

# Effect of two commercial herbicides on life history traits of a human disease vector, *Aedes aegypti*, in the laboratory setting

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**Abstract** Some mosquito species utilize the small niches of water that are abundant in farmland habitats. These niches are susceptible to effects from agricultural pesticides, many of which are applied aerially over large tracts of land. One principal form of weed control in agricultural systems involves the development of herbicide-tolerant crops. The impact of sub-agricultural levels of these herbicides on mosquito survival and life-history traits of resulting adults have not been determined. The aim of this study was to test the effect of two commercial herbicides (Beyond and Roundup) on the survivorship, eclosion time, and body mass of *Aedes aegypti*. First instar *A. aegypti* larvae were exposed to varying concentrations (270, 550 and 820  $\mu\text{g}/\text{m}^2$  of glyphosate and 0.74, 1.49, 2.24  $\mu\text{L}$  imazamox/ $\text{m}^2$ ), all treatments being below recommended application rates, of commercial herbicides in a controlled environment and resulting adult mosquitoes were collected and weighed. Exposure to Roundup had a significant negative effect on *A. aegypti* survivorship at medium and high sub-agricultural application concentrations, and negatively affected adult eclosion time at the highest concentration. However, exposure to low concentrations of Beyond significantly increased *A. aegypti* survivorship, although adult female mass was decreased at medium sub-agricultural concentrations. These results demonstrate that low concentrations of two different herbicides, which can occur in rural larval habitats as a result of spray drift, can affect the

same species of mosquito in both positive and negative ways depending on the herbicide applied. The effects of commercial herbicides on mosquito populations could have an important effect on disease transmission within agricultural settings, where these and other herbicides are extensively applied to reduce weed growth.

**Keywords** Herbicide · Roundup · Beyond · Glyphosate · Imazamox · Mosquito · *Aedes aegypti* · Agriculture

## Introduction

Agricultural systems use various forms of pesticides to control and prevent the consumption of commercial crops by many species of arthropods. These pesticides have various environmental fates when applied to crop fields. Runoff and spray drift occurs even when pesticides are used according to the safety labels and when used with formulations that reduce drift. Pesticide residues are persistent in irrigation runoff in hazardous levels (Kadoun and Mock 1978) and can enter streams and other waterways around farmland and be deposited in aquatic habitats such as ditches, small ponds, “tree-holes”, and other bodies of water, where pest insects such as the larvae of mosquitoes reside (Tsui and Chu 2008).

Some mosquito species inhabit and thrive in fragmented habitats and agricultural landscapes (Reiter and LaPointe 2007), especially in areas of cluttered vegetation (Fuentes-Montemayor et al. 2013). These habitats are susceptible to effects from agricultural pesticides (herbicides, insecticides, and fungicides), many of which are applied aerially in low-volume sprays over large tracts of land (Floore 2006). These pesticides, in turn, can produce direct and indirect effects on mosquito survival, affecting fecundity,

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adult size, and eclosion time. Additionally, these pesticides may impact mosquitoes directly through feeding on the nectar of flowering crops treated with herbicides or indirectly through systemic herbicides that reach the areas of the plant which supplement sugar for mosquitoes (Van Doorn and de Vos 2013).

The effect of some pesticides on mosquito populations have been demonstrated in recent studies, which have shown that varying concentrations of pesticide can affect mosquito life history traits and vector competence (Kesaraju et al. 2010; Muturi et al. 2011; Muturi 2013). Most studies, however, have focused on insecticides and have not tested the impact of herbicides. The studies which have evaluated effects of herbicides focus mainly on damselflies (*Enallagma*) (Janssens and Stoks 2012), *Daphnia* sp. (Cuhra et al. 2013), frogs (*Rana* sp.) (Relyea 2005) and salamanders (Allran and Karasov 2001) but have not evaluated how herbicides affect the growth, development and survival of mosquito species (Bara et al. 2014).

One principal form of weed control in agricultural systems involves the development of herbicide-tolerant crops (Brimner et al. 2005; Durkin 2010). Two main active ingredients involved in these systems are glyphosate, the active ingredient of the 'Round-up Ready' system<sup>®</sup> and imazamox, the active ingredient of the Clearfield Beyond technology system<sup>®</sup> (Armstrong et al. 2013). Both are applied aerially and via tractor spraying. Glyphosate is an herbicide used to control weeds via inhibition of the enzyme responsible for the synthesis of essential aromatic amino acids in plants and some microorganisms (Baylis 2000). A major component of control strategies in herbicide-resistant crops since the mid-1990s, glyphosate is mainly used in soybeans, cotton and corn but is also commercially available for domestic use to control unwanted weeds in yards (Battaglin et al. 2009). Studies have shown that glyphosate in aquatic environments can reduce mosquito populations that feed on aquatic plants (Serandour et al. 2011) as well as increase expression of specific enzymes which can confer resistance to a variety of insecticides (Raiz et al. 2009).

Imazamox is a newly developed herbicide also used to control weeds using an acetolactate synthase (ALS) inhibition mode of action, common in other herbicide products. Toxicity testing of *Daphnia magna* and algae demonstrated tolerance well below the application rate (Durkin 2010). Reported by the USDA (Durkin 2010), imazamox degrades rapidly in water with an average half-life of 17 days thus posing little long-term threat to non-target organisms. It does not leach into groundwater and does not bio-accumulate in tested organisms. The breakdown products are not herbicidal, but little is known about the herbicide's effects on other invertebrates, including sensitive aquatic insect species. Imazamox, marketed as Beyond<sup>®</sup> under the

Clearfield production system, is used extensively in wheat production in Oklahoma (Armstrong et al. 2013) but is also used extensively in canola, rice, sunflower and lentils (Pfenning et al. 2008).

Recently, Bara et al. (2014) demonstrated that larval development and sex ratio of emerging adults of two important mosquito vectors of arboviruses, *Aedes aegypti* and *A. albopictus*, can be affected when larvae are exposed to sublethal concentrations of the herbicides atrazine and glyphosate. This study, while important in documenting effects of herbicides on mosquito development, was nevertheless limited to testing a single standard concentration of herbicide (5 mg/mL), and utilized the active ingredient of each herbicide tested (Bara et al. 2014). As previously noted, larval mosquitoes are more likely to be exposed to concentrations below the standard agricultural application rate, as a result of spray drift from the field into adjacent aquatic habitats in which larval mosquitoes reside. Additionally, as Cuhra et al. (2013) demonstrated, the commercial formulation of glyphosate (e.g., Roundup) can have greater effects on insect populations than the isolated active ingredient (e.g. glyphosate).

The aim of our study was to expand upon the results of the Bara et al. 2014 study by focusing on how different concentrations of commercially available herbicide products utilized in the field situation would affect the life stage development of an important mosquito vector species. To achieve this, we exposed *A. aegypti* larvae to concentrations of Roundup and Beyond. Larval mosquitoes were placed in treatments of varying concentrations and effects on survival, eclosion time, and adult female mosquito masses were measured. Based upon the results obtained by Cuhra et al. (2013), we predicted that *A. aegypti* development may be negatively affected by Roundup concentrations below that of agricultural application rate. To date, no published studies have evaluated effects of imazamox or Beyond on mosquito development.

## Materials and methods

### Herbicide application

Roundup Ready-to-use (Monsanto; active ingredient glyphosate) (Lot M3114/PM/1/2) and Beyond (BASF; active ingredient ammonium salt of imazamox) (Lot 2/061/M01/MV) were maintained according to label safety instructions and applied directly to 800 mL containers according to estimated field rates. Roundup and Beyond were applied at rates much lower than the concentration that would be sprayed in an agricultural setting. These concentrations are similar to those reported in contaminated aquatic habitats (Bara et al. 2014) and provide an approximate amount that

would be found in an application drift situation in an ecotone adjacent to an agricultural field. Low, medium, and high doses were determined by one, two, and three sprays of Roundup (1.3, 2.6, 3.9 mL) respectively. As per the manufacturer's instructions, this corresponds to 37  $\mu\text{g}/\text{mL}$  (270  $\mu\text{g}/\text{m}^2$ ), 74  $\mu\text{g}/\text{mL}$  (550  $\mu\text{g}/\text{m}^2$ ), and 111  $\mu\text{g}/\text{mL}$  (820  $\mu\text{g}/\text{m}^2$ ) of active ingredient, concentrations which are considerably lower than the 4880  $\mu\text{g}/\text{m}^2$  agricultural application rate recommended by the manufacturer (Monsanto 2015) and lower than the 5000  $\mu\text{g}/\text{mL}$  tested by Bara et al. (2014). Imazamox in Beyond inhibits the production of acetolactate synthase, a necessary growth enzyme in plants (Durkin 2010). A 1:100 dilution of Beyond (1.21  $\mu\text{L}$  imazamox/1 mL) was diluted in water according to manufacturer's instructions then applied to different treatment cups using a micropipette. The treatment concentrations used consisted of 6.5  $\mu\text{L}$  (0.74  $\mu\text{L}$  imazamox/ $\text{m}^2$ ), 13.3  $\mu\text{L}$  (1.49  $\mu\text{L}$  imazamox/ $\text{m}^2$ ) and 19.5  $\mu\text{L}$  (2.24  $\mu\text{L}$  imazamox/ $\text{m}^2$ ) of the Beyond dilution applied to each treatment cup, which is half field application rate (14.6  $\mu\text{L}/\text{m}^2$ ), equal to field rate (29.2  $\mu\text{L}/\text{m}^2$ ), and 1.5 times field rate (43.8  $\mu\text{L}/\text{m}^2$ ), as recommended by the manufacturer (Clearfield® 2015). With imazamox (12.1 %) as the active ingredient in the Clearfield commercial formulation, the field application rate of 4 oz/acre correlated to 3.54  $\mu\text{L}$  imazamox/acre. This indicates that the concentrations of imazamox used in the current study were also below field application concentrations as were the glyphosate concentrations.

### Plant and protein material

Red cedar was used because of associations with mosquito breeding sites in Oklahoma agricultural areas (Reiskind and Zarrabi 2011; O'Brien and Reiskind 2013). Red cedar foliage used as vegetative matter was collected from various locations around Stillwater, OK (Payne County). It consisted of senesced leaf material and was dried at 50 °C for 72 h then weighed and stored in plastic ziplock bags until use in the assays. Protein supplements consisted of store-bought crickets from Petco which were frozen and then dried at 30 °C for 24 h then combined with dried mosquito (*A. aegypti*) and pea aphid (*Acyrtosiphon pisum*) carcasses and then crushed into a protein powder. In natural situations, mosquito larvae often develop in treeholes and containers which have a mixture of animal and plant detritus (Yee and Juliano 2006; Yee et al. 2007; Murrell et al. 2011; Getachew et al. 2015). Thus, the addition of insect detritus with the red cedar plant material is realistic and provides a suitable diet in which to test the relative impact of the herbicides based on the situations being envisioned for this study.

### Responses of cohorts to herbicide concentrations

Two independent toxicology assays, one for each herbicide, were established to test the effects of different concentrations of herbicides on *A. aegypti* larvae. For each herbicide assay, we established eight replicate containers of a control and three increasing concentrations of the herbicide (Roundup or Beyond,  $n = 32$  containers/herbicide assay). Three days prior to adding larvae, 3 g of dried red cedar leaf material and 700 mL of water were added to 800 mL food grade, plastic containers (Newspring Industrial Corporation, Kearney, NJ) and the various treatments of herbicides were applied to appropriate cups kept at room temperature, covered with modified plastic tops with plastic mesh glued on to allow the cups to circulate air without escaping mosquitoes. The evening prior to beginning the experiment, *A. aegypti* (Liverpool strain) eggs, originally from The Malaria Research and Reference Reagent Resource Center (MR4), were hatched using a nutrient broth containing 0.3 g of 1:1 yeast:albumin.

On the day of the experiment, 20 first instar larvae were counted and introduced via pipette to each container as is common for these kinds of mosquito experiments (Kesaraju et al. 2010; Muturi 2013; Bara et al. 2014). The containers were stored in a controlled environmental chamber (26 °C 14:10 L:D). Three days after initialization, all containers were pulsed with 0.1 g of the insect-based protein powder to promote survival and ensure pupation. The decision to pulse at this stage was based on earlier experiences in which the majority of the larvae in cups failed to survive through the pupation stage to adulthood.

After day 7, pupation and emergence data were recorded daily for each container. When individual larvae pupated, each were removed from the treatment containers and transferred to plastic 50 mL conical tubes with 10 mL of original media from their source cup. Tubes were covered with cheesecloth and secured with a rubber band and placed back into the controlled environmental chamber. On eclosion, adult mosquitoes allowed to live at least 24 h after which they were killed in a drying oven for at least 24 h. All mosquitoes were sexed and females were weighed using an analytical microbalance (Mettler Toledo AT20). We collected total survival (male and female), mean dry mass of females, and median days to adult eclosion for each container in each experiment.

From the demographic data collected, the estimated finite rate of increase ( $\lambda'$ ) for each container was calculated using the following equation (modified from Juliano 1998; Livdahl and Sugihara 1984):

$$\lambda' = \exp \left[ \frac{\ln \left[ (1/N_0) \sum_x A_x f(m_x) \right]}{D + \left[ \frac{\sum_x x A_x f(m_x)}{\sum_x A_x f(m_x)} \right]} \right],$$

where  $N_0$  is the initial number of females, (assumed to be 20, or 50 % of the larvae per container),  $A_x$  is the number of females eclosing on day  $x$ ,  $m_x$  is the mean dry mass of females eclosing on day  $x$ ,  $f(m_x)$  is a function predicting female fecundity based upon female mass, and  $D$  is the estimated time needed for the newly eclosed female mosquito to bloodfeed, mate, develop eggs, and oviposit. We used the mass/fecundity regression calculated for *A. aegypti* by Lounibos et al. (2002) to determine  $f(m_x)$ , and we assumed  $D$  to be 12 days for *A. aegypti* (Grill and Juliano 1996). At population equilibrium  $\lambda' = 1$ ; values of  $\lambda' > 1$  indicate population growth while values  $< 1$  indicate estimated population decrease.

For each herbicide assay, we used 1-way ANOVAs to analyze effects of pesticide treatments on proportion survivorship, median days to eclosion, mean female mass,  $\lambda'$ , and proportion females. For significant ANOVAs, we also conducted pairwise comparisons with a Tukey adjustment to determine differences among individual treatments. Data fit the assumptions for normality and equal variance for all variables except  $\lambda'$  in both experiments, which was ln-transformed for analyses, and median days to eclosion in the Beyond experiment which could not be corrected with any transformation. A randomization test was therefore performed on this variable (Cassell 2002).

## Results

### Roundup (glyphosate) effects

Exposure to Roundup at different concentrations had significant effect on survivorship from larval to adult stages ( $F = 5.20$ ;  $df = 3$ ;  $p = 0.0056$ ) (Fig. 1a) as well as adult eclosion time ( $F = 8.99$ ;  $df = 2$ ;  $p = 0.0004$ ) (Fig. 1b). *Aedes aegypti* survival rates in medium and high glyphosate concentrations were significantly lower compared to those from controls and low glyphosate concentration (Fig. 1a). Mosquitoes from the high glyphosate treatment took longer to develop compared to those from the other treatments (Fig. 1b). Roundup had no effect of on sex ratio ( $F = 1.98$ ;  $df = 3$ ;  $p = 0.1456$ ) or mass of resulting female adult mosquitoes ( $F = 1.74$ ;  $d = 3$ ,  $p = 0.1937$ ) (Fig. 1c). Mean  $\pm$  SE  $\lambda'$  was  $0.85 \pm 0.17$ , with no significant difference in  $\lambda'$  across treatment ( $F = 1.88$ ;  $d = 3$ ;  $p = 0.1667$ ) (Fig. 1d).

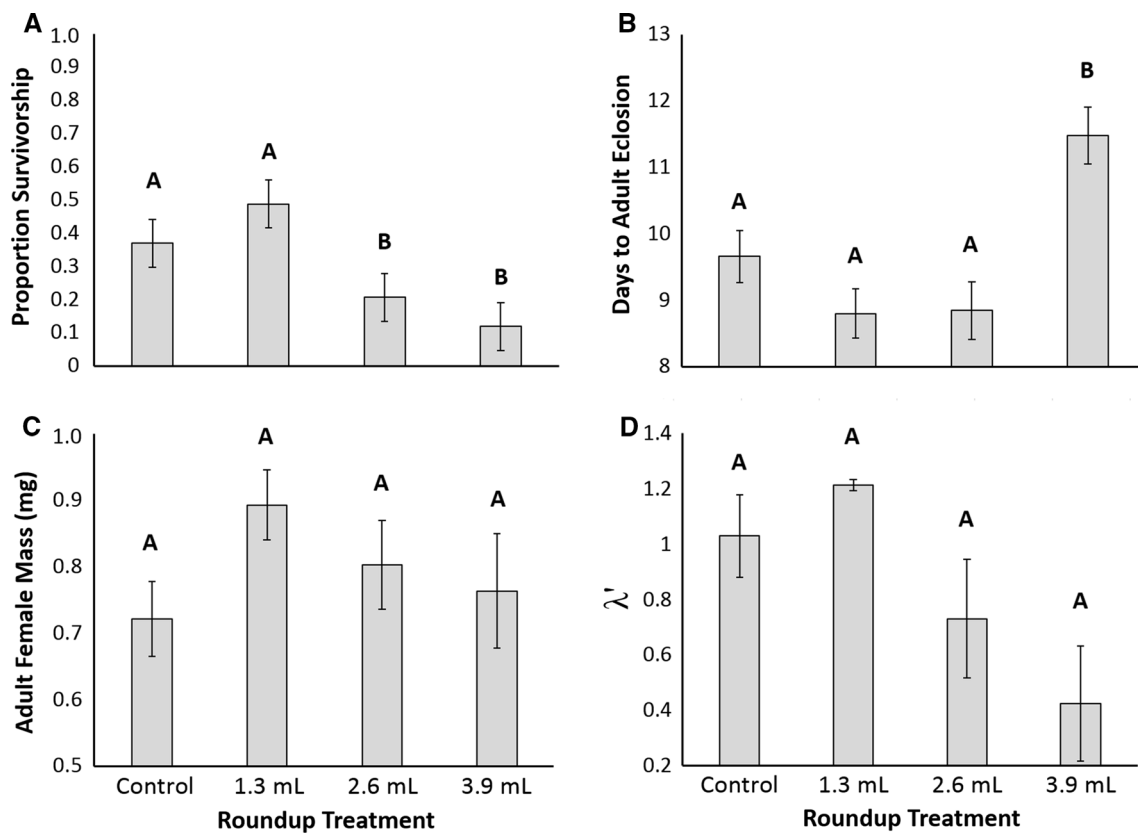
### Beyond (imazamox) effects

Exposure to Beyond at different concentrations had a significant effect on survivorship from larval to adult stages ( $F = 4.66$ ;  $df = 3$ ;  $p = 0.0092$ ) (Fig. 2a) as well as adult female mass ( $F = 4.95$ ;  $df = 3$ ;  $p = 0.0070$ ). Beyond treatments had no significant effect on proportion female survival ( $F = 0.09$ ;  $df = 3$ ;  $p \leq 0.9658$ ), or adult eclosion times (randomization test  $p = 0.7432$ ) (Fig. 2b). *Aedes aegypti* survival rates from the lowest Beyond treatment were significantly higher than the controls and medium and high treatment groups (Fig. 2a). Adult female mass in the medium treatment was significantly lower compared with the controls and other treatment groups (Fig. 2c). Mean  $\pm$  SE  $\lambda'$  was  $1.22 \pm 0.01$ , and was not significantly affected by treatment ( $F = 1.54$ ;  $d = 3$ ;  $p = 0.2263$ ) (Fig. 2d).

## Discussion

The purpose of this study was to evaluate the effect of environmentally relevant concentrations of two commercially available herbicides widely used in the agricultural sector on mosquito life-history traits. The results demonstrate that two different herbicides can affect the same species of mosquito in different ways. Roundup (with the active ingredient of glyphosate) does not appear to affect mosquito survival and development except at medium to high concentrations. These results are supported by Bara et al. (2014) who found no difference in survivorship and eclosion time between controls and glyphosate at sub-lethal concentration levels. On the other hand, Beyond (with the active ingredient ammonium salt of imazamox) appears to increase survivorship at the lowest sub-lethal concentration and produces females which weigh less than controls at a mid-level sub-lethal concentration but has no impact on adult eclosion time or female survival. This increased survivorship may have impacted the mass of resulting females, contributing to significantly lower weight at concentrations than are normally lower than those used for field-application. As smaller *A. aegypti* females are more susceptible to acquiring and disseminating viral infections (Alto et al. 2008), low doses of Beyond may produce a population of mosquitoes that is more likely to transmit disease. Collectively, the effects of different concentrations of these two commercial herbicides on mosquito populations could have an important effect on disease transmission in the agricultural sector, where both herbicides are widely employed to reduce weed growth.

Neither Roundup nor Beyond had significant effects on sex ratio. The result from our Roundup experiment corresponds with Bara et al. (2014), who found no effect of



**Fig. 1** Effect of Roundup® and control treatments on *A. aegypti*. **a** proportion survivorship to adult, **b** median time to adult eclosion, **c** mean female mass, **d** estimated finite rate of increase ( $\lambda'$ ). Error

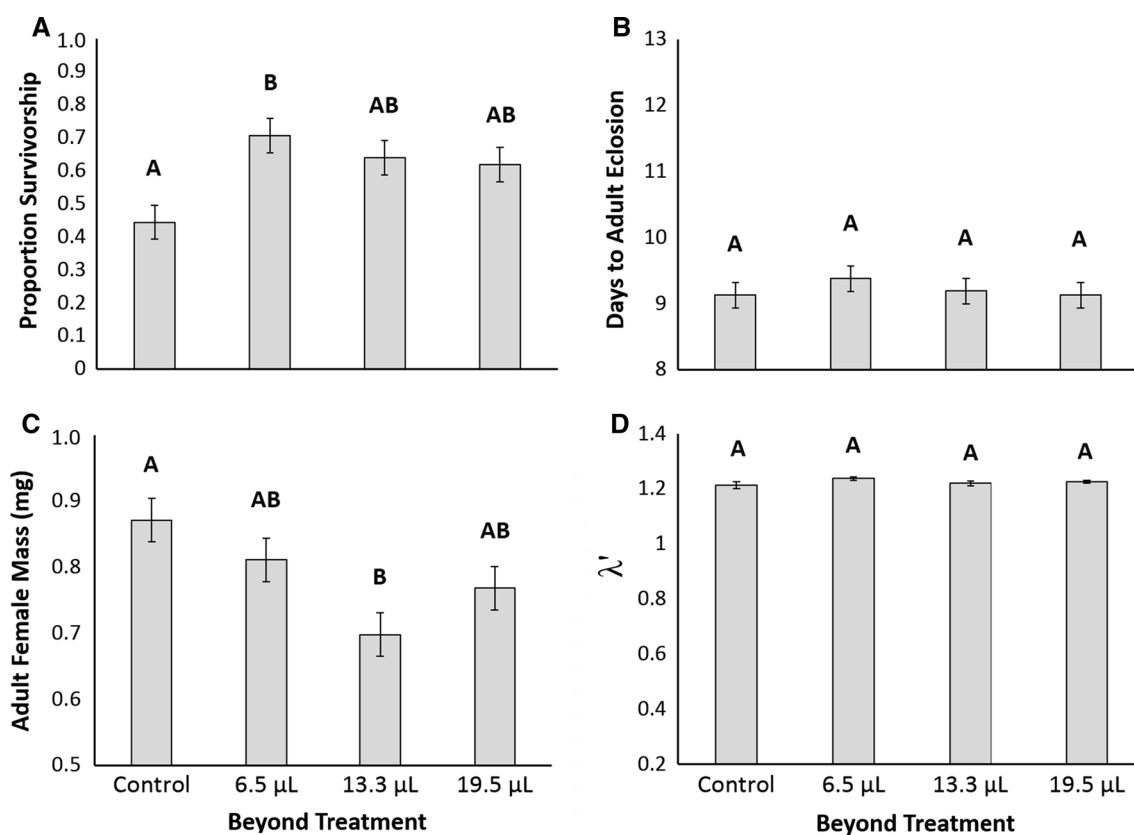
bars denote standard error. Treatment means associated with the same letters do not significantly differ

glyphosate on sex ratio. Beyond showed a similar lack of shift in sex ratio. Additionally, neither of our experiments showed a significant effect of any of the herbicide treatments on  $\lambda'$ . Estimates of population were generally at or above equilibrium ( $\lambda' = 1$ ). Together these results suggest that neither the number of mosquitoes nor the numbers of females are likely to decrease when exposed to sub-lethal, sub-application rates of herbicides.

One notable aspect of this study was the increase in the algal content of the cups as concentrations of herbicides increased. Herbicides within a given aquatic environment stimulate the increase of microbial communities, sometimes to a high level (Muturi et al. 2013). Mosquito larvae will browse or filter feed on a wider variety of heterotrophic microbial communities within a given environment. These are either bacteria within the suspended fluid substrate or growing on various surfaces like the side of the cups or the vegetative matter (Merritt et al. 1992). The significantly lower survival of larvae at the medium and high dosage of Roundup treatment indicates that, for at least *A. aegypti* mosquitoes, the higher microbial blooms observed at higher concentrations may inhibit mosquito survival and development. Similar effects were observed

by Bara et al. (2014) using sublethal concentrations of atrazine and glyphosate in the context of *A. aegypti* and *A. albopictus* larval development. While atrazine had significant effects on both mosquito species, low concentrations of glyphosate had no effect on survivorship, adult eclosion or body mass but had a positive effect on female survivorship. This positive trend, although not significant, was also observed in the current study using a considerably lower concentration than Bara et al. (2014), with higher proportion of survivorship in the medium treatment of glyphosate compared with the lowest dose but the trend collapsed at the highest concentration.

The results from this study could be important from the perspective of the aerial or tractor-based application of Beyond on the Great Plains where it is common to find remnants of trees on the edges of fields used for commercial crops (Coppedge et al. 2001). These remnants can be along roads, provide boundary delineation or form part of a watershed. Over time, these remnants often accumulate old equipment, tires and plastic containers which can serve as breeding sites for local or invasive mosquito species (Juliano and Lounibos 2005; Yee 2008) which can further develop into nidi for local zoonotic transmission of



**Fig. 2** Effect of Beyond<sup>®</sup> and control treatments on *A. aegypti*, **a** proportion survivorship to adult, **b** median time to adult eclosion, **c** mean female mass, **d** estimated finite rate of increase ( $\lambda'$ ). Error

bars denote standard error. Treatment means associated with the *same* letters do not significantly differ

arboviruses such as WNV (O'Brien and Reiskind 2013; Gardner et al. 2014). It is feasible, then, that herbicides could drift into these remnants either aerially at the end of a pass-over of a field or a turn around by a tractor application and sub-concentrations can have an effect on mosquito population development and growth (Bara et al. 2014; Egan et al. 2014). By demonstrating the effect of varying concentrations below field-application levels of actual products used in the agricultural sector, the results reinforce the idea that downstream effects of the herbicides can definitely affect aquatic insect performance.

A second component of this study indicates that herbicides may interact with other components in the substrate which are important in mosquito larvae development. In this study, we used one of the harshest forms of vegetation, the eastern red cedar flower and stems, as the detrital resource to mimic environmental conditions in tree remnants in agricultural areas of Central Oklahoma (Coppedge et al. 2001), where container *Aedes* are common (O'Brien and Reiskind 2013) and the two pesticides we tested are commonly used (Regional IPM 2007). Red cedar material has been shown to enhance *A. albopictus* larval development (Reiskind and Zarrabi 2011; O'Brien and Reiskind

2013). However, the low number of surviving *A. aegypti* in the control treatments in both experiments indicated that some component within the red cedar infused containers was detrimental to *A. aegypti* larval development. This could have involved a component of the red cedar that was either directly toxic to mosquito larvae, or indirectly affected the larvae by suppressing growth of the microbes upon which the larvae feed. What is important to note is that, when herbicides were applied to these containers, the larval response was not consistently negative. For example, higher concentrations of Roundup further compromised survival and mass of already stressed mosquito larvae, while low concentrations of Beyond improved mosquito survivorship. Further experiments could help to determine whether these herbicides are stimulating microbial growth, or interacting with the chemicals in the detritus to affect mosquito development.

The difference in response to Roundup versus glyphosate alone could explain our ability to detect negative effects of Roundup on *A. aegypti*, whereas Bara et al. (2014) detected no deleterious effects of glyphosate alone on *A. aegypti* larval performance. Given that our treatments used lower concentrations than Bara et al. (2014), it is also

possible that something else in the commercial product other than the glyphosate caused deleterious effects on mosquito larvae at higher concentrations. Our study demonstrates the necessity of conducting toxicity assessments across a range of concentrations of both the compound and the commercial product. It also emphasizes the need for future studies that examine commercial blends themselves, which are the chemical formulae that are widely applied and subsequently wind up in our waterways, in addition to studies that investigate the active ingredient alone. One of the key questions for future follow-up studies should focus on whether the active ingredient in Beyond (12.1 %) or Roundup (2 %) or any of the proprietary ingredients (87.9 % (Beyond) and 98 % (Roundup)) are producing the effect in the mosquito population. These are questions which often occur when testing the effects of a commercial product on the development or survival of specific arthropod species (Evans et al. 2010; Revay et al. 2013a, b).

In contrast to the Roundup assay, below field-application concentrations of Beyond lead to significantly higher survival of mosquitoes. Lower concentrations such as this can occur due to drift from an aerial fly-over application of the manufacturers prescribed concentration of Beyond on a particular agricultural crop (de Snoo and de Wit 1998; Reimer and Prokopy 2012). Our research shows that these sub-standard concentrations could lead to an increased likelihood of mosquito survival within an affected tree remnant beside that sprayed agricultural plot. This results in more numerous, smaller female mosquitoes which could lead to increased disease transmission potential of the population (Alto et al. 2008). As in the case of atrazine application (Bara et al. 2014), Beyond applied at wide agricultural scales may successfully suppress weeds, but at the same time has the potential to increase the risk of human infection by mosquito-borne diseases.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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