

Glyphosate induces cardiovascular toxicity in *Danio rerio*



Nicole M. Roy*, Jeremy Ochs, Ewelina Zambrzycka, Ariann Anderson

Department of Biology, Sacred Heart University, Fairfield, CT, United States

ARTICLE INFO

Article history:

Received 4 August 2016

Accepted 10 August 2016

Available online 11 August 2016

Keywords:

Zebrafish
Development
Glyphosate
Cardiac
Vasculature

ABSTRACT

Glyphosate is a broad spectrum herbicide used aggressively in agricultural practices as well as home garden care. Although labeled “safe” by the chemical industry, doses tested by industry do not mimic chronic exposures to sublethal doses that organisms in the environment are exposed to over long periods of time. Given the widespread uses of and exposure to glyphosate, studies on developmental toxicity are needed. Here we utilize the zebrafish vertebrate model system to study early effects of glyphosate on the developing heart. Treatment by embryo soaking with 50 µg/ml glyphosate starting at gastrulation results in structural abnormalities in the atrium and ventricle, irregular heart looping, *situs inversus* as well as decreased heartbeats by 48 h as determined by live imaging and immunohistochemistry. Vasculature in the body was also affected as determined using *fli-1* transgenic embryos. To determine if the effects noted at 48 h post fertilization are due to early stage alterations in myocardial precursors, we also investigate cardiomyocyte development with a Mef2 antibody and by *mef2ca* *in situ* hybridization and find alterations in the Mef2/*mef2ca* staining patterns during early cardiac patterning stages. We conclude that glyphosate is developmentally toxic to the zebrafish heart.

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1. Introduction

Glyphosate is a non-selective, broad-spectrum, post-emergent herbicide (EPA, 2016; Uren Webster et al., 2014) used heavily in agriculture as well as domestic and urban settings. Since its original introduction to farmers in the 1970s, use of glyphosate based herbicides (GBHs) have increased 100-fold and is projected to increase due to emergence of glyphosate resistant weeds (Myers et al., 2016). The EPA reported that in 1987 6–8 million pounds of glyphosate were applied in the United States and by 2007, the EPA reported yearly use in excess of 180–185 million pounds (Aspelin and Grube, 1999; Grube et al., 2011; Myers et al., 2016). One of the initial benefits of glyphosate use was that it strongly absorbs to soil and thus, limited amounts would need to be applied and environmental contamination would be negligible (Giesy et al., 2000). However, glyphosate is water soluble and environmental contamination is evident due to air contamination/rainwater, groundwater leaching and surface runoff (Coupe et al., 2012; Battaglin et al., 2014; Majewski et al., 2014; Myers et al., 2016). Furthermore, the half-life in soil and water is longer than previously thought and can range from days to years depending upon soil composition

(Szekacs and Darvas, 2012) making the risk of incremental build-up evident (Myers et al., 2016). Glyphosate residues are found in crops and processed foods (Service, 2011; Bohn et al., 2014; Myers et al., 2016) namely corn, wheat, barely, soy, and beans where GBHs are routinely applied. Glyphosate was thought to be target specific to plants as its mode of action is to inhibit the 5-enolpyruvylshikimate-3-phosphate synthase enzyme required for aromatic amino acid synthesis in the plant shikimate pathway which vertebrate species lack (Steinrucken and Amrhein, 1980; Schonbrunn et al., 2001). However mounting evidence has shown glyphosate can induce toxic effects in a number of species (Giesy et al., 2000; Uren Webster et al., 2014).

Despite the heavy use of glyphosate based herbicides globally since the 1970s, there is still a lack in the literature regarding its toxicity. Most of the early acute toxicity data in regards to glyphosate come from investigations using frogs. LC₅₀ experiments have been conducted on a number of GBHs with or without surfactants in amphibians (Mann and Bidwell, 1999; Perkins et al., 2000; Relyea and Jones, 2009). Tadpoles chronically exposed to environmentally relevant concentrations of GBH demonstrated abnormalities of the gonads, defects in tail development and decreased body length as measured from snout to vent (Howe et al., 2004). Paganelli expanded on the decreased size along the snout to vent axis showing loss of hindbrain rhombomeric domains, decrease size in the optic vesicle and general microcephaly (Paganelli et al., 2010). There have been a handful of studies investigating the toxicity

* Corresponding author at: Sacred Heart University, Biology Department, 5151 Park Ave, Fairfield, CT 06825, United States.

E-mail address: royn@sacredheart.edu (N.M. Roy).

of glyphosate in the fish model. Most studies were completed on adult fish, for example the effect of glyphosate on sperm quality in the adult zebrafish inducing decreases in sperm motility (Lopes et al., 2014), alterations to steroidogenic factor 1 gene expression in adult female ovaries (Armiliato et al., 2014) and glyphosate induced changes on a number of genes in the ovary and testis (Uren Webster et al., 2014). A study completed on juvenile Nile tilapia demonstrated histopathological changes in liver, kidney, gills and brains (Ayoola, 2008). However, there are few studies investigating developmental toxicity in fish during crucial developmental windows. Recently, glyphosate induced neurotoxicity during the early neural developmental stages was described demonstrating glyphosate induced changes similar to that seen in amphibians (Roy et al., 2016). Other studies investigating glyphosate induced toxicity include studies on protozoa and crustaceans (Tsui and Chu, 2003) and other non-target species (Giesy et al., 2000). Additionally, humans are not immune to the toxic effects of glyphosate. In farm rich areas of South America and Paraguay where GHBs are routinely used in high quantities, numerous birth defects including microcephaly and facial defects have been documented (Benitez Leite et al., 2009; Campana et al., 2010) as well as increased rates of cyclopia (Saldarriaga, 2010; Lopez et al., 2012). Furthermore, the World Health Organization's International Agency for Research on Cancer recently concluded that glyphosate is "probably carcinogenic to humans" (Myers et al., 2016).

To date, there has been a paucity of studies investigating the effect of glyphosate on the heart or heart development. Bullfrog tadpoles were exposed to the glyphosate herbicide Roundup® (41% glyphosate) for 48 h and demonstrated tachycardia and hyperactivity, but this was attributed to stress-induced adrenergic stimulation (Costa et al., 2008). In a study exposing Java medaka to glyphosate, heart rates increased from days 3–16 post-fertilization, but the authors conclude this is due to embryonic stress. A decrease in heart rate was noted after day 16 and attributed to glyphosate induced destruction of the cardiac wall leading to less heart pumping (Yusof et al., 2014). Studies in rabbits have noted low incidences of cardiovascular abnormalities (Kimmel et al., 2013), but a study in rat has noted changes in heart enzymes of mother rats and their fetuses after maternal glyphosate exposure (Daruich et al., 2001). Glyphosate use in suicide attempts in humans has increased and clinicians have found prolonged QT intervals followed by intra-ventricular conduction delay and atrioventricular block leading to mortality (Kim et al., 2014).

The zebrafish has been a long standing model of vertebrate developmental given its genetic homology to higher order vertebrates (Grunwald and Eisen, 2002; Howe et al., 2013). The model is particularly useful as zebrafish pair-wise mating produce hundreds of embryos *ex utero* that are optically transparent, developmental stages are well documented, they mature rapidly to facilitate multi-generational studies and there is a large community with a wealth of genetic tools and transgenic lines. There is also a wealth in the literature utilizing zebrafish specifically for environmental toxicological studies ranging from estrogen mimicking compounds and endocrine disruptors to heavy metals and persistent organic pollutants (Kimmel et al., 1995; Teraoka et al., 2003; Hill et al., 2005; Dai et al., 2014; Garcia et al., 2016). The zebrafish heart is particularly amenable to developmental toxicological studies given its simple anatomy compared to higher order vertebrates, yet the genes involved and the sequence of events in cardiac development are conserved with higher order vertebrate species (Liu and Stainier, 2012). Specification and differentiation of cardiac progenitor cells, heart tube morphogenesis and development of the atria, ventricle and atrioventricular canal are well documented (Staudt and Stainier, 2012; Wilkinson et al., 2014; Houk et al., 2016).

There is mounting evidence that glyphosate based herbicides are becoming a toxicological concern for non-target species. Cur-

rently, there are few studies investigating the developmental toxicity of glyphosate. Here we seek to investigate the cardiovascular effects of glyphosate during embryonic development using the zebrafish vertebrate model. We investigate architectural abnormalities by examining live gross morphology and by using immunohistochemical analysis of the heart and further investigate vascular defects in the body utilizing a transgenic approach. Heart rate upon exposure was monitored and early cardiomyocyte differentiation was investigated. We conclude that glyphosate exposure during heart development decreases heart rate, induces structural abnormalities of the heart and vasculature in the body and alters early cardiac progenitor gene expression during cardiomyocyte differentiation.

2. Methods

2.1. Adult and embryo handling and maintenance

2.1.1. Adult

Wild-type AB strain adult male and female zebrafish were contained in a zebrafish husbandry unit (ZMOD- zebrafish module, Pentair Aquatic Eco-systems®, Inc) set to a 14h:10h light:dark cycle. Water quality including levels of ammonia, nitrate, nitrite and pH values were monitored and a 10% water change performed daily. A combination of brine shimp and TetraMin® flake food was provided twice a day. Transgenic fish *fli-1 gfp* (Lawson and Weinstein, 2002) were a generous donation from the Lawson Lab at the University of Massachusetts Medical Center and cared for as described above for wild-type fish. Wild-type or transgenic embryos were produced by natural pair-wise mating (Westerfield, 1993) using standard mating boxes.

2.1.2. Embryo

Embryos were collected and placed in 30% Danieau Buffer (Westerfield, 1993) in glass petri dishes and incubated at 28.5 °C until 5hpf (hours post fertilization). Embryos were staged according to Kimmel's standard staging series (Kimmel et al., 1995). Treatment protocols were approved by the Sacred Heart University Institutional Animal Care and Use Committee (IACUC) as meeting ethical standards and responsible conduct for animal use.

2.2. Solutions and exposures

Solutions of glyphosate were prepared by diluting glyphosate (Sigma-Aldrich) in 30% Danieau Buffer to a final concentration of 50 µg/ml (Roy et al., 2016). Embryos were transferred to control (30% Danieau Buffer) or glyphosate at 5hpf just before the onset of gastrulation (Roy et al., 2016) and continuously treated at 28.5 °C in glass petri dishes until the desired endpoint (48hpf for heartbeat analysis, live morphological analysis, double immunohistochemistry and vascular analysis using transgenics or 16hpf for *mef2ca* *in situ* hybridization or Mef2 antibody staining).

2.3. Live cardiac morphology

Live images were taken under a Nikon Eclipse 400 microscope attached to a cooled CCD camera. Embryos were placed in depression slides in a lateral position using 3% methyl cellulose to aid in positioning.

2.4. Measuring heartbeats

Embryos were placed in depression slides removing them one at a time from the 28.5 °C incubator to avoid temperature changes that could alter accurate heartbeat data acquisition. Embryos were placed under a dissection microscope in a lateral position to

clearly visualize the heart and heartbeats were recorded for a 60 s timeframe. A total of 60 embryos were counted for control and glyphosate treatments. The experiment was completed in triplicate for a total n for each (control and glyphosate) of 180.

2.5. Immunohistochemistry

S46/MF20- Embryos at 48hpf were fixed overnight in 4% paraformaldehyde and transferred to 100% methanol overnight. Embryos were rehydrated in 100% phosphate buffered saline with 10% TWEEN (PBT) using stepwise rehydration for 5 min each in 75%/25%, 50%/50% and 25%/75% methanol/PBT. Embryos were treated with proteinase K (10 µg/ml in PBT) for one hour and washed three times for 5 min in PBT. Embryos were placed in block (goat serum with 1 g Bovine Serum Albumin) for 2 h at room temperature. Embryos were transferred into 500 µl of S46 (IgG1) (400 µl) and MF20 (IgG2b) (100 µl) primary antibodies (supernatant forms- Developmental Hybridoma Bank) with epitopes specific to the atrium alone (S46) and atrium/ventricle (MF20) and incubated overnight at room temperature. Embryos were washed three times each for 15 min in PBT and transferred to block for 1 h. Embryos were transferred to a 1:500 dilution of Fluorescein goat anti-mouse IgG1 (for S46) and Alexa-Fluor 594 goat anti-mouse IgG2b for (MF20) secondary antibody in PBT in dark amber tubes. Embryos were incubated in secondary antibody for 2 h, washed in PBT three times for 5 min each and imaged under a Nikon Eclipse 400 fluorescent microscope attached to a cooled CCD camera. Embryos were placed dorsally for a direct ventral view of the heart on a depression slide in 3% methyl cellulose to aid in positioning. A total of 15 embryos were imaged for control and glyphosate treatments. The experiment was completed in triplicate for a total n for each (control and glyphosate) of 45.

Mef2- Embryos were fixed at 16hpf. Procedures were followed as described above with the following exceptions- Proteinase K treatment was only 10 min, primary antibody Mef2 (Santa Cruz) was used at a dilution of 1:200 and a goat anti-rabbit Alexa-Fluor 594 secondary antibody was used at a 1:500 dilution. Embryos were placed laterally for views of the somatic muscle fibers and lateral myocardial precursor field. Shown are zoomed images of the right bilateral heart fields. A total of 15 embryos were imaged for control and glyphosate treatments. The experiment was completed in triplicate for a total n for each (control and glyphosate) of 45.

2.6. Transgenic

In the *fli-1* transgenic utilized, the zebrafish *fli-1* promoter drives expression of enhanced green fluorescent protein in all blood vessels during embryogenesis (Lawson and Weinstein, 2002). Control or glyphosate treated live transgenic embryos were treated as described above and were imaged for green fluorescent protein expression under a Nikon Eclipse 400 fluorescent microscope attached to a cooled CCD camera. Embryos were placed laterally on a depression slide in 3% methyl cellulose to aid in positioning. A total of 10 embryos were imaged for control and glyphosate treatments. The experiment was completed in triplicate for a total n for each (control and glyphosate) of 30.

2.7. *in situ* hybridization

in situ hybridizations were performed in accordance with Sagerstrom Lab protocols (Sagerstrom et al., 1996). The *mef2ca* probe utilized was a generous gift from Dr. Yaniv Hinitz King's College, London. The *mef2ca* RNA probe was a digoxigenin labeled (Roche Life Sciences) antisense RNA probe transcribed with a Promega SP6/T7 *in vitro* transcription kit. Hybridization was visualized through colorimetric staining using an anti-digoxigenin

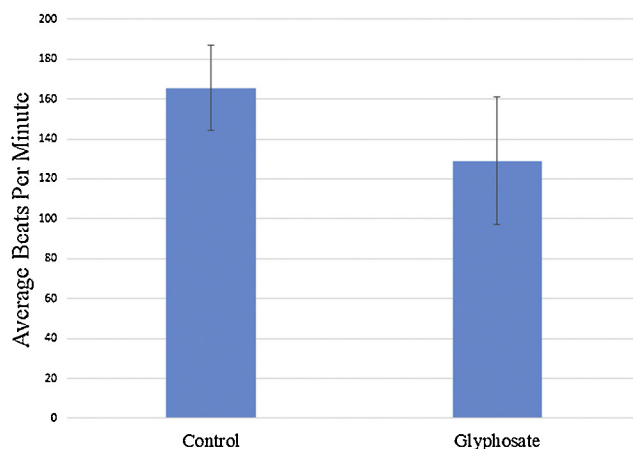


Fig. 1. Heartbeat Measurements. Control embryos averaged 166 beats per minute while glyphosate treated embryos averaged 129 beats per minute. $p < 0.05$.

antibody (Roche Life Sciences) coupled to nitroblue tetrazolium and bromo-4-chloro-indolyl phosphate (NBT/BCIP, Promega). Control and glyphosate samples were run side-by-side in 12-well plates utilizing the same solutions. A total of 15 embryos were imaged for control and glyphosate treatments. The experiment was completed in triplicate for a total n for each (control and glyphosate) of 45. Images shown are zoomed images of the right bilateral heart field as it is difficult to obtain a zoomed image of both sides of the embryo due to curvature of the embryo at this timepoint.

3. Results

3.1. Heart rate

Heartbeats per minute were determined for control and glyphosate treated embryos. Control embryos averaged 166 beats per minute (bpm), while glyphosate treated embryos averaged 129bpm at 48 h post fertilization. The difference was significant with a p value of <0.05 (Fig. 1).

3.2. Live heart morphology

To investigate heart morphology after development in glyphosate, embryos were examined at 48 h in development. In control embryos, in a lateral view, the atria and ventricle are evident and the atrioventricular boundary is apparent (Fig. 2A). In glyphosate treated embryos, phenotypes ranges from mild to moderate to severe. In mild cases, the atria and ventricle appear slightly smaller and misshapen. Slight edema as well as thickening of the endocardium is noted (Fig. 2B). In more moderate cases, the atria and ventricle show clear decreases in size, edema is more pronounced and the endocardium is thickened (Fig. 2C). In more severe cases, massive edema is noted, the atria and ventricle are severely decreased in size and difficult to distinguish (Fig. 2D).

3.3. S46/MF20 double immunohistochemistry

Live images proved difficult to properly score given the thickness of the tissue and difficulties obtaining direct ventral views of the heart under live microscopy. Thus, we performed a whole mount double immunohistochemistry utilizing the S46 antibody with an epitope specific to atrial myosin and the MF20 antibody with epitopes specific for sarcomeric myosin heavy chain in both the atria and ventricle. Control embryos demonstrate the large atria in green and the ventricle in red with the overlap at the atrioventricular boundary in yellow (Fig. 3A). In glyphosate treated embryos,

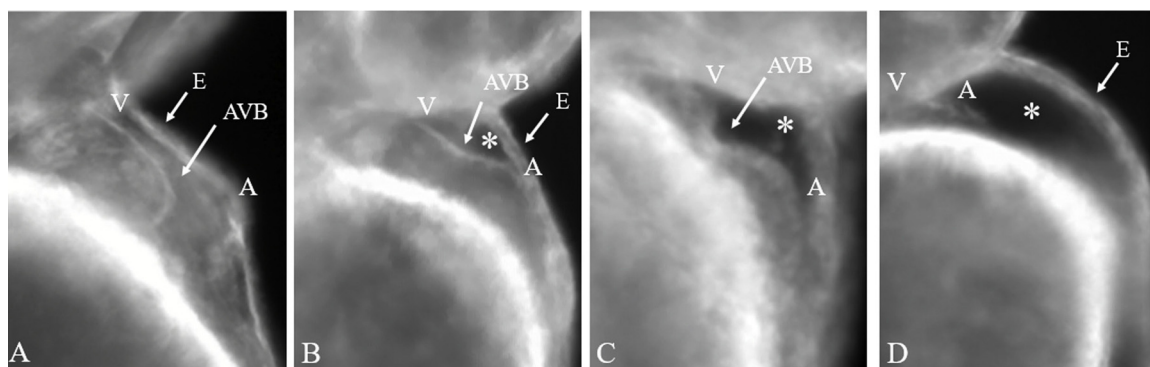


Fig. 2. Live Heart Images. (A–D) lateral views of the heart. Control (A) and glyphosate treated (B–D). V: ventricle, A: atria, AVB: atrioventricular boundary, E: endocardium. Asterisks denotes edema.

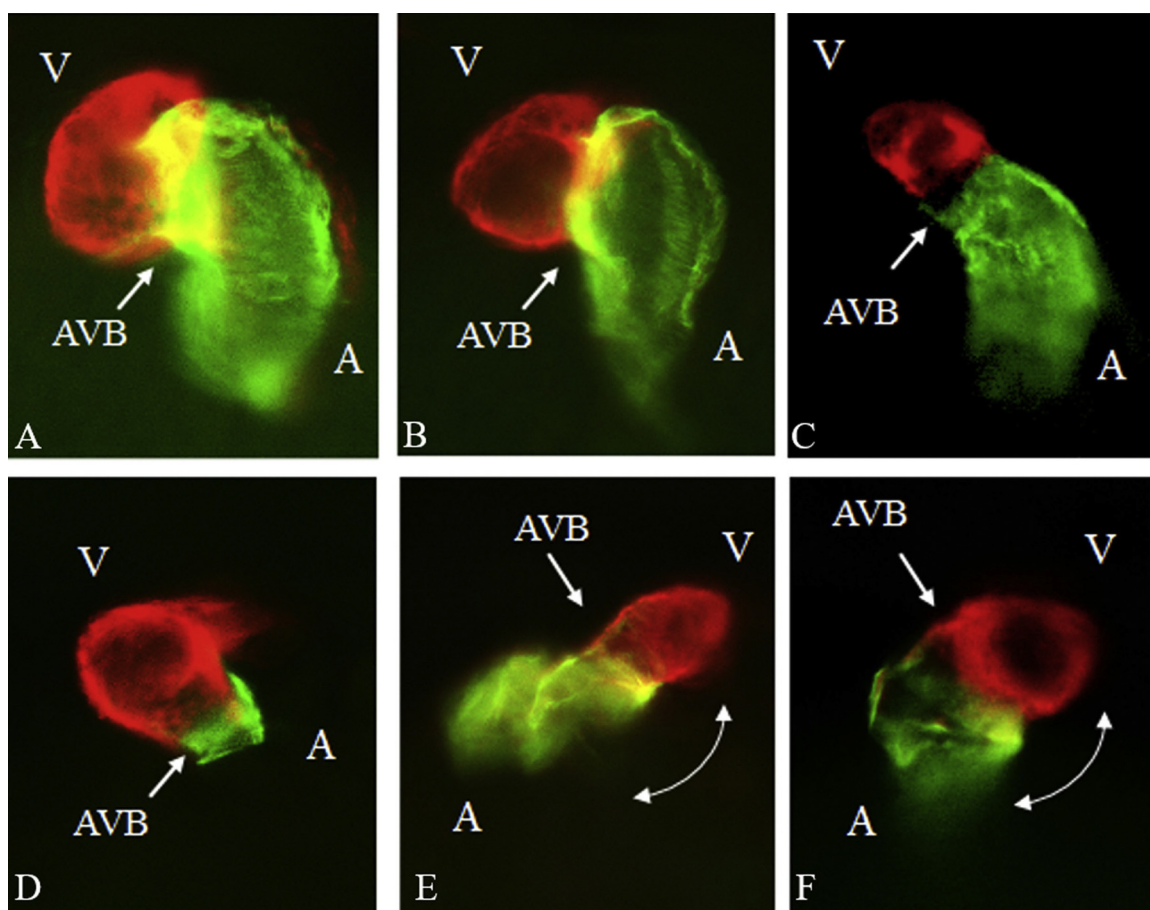


Fig. 3. Double Immunohistochemistry. Whole mount double fluorescent immunohistochemistry using an S46 antibody specific to the atria (shown in green) and an MF20 antibody specific to atria and ventricle (shown in red). Overlap at the atrioventricular boundary in yellow. (A–F) ventral views of the heart. Control (A) and glyphosate treated (B–F). V: ventricle, A: atria, AVB: atrioventricular boundary. Double headed curved arrows denote *situs inversus*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Heart morphological defects as determined by double immunohistochemistry.

Heart Defects (MF20/S46 Immunohistochemistry)					
Time	Treatment	wt atria and ventricle	Abnormally shaped and decreased atria and ventricle Decreased angular heart looping	Severe decrease/loss of atria	Abnormally shaped and decreased atria and decreased ventricle <i>situs inversus</i> heart looping
48 h	Control	43/45 (96%)	2/45 (4%)	0/45 (0%)	0/45 (0%)
	Glyphosate	9/45 (20%)	18/45 (40%)	12/45 (27%)	6/45 (13%)

n = 15 per experiment, replicate = 3, total = 45.

20% demonstrated a normal wild-type phenotype (Fig. 3B, Table 1), while 40% demonstrated abnormally shaped and small atria and ventricle along with a loss of angular heart looping (Fig. 3C, Table 1). Treated embryos also demonstrated a loss of the atria in 27% of embryos (Fig. 3D, Table 1) and in most severe cases decreases in the size of the atria and ventricle with *situs inversus* (Fig. 3E,F, Table 1).

3.4. *fli-1* transgenic

Given the defects we detected in heart development, we sought to investigate if vasculature would also be affected by glyphosate exposure. We utilized a *fli-1 gfp* transgenic which fluoresces green fluorescent protein in all blood vessels during embryogenesis and focused on trunk vasculature as the cranial and cardiac cavities were too difficult to discern due to density and thickness of the tissue. At 48hpf, the fluorescent signal was strong throughout the intersegmental vessels (ISV). In control embryos, the ISVs were evenly spaced and complete vertically through the somatic region (Fig. 4B, Table 2). Glyphosate treated embryos displayed a variety of phenotypes often within the same embryo (Table 2). Phenotypes noted included side branching of one ISV to connect to another instead of vertically through the somatic tissue (Fig. 4C, close up 4H and 4I, Table 2), incomplete ISVs through the somatic region (Fig. 4D, close up 4F and 4G, Table 2), and irregular looping within the somatic tissue (Fig. 4E, close up 4J and 4K, Table 2).

3.5. *Mef2* immunohistochemistry and *mef2ca* in situ hybridization

Control embryos demonstrated punctate nuclei of differentiated slow muscle fibers in the somatic tissue in the classic organized, chevron shaped pattern (Fig. 5A, same body segments as shown in box, Fig. 4A) and clustered lateral myocardial precursor cells along the bilateral heart fields (shown are zoomed images of right bilateral heart field) (Fig. 5F). Glyphosate treated embryos demonstrated various degrees of somatic disorganization and loss of the chevron shaped pattern (53%) (Fig. 5B,C, Table 3) and/or loss of staining (27%) (Fig. 5D,E, Table 3). Additionally, the strong clustering of bilateral myocardial precursors was lost (80%) (Fig. 5G), some with hardly any staining (11%) (Fig. 5H). *In situ* hybridization with a *mef2ca* RNA probe also demonstrates strong bilateral myocardial precursor fields (Fig. 5I) in control treatments, but glyphosate treatments demonstrate a strong loss of staining (78%) (Fig. 5J), some with hardly any staining (9%) (Fig. 5K).

4. Discussion

Glyphosate based herbicides are the most heavily applied pesticides utilized on a variety of crops in the United States with 240 million pounds applied in 2014 (Benbrook, 2012; Myers et al., 2016). Although initial testing performed by the chemical industry suggested low risk to non-target species, mounting evidence from a range of vertebrate species including frogs, fish, rats and even human epidemiological studies suggests that is simply not the case. Given the likely exposure of animals and humans from air and precipitation near farmlands, contaminated drinking water and food crops (Myers et al., 2016), investigations into glyphosate toxicity are needed.

There is limited data regarding cardiovascular toxicity in response to glyphosate exposure especially during delicate windows of development. Amphibian species like frog utilize aquatic areas often adjacent to farmland for reproduction and tadpoles are often developing in pesticide laden waters (Costa et al., 2008). Glyphosate induced toxicity to the tail, gonads and brain development have been described (Howe et al., 2004; Paganelli et al., 2010), but there is little in the literature investigating effects on the heart.

A study completed on bullfrog tadpole development while exposed to Roundup® demonstrated generation of reactive oxygen species and oxidative stress. They also concluded that Roundup® induced tachycardia most likely due to stress-induced adrenergic activity (Costa et al., 2008). Here we do not find a tachycardic response, rather a decrease in heart rate (Fig. 1). However, we utilize different concentrations of glyphosate in our experiments with ours being higher and more likely to induce a teratogenic response rather than a low concentration which could illicit a stress-induced physiological response. Additionally, we treat our embryos right at the onset of gastrulation throughout embryogenesis whereas Costa et al. treat post-embryonic bullfrogs at the tadpole stage. A recent study investigating the effects of glyphosate on early life stages of Java medaka have demonstrated morphological alterations to the tail, abdomen, fin and head as well as changes in heart rate (Yusof et al., 2014). Here, heart rate initially increased in response to glyphosate, fluctuated and finally slowed and eventually stopped over the course of 3–16 days. The authors attribute the increase in heart rate to stress, fluctuations in the heart rate to adapting to the environment and the slowing of the heart rate to cellular disruption to the heart wall leading to less pumping (Yusof et al., 2014). Here the authors utilize a concentration of Roundup® that is several concentrations lower than the manufacturers recommendation and completed a longer duration study, whereas we sought to investigate a more acute exposure to glyphosate over a shorter period of time (Roy et al., 2016). Whereas a longer exposure with a lower concentration caused the initial increase in heart rate, a shorter acute exposure with a higher concentration caused a decrease in heart rate (Fig. 1). We did find one online journal article that also notes a decrease in heartbeats at 48hpf at the concentration we used (50 µg/ml) in zebrafish embryos (Bortagaray et al., 2010).

Besides analysis of heart rate, there is very little in the literature in regards to glyphosate and heart morphology or structure, but one study has shown that maternal exposure to glyphosate leads to abnormalities in a number of enzymes of the heart, liver and brain of their pups (Daruich et al., 2001). A study performed on isolated rat aorta and hearts demonstrated endothelium-dependent vasorelaxation (Chan et al., 2007). Yet, none of these studies look at heart development and morphology. In 2002, the European Commission requested a review of seven unpublished glyphosate studies in regards to heart defects observed during rabbit development. The review concluded that there were random occurrences of cardiovascular malformations, but did not exhibit a dose-response relationship. It was thus concluded that there was little risk of cardiovascular defects resulting from maternal exposure to glyphosate in rabbit pups (Kimmel et al., 2013). In our present study, we do not investigate glyphosate exposure to adults and address cardiovascular defects on the embryos, but rather expose embryos directly as fish embryos are externally fertilized and embryos develop directly in contaminated water sources. Here we find structural abnormalities in both the atria and ventricle. In live control images, the atria, ventricle, atrioventricular boundary and endocardium are present (Fig. 2A). In treated embryos, the atria and ventricle begin to lose their shape and size. Additionally, the endocardium becomes thicker and edema is present (Fig. 2B–D). Immunohistochemistry with atria and ventricle specific antibodies corroborated live analysis again showing a decrease in the atria and ventricle, loss of angular heart looping and *situs inversus*. However, it is difficult to compare our results with other species, as there is very little literature regarding glyphosate or GHBs and heart morphology. We did find one unpublished Master's Thesis investigating pesticides on the developing zebrafish. Although the main body of the thesis focused on carbaryl, glyphosate was also studied. The author concluded that doses of 42–105 µg/ml beginning at 4hpf and testing at 28hpf induced cardiac edema, malformation in tail and debris in the chorion, but no other cardiovascular defects were seen (Lin,

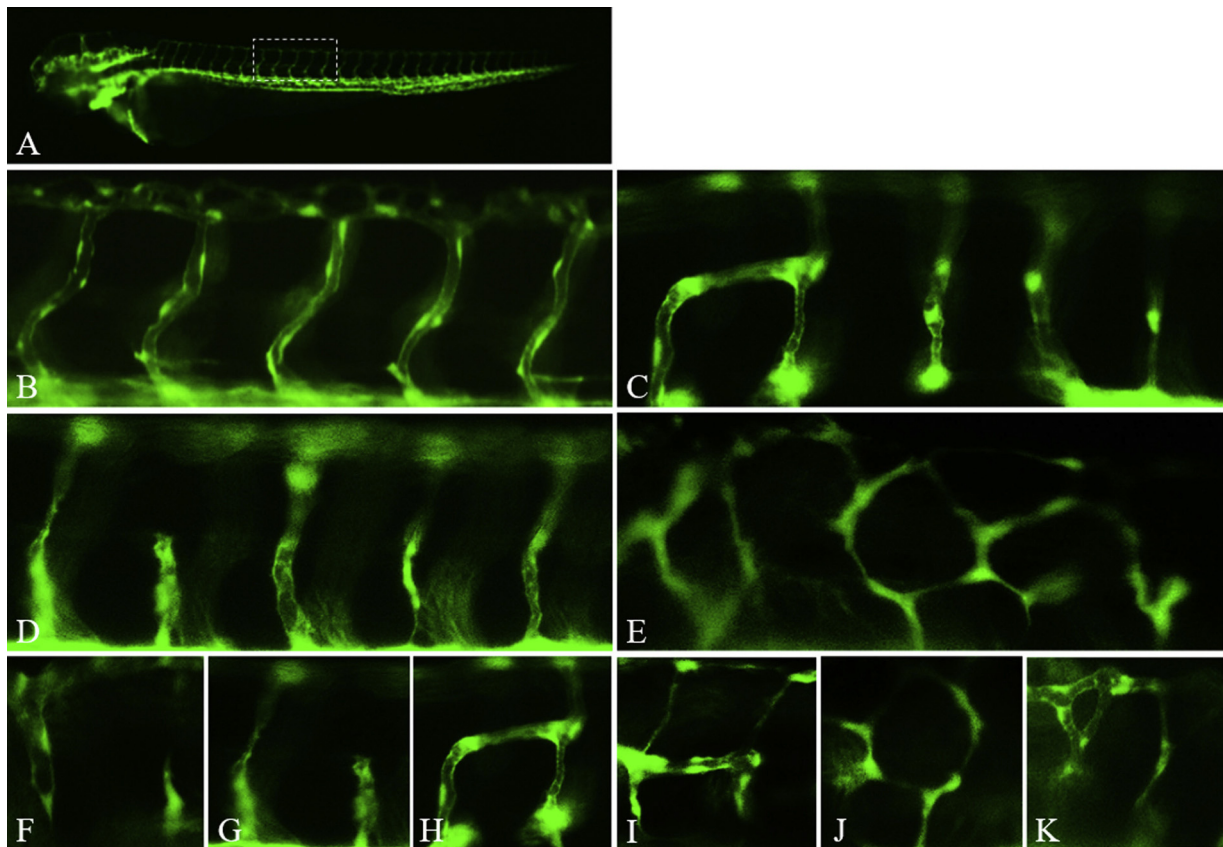


Fig. 4. *fli-1* transgenics and vasculature. Live lateral images of vasculature as seen by green fluorescent protein. Whole embryo (A) with dashed box denoting area of imaging. Control (B) and glyphosate treated (C–K). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

Vascular defects in the body as determined by *fli-1* transgenic embryos expressing green fluorescent protein.

<i>fli-1</i>				
Time	Treatment	incomplete ISV	side branching of ISV	looping of ISV
48 h	Control	1/30 (3.3%)	2/30 (6.7%)	0/30 (0%)
	Glyphosate	18/30 (60%)	22/30 (73%)	7/30 (23%)

n = 10 per experiment, replicate = 3.

Table 3

Glyphosate induced alterations in the somatic tissue and myocardial precursors. NT: not tested.

Time 19hpf	<i>Mef2</i> Antibody		<i>mef2ca</i> in situ	
	Control	Glyphosate	Control	Glyphosate
<i>Staining in somatic tissue</i>				
Strong punctate staining in nuclei of slow muscle somatic fibers in chevron shape	44/45 (98%)	9/45 (20%)	NT	NT
Disorganized pattern	1/45 (2%)	24/45 (53%)	NT	NT
Disorganized pattern and loss of staining	0/45 (0%)	12/45 (27%)	NT	NT
<i>Staining in myocardial precursors</i>				
Strong clustering of cells in myocardial precursor field	43/45 (96%)	4/45 (9%)	44/45 (98%)	6/45 (13%)
Weak clustering and decreased of staining	2/45 (4%)	36/45 (80%)	1/45 (2%)	35/45 (78%)
Loss of staining	0/45 (0%)	5/45 (11%)	0/45 (0%)	4/45 (9%)

n = 15 per experiment, replicate = 3.

2007). In our experiments, we utilize 50 $\mu\text{g}/\text{ml}$ and also treat before the onset of gastrulation at 5hpf, however, our experiments expand past the 24hr time point. We also see cardiac edema, but note the structural changes to the heart at the 48hpf time point, 20 h after Lin had completed the experiment. Additionally, we find one online journal article that notes cardiac edema at 50 $\mu\text{g}/\text{ml}$ at 48hr and also cardiac edema and circulatory delay at 75 $\mu\text{g}/\text{ml}$ (Bortagaray et al., 2010).

As Bortagaray et al. observed circulatory delay, we also wished to investigate the developing vasculature in more detail utilizing *fli-1* transgenic zebrafish embryos. The *fli-1* promoter drives expression of green fluorescent protein (GFP) in all blood vessels during embryonic development. In control embryos, the GFP signal demonstrates the normal pattern of intersegmental vessels (ISVs) running through the somatic tissue (Fig. 4B). However, glyphosate treated embryos demonstrate a poorly connected vascular network

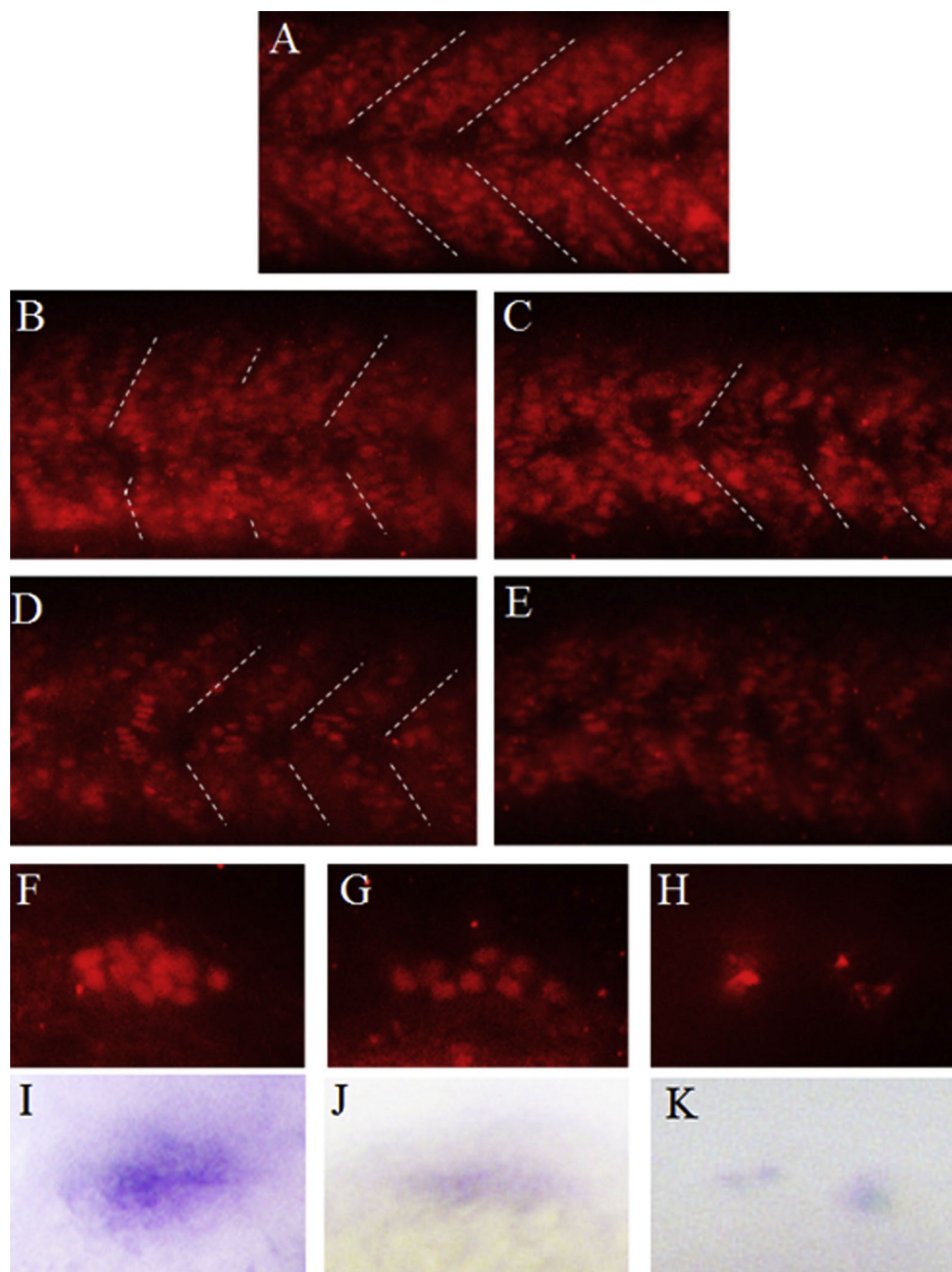


Fig. 5. Mef2 Antibody Immunohistochemistry and *mef2ca* *in situ* hybridization. Lateral view of somitic tissue (A–E, same boxed region as Fig. 4A). Control (A) and glyphosate treated (B–E). Dashed lines denoted chevron shaped somites in the body. Zoomed lateral view of right side myocardial precursors (F–K) as shown by immunohistochemistry with Mef2 antibody (F–H) or *mef2ca* *in situ* hybridization (I–K). Control (F, I) and glyphosate treated (G, H, J, K).

with irregular branching, incomplete ISVs and irregular looping in the somatic tissue. The irregular development of the intersegmental vessels leads to improper movement and slowing of red blood cells throughout the body and back to the heart contributing to edema.

As our results indicate cardiovascular defects at the 48 h time point, we also sought to investigate if these changes were attributable to earlier glyphosate induced alterations in cardiac patterning events. The zebrafish heart is derived from two pools of cardiac progenitor cells, the first and second heart fields which become the cardiac tube, ventricle, atrioventricular canal and atria and the outflow and inflow tracts respectively (de Pater et al., 2009;

Hinitz et al., 2012). The myocyte enhancer factor 2 (Mef2) transcription factor plays a key role in cardiomyocyte regulation and differentiation (Karamboulas et al., 2006). Studies in mice have shown that *Mef2ca* mutants die from gross heart defects including a single ventricular chamber, inflow and outflow tract disruption and defective cardiac looping (Bi et al., 1999; Vong et al., 2006). Additional studies in zebrafish demonstrated that Mef2c is essential for cardiomyocyte differentiation and also skeletal myogenesis (Hinitz and Hughes, 2007; Hinitz et al., 2012). Thus, we sought to investigate if glyphosate induces changes to early Mef2 expressing cardiomyocytes that would lead to the heart defects we observed at the later timepoint and also if it induced changes in skeletal myo-

genesis which could explain the vascular defects observed in the somatic tissue. It was noted that MEF2C null mice exhibit vascular malformations and reduced VEGF, a key factor in generation of the vascular network (Bi et al., 1999). We find that in the skeletal muscle, control embryos show expression of Mef2 in the classic chevron shaped pattern by 16 h (Fig. 5A). However, glyphosate treatments (Fig. 5B–E) show disorganization and a decrease in Mef2 antibody staining in the somatic tissue at 16 h (it was difficult to make any assessments on somatic tissue using *in situ* hybridizations as the embryonic body tends to curve during *in situ* processing). The vascular network is just beginning to form at 24 h when segmental vessels are just starting to sprout from the dorsal aorta and is dependent on VEGF signaling (Lawson and Weinstein, 2002). Thus, the results we see in our disorganized vascular pattern are probably due to prior changes in the somatic tissue and possibly by decreased VEGF signaling due to a decrease in Mef2. By 16 h, we also note in control embryos strong clustered cardiomyocyte expression along the bilateral heart fields (Fig. 5F,I). However, in glyphosate treatments, we note a loss of Mef2/*mef2ca* staining. The loss of Mef2/*mef2ca* expression could lead to a loss of cardiomyocyte differentiation and lead to the heart defects, including *situs inversus* noted in Fig. 3. Further investigation into genes and signaling molecules downstream of Mef2 is needed, but that is beyond the scope of this study.

Conflict of interest

The authors declare that there are no conflict of interest.

Acknowledgements

The authors wish to thank the Lawson Lab (University of Massachusetts Medical Center) for the *fli-1* transgenic fish and Dr. Yaniv Hinitz for the *mef2ca* DNA plasmid.

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