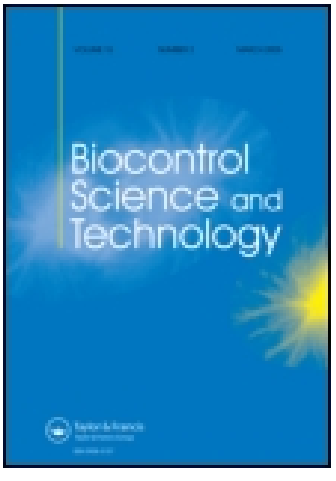

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RESEARCH ARTICLE

Identification of *Metarhizium* strains highly efficacious against *Aedes*, *Anopheles* and *Culex* larvae

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Entomopathogenic fungi, such as *Metarhizium anisopliae* and *Beauveria bassiana,* have been shown to be efficacious in killing mosquito larvae of different mosquito species. The current study compared the pathogenicity and efficacy of two formulations of three fungal strains against different instars of three mosquito species with the aim of identifying the most virulent strain for use under field conditions. Three strains of *Metarhizium*, ARESF 4556, ARSEF 3297 and V275, were assayed against early (L_{2-3}) and late (L_{3-4}) instar larvae of *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*. Two formulations of the fungi were tested, dry conidia and aqueous suspensions (i.e. 'wet' conidia). Effects of all combinations of conidia, mosquito species, instar, fungal strain and concentration on mosquito mortality were analysed using Cox regression and Kaplan–Meier analyses. Strain ARSEF 4556 was more virulent than ARSEF 3297 and V275, with LT_{50} values ranging from 0.3 to 1.1 days, with *Anopheles* and *Culex* being more susceptible than *Aedes*. Early and late instars were equally susceptible independent of species. Although the formulation did influence mortality rates, both 'wet' and 'dry' conidia applications were highly effective in killing mosquito larvae. Viable spores were more efficacious than heat killed spores. The latter did cause mortality but only at high concentrations. *Metarhizium sp*. has proved to be effective in reducing survivability of all larval stages of *Aedes*, *Anopheles* and *Culex* under laboratory conditions. *Aedes* larvae were generally more tolerant than *Anopheles* and *Culex* irrespective of fungal strain.

Keywords: *Metarhizium anisopliae*; *Aedes aegypti*; *Anopheles stephensi*; *Culex quinquefasciatus*; tolerance; larval susceptibility

1. Introduction

Mosquitoes of the genera *Aedes*, *Anopheles* and *Culex* are arthropod vectors of human and animal diseases worldwide and are increasing in importance due to their recent establishment in areas previously unrecorded within Europe (Medlock et al., 2012). This increase in range poses considerable health risks (McMichael & Lindgren, 2011; Medlock et al., 2012). Furthermore, there are increasing reports of resistance in mosquito populations to insecticides (Hemingway, Field, & Vontas, 2002; Vontas, Ranson, & Alphey, 2010). Mosquitoes are able to colonise temporary

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as well as permanent water sites and there are few aquatic habitats that do not lend themselves as a breeding site, often resulting in overlapping habitat range of these three genera (Becker et al., 2010; Yasuoka & Levins, 2007).

Insect pathogenic fungi belonging to the genus *Metarhizium* have been developed to control a wide range of arthropods, including pests of crops and vectors of human and animal diseases (Ansari, Pope, Carpenter, Scholte, & Butt, 2011; Faria & Wraight, 2007; Frazzon, da Silva Vaz Junior, Masuda, Schrank, & Vainstein, 2000; Orlando Beys Silva, Mitidieri, Schrank, & Vainstein, 2005). They are considered to offer an environmentally friendly alternative to chemical pesticides for the control of mosquitoes (Scholte, Knols, Samson, & Takken, 2004) *Metarhizium* is able to infect and kill several developmental stages (eggs, larvae and adults) of *Aedes*, *Anopheles* and *Culex* mosquitoes (Albernaz, Tai, & Luz, 2009; Bukhari, Middelman, Koenraadt, Takken, & Knols, 2010; Knols, Bukhari, & Farenhorst, 2010; Luz, Mnyone, & Russell, 2011; Scholte et al., 2004). It has been shown to work synergistically with chemical pesticides or when impregnated in bed nets in the control of adult mosquitoes. Furthermore, *Metarhizium* will also kill mosquitoes that have developed resistance to conventional pesticides (Hancock, 2009; Howard et al., 2011)

Fungal pathogenicity of arthropod hosts is influenced by a plethora of factors relating to the pathogen itself (e.g. virulence, specificity), environmental conditions (e.g. humidity, temperature) and the target host (Inglis, Goettel, Butt, & Strasser, 2001; Wekesa, Knapp, Maniania, & Boga, 2006). Previous studies have demonstrated that the hosts' developmental stage plays an important role in the efficacy of entomopathogenic fungi with early instar larvae usually being more susceptible to infection than older instars. This is clearly demonstrated for the European corn borer (*Ostrinia nubilalis*) and the diamondback moth (*Plutella xylostella*; Feng, Carruthers, Roberts, & Robson, 1985; Vandenberg, Ramos, & Altre, 1998). There are some exceptions, for example, Ekeski and Maniania found that legume flower thrips (*Megalurothrips sjostedti*) larvae were less susceptible to *Metarhizium anisopliae* than the pupae and adults (Ekesi & Maniania, 2000). Differences in susceptibility to the oomycete fungus *Lagenidium giganteum* were also noted for different instar mosquito larvae (Lord, Magalhães, & Roberts, 1987).

Various strategies have been used to control mosquito adults including release of sterile males, use of transgenic fungi with increased virulence and exploitation of synergies between the fungus and low dose insecticides (Fang et al., 2011; Farenhorst et al., 2010; Gilles et al., 2014; Nolan et al., 2011). Currently there is much interest in the use of *Metarhizium* to control mosquito larvae with some strains killing as fast as *Bti* (Boyce et al., 2013; Butt et al., 2013; Kroeger, Liess, Dziock, & Duquesne, 2013; Scholte et al., 2004). Selection of a strain of *Metarhizium* that is stable and highly efficacious against all important mosquito genera will reduce both registration and application costs.

Until recently it was assumed that the fungus propagated within the larvae, presenting the potential for inoculum to amplify within the host and horizontal transfer to occur (Bukhari et al., 2010; Lacey, Lacey, & Roberts, 1988; Riba, Keita, Soares, & Ferron, 1986). Recently we reported that the strains can kill without infection (Butt et al., 2013), highlighting the need for a more in-depth analysis of the relationship between dose and susceptibility. In light of these observations a re-evaluation of current control strategies is needed, as the route and mode of infection seriously impacts on rate and frequency of application and the overall control strategy.

This study provides the first comprehensive analysis of key parameters affecting fungus-induced mortality of mosquito larvae; it compares the efficacy of three strains of *Metarhizium* sp., known to be highly pathogenic to ticks (unpublished data), midges and crop pests (Ansari & Butt, 2012; Ansari et al., 2011), against the larvae of three major mosquito disease vectors using different doses and formulations of the fungus. We provide further evidence that the mode of pathogenesis is not the same as in a terrestrial system, although Pr1 undoubtedly plays a crucial role in pathogenesis, there are other factors that cannot be ignored, including the behaviour of the larvae and cell wall constituents.

2. Material and methods

2.2. Mosquitoes

Aedes aegypti (strain AeAe), *Anopheles stephensi* (strain Beech) and *Culex quinquefasciatus* (strain Muheza) eggs were obtained from the London School of Hygiene and Tropical Medicine, UK. All species were maintained in tap water and incubated at 25°C (±2°C) in a 16L:8D photoperiod, and were fed on Tetramin® fish-food, whilst *An. stephensi* was also supplemented with fresh grass shoots.

2.3. Fungal strains and preparation

Aerial conidia of *M. anisopliae* isolates V275, ARSEF 4556 and *Metarhizium brunneum* ARSEF 3297 (*sensu stricto*) were produced through solid state fermentation using broken Basmati rice as previously described by Ansari and Butt (2011). Conidial viability was assessed using the plate count technique on Sabouraud Dextrose Agar (Vega et al., 2009), only those with viability above 90% were used in subsequent experiments.

All experiments were performed at room temperature (22°C ± 2°C) with a 16L:8D photoperiod, in 250 ml round containers (diameter 92 mm) with perforated lids. Each treatment was replicated three times with a control for each and independently repeated three times.

2.4. Pathogenicity of V275, 4556 and 3297 using 'wet' versus dry conidia

A series of experiments were conducted to determine whether larval susceptibility to fungal infection was influenced by: (1) genera, (2) developmental stage, (3) fungus species/strain, (4) conidia concentration and (5) formulation of conidia.

Mortalities were compared of early (L_{2-3}, 3–4 days old) and late (L_{3-4}, 4–8 days old) larval stages of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* exposed to different concentrations (1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia ml^{-1}) of *M. anisopliae* and *M. brunneum* conidia.

Metarhizium spp. conidia was either applied as a dry dust powder (dry conidia) or suspended in 0.03% aqueous Tween 80 (wet conidia) to evaluate how the pathogenicity of fungal conidia and larval mortality is affected.

Ten larvae of either L_{2-3} or L_{3-4} were placed into round clear plastic containers containing 100 ml of conidia suspension or conidia dusted evenly over the water surface. Controls consisted of 10 larvae exposed to 100 mL 0.03% aqueous. Tween 80 or plain distilled water. Larval mortality was recorded daily for 12 days. To determine whether conidia caused blockage within the larvae or were required to

actively bring about mortality and whether the same process as in *Aedes* was witnessed in *Anopheles* and *Culex* larvae, additional experiments were conducted with heat-killed conidia, whereby the conidia were wrapped in aluminium foil and autoclaved for 15 min at 121°C, to denature enzymes and heat-labile toxins.

2.5. Statistical analysis

Effects of conidia, mosquito species, instar, fungal strain and concentration on mosquito mortality were analysed using Cox regression, resulting hazard ratio values were used to evaluate differential mortality rates. Survival curves were generated and Kaplan–Meier survival analysis was used to plot cumulative survival functions by treatment with pairwise comparison over log-rank test (Butt et al., 2013).

Mean lethal times, LT_{50} (for a concentration of 1×10^6) and mean lethal concentrations LC_{50} (at day 6) were estimated for all combinations of conidia, mosquito species, instar and fungal species/strain by fitting probit regression models to the quantal response data. Log transformations were applied to the dosage and time independent variables. An additional parameter was fitted to the LC_{50} model, to account for the natural mortality rate of the trial mosquitos. Differences in LT_{50} and LC_{50} were compared by analysis of variance and significance levels adjusted, where appropriate, by application of Tukey's multiple comparisons post-test.

All statistical analyses were carried out using SAS 9.3 (2011) and SPSS v16.

3. Results

3.1. Virulence strains (based on LT_{50} at 1×10^6 conidia mL^{-1})

Significant differences in LT_{50} values were observed among treatments. 'Dry' and 'wet' conidia resulted in a significantly lower LT_{50} values than heat-treated conidia [$F_{(2,60)} = 189.49$; $p < 0.001$] (Table 1). In terms of mosquito species, *Cx. quinquefasciatus* and *An. stephensi* were more susceptible to *Metarhizium* strains than *Ae. aegypti* [$F_{(2,60)} = 93.44$; $p < 0.001$]. Larval instar had no overall effect [$F_{(1,60)} = 1.91$; $p = 0.172$]. For fungal species/strains, *M. anisopliae* ARSEF 4556 was more virulent, against all three mosquito species, than *M. brunneum* ARSEF 3297 and *M. anisopliae* V275 [$F_{(4,60)} = 3.92$; $p < 0.007$]

Significant interactions were also observed among the conidia application, fungal and mosquito species treatments. Dry conidia of ARSEF 4556 were more virulent than ARSEF 3297 and V275, which had LT_{50} values ranging from 0.3 to 1.1 days, 0.3 to 3.1 days against L_{2-3} and L_{3-4} of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*, respectively (Table 1), all values being similar for heat-killed conidia [$F_{(8,60)} = 3.37$; $p < 0.003$]. Similarly, lowest LT_{50} values were recorded for ARSEF 4556 when applied to *Cx. quinquefasciatus,* there being little effect of the various fungal species on *An. stephensi* and *Ae. aegypti* [$F_{(4,60)} = 3.92$; $p < 0.007$] (Table 1).

3.2. Dose responses test (six days after treatment)

LC_{50} values of dry and wet conidia were significantly lower by 100-fold compared to heat-killed conidia [$F_{(2,62)} = 112.78$; $p < 0.001$]. In general, lower LC_{50} values were recorded against *Cx. quinquefasciatus* and *An. stephensi* compared with *Ae. aegypti* (Table 2; [$F_{(4,62)} = 5.54$; $p < 0.001$] whilst main treatment effects of instar and fungal application and strain were not significant, ([$F_{(2,62)} = 0.36$; $p = 0.697$], [$F_{(2,62)} = 0.38$; $p = 0.687$], respectively).

Table 1. Mean lethal time (LT_{50}) in days for mosquito species treated with conidial formulations of *M. anisopliae* (1×10^6 conidia/ml).

		L_{2-3} (LT_{50})			L_{3-4} (LT_{50})		
Formulation/ conidia	Fungal strains	*An. stephensi*	*Ae. Aegypti*	*Cx. quinquefasciatus*	*An. stephensi*	*Ae. aegypti*	*Cx. quinquefasciatus*
Dry	4556	1.1 (1.0–1.3)	1.5 (1.4–1.6)	0.3 (0.2–0.5)	1.2 (1.0–1.4)	3.1 (2.5–3.7)	1.0 (0.8–1.3)
	V275	1.4 (1.3–1.6)	3.2 (2.8–3.8)	4.1 (3.8–4.4)	1.2 (1.2–1.3)	4.5 (4.1–5.1)	3.5 (3.2–3.8)
	3297	2.8 (2.7–2.9)	5.7 (4.4–7.8)	1.9 (1.9–1.9)	1.2 (1.0–1.4)	6.3 (5.8–7.0)	1.8 (1.7–2.0)
Wet	4556	1.5 (0.9–1.7)	3.0 (2.2–4.9)	2.2 (1.3–3.3)	0.7 (0.5–0.9)	3.3 (2.8–4.3)	0.7 (0.6–0.8)
	V275	1.7 (1.2–2.0)	4.8 (3.9–5.6)	1.4 (0.9–1.8)	2.8 (2.6–3.0)	5.5 (4.4–7.2)	1.5 (1.2–1.7)
	3297	2.2 (1.4–2.8)	4.0 (3.1–4.9)	2.0 (0.9–3.3)	0.5 (0.2–0.8)	6.5 (6.2–6.8)	2.2 (1.6–2.8)
Heat killed	4556	15.5 (5.8 to >30)	18.2 (14.2 to >30)	4.1 (2.8–7.2)	7.2 (5.7–11.0)	>30 (nc)	3.6 (2.9–4.9)
	V275	7.9 (6.1–9.8)	15.7 (10.6–26.2)	11.6 (8.8–15.0)	5.9 (3.5–11.2)	23.1 (16.3–30.)	3.4 (2.6–4.5)
	3297	10.5 (8.2–16.0)	12.1 (8.5–17.8)	10.0 (2.0–18.6)	4.9 (2.9–10.3)	28.2 (20.8 to >30	3.6 (3.3–4.1)

Note: Mean lethal time (LT_{50}) for conidia formulations versus species, strain and larval life stage. 95% CI are given in parentheses.

Table 2. Mean lethal concentration (LC_{50}) for mosquito species treated with conidial formulations of *M. anisopliae* (1×10^6 conidia/ml).

		L_{2-3} (LD_{50})			L_{3-4} (LD_{50})		
Formulation	Fungal strains	*Ae. Aegypti*	*An. stephensi*	*Cx. quinquefasciatus*	*Ae. aegypti*	*An. stephensi*	*Cx. quinquefasciatus*
Dry	4556	5×10^4 (nc)	$<1.0 \times 10^5$ (nc)	$<1.0 \times 10^5$ (nc)	9.7×10^4 (4.7×10^4–1.5×10^5)	$<1.0 \times 10^5$ (nc)	9.2×10^4 (nc)
	V275	3.7×10^5 (1.7×10^5–6.6×10^5)	$<1.0 \times 10^5$ (nc)	2.3×10^5 (1.4×10^5–3.4×10^5)	1.8×10^5 (9.7×10^4–2.9×10^5)	$<1.0 \times 10^5$ (nc)	2.3×10^5 (1.4×10^5–3.4×10^5)
	3297	6.1×10^5 (2.4×10^5–1.1×10^6)	4.2×10^4 (nc)	1.2×10^5 (nc)	3.4×10^5 (1.4×10^5–6.4×10^5)	$<1.0 \times 10^5$ (nc)	1.2×10^5 (nc)
Wet	4556	1.8×10^5 (1.2×10^4–7.2×10^5)	6.2×10^4 (1.7×10^4–1.1×10^5)	5.1×10^4 (1.2×10^4–1.0×10^5)	7.5×10^5 (4.0×10^5–1.2×10^6)	1.1×10^5 (6.7×10^4–1.6×10^5)	9.0×10^4 (nc)
	V275	1.3×10^6 (5.0×10^5–2.6×10^6)	4.0×10^4 (4.0×10^3–1.0×10^5)	1.2×10^5 (nc)	1.2×10^6 (6.2×10^5–2.0×10^6)	1.3×10^5 (8.5×10^4–1.9×10^5)	7.2×10^4 (nc)
	3297	1.0×10^6 (4.5×10^5–2.0×10^6)	5.4×10^3 (2.9×10^3–5.3×10^4)	5.7×10^4 (1.5×10^4–1.1×10^5)	3.2×10^6 (1.5×10^6–5.5×10^6)	1.8×10^5 (1.1×10^5–2.8×10^5)	1.4×10^4 (3.6×10^2–4.7×10^4)
Heat killed	4556	5.8×10^7 (3.1×10^7–1.4×10^8)	1.0×10^7 (nc)	1.4×10^6 (6.2×10^5–2.4×10^6)	$<1.0 \times 10^8$ (nc)	1.8×10^7 (2.4×10^6–7.4×10^7)	4.7×10^5 (3.1×10^5–6.8×10^5)
	V275	1.7×10^7 (9.7×10^6–2.9×10^7)	6.0×10^6 (2.2×10^3–9.7×10^6)	7.4×10^6 (3.4×10^6–1.4×10^7)	$<1.0 \times 10^8$ (nc)	3.0×10^6 (4.1×10^5–1.4×10^7)	3.7×10^5 (1.6×10^5–6.7×10^5)
	3297	9.2×10^7 (2.1×10^7–1.7×10^9)	1.1×10^7 (nc)	1.6×10^6 (3.7×10^5–3.4×10^6)	$<1.0 \times 10^8$ (nc)	2.3×10^6 (4.2×10^5–7.2×10^6)	2.2×10^5 (7.2×10^4–4.8×10^5)

Note: Mean lethal concentration (LC_{50}) for conidia formulations versus species, strain and larval life stage. 95% CI are given in parentheses.

The interaction between conidia application and mosquito species was significant (Table 2) [$F_{(4,62)} = 11.00$; $p < 0.001$]. Under a dry application, all concentrations caused a significantly higher mortality than the control treatment ($p < 0.05$); with the exception of *Cx. quinquefasciatus* larvae exposed to 1×10^5 conidia mL^{-1} of 3297 (Figure 1, Table 3). A similar trend was observed under a wet application, all concentrations significantly reduced survivability, compared to the control ($p < 0.05$), with the exception of *Ae. aegypti* larvae treated with 1×10^5 conidia mL^{-1} of ARSEF 4556 (Figure 2, Table 3). No significant differences in LC_{50} values were recorded between mosquitoes for dry conidia, while wet conidia significantly decreased LC_{50} for both *An. stephensi* and *Cx. quinquefasciatus* [$F_{(4,62)} = 11.00$; $p < 0.001$]. The lowest LC_{50} values were observed for the combinations of dry conidia and *An. stephensi*, (all $<1.0 \times 10^5$, Table 2) whilst the highest LC_{50} values were observed for all strains against *Ae. aegypti*. (all $>1.7 \times 10^7$, Table 2). In the case where mosquitoes were exposed to heat-killed conidia, LC_{50} values for *Cx. quinquefasciatus* were significantly lower than *An. stephensi* which in turn were significantly lower than *Ae. aegypti*.

4. Discussion

All three strains of *Metarhizium* were found to be pathogenic to *Aedes*, *Anopheles* and *Culex* larvae with ARSEF 4556 being the most aggressive. Differences in susceptibility were noted between mosquito species with *Ae. aegypti* being less susceptible than either *Cx. quinquefasciatus* and *An. stephensi.* This has also been observed by other workers using different fungal species and strains (Scholte, Njiru, Smallegange, Takken, & Knols, 2003; Scholte et al., 2004; Silva, Silva, & Luz, 2004). This trend is observed in both adults and larvae, demonstrating the robustness of *Aedes* species. Mortality in *Ae. aegypti* larvae due to *Metarhizium* was shown in earlier studies to be largely due to protease-induced stress (Butt et al., 2013). Presumably *Anopheles* and *Culex* species are more easily stressed by the pathogen and, therefore, die more quickly.

Both early and late larval stages of all three mosquito species were equally susceptible to *Metarhizium,* independent of strain. Other researchers have reported differential susceptibility with early instar larvae being generally more susceptible (Bukhari et al., 2010). It is possible that in the case of the mosquito larvae, there is an increase in protease binding receptors as the insect develops and results in a proportional increase in stress.

Heat-killed conidia caused little larval mortality and this suggests that the mechanism of death is Pr1 as was reported for *Aedes* also applies to *Anopheles* and *Culex* (Butt et al., 2013). However, at relatively higher concentrations the heat-killed conidia caused significant mortality suggesting factors other than Pr1 may also contribute to larval death. It is possible that the conidia are not nutritious and cause starvation stress. Previous studies by Butt et al. showed an increase in caspase activity in mosquito larvae treated with heat-killed conidia (Butt et al., 2013), indicating that the cell wall components, other than Pr1, were causing stress. Cell surface proteins and enzymes are pivotal in defining the host-pathogen interaction.

Invasion by the fungi is typically recognised through the binding of proteins to β 1-3 glucans present within the fungal cell wall (Charnley, 2003; Jiang, Ma, Lu, & Kanost, 2004). The insect humoral response is triggered by pathogen-associated

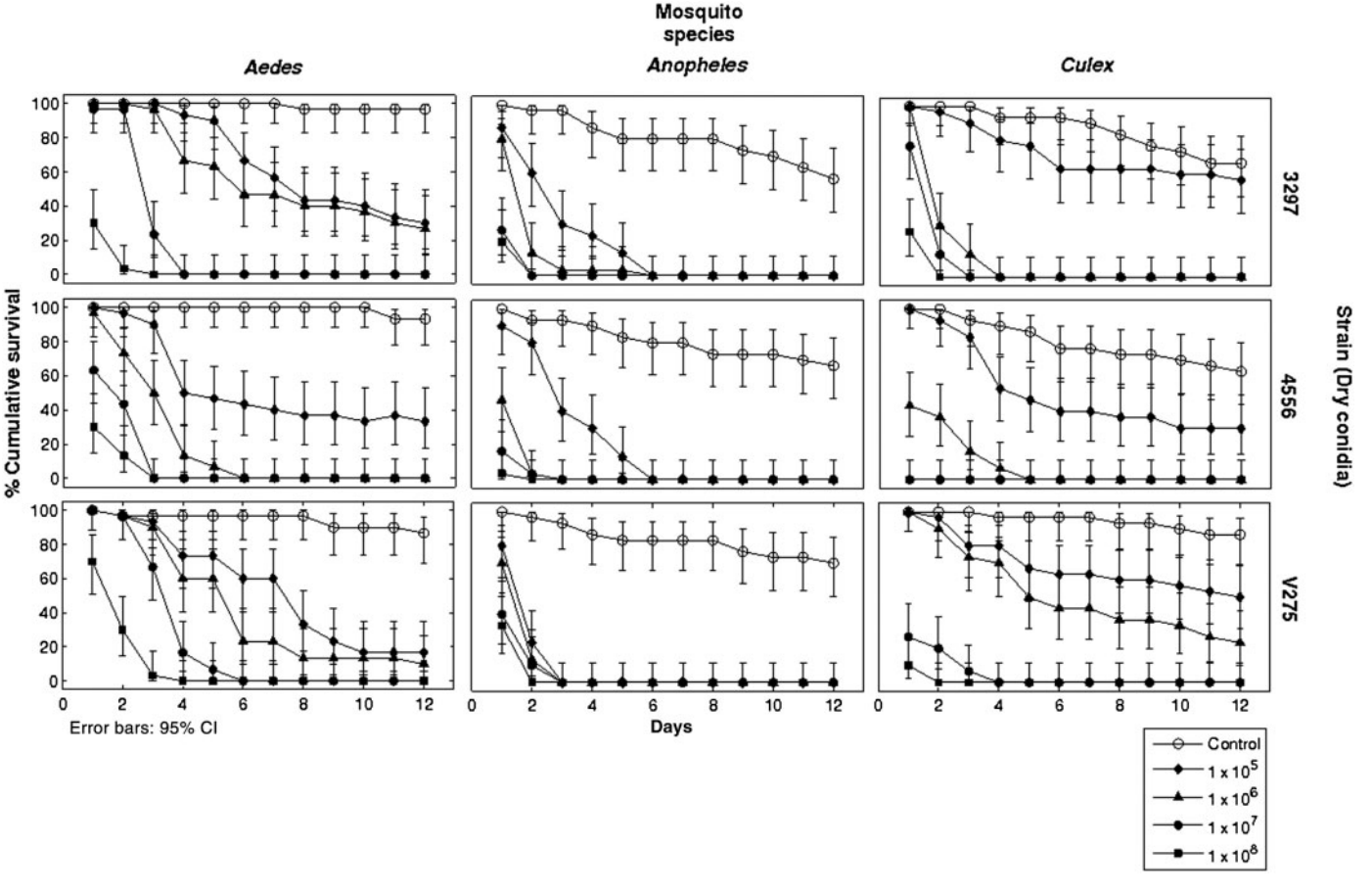

Figure 1. Survival curves of mosquito larvae exposed to different strains and concentration of 'dry' formulated *Metarhizium*. Percentage cumulative survival curves of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* (L_{3-4}) exposed to different concentrations of 'dry' formulated *Metarhizium* sp. for 12 days.

Note: Error is represented as 95% CI.

Table 3. Kaplan–Meier log rank pairwise comparisons of conidia concentration.

			Aedes				*Anopheles*				*Culex*			
Strains	Applications	Treatments	0	1×10^5	1×10^6	1×10^7	0	1×10^5	1×10^6	1×10^7	0	1×10^5	1×10^6	1×10^7
3297	Wet	1×10^5	ns				***				***			
		1×10^6	***	***			***	***			***	ns		
		1×10^7	***	***	***		***	***	*		***	***	***	
		1×10^8	***	***	***	***	***	***	**	ns	***	***	***	*
	Dry	1×10^5	***				***				ns			
		1×10^6	***	ns			***	**			***	***		
		1×10^7	***	***	***		***	***	***		***	***	*	
		1×10^8	***	***	***	***	***	***	***	ns	***	***	ns	***
	Heat killed	1×10^5	ns				ns				***			
		1×10^6	ns	ns			*	ns			***	ns		
		1×10^7	***	***	***		*	ns	ns		***	***	***	
		1×10^8	***	***	***	*	***	***	***	***	***	***	***	***
4556	Wet	1×10^5	*				***				***			
		1×10^6	***	***			***	***			***	***		
		1×10^7	***	***	***		***	***	ns		***	***	*	
		1×10^8	***	***	***	ns	***	***	**	*	***	***	**	ns
	Dry	1×10^5	***				***				**			
		1×10^6	***	***			***	***			***	***		
		1×10^7	***	***	***		***	***	*		***	***	***	
		1×10^8	***	***	***	**	***	***	***	ns	***	***	***	ns
	Heat killed	1×10^5	ns				*				**			
		1×10^6	ns	ns			*	ns			***	***		
		1×10^7	***	***	***		***	***	***		***	***	**	
		1×10^8	***	***	***	***	***	***	***	ns	***	***	***	*

Table 3 (*Continued*)

			Aedes				*Anopheles*				*Culex*			
Strains	Applications	Treatments	0	1×10^5	1×10^6	1×10^7	0	1×10^5	1×10^6	1×10^7	0	1×10^5	1×10^6	1×10^7
V275	Wet	1×10^5	***				***				***			
		1×10^6	***	***			***	**			***	**		
		1×10^7	***	***	*		***	***	*		***	***	***	
		1×10^8	***	***	***	***	***	***	***	***	***	***	***	ns
	Dry	1×10^5	***				***				**			
		1×10^6	***	ns	***	***	***	ns			***	*		
		1×10^7	***	***	***		***	**	*		***	***	***	
		1×10^8	***	***			***	***	**	ns	***	***	***	*
	Heat killed	1×10^5	ns				ns				ns			
		1×10^6	ns	ns			ns	ns			***	***	***	
		1×10^7	***	***	***		ns	ns	ns		***	***	***	ns
		1×10^8	***	***	***	***	***	***	***	***	***	***		

Note: *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* ($L_{3–4}$) exposed to different concentrations of formulated *Metarhizium*.
*$p < 0.05$; **$p < 0.01$; ***$p = < 0.001$.

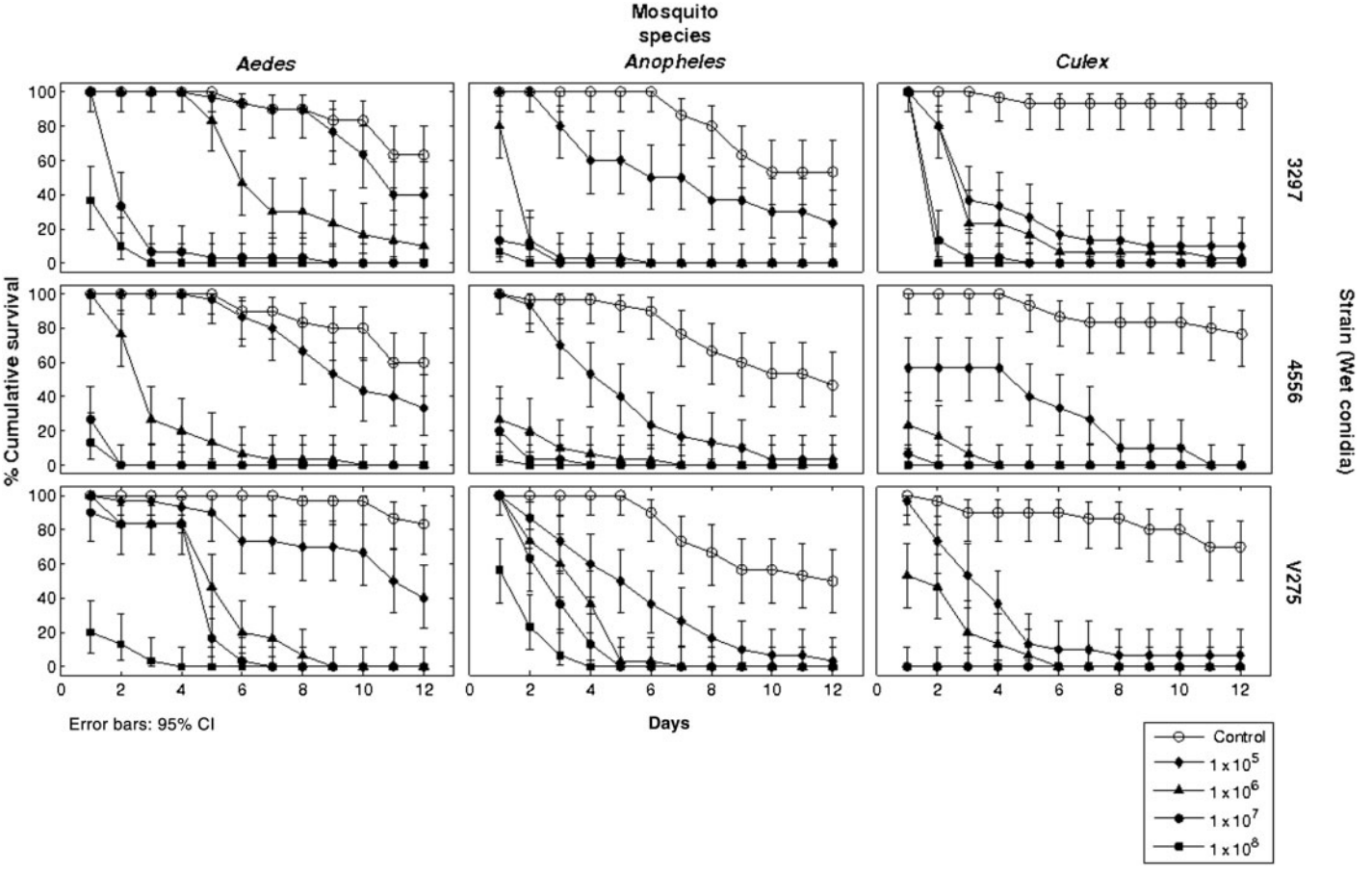

Figure 2. Survival curves of mosquito larvae exposed to different strains and concentration of 'wet' formulated *Metarhizium*. Percentage cumulative survival curves of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* (L_{3-4}) exposed to different concentrations of 'wet' formulated *Metarhizium* sp. for 12 days.
Note: Error is represented as 95% CI.

molecular patterns and in the mosquito β 1-3 glucan recognition proteins stimulate the activation of the phenoloxidase cascade (Fabrick, Baker, & Kanost, 2003; Jiang, Wang, Yu, Zhu, & Kanost, 2003; Kanost, Jiang, & Yu, 2004). Through denaturing proteins and undoubtedly provoking conformational changes in the fungal conidia, an activation of the host humoral defences by cell wall sugars (β 1-3 glucans) may exacerbate the stress leading to host death. Indeed, fungal cell wall components will activate both cellular and humoral defences in most arthropods with the response being dose-related (Charnley, 2003; Gottar et al., 2006; Jiang et al., 2004).

Slight differences in efficacy were noted between 'dry conidia' versus conidia suspensions or 'wet conidia', supporting the findings of Bukhari et al. (2010). In contrast, Alves, Alves, Lopes, Pereira, and Vieira (2002) noted the opposite for *Culex* larvae. The dry, hydrophobic conidia would largely remain concentrated at the surface, whereas the wet conidia would be suspended within the body of water. Exactly why the dry conidia should prove more virulent is unclear but this phenomenon has been reported for other insect hosts (Howard, Koenraadt, Farenhorst, Knols, & Takken, 2010; Morley-Davies, Moore, & Prior, 1996; Rangel, Braga, Anderson, & Roberts, 2005).

One possible explanation for dry conidia being more aggressive is the fact that spore bound enzymes, especially the virulence determining protease Pr1, would not have been removed by surfactants used to prepare conidia suspensions. Pr1 plays a key role in pathogenesis of both terrestrial hosts and mosquito larvae (Fang & Bidochka, 2006; Wang, Typas, & Butt, 2002) removal or dilution of this enzyme would undoubtedly influence overall pest control. Passaged conidia of *M. anisopliae* has been shown to increase virulence for mosquito larvae (Daoust & Roberts, 1982). This may be because conidia from passaged pathogens are known to have higher levels of spore-bound Pr1 (Frazzon et al., 2000; Shah, Wang, & Butt, 2005)

Our studies show that a spore suspension was more efficacious at a lower concentration than a dry formulation, especially in respect to *An. stephensi* and *Cx. quinquefasciatus*. This difference in susceptibility could be attributed to the feeding habits of the larvae. Typically *Anopheles* and *Culex* species are categorised as 'active' suspension-feeders, feeding actively on particulate matter within the water column via a collecting–filtering mechanism, as well as grazing from the surface (Dahl, Widahl, & Nilsson, 1988; Merritt, Craig, Wotton, & Walker, 1996; Merritt, Dadd, & Walker, 1992; Workman & Walton, 2003), which may contributed to their increased susceptibility. In contrast, *Aedes* preferentially feed through browsing, whereby harvesting particulates from surrounding substrates. *Aedes* species do not feed exclusively in this manner, they will also filter feed (Eisenberg, Washburn, & Schreiber, 2000).

Mortality was dose-dependent, with all strains being more efficacious at higher concentrations, Exposure of larvae to different concentrations of purified Proteinase K, which has significant homology with Pr1, also showed dose-related mortality (unpublished). In the field, *Metarhizium* is typically applied at concentrations above 1×10^9 conidia mL^{-1} (Kassa, Stephan, Vidal, & Zimmermann, 2004; Paula, Carolino, Silva, & Samuels, 2011; Scholte, Takken, & Knols, 2007), however, in this study, significant control was achieved at 1×10^6 conidia mL^{-1} suggesting the fungus would be still efficacious even when diluted in the water due to climatic and biotic factors.

5. Conclusion

Metarhizium spp are pathogenic to mosquito larvae, independent of genus and larval developmental stage, providing a greater window of control of these important vectors of disease. Strain ARESF 4556 was highly aggressive, killing *Anopheles* and *Culex* larvae within 24 hrs. The formulation of the conidia will undoubtedly influence fungal field efficacy but central to this control strategy will be retention or enhancement of Pr1 activity since this enzyme plays a key role in pathogenesis (Butt et al., 2013).

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