exclude female flies from the analyses that died during testing.

2.7. Protein carbonyl assay

The amount of protein carbonyls was quantified as decribed previously (Bednářova et al., 2017) in whole-body homogenates (25 female flies in each replicate in three bioreplicates) after 24 h of exposure to sub-lethal concentrations of chemicals: Glyphosate (100 μg/mL), RCP(15 μg/mL) and POEA (45 μg/mL). Untreated samples were run in parallel. Briefly, samples were derivatized after reaction with 2,4 dinitrophenylhydrazine (DNPH) and results were expressed as nmol.mg^{-1} protein using an extinction coefficient of 22,000 M^{-1} cm^{-1} at absorbance maxima of 370 nm in a H1M Synergy plate reader (BioTek, Winooski, VT, USA). Bovine serum albumin (BSA) standard curve was used for protein concentrations in guanidine solutions (Abs 280 nm). Protein carbonyl values were corrected for interfering substances by subtracting the Abs 370 nm mg^{-1} protein measured in control samples. Data is represented as mean of three independent replicates.

2.8. Carbonyl reductase assay

Whole body homogenates (25 female flies per replicate) of flies (untreated and treated with sub-lethal concentrations of Glyphosate (100 μg/mL), RCP (15 μg/mL) and POEA (45 μg/mL) collected 24 h after treatment) were prepared in 100 mM sodium phosphate buffer. Carbonyl reductase activity was measured as described by Botella et al. (2004). Briefly, carbonyl reductase activity was measured spectrophotometrically in 100 mM sodium phosphate, 100 mM NaCl, pH 7.4 in the presence of 0.1 mM NADPH at 25 °C in 96 well microplates in a Synergy H1M plate reader in the kinetic mode (BioTek, Winooski, VT, USA). NADPH consumption (Extinction coefficient at 340 nm 6.22 mM^{-1} cm^{-1}) was monitored using 0.4 mM of *p*-nitrobenzaldehyde as substrate. Negative controls used just 100 mM sodium phosphate buffer instead of the enzyme. One unit of carbonyl reductase was defined as the amount of enzyme that catalyzed the consumption of 1 μmol NADPH per minute. Protein concentration was determined by bicinchoninic acid reagent using BSA as standard.

2.9. Cell culture, viability and caspase assay for apotosis

For *in vitro* studies on toxicity of the chemicals to ovarian cells, ovarian stem sheath (OSS) cells were obtained from Drosophila Genomics Resource Center, Indiana University (https://dgrc.bio.indiana.edu/(Stock 190)) and the culture media for OSS cells was prepared and cells grown in 6-well dishes as described (Niki et al., 2006). The doubling time for OSS is approximately 45 h. Exposure of OSS cells to various sub-lethal concentrations of chemicals viz. Glyphosate (100 μg/mL), RCP (15 μg/mL) and POEA (45 μg/mL) was achieved by diluting stock solutions with media to obtain desired concentrations of chemicals in the medium. Additionally, we also used another surfactant polyoxyethylene sorbitan monolaureate (20-POE) (~10% solution in water diluted to a final concentration in culture media ~45 μg/mL) which is widely used in pesticides and as food additives to check for its toxicity. We checked the viability of OSS cells using trypan blue (0.04% final concentration, 5 min incubation at room temperature) as well as the MTT assay (20 μl/well) after 48 h of incubation. Untreated cells served as controls. While trypan blue incorporation was recorded in cells using hemocytometer counts, the MTT assay employed measuring absorbance of the formazan product at Abs 570 nm in a Synergy H1M plate reader (Biotek, Winooski, VT, USA). Photomicrograph of unstained cells for each treatment were taken using an inverted microscope (Vanguard, NY) for documenting morphological changes in cells associated with apoptotic events at 48 h of incubation.

The activation of caspases is an early non-reversible hallmark of cells committed to die, a caspase Asp-Glu-Val-Asp (DEVD)-based assay is routinely used to detect and quantify apoptosis (Zheng et al., 2005). This assay is based on the hydrolysis of the peptide substrate Ac-asp-glu-val-asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC), resulting in the release of the fluorescent 7-amido-4-methylcoumarin (AMC). Cells were harvested (in three independent replicates for each treatment), pelleted and homogenized in 40 μl of lysis buffer (50 mM Hepes, pH 7.5 containing 100 mM NaCl; 1 mM EDTA; 0.1% CHAPS; 10% sucrose; 5 mM DTT; 0.5% Triton X-100; 4% glycerol) and centrifuged at 13,000 g for 5 min at 4 °C. The supernatant was used to quantify caspase activity. Five microliters of extracts (3 mg/mLprotein) were incubated for 1 h at 27 °C with 25 mM Ac-DEVD-AMC (Sigma-Aldrich, St Louis, MO, USA) in lysis buffer with a final reaction volume of 50 μl. The specificity of the detection was controlled in a duplicate reaction pretreated for 15 min at 22 °C with 2.5 mM Ac-DEVD-CHO inhibitor (Sigma-Aldrich, St Louis, MO, USA). The fluorescence of this control reaction was subtracted from the test reaction. AMC fluorescence was determined by using a H1M synergy UV-VIS, fluorescence and luminescence plate reader (BioTek, Winooski, VT, USA) with the excitation and emission set at 360 nm and 460 nm, respectively. The concentration of the AMC released was calculated by using an AMC standard curve ranging from 100 nM to 20 mM. Protein concentrations in the various extracts were measured with a BCA assay. Caspase activity was expressed as nanomoles of AMC per second per milligram of protein.

2.10. Statistical analyses

Each experiment was conducted with three independent biological replicates with at least three technical replicates. For concentration-mortality studies, the mortality data was analyzed for LC_{50} and LC_{25} (sub-lethal) values using Proc Probit (SAS ver 9.2) (Table 1, Fig. 1). For lifespan measurements Kaplan-Meier survival curves were generated and Log-rank (Mantel-Cox) tests were conducted using GraphPad Prism (v.5.01, San Diego, CA, USA) (Fig. 2). All other data were subjected to One-Way ANOVA with Tukey's multiple comparison test for analysis of statistical significance using GraphPad Prism (v.5.01, San Diego, CA, USA) (Figs. 3–6). Graphs were developed in GraphPad Prism and are represented as mean of three independent replicates with SD (Figs. 3 and 5) or SEM (Figs. 4 and 6).

Table 1
Lethal concentrations (LC_{25} and LC_{50}) of glyphosate [*N*-(phosphonomethyl)-glycine], Roundup® Concentrate Plus (RCP) and the non-ionic surfactant polyethoxylated tallowamine (POEA) 15:1 to *D. melanogaster* based on concentration-mortality responses.

Chemical	Sex	Lethal concentrations		95% Fiducial limits	
				Lower	Upper
Glyphosate	Male	LC_{25}	91.04 μg/mL	5.6	1487.7
		LC_{50}	4771 μg/mL	264.9	70750.6
	Female	LC_{25}	140.9 μg/mL	24.7	801.5
		LC_{50}	5146 μg/mL	905.1	29259.8
RCP	Male	LC_{25}	14.4 μg/mL	3.1	66.8
		LC_{50}	587.7 μg/mL	126.4	2731.9
	Female	LC_{25}	20.2 μg/mL	4.3	93.8
		LC_{50}	774.4 μg/mL	166.36	3600.0
POEA	Male	LC_{25}	43.9 μg/mL	8.4	229.1
		LC_{50}	1044.9 μg/mL	200.3	5450.4
	Female	LC_{25}	58.6 μg/mL	11.2	306.1
		LC_{50}	1322.6 μg/mL	253.6	6898.8

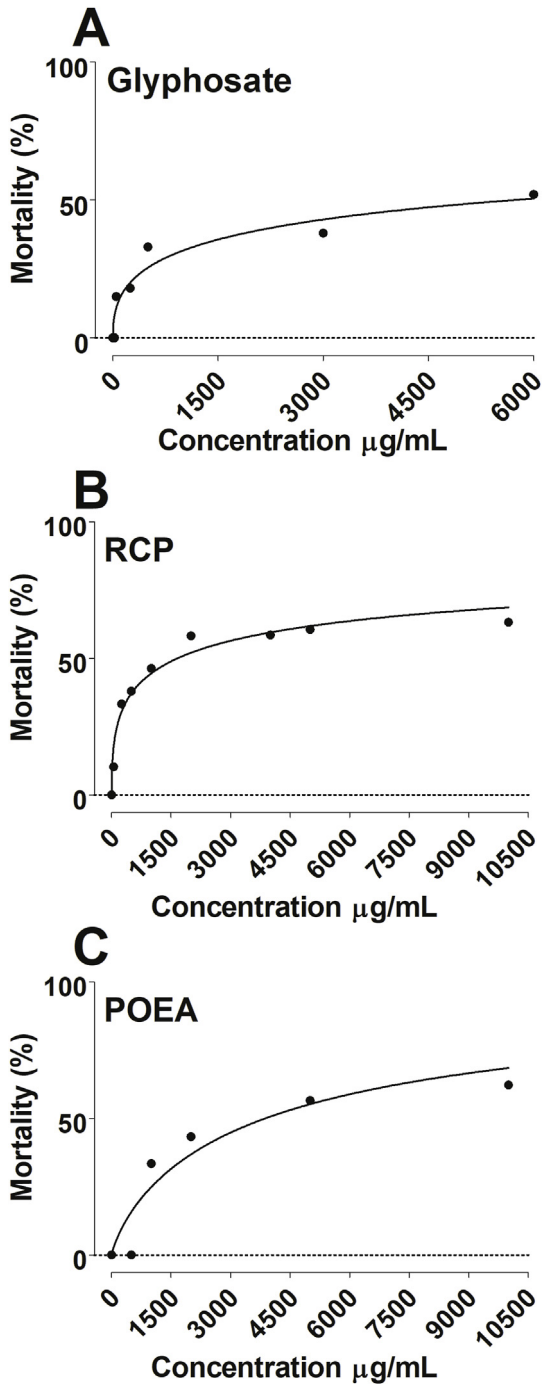

Fig. 1. Concentration-mortality (%) responses of female *D. melanogaster* to (A) Glyphosate (0, 25, 50, 500, 3000 and 6000 µg/mL), (B) Roundup® Concentrate Plus (RCP) (0, 50, 250, 500, 1000, 2000, 4000, 5000 and 10,000 µg/mL) and (C) polyethoxylated tallow amine (POEA, 15:1) (0, 500, 1000, 2000, 4000, 5000 and 10,000 µg/mL) following chronic exposure for a period of 72 h. Mean mortality (%) data (n = 90 for each chemical and concentration) are represented as dots along with the best-fitted line.

3. Results

3.1. Concentration-mortality assays

Concentration-mortality responses were conducted on male and female flies separately and the female response curve has been depicted in Fig. 1. The male flies showed a similar response albeit the mortality was slightly higher than females for each concentration of chemical

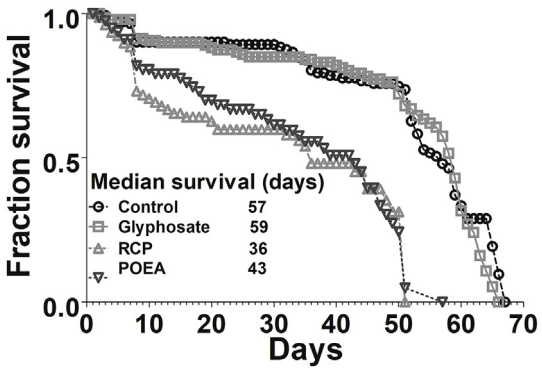

Fig. 2. Kaplan-Meier survival curves of w^{1118} wild type female *D. melanogaster* following acute exposure (for 24 h) to sub-lethal concentrations of glyphosate (100 µg/mL), Roundup® Concentrate Plus-RCP (15 µg/mL) and polyethoxylated tallowamine -POEA (45 µg/mL). Untreated females served as controls. Following exposure, the flies were maintained under 12 h light:dark (LD) cycles and ad libitum feeding conditions till end of experiment. Log-rank (Mantex-Cox test) revealed significant differences ($p < 0.0001$) between untreated control and/or glyphosate treated flies with RCP and/or POEA treated flies. Median longevity was obtained from three independent replicates of 70 females for each treatment (n = 210).

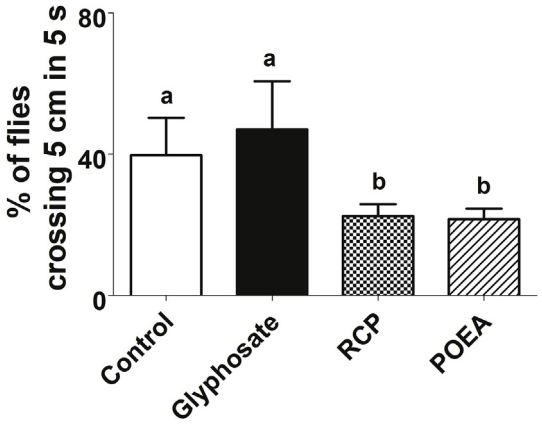

Fig. 3. Rapid iterative negative geotaxis (RINGA) assay of 5-day old female *D. melanogaster* following acute exposure to sub-lethal concentrations of glyphosate (100 µg/mL), Roundup® Concentrate Plus-RCP (15 µg/mL) and polyethoxylated tallowamine-POEA (45 µg/mL). Untreated females served as controls. Bars represent the percentage of flies crossing the 5 cm mark in a 5 s time interval after start of the assay. Error bars represent the SD (n = 150).
Data were analyzed by one-way ANOVA following transformation of percentage data. A non-significant Shapiro-Wilk's W test confirmed normality of transformed data. Tukey's multiple comparison post-test was employed for statistical significance of treatments. Bars with different superscripts (small alphabets) are significantly different at $p < 0.05$.

(data not shown). The average LC_{25} and LC_{50} values for females was slightly higher than the LC_{25} and LC_{50} values determined for males with various chemicals but within the fiducial limits (Table 1). Concentration-mortality response studies revealed that RCP has lower LC_{50} values (males: 587.7 µg/mL; females 774.4 µg/mL) compared to the pure ingredient glyphosate (LC_{50} 4771 µg/mL for males and 5146 µg/mL for females) or the non-ionic surfactant POEA (15:1, LC_{50} 1044.9 µg/mL for males and 1322.6 µg/mL for females) (Table 1, Fig. 1).

3.2. Acute exposure to RCP or POEA but not glyphosate reduces median longevity of females

Acute exposure to sub-lethal concentrations of the herbicide RCP (15 µg/mL) and POEA (45 µg/mL) significantly ($p < 0.001$) reduced the median survival of female *D. melanogaster* to 36 and 43 days

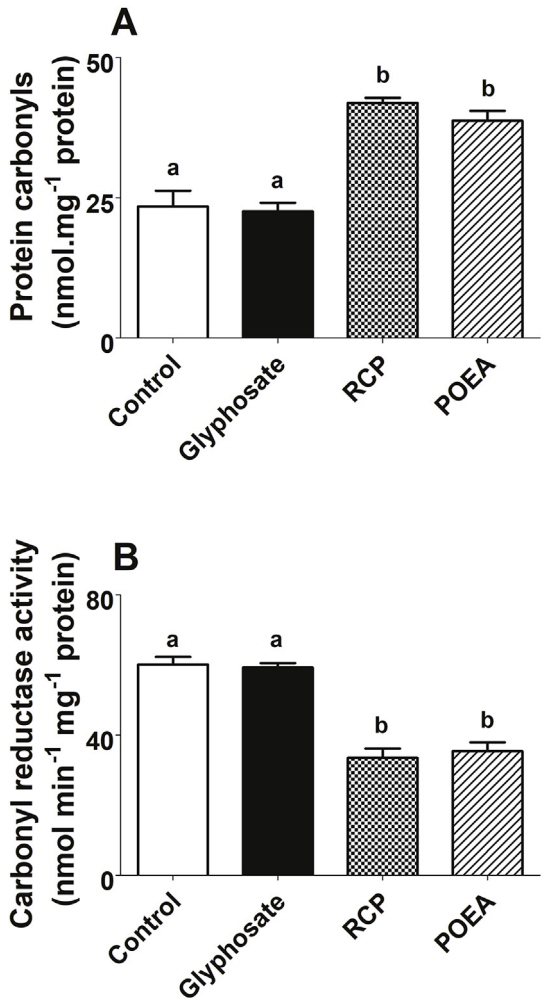

Fig. 4. Accumulation of total protein carbonyls (A) and carbonyl reductase activity (B) in 5 day old female *D. melanogaster* following acute exposure to sub-lethal concentrations of glyphosate (100 μg/mL), Roundup® Concentrate Plus-RCP (15 μg/mL) and polyethoxylated tallowamine-POEA (45 μg/mL). Untreated females served as controls. Data are mean ± SEM of assays from three independent bio-replicates. Bars with different superscripts are significantly different at $p < 0.05$ using one-way ANOVA with Tukey's multiple comparison post-test.

respectively compared to untreated controls which had a median survival of 57 days (Fig. 2). Acute exposure to pure glyphosate (100 μg/mL) on the other hand did not impair median survival (59 days) which was not significantly different from untreated controls (Fig. 2).

3.3. Negative geotaxis response is significantly impaired upon exposure to RCP or POEA but not glyphosate

Exposure to sublethal concentrations of RCP (15 μg/mL) or POEA (45 μg/mL) impaired ($p < 0.05$) the negative geotaxis ability of female flies compared to untreated controls or glyphosate exposed flies (Fig. 3). Interestingly, pure glyphosate (100 μg/mL) did not have a significant impact on this response and the results did not differ significantly from untreated controls (Fig. 3).

3.4. RCP and POEA but not glyphosate, significantly elevate accumulation of carbonylated proteins and inhibit carbonyl reductase activity

Exposure to RCP (15 μg/mL) and POEA (45 μg/mL) resulted in a 1.7-fold increase on an average ($p < 0.05$) in the level of carbonylated proteins compared to untreated controls or glyphosate (100 μg/mL)

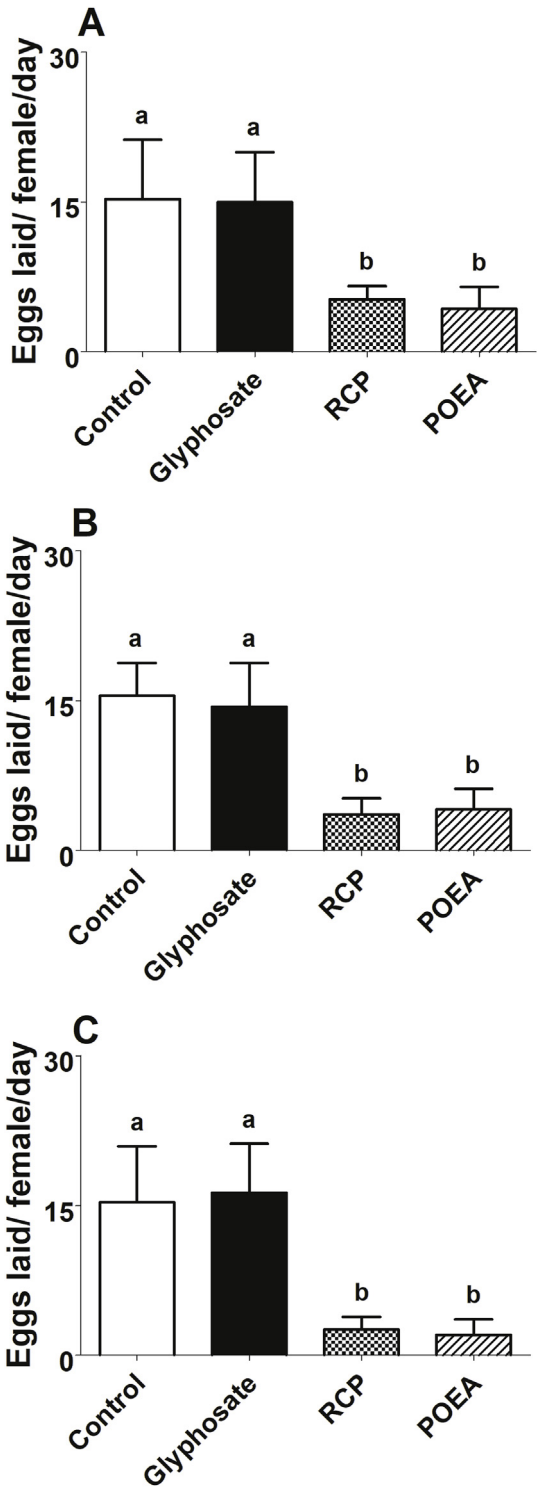

Fig. 5. Fecundity represented as mean number of eggs laid per day per female fly following acute exposure to sub-lethal concentrations of glyphosate (100 μg/mL), Roundup® Concentrate Plus-RCP (15 μg/mL) and polyethoxylated tallowamine-POEA (45 μg/mL). Untreated females served as controls. (A) Treated males mated with untreated females (B) Untreated males with treated females (C) Both males and males and females were treated and mated. Data was transformed because variance was different among groups prior to statistical testing. Data are represented as mean ± SD of mean ($n = 144$) and bars with different superscripts (small alphabets) are significantly different at $p < 0.05$ using one-way ANOVA with Tukey's multiple comparison post-test.

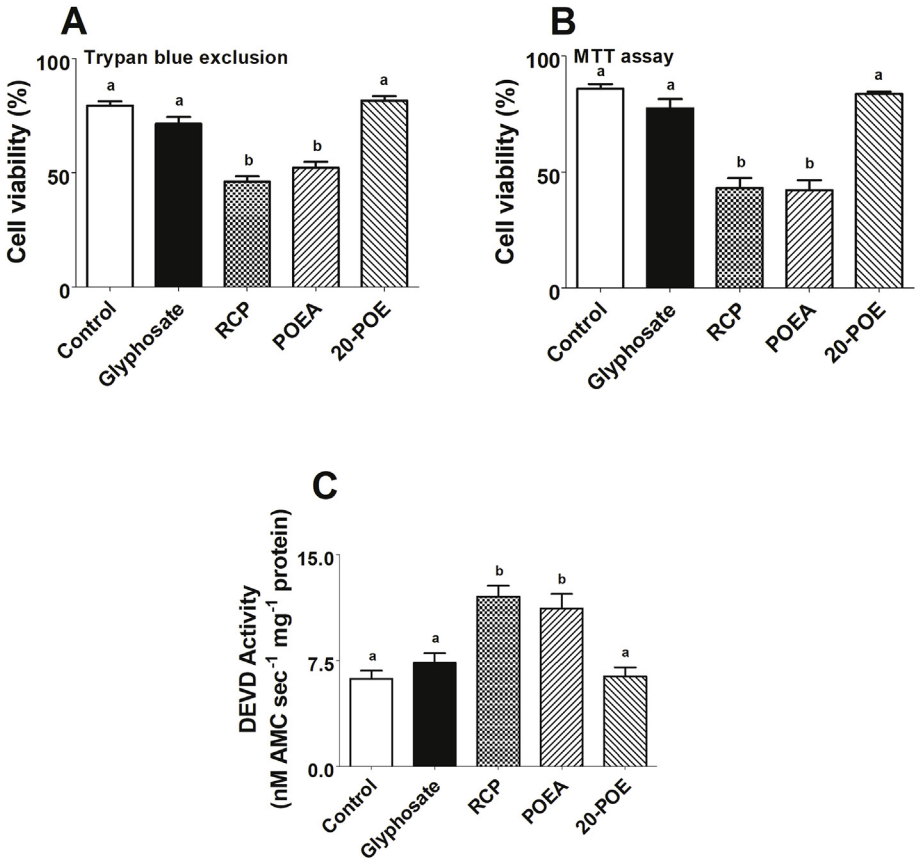

Fig. 6. Ovarian stem sheath (OSS) cell viability assays using (A) Trypan blue and (B) MTT following treatment with glyphosate (100 µg/mL), Roundup® Concentrate Plus-RCP (15 µg/mL), polyethoxylated tallowamine -POEA (45 µg/mL) and 20 POE (45 µg/mL). Cell viability was recorded at 48 h following treatment. Data are represented as mean cell viability % ± SEM from three independent replicates of cell cultures. (C) DEVD activity of OSS cells in response to treatment with glyphosate (100 µg/mL), RCP (15 µg/mL), POEA (45 µg/mL) and 20 POE (45 µg/mL). Data are represented as mean ± SEM of three independent assays. Bars with different superscripts (small alphabets) are significantly different at $p < 0.05$ following one-way ANOVA with Tukeys multiple comparison post-test.

treatment alone (Fig. 4 A). There was no difference recorded in the levels of protein carbonyls between untreated controls and pure glyphosate exposure (Fig. 4 A).

Carbonyl reductase activity was concomitantly decreased upon exposure to RCP or POEA ($p < 0.05$) compared to untreated controls whereas glyphosate exposure did not alter carbonyl reductase activity compared to untreated controls (Fig. 4 B).

3.5. Fecundity is significantly inhibited upon exposure to RCP or POEA but not glyphosate

In all experimental treatments, exposure to either RCP or POEA resulted in drastically ($p < 0.001$) reduced fecundity (Fig. 5A–C). No difference was recorded between fecundity of RCP exposed groups or POEA exposed groups. Importantly, acute exposure to pure glyphosate did not affect fecundity when compared to untreated control flies (Fig. 5A–C).

3.6. RCP and POEA but not glyphosate reduce ovarian stem sheath (OSS) cell viability and enhance caspase activity indicative of apoptosis

Treatment with sub-lethal concentrations of both RCP or POEA significantly ($p < 0.01$) reduced cell viability compared to untreated controls as evidenced by trypan blue exclusion test (Fig. 6A) and the MTT assay (Fig. 6 B). Neither pure glyphosate nor 20-POE (another surfactant, used as a check) had any inhibitory effect on cell viability (Fig. 6 A, B).

To determine the cause of reduced cell viability, we checked for caspase activity and we noted that in both RCP or POEA treatments the caspase activity was significantly ($p < 0.05$) enhanced (Fig. 6 C) compared to untreated controls. On the other hand, the caspase activity in glyphosate or 20-POE exposed cells was not significantly different from untreated controls (Fig. 6 C). This was supported lack of morphological changes in OSS cells in case of untreated controls, pure glyphosate or 20-POE exposure (Supplemental Fig. S1 A, B, E) whereas in case of RCP and POEA treatments pro-apoptotic events such as cell-shrinkage, membrane blebbing and cell fragmentation were documented (Supplemental Fig. S1 C, D).

4. Discussion

The widely used Roundup® is an aqueous solution of isopropylamine salt of glyphosate with a polyethoxylated tallow amine surfactant. In Roundup® the surfactant used is either MON 0818 or MON 0139 (Smith and Oehme, 1992). In the Roundup® Concentrate Plus that was used in this study, glyphosate isopropylamine salt was 18% and this was 1.19 lbs/gallon which works out to 143 g/L or 143,000 µg/mL. The other ingredients account for 81.27% which would include the surfactant. It has been reported that surfactant in Roundup® formulations is present at 15% (Sawada et al., 1988).

This study specifically focused on evaluating the toxicity of the active ingredient glyphosate [*N*-(phosphonomethyl)-glycine] vis-à-vis the complete herbicide formulation Roundup® Concentrate Plus and the commonly used surfactant POEA. We employed the fruit fly *D. melanogaster* as the model non-target organism. Our results suggest that pure glyphosate is less toxic than the formulated RCP or the surfactant POEA. Previous *in vitro* and *in vivo* studies have also demonstrated that the toxicity of commercially available GBH is significantly more toxic than glyphosate itself (Howe et al., 2004; Contardo-Jara et al., 2009; El-Shenawy, 2009). Importantly, our results substantiate that the surfactant POEA may be more toxic than the active ingredient glyphosate corroborating other studies on non-target organisms (Brausch et al., 2007; Moore et al., 2012; Mesnage et al., 2013, 2014; Chlopecka et al., 2017).

To our knowledge, the impact of acute exposure of the RCP or its active ingredient glyphosate on the lifespan of *D. melanogaster* has been

demonstrated for the first time in this study. A significant reduction of lifespan was observed upon acute exposure to sub-lethal concentrations of RCP and the surfactant POEA but not the active ingredient glyphosate. Developmental defects on *Chrysoperla externa* (Neuroptera: Chrysopidae) have been reported earlier (Schneider et al., 2009). There are other studies which report on the impact of GBH or its active ingredient on life-history parameters in insects such as the Coleopteran *Eriopis connexa* – leading to drastic reductions in the weight of pupae, longevity, fecundity and fertility (Mirande et al., 2010), reduced emergence of the generalist endoparasitoid *Palmistichus elaeisis* (Cruz et al., 2017), reductions in egg number in the zoophytophagous *Podisus nigrispinus* (Zanuncio et al., 2018) and increasing insecticide resistance in the malarial vector *Anopheles arabiensis* (Oliver and Brooke, 2018).

The negative geotaxis assay was conducted as a measure of the flies' ability to recover from exposure to an external stressor (chemical) or reaction to it. Such assays are commonly used in the field of aging research in *D. melanogaster*, which has long recognized that measures of both healthspan (ability to recover from stress) and longevity are critical to understanding the biology of aging (Gargano et al., 2005). We hypothesized that these metrics (longevity and negative geotaxis) could also be used to assess health of the flies following acute exposure to the chemicals being tested. The negative geotactic behavioral ability has been shown to be sensitive to oxidative stress as well as decline with age (Gargano et al., 2005; Bednářová et al., 2018). Our rationale was that exposure to the tested chemicals would cause physiological changes resulting in performance deficits. We recorded a marked decline in the negative geotaxis response of flies when exposed to sub-lethal concentrations of RCP or the surfactant POEA but not the active ingredient glyphosate.

To investigate the physiological and biochemical underpinnings of the impact of RCP and POEA on longevity and negative geotaxis, we investigated a commonly used biomarker of oxidative damage to proteins – the protein carbonyl content along with the activity of the enzyme carbonyl reductase (EC 1.1.1.184). This enzyme catalyzes the reduction of a wide variety of carbonyl compounds including preventing formation of protein carbonyls and lipid peroxidation products such as 4-oxonon-2-enal (4ONE) (Botella et al., 2004; Martin et al., 2011). Oxidative stress has been reported to be associated with exposure to GBH in both mammals and flies (El-Shenawy, 2009; de Aguiar et al., 2016; Bali et al., 2019). We found elevated levels of protein carbonyls following acute exposure to sub-lethal concentrations of RCP and POEA but not glyphosate. It is possible that chronic long-term exposure to glyphosate could also result in oxidative stress, however, a short-term acute exposure to glyphosate did not appear to have any detrimental effect on *D. melanogaster*. Importantly, the effects of oxidative damage to proteins could be linked to a significant reduction in carbonyl reductase activity upon both RCP/or POEA treatment. No changes in carbonyl reductase activity relative to untreated controls were observed upon exposure to glyphosate.

Reproductive fitness is a broad, ecological measure of health. We conducted reciprocal treatments to investigate how fecundity is affected upon exposure to RCP or the surfactant POEA, or the active ingredient of RCP – glyphosate. We found that fecundity is significantly inhibited upon exposure of *D. melanogaster* (males alone, females alone or both males and females) to sub-lethal concentrations of RCP or POEA but exposure to glyphosate alone did not have an effect on fecundity. The fecundity upon exposure to Glyfoglex 48®) was studied in the Neuropteran *Chrysoperla externa* (Schneider et al., 2009). The authors reported that fecundity and fertility were deeply reduced upon exposure to Glyfoglex 48®. Also, most eggs from glyphosate-treated cohort looked abnormal, smaller than control, dehydrated and became black 2 d after oviposition. We do not discount that glyphosate alone at higher concentrations and in a chronic exposure regimen could likely result in impairment of fecundity in *D. melanogaster*, but this remains to be demonstrated. While there are several studies testing associations between glyphosate exposure and adverse effects on development of frog (*Xenopus laevis*), chicken embryos, and pre-natal development in rats (Paganelli et al., 2010; Dallegrave et al., 2007), data from existing epidemiological studies do not lend support to the notion that glyphosate is a human reproductive and developmental toxicant (de Araujo et al., 2016).

To generate a mechanistic understanding behind the reasons of decreased fecundity upon exposure to RCP or POEA, we decided to employ *Drosophila* ovarian stem sheath (OSS) cell cultures for an *in vitro* study. Treatment of OSS cells with sub-lethal concentrations of RCP or POEA significantly reduced cell viability as revealed by trypan blue exclusion test (where live cells do not uptake the trypan blue dye whereas dead cells do) (Strober, 2015). The decreased viability was linked to enhanced apoptosis, as documented morphologically as well as with enhanced caspase activity. Apoptosis is a genetically controlled cell death process essential for development and homeostasis by removing unwanted, damaged or harmful cells. Inappropriate activation of apoptosis after a proapoptotic challenge can lead to detrimental consequences. Based on the results of our study, we hypothesize that the RCP and surfactant POEA can generate oxidative stress (as revealed by protein carbonyl accumulation) which can lead to a proapoptotic situation enhancing cell death in reproductive tissues resulting in decreased fecundity. Interestingly another oxysorbic surfactant 20 POE did not demonstrate these negative effects.

5. Conclusions

Taken together, our results suggest the possibility that toxic effects of GBH formulations may be due to the surfactants/adjuvants such as POEA along with other formulants. Moreover, our results characterize the RCP and the surfactant POEA as possible disrupters of fecundity in non-target organisms at sub-lethal concentrations. Exposure to both RCP/or POEA but not pure glyphosate alone promoted an increase in oxidative damage (as revealed by protein carbonyl levels) while reducing lifespan. Decreased fecundity is probably due to increased caspase activity indicative of proapoptotic events as revealed by *in vitro* studies.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.109883.

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