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Studies on the toxicity of glyphosate and the effect of spermidine in conferring resistance to induced toxicity in *Drosophila melanogaster*

Bachelor Thesis

Submitted by

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Annotation:
In this study *Drosophila melanogaster* is used as a model system to study the toxicological effect of pure glyphosate. Furthermore, the ability of spermidine to confer resistance against the glyphosate-induced toxicity was also studied. Important physiological parameters were tested employing fecundity assay, lifespan measurements, and negative geotaxis assay. Protein carbonyl assay was employed in this study as a marker of oxidative stress.

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Acknowledgements

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Abstract

Glyphosate is an active ingredient of some commonly used herbicides that is extensively used in agriculture and urban areas and covers a broad spectrum of plants. Its use has further increased with genetically modified plants that are resistant to its effect. However, despite the plant-specific mode of action, it was reported to induce toxicity in various species. Other studies and meta-analysis data suggest that it is non-toxic to non-target organisms. Hence, it's precise effect on non-target organisms is unclear. In this study *Drosophila melanogaster* is used as a model system to study the toxicological effect of pure glyphosate. Furthermore, the ability of spermidine, a natural polyamine that was shown to promote stress resistance and longevity across species, to confer resistance against the glyphosate-induced toxicity was also studied. The present investigation demonstrates that only high doses of pure glyphosate induce toxicity, but at sub-lethal levels it does not have a detrimental effect on longevity, health (as revealed by the negative geotaxis assay), fecundity or protein carbonyl levels (an indicator of oxidative stress). It is concluded, that glyphosate is less toxic than its end-use formulations (such as Roundup®) and only weakly induces toxicity at moderate levels. This suggests further investigations of adjuvants and surfactants used in the respective formulations of glyphosate. The role of spermidine in its ability of conferring resistance to herbicide-induced toxicity has to be further investigated, as no clear positive effects could be revealed in this work.

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1 Introduction

There is increasing interest in the environment both at the professional level and also among the general public, particularly with respect to pollution and its effects on humans as well as other species. The unfavorable subtle effects of dichloro diphenyl trichloroethane (DDT) on certain organisms and its practically universal distribution in the environment has stimulated questions concerning the possibility of similar effects from other agricultural chemicals. Herbicides are common today and their use is growing rapidly. However, there has also been increasing concern on the un-wanted side-effects of these weed-killers on non-target organisms as well as the fate of these chemicals in our environment. The non-target effects of the active ingredients of these herbicides have been the topic of active research and many regulatory and policy decisions have been based upon the results of such studies.

1.1 Chemical structure and mode of action of glyphosate-based herbicides:

Among the most widely used herbicides in the world, weed-control products with glyphosate as the active ingredient are the most common. The herbicidal properties of glyphosate were discovered by Monsanto Company scientists in 1970. Glyphosate (*N*-phosphomethylglycine) (**Figure 1**) is a widely used non-selective herbicide, which inhibits plant growth through interference with the production of essential aromatic amino acids by inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase which is responsible for the biosynthesis of chorismate, an intermediate in phenylalanine, tryptophan and tyrosine biosynthesis (Vencill, 2002; Tomlin, 2006) (**Figure 2**), and is effective against a broad spectrum of plants. Since the pathway for biosynthesis of aromatic amino acids is not encountered in members of the animal kingdom, the rationale for its use as an effective inhibitor of the amino acid biosynthesis pathway exclusive to plants makes a strong argument for its use as a herbicide (Steinrüken and Amrhein, 1980). The herbicidal action of glyphosate is expressed most effectively through direct contact with foliage with its subsequent systemic translocation throughout the plant. Glyphosate is thought to be predominantly degraded in the environment by microorganisms and also to a certain extent by metabolization by plants leading to innocuous substances such as carbon dioxide and phosphoric acid.

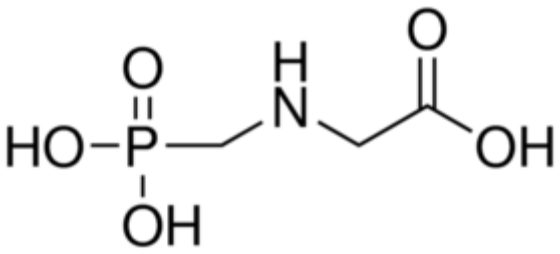

Figure 1: Chemical structure of glyphosate

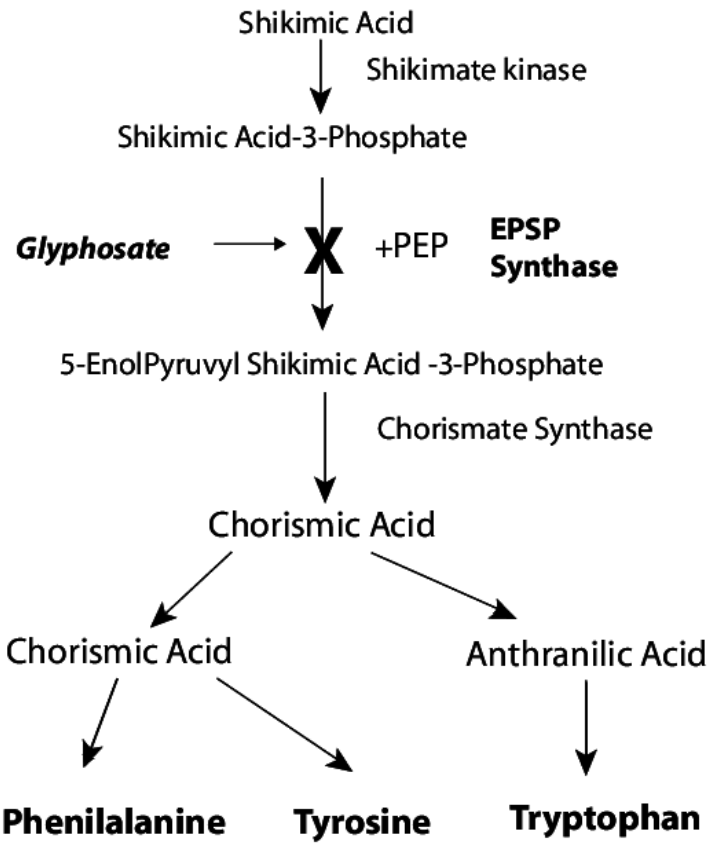

Figure 2: Mode of action of glyphosate-based herbicide (Dill, 2005).

1.2 Environmental impact:

Roundup® herbicide containing glyphosate as the active ingredient was first introduced in 1974 for nonselective weed control (Franz et al., 1997). The use of glyphosate-based products by farmers has continued to increase since then this use is further augmented in agricultural applications with the introduction of genetically modified plant varieties that are tolerant to glyphosate (Roundup-Ready®). It is currently, and most likely will be for a long time, the most used herbicide globally, being registered in more than 130 countries and available under different brand names. Glyphosate, the main ingredient in formulations

including Roundup®, Rodeo® and Touchdown®, is adsorbed strongly by the soil, but is susceptible to microbial degradation (Uren Webster et al., 2014). Since glyphosate exhibits low persistence, repeated applications are necessary for weed control (Ayoola, 2008). The fact that glyphosate is water soluble has been seen in rivers near urban runoff and wastewater treatment effluents (Botta et al., 2009; Uren Webster et al., 2014) and with increased concentrations in river sediment loads, after heavy rainfall and flooding events (Botta et al., 2009; Giesy et al., 2000); Uren Webster et al., 2014). Environmental analyses regarding the presence of this herbicide have shown that there are significant levels in areas located close to application sites, such as river water (0.1 mg/L and 0.7 mg/L), sediments (upto 4.9 mg/Kg) and soil (0.5 mg/ Kg to 4.3 mg/Kg) (Peruzzo et al., 2008). While in faster moving and more diluting bodies of water glyphosate concentrations generally average around 10-15 µg/L (Byer et al., 2008; Struger et al., 2008; Uren Webster et al., 2014), in stagnant water bodies such as ponds and wetlands higher levels of glyphosate have been recorded which has led to contamination of the soil, surface and ground waters, the atmosphere and even food and objects of daily use (Torretta et al., 2018).

1.3 Eco-toxicological effects on non-target organisms:

Although it was thought that the mode of action of glyphosate was plant-specific (Sandrini et al., 2013) it has been shown that non-target animals are affected by it, such as rats, oysters, zebra fish and frogs (Giesy et al., 2000; Roy et al., 2016; Howe et al. 2004; Mottier et al., 2015). These effects due to exposure to glyphosate include physical deformities, endocrine disruption, behavioral disorders and oxidative stress (OS) (Larsen et al., 2012). The acute toxicity and teratogenic effects of glyphosate were first noted in amphibian species since their reproduction and early developmental stages require an aquatic environment, thus increasing their risk of exposure and susceptibility (Howe et al., 2004; Mann and Bidwell, 1999; Perkins et al., 2000). De Aguiar et al. (2016) have also shown, that glyphosate exposure can lead to an oxidative imbalance in the tissues of the fruit fly *Drosophila melanogaster*, which leads to a significant increase in the activity of antioxidant systems, and an increase of gene expression of respective genes. Furthermore, it has also been shown, that glyphosate-based herbicides can negatively impact fecundity and fertility in neuropteran species (Schneider et al., 2009).

1.4 Induction of reactive oxygen species and oxidative stress by herbicides:

Reactive oxygen species (ROS) consist of molecular oxygen, singlet oxygen, superoxide anion, hydrogen peroxide, hydroxyl radical and some of their derivatives. While hydrogen peroxide (H_2O_2) is not a radical, it is categorized as a reactive species since it has higher activity than molecular oxygen. While in general in biological systems the ROS levels are maintained at steady state levels by the action of antioxidant systems, under certain circumstances such homeostasis is unbalanced resulting in excessive generation of ROS. This can result in situations of oxidative stress where ROS can damage various macromolecules including proteins, lipids and even DNA. While there are several oxidative stress markers, the primary markers involve identification of protein oxidation, lipid peroxidation and presence of oxidized bases in DNA.

1.4.1 Protein oxidation as a marker of oxidative stress

ROS-induced oxidation or modification of proteins has become a popular measure of oxidative stress. This is because of an easy spectrophotometric assay of protein carbonyl content using dinitrophenylhydrazine (Levine et al., 2000). Proteins have many reactive sites that can be modified or damaged during OS. Modification of proteins then leads to the formation of carbonyl derivatives by direct oxidation of certain amino acid side chains and oxidation-induced peptide cleavage (Stadtman 1992, 2004). Mechanisms involved in the oxidation of proteins by ROS were elucidated in studies in which amino acids, simple peptides and proteins were exposed to ionizing radiations under conditions where hydroxyl radicals are formed. The side chains of all amino acid residues of proteins are susceptible to oxidation by the action of ROS/RNS (Stadtman 2004). Oxidation of cysteine residues may lead to the reversible formation of mixed disulphides between protein thiol groups (-SH) and low molecular weight thiols, in particular, glutathione (GSH). The concentration of carbonyl groups generated by many different mechanisms is a good measure of ROS-mediated protein oxidation.

1.4.2 Induction of ROS by herbicides:

Several different classes of herbicides may induce oxidative stress by several different mechanisms: (a) By themselves undergoing redox-cycling by accepting or donating electrons to cellular components, thereby increasing ROS levels (b) can impair antioxidant

systems, thereby contributing to decreased elimination of ROS (c) interference with energy-generating processes, thereby impairing metabolism and detoxification processes and (d) have a direct effect on core-transcriptional or translation processes, thereby enhancing ROS levels. For example, glyphosate is responsible for the inhibition of biosynthesis of chorismate, an intermediate in phenylalanine, tyrosine and tryptophan biosynthesis in plants. In goldfish, Roundup® a glyphosate-based herbicide induced oxidative stress (Lushchak et al., 2009).

While several studies have demonstrated the effect of glyphosate-based herbicide on non-target organisms, very few studies have focused on the effect of pure glyphosate (the active ingredient) on non-target organisms. Thus, one of the objectives of this study was to investigate the direct toxicity of pure glyphosate on a model organism – *Drosophila melanogaster*.

1.5 Role of polyamines (Spermidine) in response to environmental toxicants:

In response to herbicide toxicity an organism would normally exhibit a defensive response. This response can involve a number of different pathways ranging from a generalized response to stress to specific response to the physiological effects of the stressor namely oxidative stress. Polyamines are small aliphatic polycations widely distributed in nature. They were first described by Anton Van Leewenhoek in 1678 in the seminal fluid of animals resulting in the naming of two of its members spermine and spermidine. Now, it has been found that polyamines are present in all living organisms with the most common polyamines being spermine, spermidine and putrescine. Polyamines are important for gene expression due to their ability to bind to nucleic acids and proteins; thus, these molecules can remodel and stabilize the chromatin structure. Cells are continuously exposed to different types of stress, either by products of their own metabolism or by environmental pollutants that result in the generation of reactive oxygen species (ROS), pH, osmotic

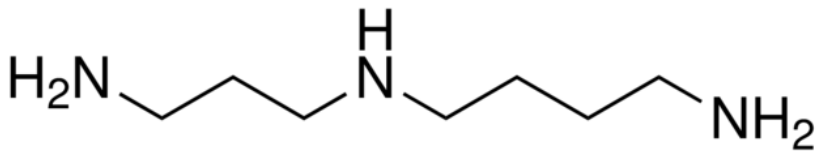

Figure 3: Chemical structure of Spermidine

pressure and temperature. Extensive literature has demonstrated that polyamines are associated with the response and protection to different types of stress which might involve multiple properties (Miller-Fleming et al., 2015). One of the functions of polyamines include scavenging of ROS (Na et al., 1998; Das et al., 2004; Fujisawa and Kadoma, 2005; Rider et al., 2007). Spermidine (**Figure 3**), a natural polyamine, has been shown to promote stress resistance in *D. melanogaster* through both autophagy-dependent and -independent pathways (Minois et al. 2012). Spermidines mode of action is in general hypoacetylation of proteins, which in turn induces autophagy (**Figure 4**) (Minois, 2014). It has also been shown that spermidine can confer longevity in humans, where especially the test group with people aged from 90-106 years shows significantly higher levels of spermidine (Pucciarelli et al., 2012).

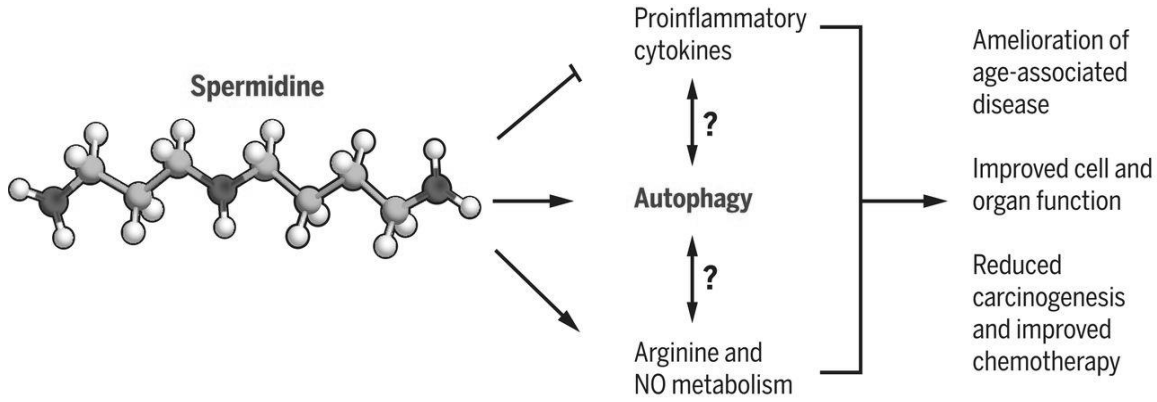

Figure 4: Spermidine mode of action (Madeo et al. 2018)

Given the protective effects of spermidine, we also investigated if exogenous supplementation of spermidine would confer protection against any toxicity induced by glyphosate or if spermidine by itself could result in beneficial effects to the model organism – *Drosophila melanogaster*.

2 Hypotheses and Objectives

The present investigation was based on two hypotheses:

Null Hypothesis (H_0) 1: Glyphosate is non-toxic to organisms.

Null-Hypothesis (H_0) 2: Spermidine does not confer protection against stress.

To test these two hypotheses the following objectives were formulated:

Objective 1: To determine the effect of pure glyphosate on some physiological parameters in the fruit fly *Drosophila melanogaster*.

Objective 2: To determine if exogenous spermidine supplementation has beneficial effects either alone or when co-treated with glyphosate.

To fulfill these two objectives the following physiological parameters were targeted:

a) Glyphosate dose-mortality response.
b) Longevity of male and female *Drosophila* following treatment with one selected dose of glyphosate (based on 1) alone or in combination with a pre-determined dose of spermidine (based on available literature, Minois et al. 2012). The effect of spermidine alone was also evaluated.
c) Rapid-iterative negative geotaxis assay as a measure of performance ability in males and females separately following treatment with glyphosate or spermidine alone or in combination.
d) Fecundity as a measure of fitness following reciprocal treatments: Males treated with glyphosate and females untreated, males untreated and females treated with glyphosate, both males and females treated with glyphosate.
e) Assay of protein carbonyl levels in *Drosophila* as a measure of oxidative damage following treatment with glyphosate or spermidine alone or in combination in males and females separately.

Thus, in this thesis, the potential of glyphosate to induce toxicity as well as the ability of spermidine to confer a resistance to glyphosate is extensively examined. The fruit fly *D. melanogaster* was used as a model organism, as it has been shown to be an effective tool in toxicological test and in general facilitates scientific research in many ways.

3 Materials and Methods

3.1 Reagents and Chemicals:

Chemicals used in this work were obtained from Sigma-Aldrich.

3.2 Drosophila stock and husbandry

For the scope of this work, only 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 12h-light/12h-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 hatching for testing.

3.3 Dose-mortality response

For establishment of a LD_{50} and LD_{25} curve of glyphosate, testing was done in cohorts of 10 male flies respectively with three biological repetitions. The flies were kept in glass vials with cotton plugs and a filter paper at the bottom. Continuous exposure over the testing period was done by pipetting sucrose-solutions with varying glyphosate content on the filter papers with a frequency of 12h of feeding. Mortality was scored every 12h with a total span of 96h of testing. Topical exposure was also tested for male flies, by submerging the flies in droplets of the respective sucrose-solutions for a one-time exposure. Mortality was scored every 12h

3.4 Treatment groups

For the treatment groups (glyphosate or spermidine alone or in combination as well as control) standardized solutions were used. For spermidine a concentration of 0.145 µl/ml was chosen and for glyphosate 100 µg/ml was chosen based on the LD_{25}-value obtained for the glyphosate dose-mortality response testing. For the combination the same concentration of both was chosen to minimize deviations between treatment groups. The solutions can be seen in Table 1 below.

Table 1: Used solutions for the different treatment groups.

Test group	**Used solution**
Control	5 %-sucrose
Glyphosate	100 µg/ml in 5 %-sucrose
Spermidine	0.145 µl/ml in 5 %-sucrose
Glyphosate + Spermidine	0.145 µl/ml Spermidine in 100 µg/ml glyphosate solution

3.5 Lifespan measurements

Lifespan measurements were conducted in one cohorts of ca. 70 males and 70 females respectively for each treatment group. Following a two times exposure over the period of 24h, they were kept in polypropylene bottles with tissue culture dishes serving as lids and containers for the diet. The dishes were replaced daily after tapping the flies down to the bottom of the bottle. The mortality was also scored daily.

3.6 Rapid iterative negative geotaxis assay (RING)

The negative geotaxis of the different treatment groups was assessed using RING-assay (Gargano et al. 2005). For each iteration a maximum of 30 flies per tube were used for the assay. The flies were loaded into the tubes after a 24 h exposure period and were given an acclimatization period of 5 minutes. 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 with both male and female flies of each group with a total amount of tested flies of ca. 140 flies for each group. 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.

3.7 Fecundity assay

Fecundity was assessed as number of eggs laid daily per female. Testing was done for the following: Males treated and females untreated, males untreated and females treated, both males and females treated (see Table 1). Cohort size was between 4 and 6 virgin females and males for each technical and biological repetition. 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 taken as a measurement for fecundity. For the evaluation special care was taken to exclude female flies that died during testing.

3.8 Protein Carbonyl assay

Carbonyls were analyzed quantitatively after their reaction with 2,4-dinitrophenylhydrazine (DNPH) (Levine et al. 2000). To the homogenates of the whole bodies of the samples 7mM DNPH (in 2M HCl) and to those of the control groups 2M HCl was added. Subsequently the samples were incubated in the dark for 1 h and the proteins were then precipitated using 28 % trichloro acetic acid (TCA). After centrifugation the pellets were resuspended in 5 % TCA. After a subsequent centrifugation the pellet is resuspended in a 1:1 ethanol/ethylacetate solution and washed three times. The pellets are then resuspended in 6 M guanidine hydrochloride by vortexing. For the spectrophotometrical detection a Corning Costar 3635 transparent UV plate with flat bottom was measured at 370 nm. The results were expressed as ng mg^{-1} using an extinction coefficient of 22.000 M^{-1} cm^{-1}. The values were corrected for interfering substances by subtracting the absorbance of the respective controls.

3.9 Statistical analysis

For obtaining the LD_{25} and LD_{50} values of the dose-mortality response variable slope model (nonlinear-fit, four parameters, 95 % confidence interval) was used based on Probit analysis. Longevity was assessed using Kaplan Meier curves and subsequent comparison using Log-rank (Mantel-Cox) Test. Rapid iterative negative geotaxis (RING) data and Fecundity data were both assessed using One-Way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison test. Protein carbonyl data was assessed using Two-Way ANOVA followed by Bonferroni's post-hoc test. Statistical Analysis was done in GraphPad Prism v. 5.0 (San Diego, CA)

4 Results

4.1 Dose-mortality response

Male Drosophila were exposed to various concentrations of pure glyphosate in 5% sucrose. Following exposure, mortality was evaluated after 72 hours and based on this, a dose-response curve was generated. Instead of a typical sigmoidal dose-response curve, a non-linear curve fit was obtained where after a slight initial increase in mortality with increased concentration of glyphosate, it plateaued. However, based on the dose response curve the LD_{25} and LD_{50} values were estimated (**Table 2, Figure 5**).

Table 2: Dose-response variable slope (nonlinear-fit, four parameters) for LD_{50} value.

Best-fit values	
Bottom	= 0.0
Hillslope	0.3757
Top	= 100.0
EC50	4771
logEC50	3.679
Span	= 100.0
Std. Error	
Hillslope	0.1633
EC50	5383
95% Confidence Intervals	
Hillslope	-0.1439 to 0.8953
EC50	0.0 to 21903
logEC50	-infinity to 4.341
Goodness of Fit	
Degrees of Freedom	3
R square	0.8104
Absolute Sum of Squares	303.3
Sy.x	10.05
Constraints	
Bottom	Bottom = 0.0
Top	Top = 100.0
EC50	EC50 > 0.0
Number of points	
Analyzed	5

The LD_{25} was calculated as 91.04 µg/ml and in all subsequent experiments approximately 100 µg/ml was used as the treatment dose for glyphosate.

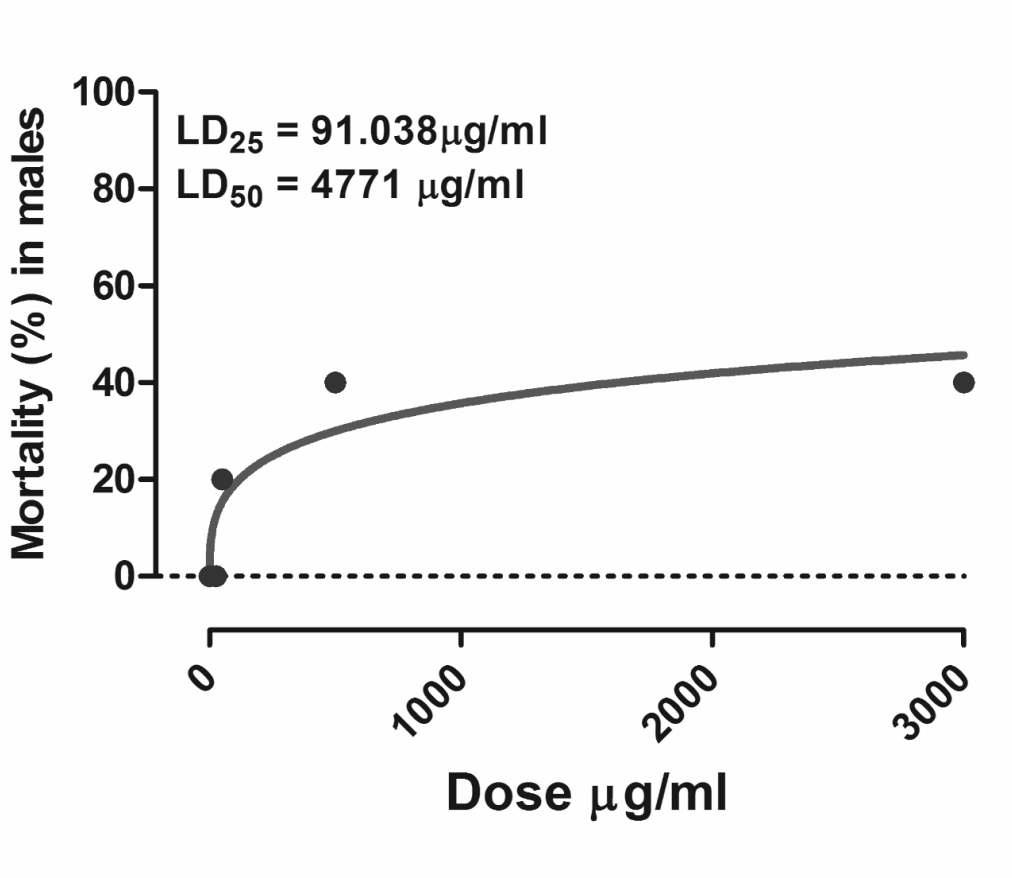

Figure 5: Dose response curve for glyphosate in males.

4.2 Lifespan measurements

Longevity of both males and females were assessed using Kaplan-Meier survival curves following different treatments: No-treatment (Control), glyphosate alone, spermidine alone or a combination of both. The median survival of untreated males was 35 days whereas females which were not treated had a median survival of 57 days (**Figure 6 A, B**). Glyphosate treatment resulted in slightly enhanced survival (non-significant) in males with a median survival of 46 days whereas in females the median survival was 59 days. Spermidine treatment alone resulted in a median survival of 33 days in males whereas in females the median survival was 58 days. Co-treatment of glyphosate with spermidine resulted in a median survival of 33 days in males and 55 days in females (**Figure 6 A, B**). Log-Rank (Mantel-Cox) test did not reveal any significant difference in any of the survival curves following treatments in either males or females (**Table 3, Table 4**).

Table 3: Comparison of survival curves for males.

Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	4.104
df	3
P value	0.2505
P value summary	ns
Are the survival curves sig different?	No
Logrank test for trend	
Chi square	1.986
df	1
P value	0.1588
P value summary	ns
Sig. trend?	No

Table 4: Comparison of survival curves for females.

Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	7.747
df	3
P value	0.0515
P value summary	ns
Are the survival curves sig different?	No
Logrank test for trend	
Chi square	6.208
df	1
P value	0.0127
P value summary	*
Sig. trend?	Yes

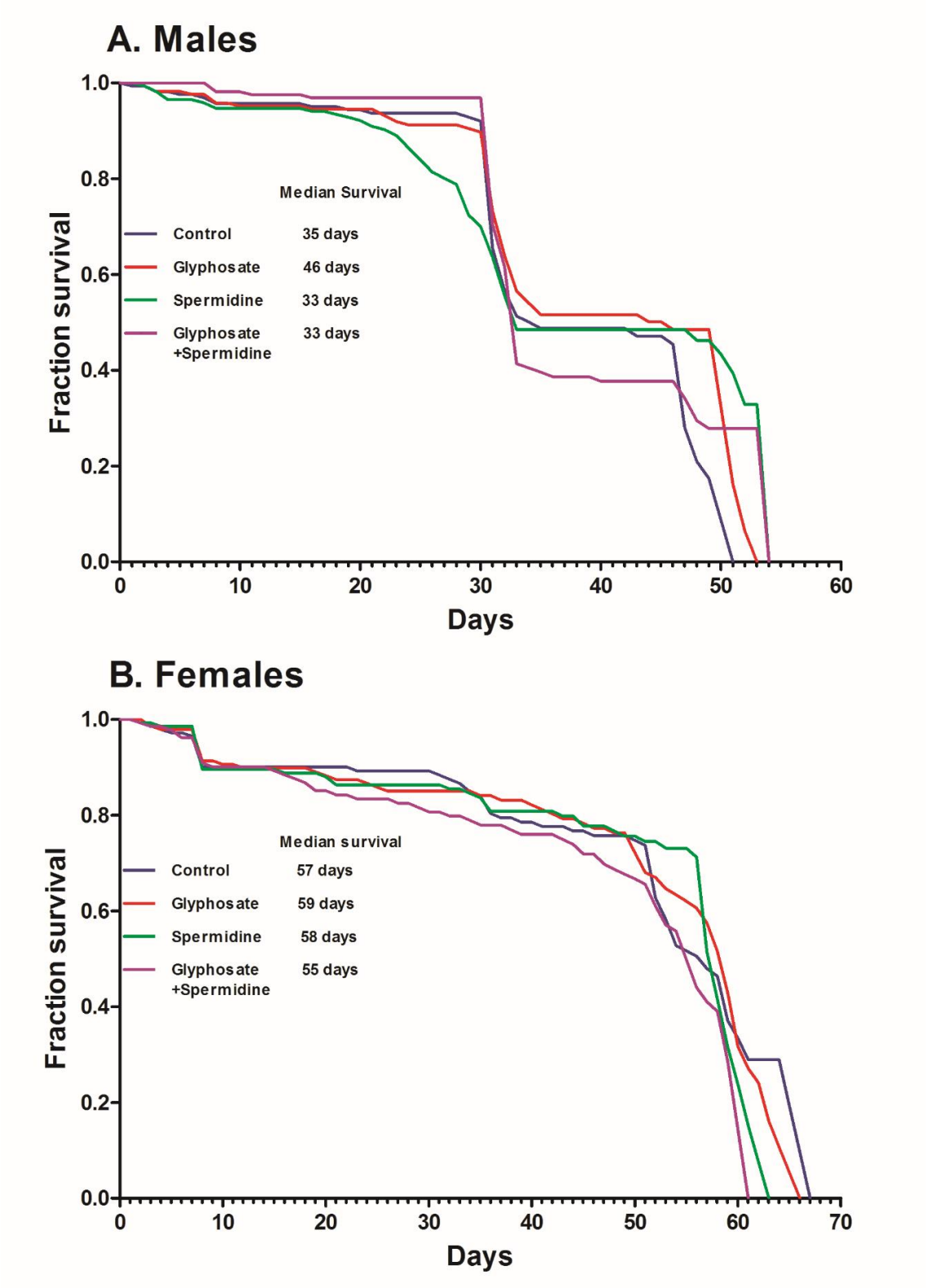

Figure 6: Kaplan-Meier survival curves for males and females of *Drosophila melanogaster* with no-treatment (Control), glyphosate alone, spermidine alone, or a combination of glyphosate and spermidine.

4.3 Rapid-iterative negative geotaxis (RING) assay:

The RING assay was performed on both males and females following different treatments: No-treatment (Control), glyphosate (Gly) alone, spermidine (Spd) alone or a combination of both (Gly+Spd). However, none of the treatments resulted in any significant difference in the negative geotaxis ability in either males or females as observed after statistical analysis of data with One-Way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison test (**Table 5, Table 6 and Figure 7**).

Table 5: One-Way ANOVA of RING assay for males.

One-way analysis of variance					
P value	0.6518				
P value summary	ns				
Are means signif. different? (P < 0.05)	No				
Number of groups	4				
F	0.5675				
R square	0.1755				
ANOVA Table	SS	df	MS		
Treatment (between columns)	381.1	3	127.0		
Residual (within columns)	1791	8	223.9		
Total	2172	11			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Gly	-1.169	0.1354	No	ns	-40.29 to 37.95
Control vs Spd	-11.47	1.327	No	ns	-50.59 to 27.66
Control vs Gly+Spd	-12.16	1.408	No	ns	-51.29 to 26.96
Gly vs Spd	-10.30	1.192	No	ns	-49.42 to 28.83
Gly vs Gly+Spd	-10.99	1.273	No	ns	-50.12 to 28.13
Spd vs Gly+Spd	-0.6977	0.08077	No	ns	-39.82 to 38.43

Interestingly, females in general showed significantly more negative geotaxis response compared to males.

Table 6: One-Way ANOVA of RING assay for females.

One-way analysis of variance					
P value	0.7718				
P value summary	ns				
Are means signif. different? (P < 0.05)	No				
Number of groups	4				
F	0.3776				
R square	0.1240				
ANOVA Table	SS	df	MS		
Treatment (between columns)	208.1	3	69.38		
Residual (within columns)	1470	8	183.7		
Total	1678	11			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Gly	-7.328	0.9364	No	ns	-42.77 to 28.11
Control vs Spd	-8.752	1.118	No	ns	-44.19 to 26.69
Control vs Gly+Spd	0.4446	0.05682	No	ns	-35.00 to 35.89
Gly vs Spd	-1.424	0.1820	No	ns	-36.87 to 34.02
Gly vs Gly+Spd	7.773	0.9932	No	ns	-27.67 to 43.21
Spd vs Gly+Spd	9.197	1.175	No	ns	-26.25 to 44.64

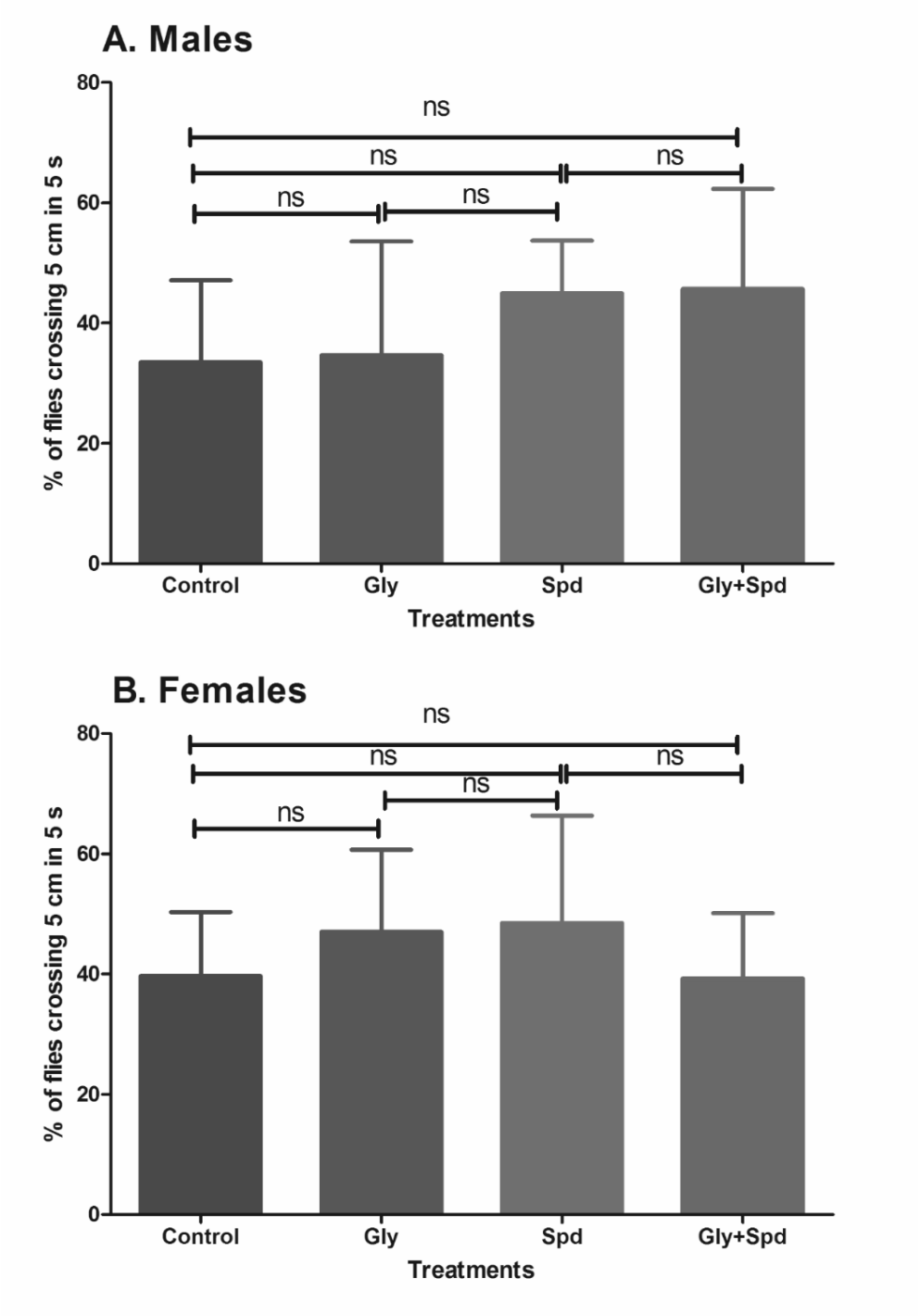

Figure 7: RING assay for males and females of *Drosophila melanogaster* following various treatments.

4.4 Fecundity assays

The fecundity of females following various treatments was assayed: Treated males mated with untreated females, treated females mated with untreated males and both males and females treated and mated. One-Way ANOVA with Tukey's multiple comparison test revealed no significant differences in fecundity in any of the treatments or the reciprocal treatment and mating schemes (**Figure 8**). Interestingly it was seen that treatment of females alone did result in increased fecundity in general.

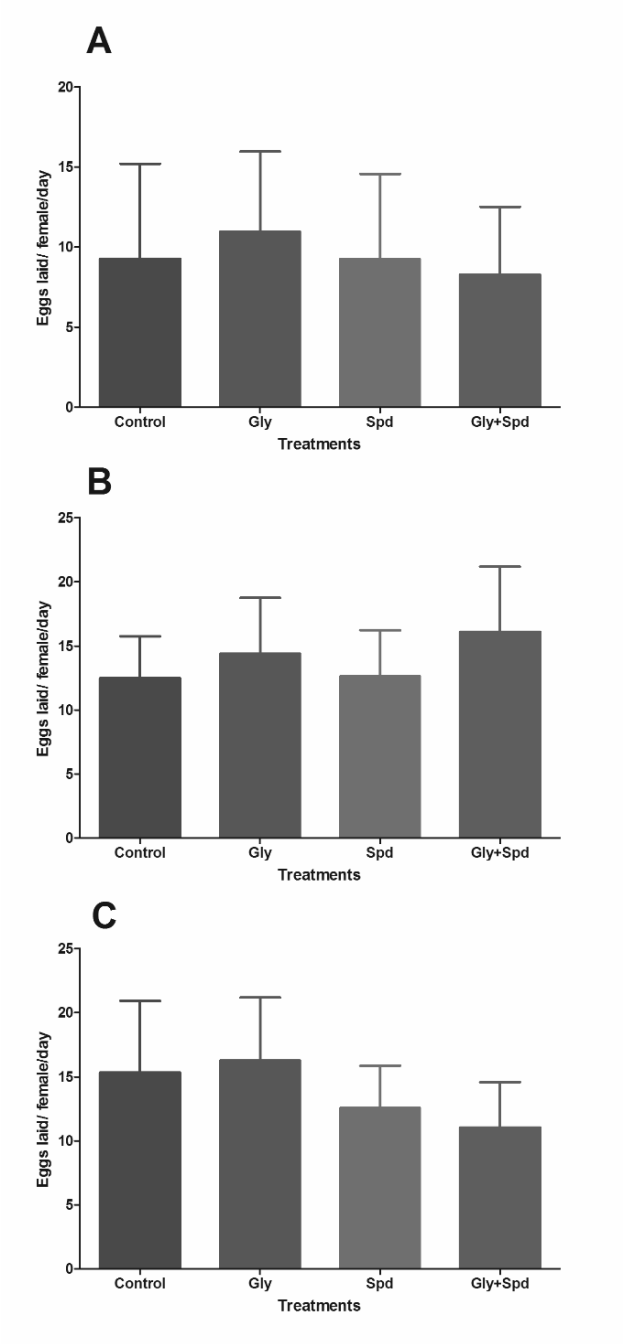

Figure 8: Fecundity analysis following different treatments.

4.5 Protein carbonyl assay

The protein carbonyl levels were assayed in whole body homogenates of males and females separately following different treatments. Interestingly, there was a significant difference in the response between males and females in protein carbonyl levels to glyphosate and spermidine treatment but not a combination of both (**Table 7, Figure 9**).

Table 7: Two-way ANOVA of protein carbonyl content in males and females of *Drosophila melanogaster* in response to different treatments.

Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	28.78	< 0.0001		
Gender	16.71	< 0.0001		
Treatments	19.35	0.0005		
Source of Variation	P value summary	Significant?		
Interaction	****	Yes		
Gender	****	Yes		
Treatments	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	3	778.3	259.4	10.91
Gender	1	451.9	451.9	19.01
Treatments	3	523.4	174.5	7.337
Residual	40	951.1	23.78	
Number of missing values	0			
Bonferroni multiple comparisons	Number of comparisons: 4			
Male vs Female				
Treatments	Male	Female	Difference	95% CI of diff.
Control	31.89	23.44	-8.450	-15.81 to -1.086
Gly	29.96	14.81	-15.16	-22.52 to -7.795
Spd	30.42	22.59	-7.832	-15.20 to -0.4679
Gly+Spd	15.94	22.84	6.894	-0.4699 to 14.26
Treatments	Difference	t	P value	Summary
Control	-8.450	3.001	P < 0.05	*
Gly	-15.16	5.385	P < 0.0001	****
Spd	-7.832	2.782	P < 0.05	*
Gly+Spd	6.894	2.449	P > 0.05	ns

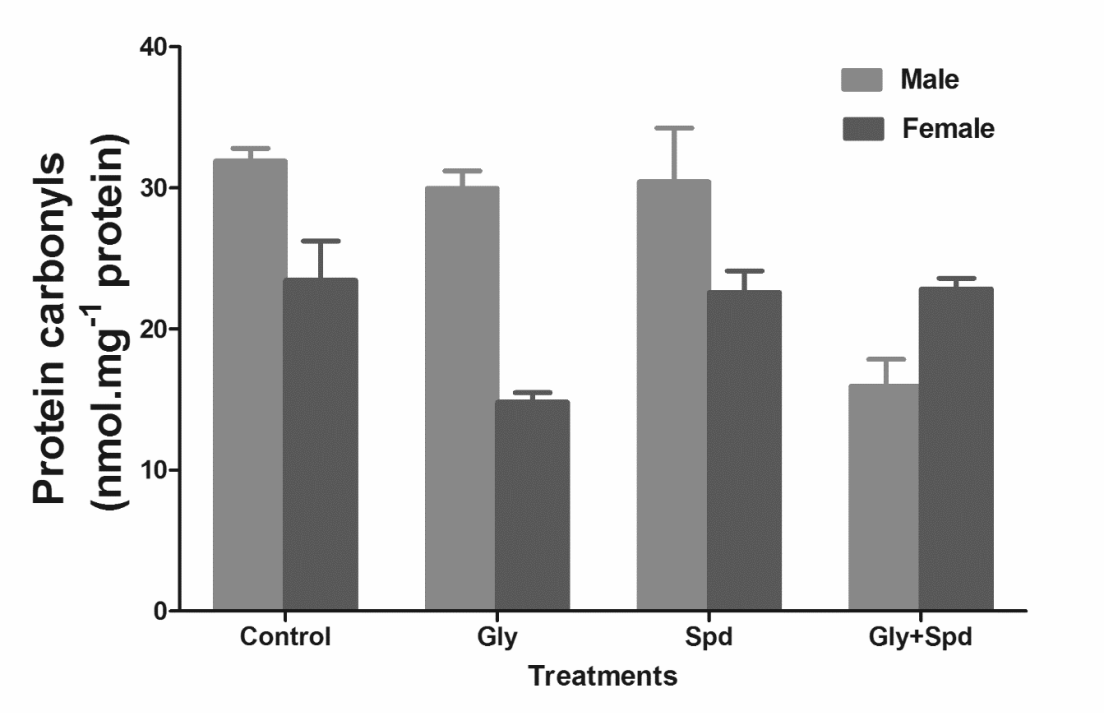

Figure 9: Protein carbonyl content in males and females of *D. melanogaster* following different treatments.

Among males, there was no significant difference between control, glyphosate alone or spermidine alone treatments whereas co-treatment with both resulted in significantly reduced protein carbonyl formation. In females on the other hand, glyphosate treatment alone resulted in significantly reduced protein carbonyl content compared to controls and spermidine alone or co-treatment with glyphosate and spermidine, which were not significantly different from each other.

5 Discussion

This study revealed that pure glyphosate at very high concentrations results in toxicity to *Drosophila* and sub-lethal levels of glyphosate do not have a detrimental effect on longevity, health (as revealed by the negative geotaxis assay), fecundity or protein carbonyl levels. A distinct sexually dimorphic difference was however recorded in the response to glyphosate in *Drosophila*. A number of studies have examined the toxicity of Roundup® herbicide to non-target organisms (as reviewed in Green and Beestman, 2007). Much of the available information reveals that end-use (formulated) glyphosate products are more toxic to non-target organisms than the glyphosate active ingredient alone (as also seen from this present study) and this result in toxicity is probably due to the surfactants used in the formulation (Hazen, 2000; Green, 2000; Karande et al., 2005). It is generally accepted that the toxicity of commercial glyphosate herbicides exceeds significantly the toxicity of glyphosate. This has been confirmed in numerous in vivo and in vitro studies (Contardo-Jara et al., 2009; El-Shenawy, 2009; Howe et al., 2004; Mesnage et al., 2015; Richard et al., 2005). While exposure to high doses of Roundup®-type products causes serious poisonings in human (Chang et al., 1999; Roberts et al., 2010; Stella and Ryan, 2004), the toxicity of glyphosate alone towards mammals is very low. This effect is borne out in this study because pure glyphosate did not result in any deleterious consequences to either longevity, robustness of the organism, fitness (fecundity) or damage to proteins, due to oxidative stress.

When discussing the toxicity of herbicides to non-target species, the emphasis is mostly on the active substance. However, herbicides are formulated products and usually contain additives (e.g. surfactants) which enhance their effectiveness and at the same time increase the toxicity to non-target biota (Tsui and Cgu, 2003; Edginton et al., 2004). These so-called inert ingredients are the probable cause of enhanced toxicity of the commercial formulations (Brausch et al., 2007; Mann et al., 2009; Mesnage et al., 2013, 2014, 2015; Moore et al., 2012). Roundup® is an aquatic solution of glyphosate, used in the form of isopropylamine salt and other co-formulants which are confidential for regulatory purposes but include polyethoxylated tallow amine (POEA). The data obtained in numerous studies point out to a very high toxicity of POEA towards animals which clearly exceeds the toxicity of glyphosate and its commercial products.

Some marginal enhancement in longevity and other parameters though non-significant might be attributed to hormesis whereby a stressor can cause a positive rather than a negative effect by enhancing certain attributes. Spermidine on the other hand did not have a beneficial effect in any of the parameters studied either alone or when co-treated with glyphosate. As discussed earlier, spermidine was reported to confer resistance to stress (Minois et al. 2012) and thus increasing lifespan and negative geotaxis, which could not be shown here. This could be caused by several reasons. First, it would probably have been necessary to conduct a dose-response study also with spermidine to determine at which dose it is most effective. Second, since glyphosate per se did not reflect any toxicity at the dose in which the experiments were conducted, the action of spermidine would not be clear in this situation.

6 Conclusions and future perspectives

Based on this study it can be concluded that glyphosate by itself is weakly toxic to *Drosophila* and does not have any deleterious effects as reported by previous studies that used the commercial formulation Roundup®. Future studies should focus on testing the adjuvants/ surfactants such as POEA to evaluate its toxicity to non-target organisms. The role of spermidine and the physiological levels that result in a beneficial effect need to be evaluated more thoroughly.

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