THE EFFECTS OF GLYPHOSATE BASED HERBICIDES ON CHICK EMBRYO DEVELOPMENT

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Glyphosate based herbicides are among the most widely used herbicides in the world. The purpose of this study was to determine developmental toxicity of glyphosate, the active ingredient in the common herbicide Roundup, on developing chicken embryos. Few studies have examined toxic effects of glyphosate alone versus the full compound formulations of Roundup, which include adjuvants and surfactants. Adjutants and surfactants are added to aid in solubility and absorption of glyphosate. In this study chicken embryos were exposed at the air cell on embryonic day 6 to 19.8 or 9.9 mg / Kg egg mass of glyphosate in Roundup or glyphosate only.

Chickens treated with 19.8 and 9.9 mg / Kg glyphosate in Roundup showed significant reduction in survivability compared to glyphosate alone treatments and controls. On embryonic day 18, embryos were sacrificed for evaluation of developmental toxicity using wet embryo mass, dry embryo mass, and yolk mass as indicators. Morphology measurements were taken on liver mass, heart mass, tibiotarsus length and beak length. Embryos treated with 19.8 mg / Kg glyphosate and 9.9 mg / Kg glyphosate in Roundup showed significant reductions in wet and dry embryo mass and yolk mass. Tibiotarsus length in 9.9 mg / Kg glyphosate in Roundup treatments were significantly reduced compared to 9.9 mg / Kg glyphosate treatments. Beak length was significantly reduced in 9.9 mg /Kg glyphosate in Roundup treatments compared to all other groups.

ACKNOWLEDGEMENTS

First, I would like to thank Dr. Edward Dzialowski for giving me the opportunity to be apart of his laboratory. I have grown considerably as an independent thinker and researcher while working under his guidance over the past several years. I thank my committee members, Dr. Duane Huggett and Dr. Aaron Roberts for their insightful guidance and contributions throughout the project. I would also like to thank all of my lab mates for their continued support and helpful suggestions, as well as the University of North Texas Developmental Integrative Biology Group whose suggestions and feedback throughout my time as a graduate student have proven invaluable to the success of this project.

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INTRODUCTION

Glyphosate Based Herbicides

Glyphosate based herbicides are among the most widely used in the world. Glyphosate is effective, economical, and considered environmentally safe (Powles, 2008). Glyphosate is very effective in controlling weeds, particularly when used with transgenic crop varieties resistant to the herbicide. Glyphosate was first developed by the Monsanto Company and introduced for weed control in 1974. Annual usage of Glyphosate based herbicides have steadily increased, in part because of increased herbicide resistance in weeds (Williams et al., 2000). As of 2005, the United States had the most herbicide resistant crops planted per hectare in the world which resulted in increased usage of glyphosate from 1.8 million kg in 1995 to 45 million kg in 2005 (Gianessi, 2008). Glyphosate is a nonselective herbicide that inhibits plant growth through shikimate pathway interference. This pathway found only in bacteria and plants is involved in production of essential aromatic amino acids. Glyphosate acts by competitive inhibition of the enzyme, enolpyruvylshikimate phosphate (EPSP) synthase (Herrmann, 1995). Inhibition of this enzyme prevents phenylalanine, tyrosine, and tryptophan biosynthesis ultimately killing the plant. Glyphosate inhibits the pathways because it is a molecular analogue of phosphoenol pyruvate, a vital substrate in the pathway (Williams et al., 2000). Glyphosate is polar and a very water soluble compound that comes in a variety of salts but primarily in the isopropylalamine form (Peruzzo et al., 2008). Glyphosate's environmental fate is degradation by soil microorganisms. When applied in a field, glyphosate binds very tightly to soil, however there are an increasing number of studies that show glyphosate water contamination (Peruzzo et al., 2008). It can persist in the environment for 170 days and has a half-life of 45-60 days (Peruzzo et al., 2008). A study in Argentina found levels of glyphosate in waters that ranged from 0.10 to

0.70 mg/L, while in sediments and soils values ranged between 0.5 and 5.0 mg/Kg. Increased rainfall events also significantly raised glyphosate levels in surface waters (Peruzzo et al., 2008).

Pesticide formulations are divided into two categories: active and inactive ingredients, which only the active compounds are labeled because inert ingredient information is considered confidential business information. Most of the tests for pesticide registration require testing on the active compound only. In numerous studies the presence of inert of inactive ingredients have been shown to significantly increase the toxicity of pesticides (Cox and Surgan, 2006). Glyphosate is commercially sold as Roundup which is combined with the surfactant polyethoxylated tallowamine (POEA) or MON 0818. It has been confirmed by numerous independent studies that the majority of toxicity from Roundup is attributed to POEA in fish, aquatic invertebrates and amphibians (Moore et al., 2012).

Glyphosate Toxicity: Mammals

Several multispecies studies have investigated toxicity of glyphosate in mammals. Human placental cells were exposed to 1% and 2% concentration of Roundup and glyphosate alone displayed signs of cytotoxicity. In all experiments, Roundup formulations were consistently more toxic than treatments using the active ingredients alone. They found that glyphosate acted as a disruptor of mammalian cytochrome P450 aromatase activity. Concentrations used were 100 times lower than those used in agricultural purposes (Richard et al., 2005). Glyphosate has been shown to disrupt aromatase activity, the enzyme responsible for estrogen biosynthesis in Wister pre-pubertal rats. Glyphosate altered progression of puberty in a dose dependent manner using 5, 50, and 250 mg/kg of body mass. Significant changes in body development and reduced testosterone levels in seminiferous tubules were also observed in

treated animals (Romano et al..2010). Glyphosate also induced oxidative stress in pregnant rats and their fetuses. Reduction in endogenous antioxidant defense enzymes as well as increased occurrence of lipid peroxidation were seen in mother rats and their fetuses (Beuret et al., 2005).

Understanding the toxicity mechanisms of Roundup and glyphosate may not only help shed light on ecological factors of exposure, there are clinical implications as well. In southern Taiwan, suicide with agrochemicals is becoming increasingly common. In one study of 131 patients suffering from POEA intoxication, individuals displayed several physiological alterations and symptoms including: sore throat (79.5%) and nausea with or without vomiting (73.8%). The most common laboratory findings were leukocytosis (68.0%), low serum bicarbonate (48.1%), and acidosis (35.8%). 11 out of 131 patients died after intoxication. The mean ± SEM time to death was 2.8 ± 0.8 days. The patients also suffered from respiratory distress, pulmonary edema, blood pressure values less than 90 mm Hg, altered consciousness, abnormal chest x-ray and renal failure necessitating hemodialysis (Lee et al., 2000). Another clinical study consisting of fifty-eight patients (19 men and 39 women) observed the side effects of glyphosate herbicide intoxication. Over the course of the study, forty-one patients survived and 17 died. The patients suffered from respiratory distress, metabolic acidosis, tachycardia, elevated creatinine levels, abnormal chest x-rays and hyperkalemia were found to be highly associated with mortality (Lee et al., 2008). This study's finding of respiratory distress and edema is consistent with other animal studies that have shown that the surfactant in Roundup damages respiratory surfaces (Brausch and Smith, 2007). Glyphosate has also been shown to be cytotoxic when combined with the surfactant POEA. Expression of mitochondrial proteins Bcl-1, Bcl-2 and Bax were measured in the rat heart cell line H9c2 exposed to 5μM and 10μM solutions of glyphosate, POEA and glyphosate and POEA mixtures. Bcl-1 decreased while Bax

increased with exposure to increasing POEA and/or glyphosate concentrations. Kim (2013) used immunological methods to test for translocation of cytochrome C and luminometric measurements to determine activity of caspases 3/7 and 9; and tetra- methyl rhodamine methyl ester assay to measure mitochondrial membrane potentials. Caspase activity increased and mitochondrial membrane potential decreased only when the cells were exposed to a mixture of both POEA and glyphosate, but not after exposure to glyphosate or POEA individually. The results support the possibility that mixtures of glyphosate and POEA induce mitochondrial damage that result in apoptosis and necrosis by possibly uncoupling the mitochondrial membrane and induced glyphosate mediated toxicity (Kim et al., 2013).

Rats exposed to Roundup concentrations of 0 to 15 mM showed decreased mitochondrial oxidative respiration. Rat livers treated with Roundup showed increased succinate-supported respiration while simultaneously collapsing the trans membrane electric potential; while glyphosate treated animals displayed neither of these phenomena. Roundup treatment depressed state 3 respiration by 40%, at 15 mM, whereas uncoupled respiration in the presence of FCCP was depressed by 50%. Glyphosate treated rat livers did not display any of the effects on mitochondrial bioenergetics observed with Roundup treatments. These results are consistent with other studies that show Roundup in conjunction with glyphosate is cytotoxic and influences function of mitochondria and oxidative phosphorylation (Peixoto, 2005). Szarek (2000) used electron microscopy to view changes in hepatocyte structure in carp (*Cyprinus carpio)* fish exposed to Roundup (205 mg of glyphosate/l or 410 mg of glyphosate/l). Treated animals showed swelling of mitochondria as well as disappearance of inner mitochondrial membrane (Szarek et al., 2000).

Several studies have shown that glyphosate has the ability to alter enzymatic activity. In pregnant rats, there was a dose dependent change in glucose-6-phosphate dehydrogenase and NADP dependent isocitrate dehydrogenase activities. In liver, 0.5% glyphosate exposure significantly decreased isocitrate dehydrogenase activity and significantly increased activity with 1% glyphosate exposure. Glucose-6-phosphate dehydrogenase produced the same trend with 0.5% and 1% glyphosate exposure respectively (Daruich et al.,2001).

Glyphosate Toxicity: Amphibians

The global decline of amphibian populations has raised international attention. There are many possible causes for this, one being increased use of agrochemicals (Realya, 2005). Several studies have looked at toxicity in amphibians, focusing on tadpoles and critical developmental stages. *Xenopus laevis* embryos incubated with 1/5000 dilutions of a commercial Glyphosate based herbicide showed increased levels of retinoic acid activity. Treated embryos had abnormal cephalic and neural crest development and a shortened anterior-posterior (A-P) axis. These phenotypes were observed when using glyphosate alone, suggesting that glyphosate alone caused the effects and not the added adjuvants (Paganelli et al., 2010). Another study looked at the lethality of Roundup on three frog species during pre- and post-metamorphosis after directly applying Roundup with 25.2% glyphosate to outdoor pond mesocosms with three different soil types to test specific glyphosate interaction. The results found 96%-100% lethality in larval stages and 68%-86% in juveniles. These values were consistent across all three types of soil (Realya, 2005). Xenopus embryos appear to be extremely sensitive to the POEA surfactant in Roundup. LC5 and LC50 of glyphosate alone were 3,779 and 5,407 mg/L. However with the surfactants the LC5 and LC50 were 2.2 and 2.7 mg/L showing a significant increase in

glyphosate toxicity with the surfactant (Erkins et al., 2000). Glyphosate exposure in amphibians may also affect the ability to cope and respond to predatory stresses. Realya (2005) investigated effects of predatory chemical cues from newts and glyphosate exposure in six North American tadpole species. The LC50 values varied from 0.55 to 2.52 mg of active ingredient (AI)/L in Roundup. In one of the six species tested (*R. sylvatica*), the addition of predatory stress made Roundup twice as lethal. This discovery suggests that there are synergistic interactions between predatory stress and Roundup exposure (Realya, 2005).

Amphibians exposed to POEA alone showed decreased snout-vent length at metamorphosis, increased time to metamorphosis, tail damage and gonadal abnormalities. Ten tadpoles were exposed to Roundup Original for 96 h at concentrations of 12.9, 19.3, and 25.8 mg/L, with three replicates per concentration. These exposure concentrations were equivalent to 4, 6, and 8 mg glyphosate/L, respectively. LC50 values for glyphosate only treatments were greater than 39.5 mg/L. LC 50 values for Roundup were 6.5 mg/L (2 mg glyphosate/L). Exposure to the POEA surfactant or the glyphosate formulations within Roundup resulted in an increased time to metamorphosis in many cases. Concomitantly, exposure to these compounds caused an increased frequency of tail damage. Tail damage was characterized by necrosis of the tail tip, flexure of the tail tip, fin damage, abnormal growths on the tail tip, and/or blistering on the tail fin. These phenotypes were not observed in tadpoles exposed to glyphosate alone. Frequency of these morphological alterations exposed to high concentrations of POEA and Roundup reached 94%. This may have been partly due to disruption in hormonal signaling suggested by elevated levels of thyroid hormone receptor mRNA levels from POEA treated animals (Howe et al., 2004).

Glyphosate Toxicity: Fish

Several studies have looked at toxicity of glyphosate in fish. Glyphosate and Roundup exposure in fish has been shown to alter enzymatic activity, cause oxidative stress and alter mitochondrial function. Silver catfish (*Rhamdia quelen*) were exposed to Roundup herbicide at concentrations of 0 (control), 0.2 or 0.4 mg/L for 96 h. Fish exposed to glyphosate had an increase in hepatic glycogen, but a reduction in muscle glycogen at both concentrations tested. Glucose levels decreased in liver and increased in muscle of fish, while lactate levels increased in the liver and white muscles. Protein levels increased in liver and decreased in white muscle while levels of ammonia in both tissues increased at both glyphosate concentrations. Exposure also resulted in decreased acetylcholinesteerase activity in the brain and increased levels of lipid peroxidation at both concentrations (Glusczak et al., 2007). Carp (*Cyprinus carpio*) exposed to Roundup (205mg/L and 410mg/L of glyphosate) resulted in the appearance of myelin-like structures in carp hepatocytes, swelling of mitochondria and disappearance of internal membrane of mitochondria at both concentrations (Szarek et al., 2000). Uchida (2012) examined changes in gene expression in liver tissues of adult medaka (*Oryzias latipes*) using microarray. Adult male medaka fish were exposed to glyphosate and the fatty acid alkanolamide surfactant (DA) for 48 hr at the following concentrations: 16 mg/l of glyphosate, 0.5 mg/L of DA, and 16 mg/l-glyphosate/0.5 mg/l-DA mixture. The purpose of the study was to compare the toxicity of glyphosate alone with that of the surfactant DA. There were significant changes in 78 and 138 genes in DA and glyphosate/DA mixture respectfully. However glyphosate alone did not induce any significant changes in expression (Uchida et al., 2012).

Glusczak (2006) showed that teleost fish, *Leporinus obtusidens* (piava) when exposed to different concentrations of Roundup; 0 (control), 3, 6, 10, and 20 mg/L for 96 h significantly

decreases AChE activity. Acetylcholinesterase (AChE) activity was significantly decreased in brain tissues of animals treated with Roundup at all concentrations used, but AChE levels were not altered in skeletal muscle. In addition, fish exposed to all glyphosate concentrations had significantly increased hepatic glycogen and glucose, but significantly reduced muscle glycogen and glucose. Ammonia levels were also significantly higher in fish treated with all Roundup concentrations (Glusczak et al., 2006). Cavalcante et al. (2008) exposed neotropical fish, *Prochilodus lineatus* to Roundup to see if exposure altered ion balance and cortisol levels. The 96 h-LC50 of Roundup was 13.69 mg/L. The study consisted of three Short-term toxicity tests (6, 24 and 96 h). The concentrations used for toxicity tests were 7.5 and 10 mg/L. Roundup treatments did not have an effect on maintenance of ionic balance and there was no significant alteration in plasma cortisol levels. However there was an increase in plasma glucose levels of fish exposed to 10 mg/L of Roundup (Cavalcante et al., 2008).

Glyphosate Toxicity: Invertebrates

Several studies have looked at the toxicity of glyphosate and Roundup on invertebrates and have shown that invertebrates are sensitive to the surfactant, POEA. Acute toxicity was studied on the fresh water mussel, *Lampsilis siliquiodea* using Roundup, its active ingredient glyphosate (the isopropryamine (IPA) salt form), IPA alone, and MON 0818 (the surfactant in Roundup) on early life stages. Exposures for all treatments were 48-h with concentrations of 0.5 mg/L. Juvenile *L. siliquoidea* were most sensitive to the surfactant and Roundup compared to glyphosate and IPA salt (Bringolf et al., 2007).

Mottier (2013) compared toxicity of glyphosate vs. Roundup formulations on the pacific oyster, *Crassostrea gigas* during critical developmental stages. Larva were

exposed to Roundup for 24 hours and then 48 hours during development to test for metamorphosis toxicity. The EC50 values were 28.315 and 40.617 mg/L for glyphosate and its metabolite, respectively, and 1.133 and 1.675 mg/L for Roundup formulations. LC50 values for embryos exposed to glyphosate during metamorphosis exceeded values of 100 mg/L for glyphosate and AMPA but were as low as 6.37 and 6.06 mg/L for commercial formulations (Mottier et al., 2013). Another study on the snail, *Biomphalaria alexandrina* examined effects of Roundup exposure on cellular mechanisms of hemocytes. Concentrations used in the experiment were 10 mg/L Roundup with 48% glyphosate over a 7-day period. The study showed that exposure to Roundup significantly increased total hemocytes. Also a single-cell gel electrophoresis or comet assay revealed that Roundup exposure lead to DNA damage in *B. alexandrina* (Mohamed, 2011).

Glyphosate Toxicity: Microorganisms

Some work has been done on microorganisms that show dose dependent changes in biochemistry and metabolic activity as a result of glyphosate and Roundup exposure. One study done on algal species, *Microcystis aeruginosa*, looked at the physiological and biochemical responses to glyphosate and Roundup exposure. The results showed that both the cell numbers and chlorophyll-a content of *M. aeruginosa* increased when the glyphosate concentration increased from 0.01 to 5 mg/L. Roundup exposure below 1 mg/L displayed growth stimulation, however exposure to concentrations exceeding 1mg/L exhibited inhibition on *M. aeruginosa* cell density and Chlorophyll-a content. Further analysis of photosystem II indicated that glyphosate induce stimulation of photosynthesis process whereas Roundup inhibited photosynthesis (Qiu et al., 2013).

The Chicken as a Model Organism

Gallus gallus. The chicken poses a great developmental toxicological testing model because of its short, 21 day embryonic development, available genomic data, and small economic burden. The chicken is also a fantastic developmental model. Avian development is well documented and staged using Hamburger Hamilton methods. Many studies use mammals for drug delivery analysis but because of high costs, ethical issues, difficulties in set up and evaluation more studies are using the chick embryo as a drug delivery system taking advantage of the chorioallantoic membrane for effective drug delivery (Vargas et al,. 2007).

Research Objectives and Hypotheses

The main focus of this study was to determine toxicity of glyphosate and Roundup on developing chicken embryos. Results from this experiment provide insight about effects of glyphosate based herbicides on avian development. I focused on comparative toxicity of Roundup by comparing it with just the active compound glyphosate and observing alterations in morphology throughout development. Few studies have examined the effects of glyphosate alone versus the full compound formulations of Roundup, which include adjuvants and surfactants (Surgan and Séralini, 2005). Adjutants and surfactants are added to aid in solubility and absorption of glyphosate, possibly by making it less water soluble and more lipophilic (Cox and Surgan, 2006). One of the questions of this study is whether the inactive ingredients or adjuvants make glyphosate-based herbicides more toxic to chicken embryos. Therefore my first hypothesis is that chicken embryos exposed to complete formulations of Roundup will have decreased survivability as compared to glyphosate only treatments.

Once survivability was observed, I then evaluated embryo morphology using indicators of growth in response to Roundup and glyphosate exposure. Reduction in embryonic weight is a indicator of developmental toxicity and is commonly used in risk assessment for registration of chemical compounds (Chahoud et al., 1999). My second hypothesis is chicken embryos treated with Roundup and glyphosate on embryonic day 6 have reduced embryo mass, yolk mass, organ masses, tibiotarsus length, and beak length on embryonic day 18.

MATERIALS AND METHODS

Materials

All chicken eggs were received from Texas A&M University. Upon delivery eggs were weighed and incubated at 37.5 degrees Celsius with 60% relative humidity. All eggs were placed in G.Q.F. Manufacturing Co. circulating air incubators that rotate eggs every four hours. Eggs are weighed and candled to ensure eggshell integrity and quality prior to the start of incubation. The egg mass on embryonic day zero was used to calculate egg mass specific dosage for treatments. Incubators were checked daily to ensure that temperature and humidity remained constant throughout incubation. Eggs were incubated for six days, removed, and candled to assess proper growth and development. This is a critical developmental period because the vessels of the allantois begin to enlarge between embryonic days 4-10 of incubation and the allantois fuses with the chorion (Ribatti et al., 2001). On embryonic day 6 (E6) eggs were removed for injection from main incubator and placed into a desktop incubator set at 37.5^{o}C and 60% RH to control for humidity and temperature while injections were made. The egg shell over the blunt end of the eggs were sanitized with 70% ETOH.

Glyphosate and Roundup solutions were prepared using serial dilutions. Dosing was based on embryonic day 0 egg mass. The concentration of Roundup solution was 19.8 and 9.9 mg of active ingredient glyphosate / Kg egg mass. Glyphosate only solutions were prepared using commercial glyphosate with DI water with concentrations of 19.8 and 9.9 mg glyphosate / Kg egg mass. Eggs were injected on embryonic day 6 (E6) because of choroallantoic membrane formation as well as the position of the embryo just beneath the air cell. On incubation day 6, embryo mass is 0.39 ± 0.029 g (Chan and Burggren, 2005). Based on this E6 embryo mass, the average dose for a day 6 embryo was 1538 mg and 3076 mg glyphosate / Kg embryo mass. A

small hole was made into the air cell of the egg using a sterile 21-gauge needle. Egg mass specific injections were made using a 100-microliter syringe. . Sham control eggs had a hole made into the air cell and received injections of sterile DI water. Injections were made onto the inner membrane in the air cell directly above the developing embryo. Twelve embryos were used for every treatment concentration, including control eggs. Embryos were treated on embryonic day six because the embryo is oriented beneath the air cell as well as distinct chorioallantoic membrane formation. Embryonic day 18 was chosen as a stopping point because of the time period just before the chicken begins to hatch. After all injections were made, the holes were sealed using dental wax. All injections were done under sanitized conditions. Eggs were placed into the incubator until embryonic day 12 (E12) and day 18 (E18), when they were candled to determine mortality. On E18 embryos were euthanized by isoflurane overdose and then dissected. All embryos were removed from the egg and weighed to determine embryo wet mass. Yolk, liver, and heart were removed and weighed. After the liver and heart had been weighed they were placed back into the embryo. Tibia and beak length were measured as indicators of growth. Once all measurements were made, the embryos were placed in weigh boats and placed in a drying oven at 60^{o}C for 2 days to obtain dry mass values. The above was repeated for a total of five trials with a total of 430 eggs (Control N = 77; 9.9 mg / Kg glyphosate in Roundup N = 98; 19.8 mg / Kg glyphosate in Roundup N = 80; 9.9 mg / Kg glyphosate N = 95; 19.8 mg / Kg glyphosate N = 80).

Statistical Analysis

The data was combined from the five different experimental runs with at least twelve animals in each treatment group per trial. Survivorship was documented every six days of

incubation until embryonic day 18. Statistical analysis of survival was performed using the log-rank survivability test using Prism 6. All data used from morphometric data were analyzed using mixed models ANOVA and ANCOVA on SAS using egg and embryo masses as covariates to normalize for differences between egg masses. This was followed by a Holm-Sidak post-hoc test. Significant values were taken as $P<0.05$.

RESULTS

Survivability

Survivorship of chicken eggs treated with 19.8 mg / Kg glyphosate in Roundup, 9.9 mg / Kg glyphosate in Roundup, 19.8 mg / Kg glyphosate and 9.9 mg / Kg glyphosate on embryonic day 6 through embryonic day 18 are presented in figure 1. Survivorship was significantly higher in control and glyphosate only treatments versus Roundup treatments ($P < 0.0001$). Survivorship was also significantly higher in animals that were treated with glyphosate alone versus Roundup formulations with the same active ingredient concentration ($P < .0001$).

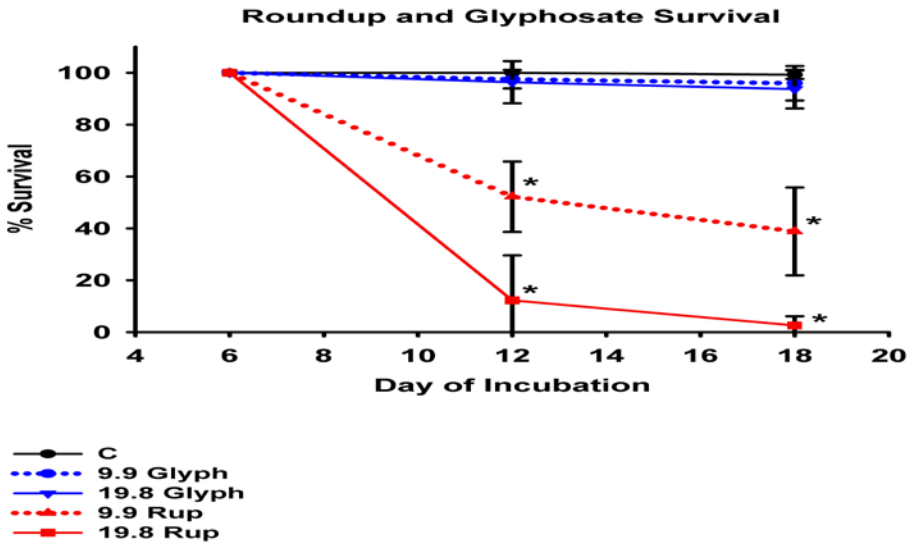

Figure 1. Percent survival of chicken eggs treated with 9.9 mg / Kg glyphosate in Roundup, 19.8 mg /Kg glyphosate in Roundup, 9.9 mg /Kg glyphosate and 19.8 mg /Kg glyphosate on embryonic day 6. An * indicates groups with significant differences compared with control. Data are presented as mean ± SD. Control N = 77; 9.9 mg glyphosate in Roundup/Kg N = 98; 19.8 mg glyphosate in Roundup/Kg N = 80; 9.9 mg glyphosate/Kg N = 95; 19.8 mg glyphosate/Kg N = 80.

Morphometric Measurements: Embryo Mass, Yolk Mass, Heart Mass and Liver Mass

On embryonic day 18, embryos were sacrificed and embryo mass and yolk mass were measured (Figure 2). Changes in embryo mass were used as an indication of developmental toxicity. No morphometric data was obtained for 19.8 mg / Kg glyphosate in Roundup treatments because all embryos died. Chicken eggs treated with 9.9 mg / Kg glyphosate in Roundup and 19.8 mg / Kg glyphosate had significant reductions in wet embryo mass when egg mass was used as the covariate as compared to controls and 9.9 mg / Kg glyphosate treatments (Figure 2A; $P < 0.05$). Embryo dry masses from 9.9 mg / Kg glyphosate in Roundup animals were significantly smaller than controls and 9.9 mg/Kg glyphosate treatments (Figure 2B; $P < 0.05$). Masses from 19.8 mg / Kg glyphosate treated embryos were significantly smaller than control animals ($P < 0.05$). Yolk masses from 9.9 mg / Kg glyphosate in Roundup and 19.8 mg / Kg glyphosate treated animals were significantly reduced when compared to 9.9 mg / Kg glyphosate and control animals (Figure 2C; $P < 0.05$). Yolk masses, correcting for embryo mass, in 9.9 mg / Kg glyphosate in Roundup treated animals were significantly reduced when compared to 9.9 mg / Kg glyphosate treated animals (Figure 2D; $P < 0.05$). Also, 19.8 mg / Kg glyphosate treated animals had significantly smaller yolk masses than 9.9 mg / Kg glyphosate and control animals (Figure 2D; P values < 0.05). The predicted relationship between embryo mass and yolk compared to embryo mass and yolk mass of treated animals is represented in (Figure 5)..

Heart and liver of chickens were removed and weighed on embryonic day 18. Using embryo mass as a covariate revealed that heart and liver masses were not significantly different amongst the different treatment groups and controls (Figure 3A and 3B).

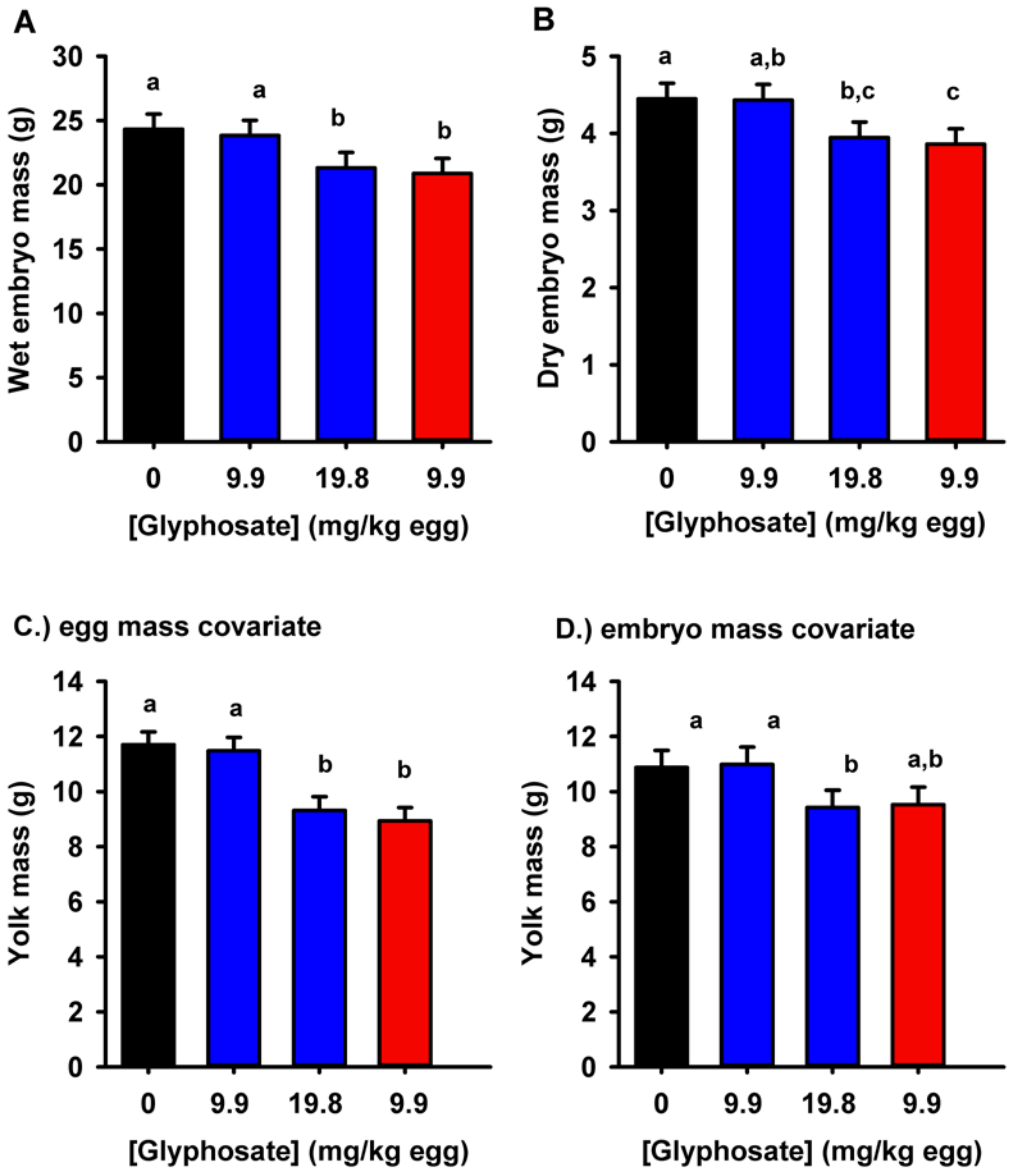

Figure 2. Embryo and yolk masses in response to glyphosate and Roundup treatment at day 18 of incubation. (A) Embryo wet mass (g) using egg mass as covariate, (B) Embryo dry mass (g) using egg mass as covariate, (C) Yolk mass (g) using egg mass as covariate, (D) Yolk mass (g) using embryo as covariate shows affects of treatments on embryo mass. Different letters indicate significant differences between groups using mixed model analysis, followed by sidek post hoc test at $p < 0.05$. Data presented as mean ± SE. Control N = 34; 9.9 mg / Kg glyphosate N = 32 ; 19.8 mg / Kg glyphosate N = 33; 9.9 mg / Kg glyphosate in Roundup N = 35.

Morphometric Measurements: Tibiotarsus Length and Beak Length

Tibiotarsus length and beak length were measured on embryonic day 18 as indicators of growth. Glyphosate and Roundup treatments caused significant reductions in tibiotarsus length when using egg mass as a covariate. Animals treated with 9.9 mg / Kg glyphosate in Roundup had significantly smaller tibiotarsus lengths versus 9.9 mg / Kg glyphosate and control animals (Figure 4A; $P < .0001$). 9.9 mg / Kg glyphosate in Roundup treated animals also had significantly smaller tibiotarsus length than 9.9 mg /Kg glyphosate treated animals using embryo mass as a covariate (Figure 4C; $P < 0.05$). 19.8 mg / Kg glyphosate treated animal's tibiotarsus were also significantly smaller than control animals using egg mass a as a covariate (Figure 4A; $P < 0.002$). 19.8 mg / Kg glyphosate treated animals also showed a significant reduction in tibiotarsus length versus 9.9 mg / Kg glyphosate treated animals (Figure 4A; $P < 0.001$).

Beak length was also impacted negatively by Roundup and glyphosate treatments when using egg mass as a covariate on embryonic day 18. Beak lengths were significantly reduced in 9.9 mg / Kg glyphosate in Roundup treated animals versus control animals (Figure 4B; $P < 0.0001$). Beak lengths in Roundup treated animals were also significantly smaller than 9.9 mg glyphosate / Kg treated animals (Figure 4B; $P < 0.0001$). 19.8 mg glyphosate/Kg treated animals showed a significant reduction in beak length versus control animals ($P < 0.002$). In addition, 19.8 mg / Kg glyphosate treated animals had significantly smaller beaks than 9.9 mg / Kg glyphosate treated animals (Figure 4B; $P < 0.01$). Roundup treatments resulted in a significant reduction in beak length compared to all other treatment groups and controls using embryo mass as a covariate (Figure 4D; $P < 0.001$).

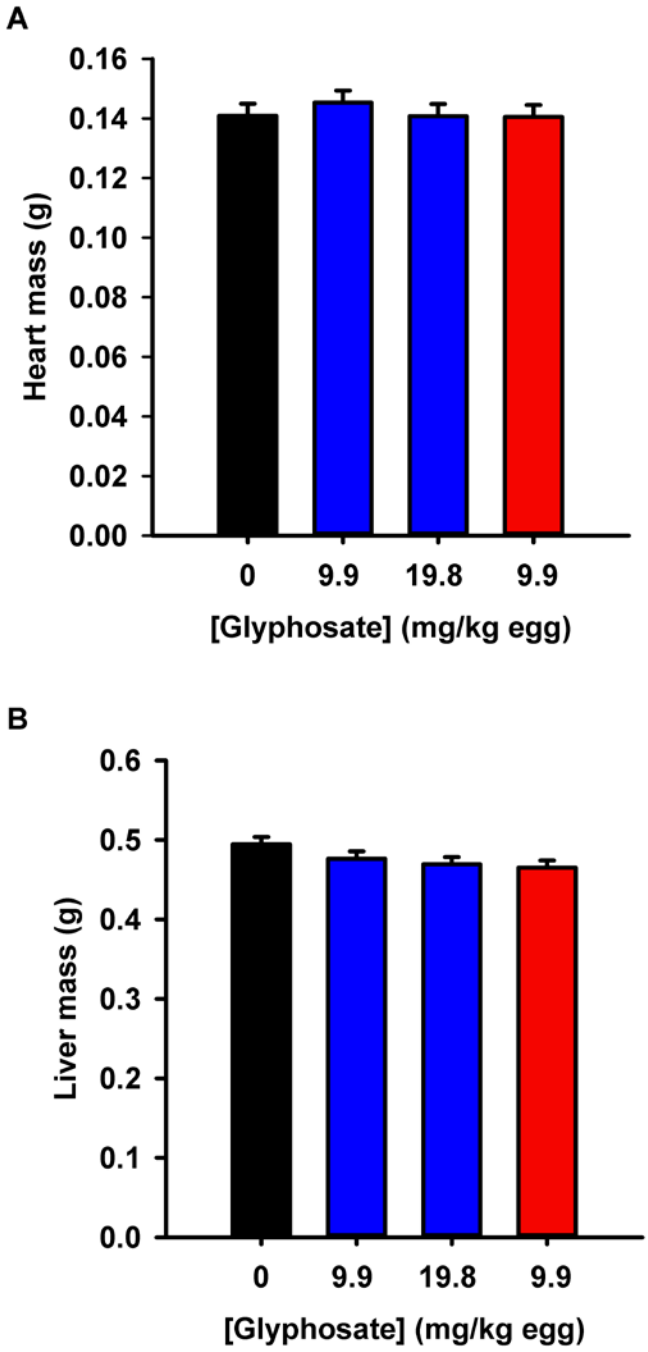

Figure 3. (A) Heart wet mass (g), and (B) Liver wet mass (g) of embryonic day 18 chickens treated with 9.9 mg/Kg Roundup, 9.9 mg/Kg Glyphosate and 19.8 mg/Kg Glyphosate. Statistical analysis was performed using mixed models. There were no significant differences between groups. . Control N = 34; 9.9 mg / Kg glyphosate N = 32 ; 19.8 mg / Kg glyphosate N = 33; 9.9 mg / Kg glyphosate in Roundup N = 35.

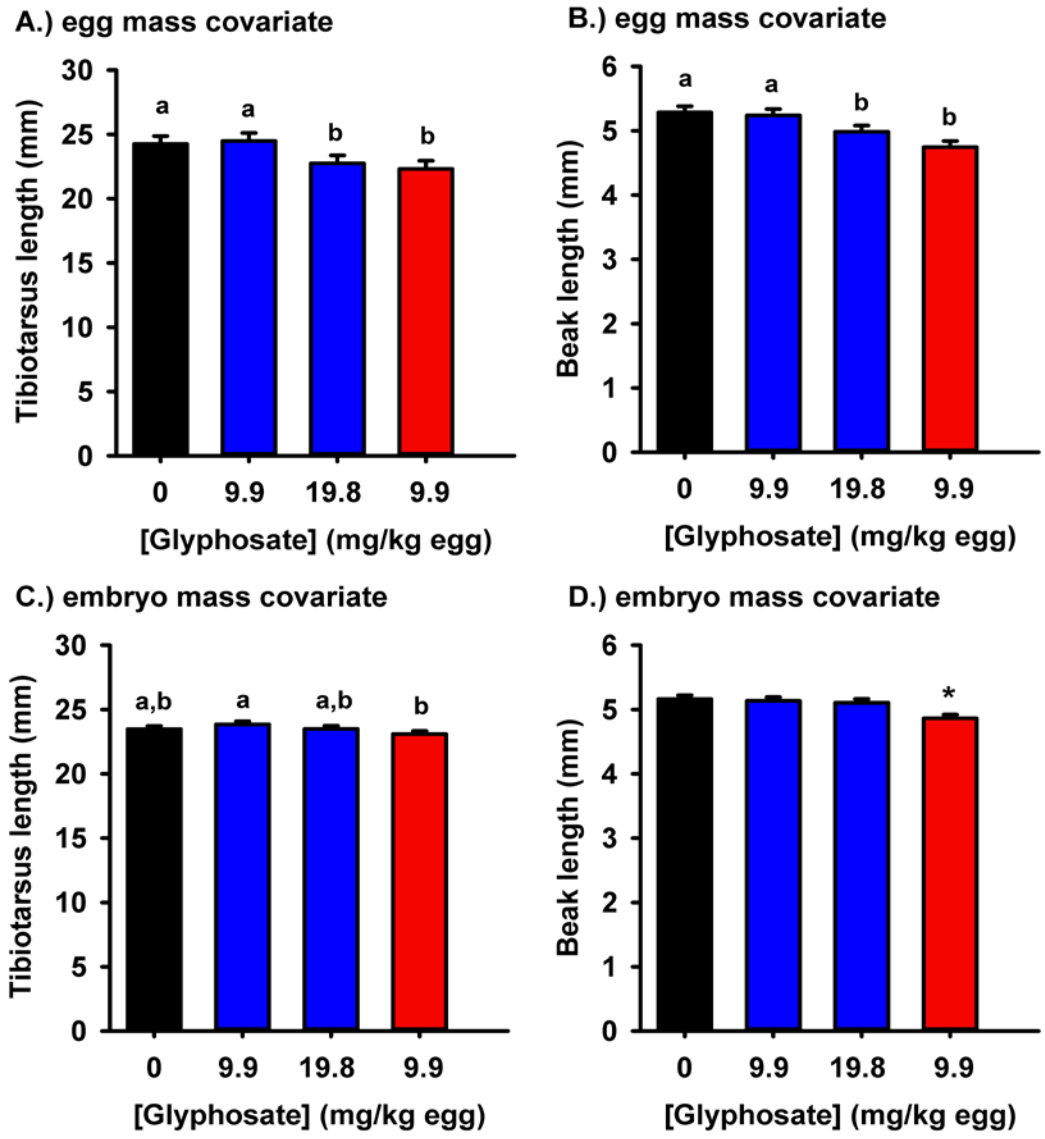

Figure 4. Structural measures of day 18 embryo size in response to glyphosate and Roundup. (A) Tibiotarsus length (mm) using egg mass as a covariate, (B) Beak length (mm) using egg mass a covariate, (C) Tibiotarsus length using embryo mass as a covariate and (D) Beak length (mm) using embryo mass as a covariate. All measurements were taken on embryonic day 18. Letters A, B,C,D and *denote significance differences ($p < .05$) Data is presented as mean ± SE. . Control N = 34; 9.9 mg / Kg glyphosate N = 32 ; 19.8 mg / Kg glyphosate N = 33; 9.9 mg / Kg glyphosate in Roundup N = 35.

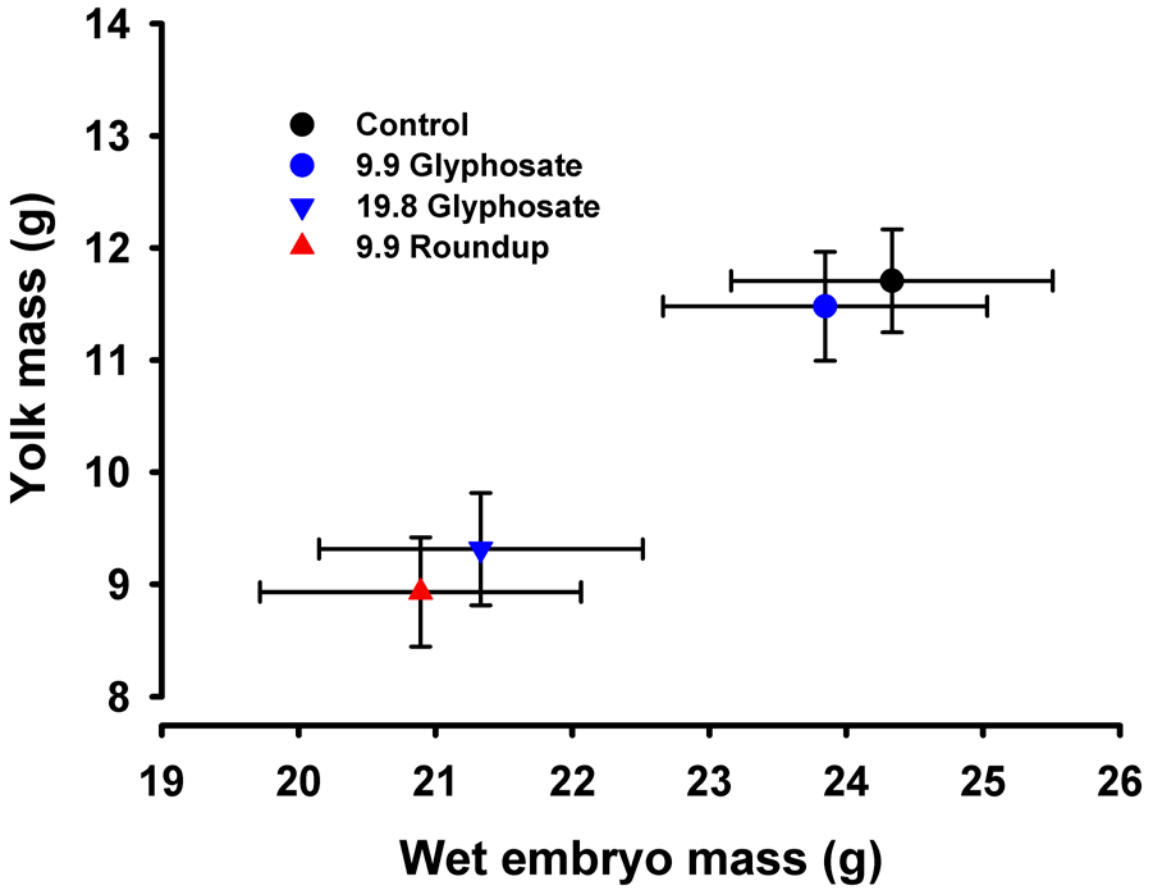

Figure 5. Predicted relationship between yolk mass and wet embryo mass. 19.8 mg/kg glyphosate and 9.9 mg/kg glyphosate inRoundup treatments resulted in simultaneous reductions in wet embryo and yolk masses. Data presented as mean ± SE. Control N = 34; 9.9 mg / Kg glyphosate N = 32 ; 19.8 mg / Kg glyphosate N = 33; 9.9 mg / Kg glyphosate in Roundup N = 35.

DISCUSSION

In this study, I found that glyphosate in conjunction with the inert ingredients of Roundup are more toxic to chicken embryos than the active compound glyphosate alone. Survivability in chickens treated with 9.9 mg/Kg and 19.8 mg/Kg glyphosate in Roundup were significantly lower than in chickens treated with the same concentration of only glyphosate. Results of the study also showed marked differences in morphology. 9.9 mg /Kg glyphosate in Roundup treated groups showed significant reductions in embryo and yolk masses. 19.8 mg/Kg glyphosate treated animals also showed significant reductions in embryo and yolk masses. I also observed changes in beak and tibiotarsus length. 9.9 mg /Kg glyphosate in Roundup treated groups showed significant reduction in tibiotarsus length vs. 9.9 mg/Kg glyphosate treated groups. 9.9 mg/Kg glyphosate in Roundup treated groups also showed a significant reduction in beak length versus all other groups. It is important to note that dosing was based on egg mass on embryonic day 0. In this study the average mass for an egg was 61 grams therefore the average amount of glyphosate that with injected into the egg for 9.9 mg/kg was 0.6 mg and the 19.8 mg/kg was 1.2 mg.

Survivability of Roundup and Glyphosate Treatments

A number of studies have shown that glyphosate with surfactants significantly increases toxicity from exposure. This is consistent with the data and findings of this study. To date there are several studies on amphibians that show the surfactant POEA in Roundup is responsible for the toxic effects of the herbicide (Govindarajulu, 2008). There are several studies that show the majority of toxicity from Roundup is directly linked to the presence of the surfactant POEA. This is consistent with my survivability findings. Xenopus embryos have been shown to be

extremely sensitive to the POEA surfactant in Roundup. LC5 and LC50 of glyphosate alone were 3,779 and 5,407 mg /L. Adding the surfactants decreased the LC5 and LC50 values to 2.2 and 2.7 mg/L, showing a significant increase in glyphosate toxicity with the surfactant (Erkins, et al., 2000) My results are also consistent with known toxicity data on adult bobwhite quail which reports LD50 values of glyphosate only doses exceeding 4500 mg/Kg body weight (Sparling et al,. 2006). Unfortunately I was unable to obtain the POEA surfactant to discount synergistic toxicity with commercial glyphosate.

Mottier et al. (2013) found that LC50 values for oyster embryos exposed to glyphosate during metamorphosis exceeded values of 100 mg/L, but were as low as 6.37 and 6.06 mg/L for commercial formulations (Mottier et al., 2013). Similarly, Erkins et al. (2000) showed that LC 50 values for xenopus embryos exposed to glyphosate exceeded 3,779 mg / L and for Roundup LC 50 values were 2.7 mg / L . (Erkins, et al., 2000).

Acute toxicity studies required to register glyphosate as a pesticide with the EPA found that glyphosate is practically non-toxic to birds. LD 50 values for adult bobwhite quail orally fed glyphosate exceeded 4640 mg / Kg body weight. In this study 19.8 mg / Kg glyphosate treatments were equivalent to 3076 mg / Kg embryo mass on embryonic day 6 which resulted in 93.7% survival in glyphosate only treatments compared to 2.6% survival in equivalent Roundup treatments on embryonic day 18. Percent survival for 9.9 mg / Kg glyphosate was 96.0 % versus 38.1% in 9.9 mg / Kg glyphosate in Roundup treatments. This difference in survivability can be attributed to the surfactant POEA and other inert ingredients of Roundup and not the active compound glyphosate.

Effects on Morphology

Chickens treated with 9.9 mg/Kg Roundup and 19.8 mg/Kg glyphosate were significantly smaller than control and 9.9 mg/Kg glyphosate animals. Paradoxically, these same embryos also had significantly smaller yolks. This implies that glyphosate and Roundup may act as metabolic toxin at these concentrations. Sparline et al (2006)found that red eared slider turtles eggs (*Trachemys scripta elegans*) dipped in 11,206 ppm (mg/L) glyphosate solution for 30 seconds resulted in reduction in body mass and was negatively related to glyphosate concentration. Exposure also resulted in decreased timing to hatching as well as changes in righting behaviors when placed on their backs post hatch (Sparling et al., 2006).

The reduction of yolk mass in conjunction with reduced embryo mass was also observed (Figure 5). However when taking into account the physiological and biochemical alterations as a result of Roundup exposure in other animals, may shed some light on this paradoxical phenomenon. Glusczak's study on catfish found that exposure to roundup resulted in decreased hepatic glycogen, increased muscle glycogen, increased blood glucose levelas and inhibtion of acetylcholineesterase activtiy may possibly be responsible for the increased yolk utilization observed in this study. However, futher testing is needing to see if similar responses are observed in avian species. Changes in cellular enzymatic activity as well as alterations in biochemistry may account for the combined effects of reduced embryo mass and yolk mass.

As discussed previously, exposure to Roundup has been found to alter mitochondrial structure and function. Increased yolk utilization may be a result of permanent damage to mitochondria due to bioenergetic inefficiency. Szarek showed that in catfish exposed to 205 mg/L of Roundup resulted in swollen mitochondria as well as disappearance of the inner mitochonridal membrane. Peixoto showed that rats exposed to 15mM Roundup increased

succinate supported respiration with simultaneous decrease in mitochondrial membrane potential. Further testing needs to be done to see if this also occurs in avian species.

Morphology Measurements: Tibiotarsus Length and Beak Length

In this study I found that exposure to Roundup during development negatively impacted growth of tibiotarsus length and beak length. Using embryo mass as a covariate, tibiotarsus length was significantly smaller than 9.9mg / Kg glyphosate treatments. Embryos treated with 9.9 mg/Kg Roundup displayed significant reduction in beak length compared to control and other treatment animals using embryo mass as a covariate. Chickens and amphibians exposed to low concentrations of glyphosate and Roundup have various teratogens. Chicken embryos were injected on embryonic day 0 with 1/3500 and 1/4500 dilutions of Roundup and analyzed on Hamburger Hamilton stage 9 (8 somites) (Paganelli et al., 2010). There was a dose dependent decrease in Pax6 and *Shh* gene expression from treatments. The treated embryos had alterations in cephalic and neural crest development and shortening of the anterior-posterior axis. Embryos injected with glyphosate alone showed very similar phenotypes. The embryos experienced reduction in optic vesicles and microcephaly. Treated frog and chicken embryos also had increased endogenous retinoic acid activity, which was consistent with the decrease in Sonic hedgehog signaling from the dorsal midline. There was also inhibition of otx2 expression and disruption of the neural crest development. Increased Retinoic acid signaling was attributed to teratogens because a retinoic acid signaling antagonist reversed the teratogenic effects in the treated embryos (Paganelli et al., 2010).

Studies have shown that retinoic acid (RA) has been considered as a morphogen in the chicken limb formation and has also been suggested to be involved in early embryonic

development (Chen et al., 1992). Site specific increases in retinoic acid in the anterior margin of developing chicken embryos leads to cranial-facial malformations and affects the pattern formation in the limbs. This was characterized by the failure of the frontonasal mass of the beak to enlarge (Tamarin et al,.1984). One study done on chickens suggests that retinoic acid itself is not responsible for the discussed abnormalities. Rather retinoic acid helps maintain gradients of other signaling molecules like Sonic hedgehog (SHH) and fibroblast growth factor 8 (FGF8). The decrease in these molecules leads to increased programmed cell death and decreased proliferation in the forebrain and frontonasal process. This study also showed that the morphological defects can be rescued by re-introducing a retinoic acid antagonist, fibroblast growth factor 8 and sonic hedgehog back into the embryo (Schneider et al., 2001). The observed phenomenon that glyphosate exposure alters retinoic signaling activity is a possible explanation for the observed affects in this study, in which treated embryos showed a significant reduction in beak length.

Future Work

The findings from this study are consistent with other studies test for developmental toxicity of glyphosate and Roundup exposure. It would be very beneficial to test for alterations in enzymatic activity such as that performed on the Daruich study that looked at isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase activities to confirm these same phenomenon can be observed in avian species. More work is also needed to understand the specific mechanisms of toxicity of the surfactant POEA used in Roudup. It would also be interesting to see if the same dose dependent increase and decrease of metabolic activity is observed in vertebrates as it is in microorganisms. Further testing of Roundup exposure on other

amniotic organisms is needed to see if the same phenomenon of reduced yolk size and embryo mass occurs. It would also be beneficial to test liver function enzymes such as serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase on embryonic day 18 to test for liver damage as a result from treatments. Histological sections of tussues can be obtained to check for toxicity at the tissue and cellular level. also might be responsible for the increased consumption of yolk, indicating a increased metabolic response due to detoxification.

Because all measurement parameters for this experiment were obtained between embryonic day 6 and day 18, it would be interesting to test the effects of glyphosate and POEA exposure on different developmental stages to check for critical windows of exposure and sensitivity to the compounds. Sparling has shown that red eared slider turtles experience a delay in hatching when exposed to glypphosate during embryonic development. Future studies should be conducted on avian species to see if there is a delay in hatching upon Roundup and glyphosate exposure (Sparling et al., 2006).

CONCLUSION

In this study I have shown that glyphosate in conjunction with the inert ingredients of Roundup are more toxic to chicken embryos than the active compound glyphosate alone. Roundup treatments of 19.8 mg/Kg and 9.9 mg/Kg were significantly more toxoic than equivalent concentrations of glyphosate only treatments, which were indicated by decreased survivability. Further, I have shown that glyphosate and Roundup cause morphological changes in development. 19.8 mg/Kg glyphosate treatements resulted in a significant reduction in wet and dry masses as well as a significant reduction in yolk mass on embryonic day 18. Embryonic wet and dry masses as well as yolk mass were also significantly reduced in 9.9 mg/Kg glyphosate in Roundup treatments on embryonic day 18.

Embryos treated with Roundup resulted in morphometric alterations in tibiotarsus and beak length. Chickens treated with 9.9 mg/Kg glyphosate in Roundup had significantly smaller tibiotarsus compared to respective glyphosate treatments. Also, chickens treated with 9.9 mg/Kg glyphosate in Roundup had significantly smaller beaks when compared to all other treatments and control animals.

The findings of this study are consistant with other studies on vertebrates that show that glyphosate in conjuntion with inert ingredients of Roundup are significantly more toxic than the active compound glyphosate alone. These and other studies will shed light on the mechanisms of toxicity from exposure to this herbicide, which very little is known. This information is valuable especially due to the increasing number of clinical cases of herbicide intoxication, where very limited treatment options are known and available. Aquatic organisms have been shown to be extreemly sensitive to the herbicide. This is of major concern due to increased application of glyphosate based herbicides worldwide. Therefore more testing is needed to fully understand

the effects of glyphosate exposure to aquatic and terrestial organisms and to determine the overall safety and use of glyphosate based herbiceds with their respective surfactants.

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