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Differential Pathogenicity of *Metarhizium* Blastospores and Conidia against Larvae of Three Mosquito Species

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Abstract

Biorational insecticides are being increasingly used in integrated pest management programs. In laboratory bioassays the pathogenicity of blastospores and conidia of the entomopathogenic fungus *Metarhizium brunneum* ARSEF 4556 were evaluated against larvae of three mosquito species. Three propagule concentrations ($1x10^6$, $1x10^7$, and $1x10^8$ spores ml^{-1}) were used in the bioassays. Results showed that *Aedes aegypti* had lower survival rates when exposed to blastospores than when exposed to conidia, whereas the converse was true for *Culex quinquefasciatus* larvae. *Anopheles stephensi* larvae survival rates were similar when exposed to blastospores and conidia except at the higher doses where blastospores were more virulent. Several assays showed little difference in mortalities when using either $1x10^7$ or $1x10^8$ spores ml^{-1}, suggesting a threshold above which no higher control levels or economic benefit would be achieved. When tested at the lowest dose, the LT_{50} of *Cx. quinquefasciatus* using blastospores, wet, and dry conidia was 3.2, 1.9, and 4.4 days respectively. The LT_{50} of *Ae. aegypti* using blastospores, wet, and dry conidia was 1.3, 3.3, and 6.2 days, respectively. The LT_{50} of *An. stephensi* using blastospores, wet, and dry conidia was 2.0, 1.9, and 2.1 days respectively. These observations suggest that for optimized control, two different formulations of the fungus may be needed when treating areas where there are mixed populations of *Aedes*, *Anopheles,* and *Culex.*

Key Words: *Metarhizium, Aedes, Culex, Anopheles,* conidia, blastospores, bioassays.

There are over 3200 species of mosquito worldwide of which the three most important genera are *Aedes* (= *Stegomyia)*, *Anopheles* and *Culex* (Becker *et al*. 2010). Mosquitoes are vectors of a wide range of diseases affecting human and animal health. Some of the notable diseases include malaria, dengue, yellow fever, heartworm, lymphatic filariasis, zika, Western Nile fever, and chikungunya. Mosquitoes impact on over half the world's population (Cancrini and Kramer 2001, Tolle 2009, Marcondes and Ximenes 2016). The mosquito range is gradually increasing due to climate change, globalization of cargo transport, and their ability to rapidly adapt to local environments (Medlock *et al*. 2012, Medlock *et al*. 2015). Exotic species such as *Aedes albopictus* and *Aedes japonicas* have now become firmly established in the USA and Europe (Kaufman and Fonseca 2014, Kraemer *et al*. 2015, Akiner *et al*. 2016). Mosquitoes pose both an economic (e.g. tourism, land usage, trade) and public health threat. For example, the cost of treating dengue alone is estimated to be several billion dollars per annum (Schaffner and Mathis 2014, Guzman and Harris 2015).

Mosquitoes will breed in disparate habitats where water is available for larval development. *Aedes* species will lay eggs, which can survive desiccation, near polluted and unpolluted water, in natural and artificial containers whether indoors or outdoors, while *Culex* oviposit in stagnant dirty water (Hamdan *et al*. 2005). *Anopheles* species usually prefer clean water for oviposition but have also been known to lay eggs in mud (Gimnig *et al*. 2001, Miller *et al*. 2007). All mosquito species will utilise permanent and temporary bodies of water and have overlapping habitat ranges (Lounibos 1981, Yasuoka and Levins 2007, Becker *et al*. 2010).

One major strategy in mosquito control is larval source management (LSM) which is indiscriminate of species and provides the benefits of reducing numbers of both house-

entering mosquitoes and those that bite outdoors (Fillinger and Lindsay 2011). Currently, the most common interventions for mosquito larval control are the application of entomopathogenic bacteria (e.g. *Bacillus thuringiensis israelensis* , *Bacillus sphaericus*), chemical insecticides (e.g. temephos and diflubenzuron), habitat management (e.g. land filling, drainage, covering water container etc.) and the introduction of predatory fish into mosquito breeding sites. Each has its limitations. For example, chemical pesticides are discouraged because of the risk they pose to human health, pollution of the environment and increasing incidence of insect resistance. Entomopathogenic bacteria are environmentally friendly but there are reports of resistance developing to these agents in mosquito populations (Hongyu *et al*. 2004, Liu *et al.* 2004, Paul *et al*. 2005).

Entomopathogenic fungi (EPF) such as *Tolypocladium cylindrosporum*, *Beauveria bassiana* and *Metarhizium anisopliae* show promise for mosquito control (Goettel 1988, Scholte *et al*. 2004, Bukhari *et al*. 2011). One of the advantages of using EPF against mosquitoes is that they can infect and kill eggs, larvae, and adults (Scholte *et al.* 2007, Luz *et al.* 2008, Greenfield *et al.* 2015). Entomopathogenic bacteria can only infect the mosquito larval stages as they need to be ingested to cause death, whereas EPF infect their hosts primarily by penetrating the integument (Shah and Pell 2003, Sanahuja *et al*. 2011).

The use of EPF against the adult stage of the mosquito life cycle is highly promising. One of the current strategies for deployment of EPF against adult mosquitoes is lure and kill. This approach normally involves the use of fungus impregnated surfaces onto which mosquitoes land and following brief contact with the fungal inoculum, become infected and die. Black cloths impregnated with *M. anisoplaie* have been show to significantly reduce *Aedes aegypti* survival rates in simulated field conditions (Paula *et al*. 2013). In Africa, bait stations

impregnated with *M. anisoplaie* were efficient at reducing mosquito survival. Ninety-five percent of *Anopheles arabiensis* mosquitoes that visited the bait stations died within 14 days (Lwetoijera *et al*. 2010).

To date two forms of EPF inoculum have been tested for larval mosquito control namely conidia and blastospores. Conidia are commonly used for control of agricultural pests and are the natural dispersal form of many EPF, produced by structures known as conidiophores on the surface of infected hosts. Conidia are generally resistant to desiccation and can remain dormant in the soil for long periods (Fuxa 1987, Scheepmaker and Butt 2010). Blastospores on the other hand are produced "naturally" only in the hemolymph of the infected host insect (Pendland *et al*. 1993). Blastospores possess thin cell walls and do not readily withstand desiccation therefore they could be more suitable for use in aquatic environments. When comparing the pathogenicity of *Metarhizium brunneum* blastospores and conidia against *Aedes aegypti* larvae, it was found that conidia did not readily adhere to the larval integument, whereas the blastospores adhered and rapidly infected this host (Alkhaibari *et al*. 2016). However, *M. brunneum* conidia killed *Ae. aegypti* larvae following ingestion as a result of the toxicity of proteolytic enzymes on the surface of the conidia (Butt *et al*. 2013)

Both conidia and blastospores have their merits and drawbacks. For example, liquid production of blastospores is cheaper and more rapid (2-3 days) than production of conidia on solid substrates (15 days) such as rice (Jackson 1997). Conidia are hydrophobic and need a surfactant to suspend them in water, while blastospores are hydrophilic and readily suspend in water (Holder and Keyhani 2005, Holder *et al*. 2007). EPF can be applied using a range of delivery systems. Furthermore, they can be deployed in cryptic breeding habitats

including hollows in trees and epiphytic plants (e.g. bromeliads) that retain pockets of water (Berti *et al*. 2014).

Since control programmes will require extensive fungal applications, often in countries where resources are limited, it is important to develop the most virulent yet least expensive product. The current study focuses on a strain of *Metarhizium brunneum* (ARSEF 4556) which meets these criteria. Firstly, ARSEF 4556 has been shown to be high yielding as regards conidia and blastospores (Ansari and Butt 2011, Riaz *et al*. 2013, Greenfield *et al*. 2015). Secondly, conidia of this strain are virulent against *Aedes, Anopheles* and *Culex* larvae and other disease vectors such as midges and ticks (Ansari *et al*. 2010, Ansari *et al*. 2011, Butt *et al*. 2016). However, there is much controversy about which form of inoculum is more efficient for mosquito control. Some studies have shown blastospores to be slightly more virulent than conidia, whilst others show no difference or even lower virulence against mosquito larvae (Soarés Jr 1982, Riba *et al*. 1986, Miranpuri and Khachatourians 1990, Nadeau and Boisvert 1994) . Since studies often targeted different mosquito species and different larval stages, it is difficult to draw conclusions as to which formulation is more appropriate for mosquito larval control. This study compared blastospores and two formulations of conidia of *M. brunneum* ARSEF 4556 against three mosquito species. Both blastospores and conidia were virulent against the three mosquito species investigated here but differences in mosquito survival were seen between species and type of inoculum used. The implications of these findings as regards use of fungi for larval mosquito control are discussed.

Methods

Mosquitoes

Aedes aegypti, *Culex quinquefasciatus,* and *Anopheles stephensi* eggs were obtained from the London School of Hygiene and Tropical Medicine, UK. All eggs were hatched in tap water and incubated at room temperature (25±2°C). The larvae were fed on rabbit food (Burgess®) except *Anopheles* larvae where were fed on fish food (Tetra pro®).

Fungal production

Aerial conidia of *Metarhizium brunneum* isolate ARSEF 4556 were produced in Sabouraud dextrose agar (SDA) and incubated in the dark at 27±1 °C for 15 days, whilst blastospores were produced in Adamek's medium which was inoculated with 1×10^7 conidia ml^{-1} and incubated in a rotary shaker at 130 rev min^{-1} at 27±1 °C FOR 72 hr (Adamek 1963). The viability of conidia and blastospores was over 95%. An improved Neubauer haemocytometer was used to quantify conidial and blastospore concentrations.

Pathogenicity of *M. brunneum* blastospores and conidia

Experiments were performed to assess fungal virulence against larvae by investigating three factors; 1) fungal formulation [blastospores; wet conidia; dry conidia], 2) spore concentrations, and 3) mosquito species. Experiments were carried out on *Ae. aegypti, Cx. quinquefasciatus* and *An. stephensi* larvae . Three replicate groups of ten 3^{rd} or 4^{th} instar larvae (n=30) of each species were exposed to the fungal concentrations of 10^6, 10^7, 10^8 propagules ml^{-1} in plastic cups containing 100 ml of water. The conidia were applied either as wet-formulation following suspension in 0.03% aqueous Tween 80 or as dry conidia (dry weights equivalent to the above aqueous suspensions) by dusting onto the surface of the water. The blastospores were suspended in distilled water. In the control treatment, the

larvae were treated with either distilled water or 0.03% aqueous Tween 80. Mortality was recorded daily for 7 days. In total, 900 insects were used in this study: 3 mosquito species x 3 fungal formulations x 3 spore concentrations x 10 insects x 3 replicates (= 810) + controls of 10 insects x 3 replicates for each mosquito species (= 90).

Statistical Analysis

The proportion of batches of ten insects surviving for up to seven days post infection were visualised using Kaplan-Meier plots. Any insects surviving beyond this time were regarded as 'censored'. Hazard ratios (HR) were calculated to evaluate differences in mortality rate probability between fungal spore concentrations and formulations (Bukhari *et al*. 2010, Greenfield *et al*. 2015), with pairwise comparisons carried out using Log-rank tests (Butt *et al*. 2013). The median lethal time to death, LT_{50}, was estimated using parametric survival regression for combinations of fungal formulation, spore concentration, and mosquito species (Crawley 2012). Preliminary analysis showed that the best fitting parametric survival function was conditional on the specific mosquito species and spore formulation (exponential, Rayleigh, Weibull and lognormal were compared). In all cases, either Weibull or lognormal were optimal, consistent with the expected sigmoidal survival curve. Therefore, survival regression was performed separately for each mosquito x formulation combination. In each case, fungal concentration was fitted as a categorical fixed effect, with replicate sets of mosquitoes included as random effects. This type of mixed-effect model has been shown to be appropriate for survival analysis of replicated insect bioassays previously (Bull *et al*. 2012).

All statistical analyses were carried out using SPSS v22.0 (Morgan *et al.* 2012) and R Version 3.3.1 (RCore 2012).

185 **Results**

186 This study shows that the larvae of all three mosquito species were susceptible to infection
187 by both conidia and blastopores of *M. brunneum* (ARSEF 4556).). Overall mortality for
188 *Aedes aegypti* is shown in Figure 1, *Culex quinquefasciatus* in Figure 2, and *Anopheles*
189 *stephensi* in Figure 3. Responses to different propagule concentrations were conditional on
190 specific combinations of mosquito species and fungal formulation. Median lethal times, LT_{50},
191 are shown in Table 1.

192 The effects of fungal spore concentration are reported in Table 2. Kaplan Meier Log-rank
193 pair-wise comparisons of survival curves showed that *M. brunneum* (ARESF 4556), at all
194 concentrations independent of formulation, caused significantly higher mortalities than the
195 controls ($P < 0.001$) and mortality was dose dependent (Table 2). In *Ae. aegypti,* mortality
196 increased between 10^6 and 10^7 propagules ml^{-1} for all fungal formulations. However, this
197 response plateaued at higher doses, especially when treated with blastospores (Figure 1).
198 This plateau pattern was only observed for *Cx. quinquefasciatus* when exposed to dry
199 conidia at higher doses (10^7 and 10^8 conidia, Table 2, Figure 2). In, *An. stephensi* larvae had
200 similar mortality rates at all conidia concentations (10^6, 10^7, and 10^8 conidia) and at the
201 higher doses of blastospores (10^7 and 10^8 blastospores) (Table 2, Figure 3).

202 Differences in mortality between formulations of fungal spores are reported in Table 3.
203 Significant differences in hazard ratios were seen when comparing between blastospores
204 and conidia but the nature of these differences was conditional on the mosquito species.
205 Generally, larvae of *Ae. aegypti* were significantly more susceptible to infection by
206 blastospores (BS) than by wet or dry conidia (BS vs. Wet conidia: HR = 0.154, P < 0.001 ; BS
207 vs. Dry conidia: HR = 0.134, P < 0.001). Hazard Ratio's in Table 3 show that *Aedes* larvae

208 exposed to wet or dry conidia of *M. brunneum* had a lower mortality rate as compared to
209 those exposed to blastospores (reference formulation) at all concentrations ($P < 0.001$). This
210 pattern was also observed for *An. stephensi* (BS vs. Wet conidia: HR = 0.197, $P < 0.001$; BS
211 vs. Dry conidia: HR = 0.202, $P < 0.001$). However, in the case of *An. stephensi* larvae this was
212 apparent only at the highest concentrations (Table 3, 10^7 and 10^8 spores ml^{-1}). At the lowest
213 dose of 10^6 spores ml^{-1} no significant differences between blastospores and conidia were
214 observed (Table 3; BS vs. Wet conidia: HR = 0.872, $P = 0.597$; BS vs. Dry conidia: HR = 0.725,
215 $P = 0.215$). In contrast, *Cx. quinquefasciatus* larvae have been found to be highly susceptible
216 to conidial infection when compared with blastospores (BS vs. Wet conidia: HR = 5.143, $P <$
217 0.001; BS vs. Dry conidia: HR = 2.054, $P = 0.007$). The hazard ratios of wet and dry
218 formulations of conidia were significantly higher than blastospores at all concentrations ($P <$
219 0.001), with the exception of dry conidia at concentration 10^6 spores ml^{-1} where the hazard
220 ratio was similar to that of blastospores (HR = 0.941, $P = 0.817$).

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Table 1. Median lethal time (LT_{50}) in days of three mosquito species treated with different formulations of *M. brunneum ARSEF 4556* (10^6, 10^7, and 10^8 spores ml^{-1}). Median lethal time (LT_{50}) of different formulations versus species. The 95% confidence intervals are given in parenthesis.

Concentration	Formulation	Mosquito species		
		Ae. aegypti	*Cu. quinquefaciatus*	*An. stephensi*
10^6	Wet conidia	3.33 (2.89-3.76)	1.92 (1.72-2.12)	1.90 (1.76-2.04)
	Dry conidia	6.17 (5.56-6.79)	4.35 (3.96-4.74)	2.12 (1.97-2.28)
	Blastospores	1.28 (1.11-1.45)	3.24 (2.80-3.68)	2.01 (1.88-2.13)
10^7	Wet conidia	2.83 (2.46-3.20)	1.81 (1.61-2.00)	1.86 (1.73-2.00)
	Dry conidia	3.58 (3.26-3.89)	2.10 (1.92-2.27)	1.97 (1.83-2.11)
	Blastospores	1.05 (0.95-1.16)	3.02 (2.69-3.35)	1.00 (0.94-1.06)
10^8	Wet conidia	2.90 (2.55-3.25)	1.09 (0.98-1.20)	2.10 (1.94-2.25)
	Dry conidia	3.43 (3.13-3.73)	1.92 (1.76-2.08)	2.15 (1.99-2.31)
	Blastospores	1.13 (1.01-1.26)	2.38 (2.12-2.64)	1.00 (0.94-1.06)

Table. 2 Kaplan Meier Log-rank pairwise comparisons of survival curves of three mosquito species (*Ae. aegypti, Cx. quinquefasciatus* and *An. stephensi*) exposed to different concentrations of conidia (wet & dry) and blastospores ($1x10^6$, $1x10^7$ and $1x10^8$ ml^{-1}) of *M. brunneum* ARSEF 4556 for 7 days. $P < 0.01$ shown in bold.

Mosquito species	Fungal formulation		Dose (spore ml^{-1})		
			10^6	10^7	10^8
Ae. aegypti	Wet Conidia	Control	**$X^2 = 58.893$ $P < 0.001$**	**$X^2 = 65.530$ $P < 0.001$**	**$X^2 = 64.335$ $P < 0.001$**
		10^6	-	$X^2 = 4.173$ $P = 0.041$	$X^2 = 5.171$ $P = 0.023$
		10^7	-	-	$X^2 = 0.192$ $P = 0.661$
	Dry Conidia	control	**$X^2 = 34.597$ $P < 0.001$**	**$X^2 = 64.168$ $P < 0.001$**	**$X^2 = 64.091$ $P < 0.001$**
		10^6	-	**$X^2 = 29.426$ $P < 0.001$**	**$X^2 = 32.715$ $P < 0.001$**
		10^7	-	-	$X^2 = 0.452$ $P = 0.501$
	Blastospores	control	**$X^2 = 67.078$ $P < 0.001$**	**$X^2 = 66.264$ $P < 0.001$**	**$X^2 = 64.893$ $P < 0.001$**
		10^6	-	$X^2 = 6.501$ $P = 0.011$	$X^2 = 2.538$ $P = 0.111$
		10^7	-	-	$X^2 = 1.639$ $P = 0.200$
Cx. quinquefasciatus	Wet Conidia	control	**$X^2 = 63.271$ $P < 0.001$**	**$X^2 = 61.393$ $P < 0.001$**	**$X^2 = 66.053$ $P < 0.001$**
		10^6	-	$X^2 = 0.861$ $P = 0.353$	**$X^2 = 21.890$ $P < 0.001$**
		10^7	-	-	**$X^2 = 17.239$ $P < 0.001$**
	Dry Conidia	control	**$X^2 = 57.535$ $P < 0.001$**	**$X^2 = 62.129$ $P < 0.001$**	**$X^2 = 56.497$ $P < 0.001$**
		10^6	-	**$X^2 = 42.246$ $P < 0.001$**	**$X^2 = 47.070$ $P < 0.001$**
		10^7	-	-	$X^2 = 1.708$ $P = 0.191$
	Blastospores	control	**$X^2 = 62.137$ $P < 0.001$**	**$X^2 = 57.676$ $P < 0.001$**	**$X^2 = 63.094$ $P < 0.001$**
		10^6	-	$X^2 = 0.999$ $P = 0.318$	**$X^2 = 12.777$ $P < 0.001$**
		10^7	-	-	**$X^2 = 10.864$ $P = 0.001$**
An. stephensi	Wet Conidia	control	**$X^2 = 56.478$ $P < 0.001$**	**$X^2 = 56.840$ $P < 0.001$**	**$X^2 = 61.466$ $P < 0.001$**
		10^6	-	$X^2 = 0.335$ $P = 0.563$	$X^2 = 1.309$ $P = 0.253$
		10^7	-	-	$X^2 = 2.640$ $P = 0.104$
	Dry Conidia	control	**$X^2 = 62.129$ $P < 0.001$**	**$X^2 = 57.467$ $P < 0.001$**	**$X^2 = 62.560$ $P < 0.001$**
		10^6	-	$X^2 = 0.162$ $P = 0.687$	$X^2 = 0.519$ $P = 0.471$
		10^7	-	-	$X^2 = 0.274$ $P = 0.601$
	Blastospores	control	**$X^2 = 60.689$ $P < 0.001$**	**$X^2 = 59.000$ $P < 0.001$**	**$X^2 = 59.000$ $P < 0.001$**
		10^6	-	**$X^2 = 25.286$ $P < 0.001$**	**$X^2 = 25.286$ $P < 0.001$**
		10^7	-	-	$X^2 = 0.000$ $P = 1.000$

239 **Table 3. Hazard ratios (95% CI) of mosquito larvae (*Ae. aegypti, Cx. quinquefasciatus,* and *An. stephensi*) treated with**
240 **(wet or dry conidia and blastospores) and different concentrations (10^6, 10^7, and 10^8 spores ml^{-1}) of *M. brunneum* AR**
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		[HR, (lower , upper 95% C.I.),Z,P value]		
		Ae. aegypti	***Cx. quinquefasciatus***	***A***
10^6	**BS-DRY C**	[0.077, (0.038, 0.156), -7.081, *P*<0.001]	[0.941, (0.561, 1.578), -0.232, *P*=0.817]	[0.725, (0.436
	BS-WET C	[0.181, (0.097, 0.337), -5.394, *P*<0.001]	[3.833, (2.115, 6.947), 4.429, *P*<0.001]	[0.872,(0.524
10^7	**BS-DRY C**	[0.096, (0.046, 0.203), -6.163, P<0.001]	[3.065, (1.673, 5.613), 3.627, P<0.001]	[0.162,(0.077
	BS-WET C	[0.110, (0.053, 0.229), -5.921, *P*<0.001]	[3.549, (1.946, 6.474), 4.131, P<0.001]	[0.205,(0.099
10^8	**BS-DRY C**	[0.094, (0.044, 0.198), -6.198, *P*<0.001]	[2.028, (1.118, 3.677), 2.328, *P*=0.020]	[0.203, (0.099
	BS-WET C	[0.109, (0.052, 0.228), -5.889, *P*<0.001]	[4.680, (2.514, 8.714), 4.867, P<0.001]	[0.197, (0.096

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243 HR: the hazard ratio for wet and dry conidia versus blastospores. If the ratio is above 1, the risk of the event occurring
244 is higher than for blastospores. Z: calculated by dividing the coefficient by its standard error. BS: Blastospores; C: Conidi
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247 **Discussion**

248 This study shows that both conidia (wet and dry) and blastospores of *Metarhizium*
249 *brunneum* ARSEF 4556 are pathogenic to larvae of *Ae. aegypti*, *Cx. quinquefasciatus,* and *An.*
250 *stephensi.* However, there are significant differences in their respective larvicidal efficacy or
251 virulence, with mosquito species, fungal concentration and formulation, which are
252 important factors when considering potential for biological control.

253 The differential susceptibility of mosquito species to conidia of the same strain of
254 entomopathogenic fungus has previously been observed (Geetha and Balaraman 1999,
255 Greenfield *et al.* 2015) but the current study shows that this is also the case for
256 blastospores. One of the most important findings of this study was the high susceptibility of
257 *Ae. aegypti* larvae to infection by blastospores of *M. brunneum*, when compared to conidia
258 of the same fungus, with over 90% mortality being achieved within 24 hrs when using
259 blastospores, compared to conidia, which caused similar rates of mortality only after 3-5
260 days. The blastospores continued to be highly efficacious even when used at 10 fold and 100
261 fold lower concentrations than conidia, offering substantial cost reductions when
262 considering field applications. This phenomenon was not observed for *Cx. quinquefasciatus*,
263 with conidia being more virulent than blastospores. However, *An. stephensi* appeared to be
264 equally susceptible to conidia or blastospores, except at the higher doses where
265 blastospores were seen to be more virulent. There are very few studies comparing the
266 efficacy of blastospores and conidia with most reporting the former to be more virulent. For
267 example, blastospores of *Beauveria bassiana, Beauveria tenella* and *Tolypocladium*
268 *cylindrosporum,* were more virulent than conidia against a range of mosquito species
269 including *Ae. aegypti, Aedes sierrensis, Ae. triseriatus* and *Culex taraslis* (Soarés Jr 1982, Riba
270 *et al*. 1986, Miranpuri and Khachatourians 1990, Nadeau and Boisvert 1994). Interestingly,

Riba *et al*. (1986) found conidia of *M. anisopliae* to be more virulent than blastospores against *Ae. aegypti.* These observations suggest that factors, such as fungal strain/isolate, inoculum dose and culture conditions need to be taken into account (Daoust and Roberts 1983, Maldonado-Blanco *et al*. 2014, Greenfield *et al*. 2015). Most studies show that *Aedes* species are generally more tolerant of conidia than other mosquito species, independent of fungal species or strain (Clark *et al*. 1968, Geetha and Balaraman 1999, Greenfield *et al*. 2015). However, *C. tarsalis* was less susceptible to conidia of *T. cylindrosporum* than *Ae. sierrensis* but both species were rapidly killed by blastospores of this fungus (Soarés Jr 1982).

It is advantageous in biological control programs for the fungus to infect and kill mosquito larvae rapidly. Virulent isolates with fast kill times are an important consideration when choosing candidates for field trials. A faster kill rate may not allow the mosquitoes' immune system to be activated in time to stave off the attack (Alkhaibari *et al.* 2016). Another important factor to consider here is the possibility that the host could free itself from the invading fungal inoculum when shedding the exuvia during the moulting process. Larvae surviving fungal infection to reach the pupal stage do not necessarily develop into adults. Following infection of *Ae. aegypti* larvae with *M. anisopliae*, Pereira *et al*. (2009) found that of the larvae that survived to form pupae, 20% did not become adults.

Alkhaibari and co-workers (2016) studied the pathogenicity processes leading to the higher virulence of blastospores when compared to conidia against *Ae. aegypti*. Their findings showed that blastospores can infect larvae through the integument and gut. . Higher virulence of bastospores *v.* conidia has also been reported for different EPF species attacking disparate terrestrial insects (Hall 1979, Hegedus *et al*. 1992, Nadeau and Boisvert

1994, Jackson *et al*. 1997, Vandenberg *et al*. 1998, Holder *et al*. 2007, Wang *et al*. 2013). Vega and colleagues (1999) suggested that blastospores possess pathogenicity attributes absent or less pronounced in conidia such as rapid germination.

In the case of aquatic mosquito larvae exposed to conidial suspensions, it was found that mortality was caused not by a "normal" infection process involving propagule adhesion, germination, penetration and colonization of the host, as *Ae. aegypti* larvae were killed by protease-induced stress following ingestion of huge quantities of conidia (Butt *et al*. 2013). Conidia neither adhere to *Ae. aegypti* larval cuticle nor germinate inside the gut lumen following ingestion (Butt *et al*. 2013, Greenfield *et al*. 2014). In contrast, blastospores rapidly adhere to and penetrate the cuticle and also penetrate the gut lumen, the multiple entry routes accelerating death (Alkhaibari *et al*. 2016). What is unclear in the current study is why blastospores were less effective against *Cx. quinquefasciatus.* It is tempting to speculate that differences in susceptibility are linked with feeding behaviour since "collector-filterer" *Culex* and *Anopheles* larvae feed within the water column whereas "collector-gatherer" *Aedes* larvae obtain resources from organic compounds on surfaces and sediments (Merritt *et al*. 1992). Yee *et al*. (2004) found that *Culex* tend to remain at the top of water containers, where hydrophobic conidia would be located, whereas *Aedes* spend more time in the middle or at the bottom of water containers, where blastospores would be mostly located. However, other factors could be involved in the susceptibility of larvae to different inoculum types, especially when comparing *Aedes* or *Anopheles* to *Culex*. Insect defence responses could be different between species, although we can only ascertain that blastospores and conidia elicit similar defence responses in *Aedes*, and that these responses especially in the case of blastospores were not able to slow down the rapid infection process (Alkhaibari *et al.* 2016). We are currently studying the infection process of

blastospores when attacking *Culex* larvae and hope this will shed some light on the differential virulence between species.

What has been made clear by this study is that in niches where *Ae. aegypti* and *An. stephensi* predominate, blastospores could provide rapid control of larvae. However, where *Cx. quinquefasciatus* is abundant, then conidia would be a better control option. From a commercial perspective, strain ARSEF4556 has considerable potential because of high conidia and blastospore yields in solid and liquid production systems, respectively (Ansari and Butt 2011, Riaz *et al*. 2013). The use of blastospores against *Ae. aegypti* larvae is not only interesting in respect of the high virulence shown by this form of inoculum, but also for the potential in field applications. This mosquito species lays its eggs in a variety of water containers, normally with relatively low volumes, to which formulated blastospores could be applied. This behaviour is different to that of *Culex*, which can lay eggs in large bodies of water, making any type of control strategy against *Culex* larvae more complicated.

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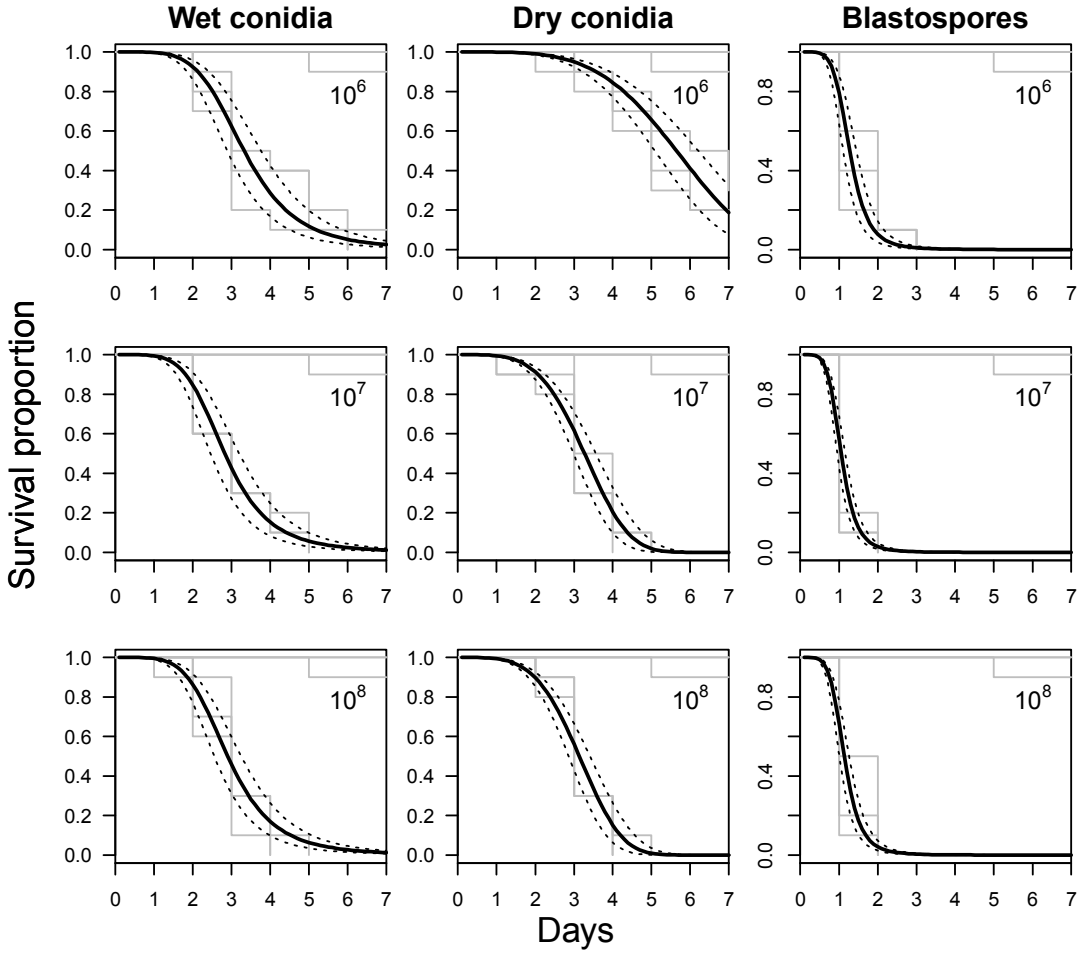

Figure 1. *Aedes aegypti* larvae survival when exposed to three different formulations and three concentrations of *Metarhizium brunneum* propagules. Kaplan-Meier step functions after treatment with 10^6, 10^7, or 10^8 propagules ml^{-1} are shown in grey (including uninfected controls). Fitted survival curves are shown in black, with 95% confidence intervals shown as dotted lines.

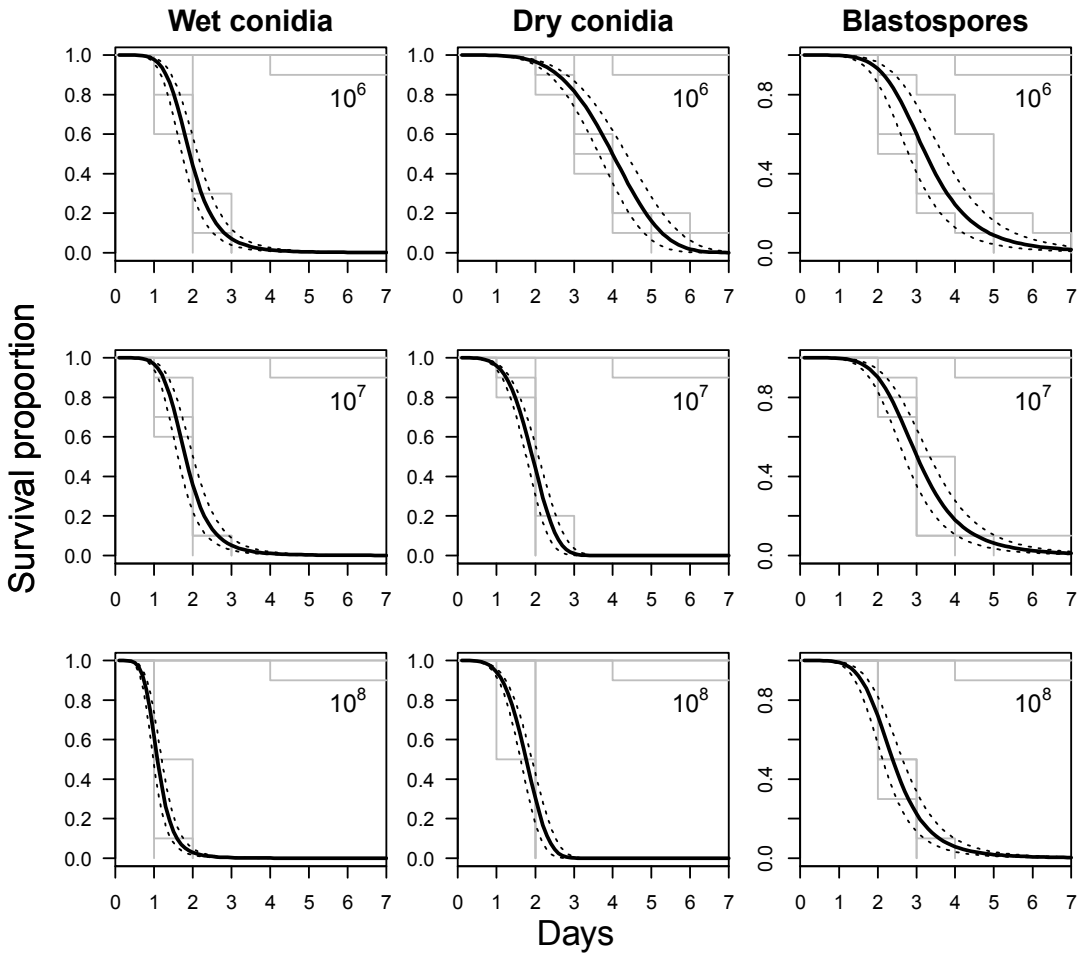

Figure 2. *Culex quinquefaciatus* larvae survival when exposed to three different formulations and three concentrations of *Metarhizium brunneum* propagules. Kaplan-Meier step functions after treatment with 10^6, 10^7, or 10^8 propagules ml^{-1} are shown in grey (including uninfected controls). Fitted survival curves are shown in black, with 95% confidence intervals shown as dotted lines.

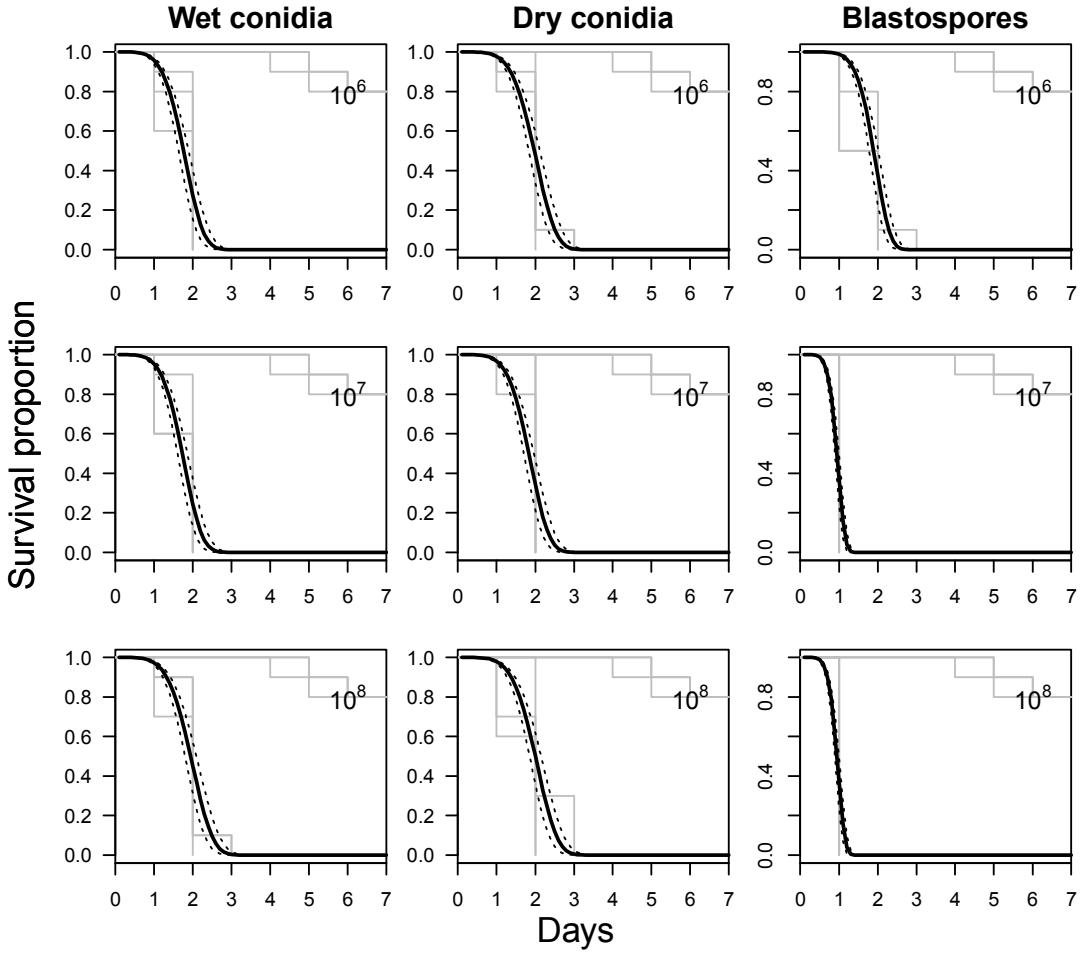

Figure 3. *Anopheles stephensi* larvae survival when exposed to three different formulations and three concentrations of *Metarhizium brunneum* propagules. Kaplan-Meier step functions after treatment with 10^6, 10^7, or 10^8 propagules ml^{-1} are shown in grey (including uninfected controls). Fitted survival curves are shown in black, with 95% confidence intervals shown as dotted lines.