Combined use of the entomopathogenic fungus, *Metarhizium brunneum*, and the mosquito predator, *Toxorhynchites brevipalpis*, for control of mosquito larvae: Is this a risky biocontrol strategy?

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**Abstract**

Mosquitoes transmit several diseases, which are of global significance (malaria, dengue, yellow fever, Zika). The geographic range of mosquitoes is increasing due to climate change, tourism and trade. Both conidial and blastospore formulations of the entomopathogenic fungus, *Metarhizium brunneum* ARSEF 4556, are being investigated as mosquito larvicides. However, concerns have been raised over possible non-target impacts to arthropod mosquito predators such as larvae of *Toxorhynchites brevipalpis* which feed on larvae of mosquito vector species. Laboratory-based, small container bioassays showed that *T. brevipalpis* larvae are susceptible to relatively high concentrations (i.e. \( \geq 10^5 \text{spores ml}^{-1} \)) of inoculum with blastospores being significantly more virulent than conidia. At lower concentrations (e.g. \(< 10^5 \text{spores ml}^{-1} \)), it appears that *M. brunneum* complements *T. brevipalpis* resulting in higher control than if either agent was used alone. At a concentration of \(10^5 \text{spores ml}^{-1} \), the LT50 of for conidia and blastospores alone was 5.64 days (95% CI: 4.79–6.49 days) and 3.89 days (95% CI: 3.53–4.25 days), respectively. In combination with *T. brevipalpis*, this was reduced to 3.15 days (95% CI: 2.82–3.48 days) and 2.82 days (95% CI: 2.55–3.08 days). Here, combined treatment with the fungus and predator was beneficial but weaker than additive. At \(10^7\) and \(10^8\) blastospores ml\(^{-1}\), mosquito larval mortality was mostly due to the fungal pathogen when the predator was combined with blastospores. However, with conidia, the effects of combined treatment were additive/synergistic at these high concentrations. Optimisation of fungal concentration and formulation will reduce: (1) risk to the predator and (2) application rates and costs of *M. brunneum* for control of mosquito larvae.

**Keywords:**

Aedes

Metarhizium

Toxorhynchites

Predator

Fungal pathogen

Blastospores

Conidia

Risk assessment

Interaction

**1. Introduction**

Mosquitoes belonging to the genera *Aedes*, *Anopheles* and *Culex* vector a range of diseases (e.g. malaria, Zika, dengue, yellow fever), which have significant medical and economic impacts for over half the world’s population (Tolle, 2009). *Aedes* mosquitoes will oviposit in extremely small, ephemeral bodies of water since their eggs can tolerate desiccation (Faull et al., 2016; Juliano et al., 2002). Current control methods targeting adult mosquitoes include persistent insecticide-treated nets and indoor residual spraying. However, targeting adults alone is insufficient in preventing disease transmission, and integrated vector management (IVM) focuses on management of both larval and adult mosquito populations (Fillinger et al., 2009; Thomas, 2017). Various tools are available to control mosquito larvae in large expanses of water such as larvivorous fish and chemical pesticides including growth regulators such as methoprene (Becker et al., 2003). More selective insecticides based on the bacteria *Bacillus sphaericus* and *Bacillus thuringiensis* are also widely used especially in urban and environmentally sensitive areas (Lacey, 2007; Mulla, 1990). However, when dealing with transient or small bodies of water (e.g. water collected at the bottom of used tyres or in leaf clusters of epiphytic plants such as bromeliads) the products and strategies are more limited (Ceretti-Junior et al., 2016).

There is a reluctance to use chemical insecticides, even though they are relatively fast acting, because of the risks they pose to human health and pollution of the environment even at relatively low concentrations (Liess et al., 2013). Furthermore, extensive use of agricultural chemical pesticides can select for insecticide resistance in mosquito disease vectors (Nkya et al., 2014). Indeed, use of both chemical and bacterial insecticides is under threat due to increasing reports of mosquitoes
developing resistance to these agents (Boyer et al., 2012; Hemingway and Ranson, 2000). These factors are prompting the search for safe alternatives such as the entomopathogenic fungi (EPF) (Shah and Pell, 2003). Laboratory studies show that *Metarhizium brunneum* can cause up to 100% mortality of mosquito larvae in <24 h depending on the fungal strain, formulation and concentration (Alkhaibari et al., 2017; Greenfield et al., 2015). However, there are many other EPF species which have been shown to infect mosquito eggs, larvae and adults including species of *Tolypocladium cylindrosporum*, *Beauveria bassiana* and *Metarhizium anisopliae* (Scholte et al., 2004).

Conidia and blastospores of *M. brunneum* differ in their mode of pathogenesis (Alkhaibari et al., 2016; Butt et al., 2013). Conidia are unable to infect through the cuticle due to their failure to adhere to the surface of the mosquito larval cuticle (Greenfield et al., 2014). However, conidia are readily ingested and although they do not germinate in the gut lumen, they can cause death through stress-induced apoptosis triggered by the spore bound protease Pr1 (Butt et al., 2013). In contrast, blastospores readily adhere to the host cuticle and are also ingested. These propagules quickly germinate with death resulting from simultaneous penetration of the cuticle and gut and subsequent colonisation of the haemocoel (Alkhaibari et al., 2016).

The use of EPF offers reduced risk to aquatic systems compared with many alternatives, for example through reduced “run off” from forest slopes or agricultural land (Ippolito et al., 2015). However, some

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**Fig. 1.** Survival curves of *Toxorhynchites brevipalpis* larvae exposed to different concentrations of conidia and blastospores of *Metarhizium brunneum* ARSEF 4556. Percentage cumulative survival of *Tx. brevipalpis* (L4) exposed to different concentrations of *M. brunneum* ARSEF 4556 over a 7 day period. Kaplan–Meier step functions after treatment with $10^5$, $10^6$, or $10^7$ propagules ml$^{-1}$ are shown in gray (including uninfected controls). Fitted survival curves are shown in black, with 95% confidence intervals shown as dotted lines.
concerns over non-target impacts of EPF have been raised. Toxicology studies show that the risk posed by *M. brunneum* conidia to the aquatic invertebrates *Artemia salina* and *Daphnia pulex* is concentration-dependent, that is, mortality increased with spore concentration (Garrido-Jurado et al., 2015). Since these invertebrates were far more tolerant of *M. brunneum* than mosquito larvae it was possible to identify a concentration which gave effective control of the pest with significantly reduced risk to the non-target invertebrates (Garrido-Jurado et al., 2015). No study has been conducted to date to determine the risk posed by EPF to the aquatic invertebrate predatory mosquito *Toxorhynchites* even though this genus is widely recognised as an important biological control agent (BCA) (Shaalan and Canyon, 2009). In fact, there are no studies on the combined use of EPF and predacious insects for mosquito control even though the potential exists to enhance mosquito control using combinations. In contrast, there are several studies on the combined use of EPF and other BCAs for control of agricultural pests (Dogan et al., 2017). The combined used of EPF with these BCAs is increasingly being used within integrated pest management (IPM) programmes partly because these agents may act in concert, allowing each agent to be used at reduced application rates. For example, co-application of *M. brunneum* with EPN resulted in higher mortality of black vine weevil (*Otiorhynchus sulcatus*) larvae than if either agent was used alone (Ansari et al., 2008). Similarly, other researchers have reported pest control being enhanced when using EPF-predator combinations.
Mean lethal time (LT50) for conidia and blastospores against
Kaplan-Meier log rank pairwise comparisons of conidia and blastospores concentrations for treatments against
Table 2
verse habitats feeding on vector prey species (Collins and Blackwell,
concomitantly reduce risks to non-target organisms.
and bene
programmes aim to exploit compatible, synergistic combinations of EPF
2017; Meyling and Pell, 2006; Ormond et al., 2011). Successful IPM
4556 and (2) establish if
susceptibility of this study to the development of
M. brunneum
arsenic, and other EPF. The aims of this study were to: (1) determine the suscept-
modified beetle predators to reduce application rates and costs and concomitantly reduce risks to non-target organisms.
Species of the predatory mosquito, Toxorhynchites, are found in di-
Medicine and hatched in 1L and 3L tap water, respectively. Larvae of
(Toxo) were obtained from the London School of Hygiene and Tropical
T. brevipalpis
were fed guinea pig pellets (PetsAtHome, Swansea, UK). Larvae
instar as the predator (Mohamad and Zuharah, 2014). The insects were
larvae daily as food. Throughout
the study, T. brevipalpis
larvae of the same
Species of the predatory mosquito,
are efficient predators and can eliminate mosquito larvae where present (Shaalan and Canyon, 2009). However,
to date, no studies have investigated the compatibility of Toxorhynchites
with EPF. The aims of this study were to: (1) determine the suscept-
bility of Toxorhynchites brevipalpis to Metarhizium brunneum ARSEF 4556 and (2) establish if M. brunneum and T. brevipalpis could work
1×105 6.05 (5.29–6.82) 3.81 (3.26–4.35)
1×106 4.18 (3.72–4.64) 2.60 (1.70–2.30)
1×107 2.66 (2.37–2.95) 1.22 (1.04–1.39)

| Mosquito species | Concentration | Conidia | Blastospores |
|------------------|---------------|---------|--------------|
| Tx. brevipalpis  | 1×10⁵         | 10.91 (8.16–13.65) 7.02 (6.08–7.97) |
|                  | 1×10⁶         | 8.44 (7.45–9.42) 3.85 (3.39–4.30) |
|                  | 1×10⁷         | 5.50 (5.15–5.84) 2.45 (2.17–2.73) |
| A. aegypti       | 1×10⁵         | 6.05 (5.29–6.82) 3.81 (3.26–4.35) |
|                  | 1×10⁶         | 4.18 (3.72–4.64) 2.60 (1.70–2.30) |
|                  | 1×10⁷         | 2.66 (2.37–2.95) 1.22 (1.04–1.39) |

Table 1
LT50 values estimated for Toxorhynchites brevipalpis and Aedes aegypti larvae versus three concentrations of conidia and blastospores of Metarhizium brunneum ARSEF 4556.

whether targeting foliar or subterranean pests (Roy and Pell, 2000; Saito and Brownbridge, 2016). Most often the success of these combi-
nations has been attributed to predators either avoiding the pathogen or being less susceptible to it compared with the target pest (Dog an et al.,
2017; Meyling and Pell, 2006; Ormond et al., 2011). Successful IPM
programmes aim to exploit compatible, synergistic combinations of EPF
and beneficial predators to reduce application rates and costs and concomitantly reduce risks to non-target organisms.

Eggs of both Aedes aegypti (AEAE) and Toxorhynchites brevipalpis (TOXO) were obtained from the London School of Hygiene and Tropical
Medicine and hatched in 1L and 3L tap water, respectively. Larvae of A. aegypti were fed guinea pig pellets (PetsAtHome, Swansea, UK). Larvae
of T. brevipalpis were isolated in 100 ml water within 2 days. A total of 240 T. brevipalpis larvae were used across all experi-
ments. Assays were also conducted to determine A. aegypti susceptibility to both conidia and blastospores of M. brunneum as described by Alkhai bari et al. (2017). Briefly, three replicates of ten larvae (n = 30)
per treatment were transferred to plastic cups containing 100 ml of water with 30 larvae per treatment i.e. per concentration. Conidia and blastospores were suspended in 0.03% Aqueous TWEEN 80 and distilled water, respectively, before applying to the bioassay cups for a final concentration of 10⁵, 10⁶, 10⁷ spore ml⁻¹.
Each larva of T. brevipalpis was provided ten A. aegypti larvae at the start of each assay. Controls consisted of either distilled water or TWEEN 80 at final concentration 0.0003% (v/v). Mortality was recorded daily over 7 days. A total of 240 T. brevipalpis larvae were used across all experi-
ments.

2.3. Susceptibility of T. Brevipalpis and A. Aegypti Larvae to M. Brunneum

The susceptibility of T. brevipalpis larvae to conidia and blastospores suspensions of M. brunneum ARSEF 4556 was tested in 200 ml plastic
cups containing 100 ml of water with 30 larvae per treatment i.e. per concentration. Conidia and blastospores were suspended in 0.03%
Aqueous TWEEN 80 and distilled water, respectively, before applying to the bioassay cups for a final concentration of 10⁵, 10⁶, 10⁷ spores ml⁻¹.
Mortality was assessed daily for 7 days. In total, 420 A. aegypti larvae were used in this study. Each experiment was repeated
three times.

2.4. Microscopy studies

The infection and developmental processes of M. brunneum in T. brevipalpis larvae was investigated using a combination of low-tem-
perature scanning electron microscopy (Cryo-SEM) and fluorescence microscopy. For Cryo-SEM, larvae were inoculated with conidia and blastospores of M. brunneum ARSEF 4556 as described above (at concentration 10⁵ spores ml⁻¹ for 24 h) then examined using a Hitachi S4800 field emission microscope equipped with a Quorum PPT2000

Table 2
Kaplan-Meier log rank pairwise comparisons of conidia and blastospores concentrations for treatments against Toxorhynchites brevipalpis and Aedes aegypti larvae.

| Mosquito species | Formulations | Conidia | Blastospores |
|------------------|--------------|---------|--------------|
|                  | Concentrations | 10⁵ | 10⁶ | 10⁷ | 10⁸ | 10⁹ |
| Tx. brevipalpis  | Control       | χ² = 2.03 | χ² = 10.40 | χ² = 66.39 | χ² = 32.45 | χ² = 68.19 | χ² = 65.38 |
|                  | P = .154      | P = .001 | P < .001 | P < .001 | P < .001 | χ² = 63.63 |
|                  | 10⁵           | – | χ² = 5.27 | – | – | χ² = 49.63 |
|                  | P = .022      | – | P < .001 | – | – | P = .001 |
|                  | 10⁶           | – | – | – | – | χ² = 10.54 |
|                  | –             | P < .001 | – | – | – | P = .001 |
| A. aegypti       | Control       | χ² = 35.69 | χ² = 65.62 | χ² = 61.57 | χ² = 65.73 | χ² = 69.70 | χ² = 66.26 |
|                  | P < .001      | P < .001 | P < .001 | P < .001 | P < .001 | P < .001 |
|                  | 10⁵           | – | χ² = 10.48 | – | – | χ² = 26.70 |
|                  | P = .001      | – | P < .001 | – | – | χ² = 47.65 |
|                  | 10⁶           | – | – | – | – | χ² = 7.51 |
|                  | –             | P < .001 | – | – | – | P = .006 |

χ² = Chi-square value.

Tx. brevipalpis and A. aegypti exposed to different concentrations of conidia and blastospores of M. brunneum. χ² = Chi-square value.
cryogenic stage and preparation chamber, as outlined by Alkhaibari et al. (2016). For fluorescence microscopy, T. brevipalpis larvae (n = 5) were fed Aedes larvae infected with conidia and blastospores of a GFP-transformed strain of M. brunneum (10^7 spores ml^-1). This facilitated visualisation of the inoculum in the digestive tract and faecal pellets and concomitantly allowed the viability of inoculum to be determined. The surface and gut contents of infected A. aegypti larvae as well as faecal pellets were examined using a Zeiss fluorescence microscope, as outlined by Butt et al. (2013).

2.5. Interactions between M. Brunneum and T. Brevipalpis in control of A. Aegypti larvae

Interactions between the predator and fungal pathogen were
Fig. 5. SEM of Metarhizium brunneum conidia on Toxorhynchites brevipalpis larvae, 24 h post inoculation, 24 h post inoculation. Conidia readily adhered to the cuticle surface either individually or in clusters (A). Close examination of the conidia showed that they had not germinated (B, C). Conidia often attached to or near the base of setae (C).

Fig. 6. SEM of cross section of infected Toxorhynchites brevipalpis larvae with conidia of Metarhizium brunneum. (A) Conidia were present in very low quantities in the gut of the predator. (B–C) Large quantities of conidia were found in the gut of A. aegypti larvae that had been ingested by Tx. brevipalpis larvae.

Fig. 7. Metarhizium brunneum blastospores expressing GFP in the Aedes aegypti cuticle surface and the gut. Larvae inoculated with blastospores of a GFP transformed strain of M. brunneum were examined 24 h hr pi. Blastospores were attached to the head (A). They were visible at the surface of the abdomen (arrow) and in the gut (*) of ingested Aedes larvae (B). The blastospores also adhered to the surface of the siphon (C) and anal gills (D).
investigated using different concentrations and formulations of the fungus. Briefly, concentration mortality studies were performed as outlined above using four different concentrations (10^5, 10^6, 10^7, 10^8 spores ml^-1) of conidia and blastospores in absence of the predator T. brevipalpis. An additional study was conducted using the above concentrations of conidia and blastospores with only a single larva of T. brevipalpis being added to each treatment. Control insects were exposed to carrier (distilled water or 0.3% Aq Tween) only. Mortality was recorded daily for 5 days. In total, 600 A. aegypti larvae and 30 T. brevipalpis larvae were used in this study. The experiments were repeated three times.

2.6. Statistical analyses

Survival rates of (1) T. brevipalpis and A. aegypti larvae exposed to the different concentrations of M. brunneum ARSEF 4556 conidia and blastospores and (2) A. aegypti larvae exposed to four concentrations of fungal spores (blastospores and conidia) in presence and absence of T. brevipalpis were visualised by plotting Kaplan-Meier survival cumulative survival functions by treatment, with pairwise comparisons assessed using log-rank tests (Kaplan and Meier, 1958) The median lethal time to death, LT50, was estimated using parametric survival regression for combinations of fungal formulation, spore concentration, predator (presence/absence). By comparing observed survival following combined treatment with expected survival, based on the additive effects of the fungus and predator alone, we tested whether combined treatment was (a) antagonistic (higher A. aegypti survival than expectation), (b) additive, or (c) synergistic (lower A. aegypti survival than expectation).

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

3. Results

3.1. Susceptibility of T. Brevipalpis and A. Aegypti larvae to M. Brunneum

Both T. brevipalpis and A. aegypti were susceptible to M. brunneum ARSEF 4556 with mortality being dependent upon the concentration and formulation (Figs. 1 and 2). Larvae of A. aegypti were significantly more susceptible to ARSEF4556 compared with T. brevipalpis, with the blastospores generally being more virulent than the conidia (Table 1; Figs. 1 and 2). For example LT50 values for A. aegypti and T. brevipalpis when exposed to conidia at the highest concentration (10^7 spore ml^-1) was 2.7 and 5.5 days, respectively whereas that of blastospores was 1.2 and 2.5 days, respectively (Table 1). A. aegypti larvae were generally twice as susceptible to conidia or blastospores than the predator at each concentration tested (Table 1), with pairwise concentration comparisons being statistically significant (Table 2). Both conidia and blastospore applications caused mortalities in both mosquito species significantly higher than the control (P < .001). However, for T. brevipalpis larvae exposed to conidia at the lowest concentration (10^6 spores ml^-1) there was no significant difference with the control (P = .154; Table 2; Fig. 1).

3.2. Microscopy studies of conidia and blastospore interactions in the gut and cuticle surface of T. Brevipalpis larvae

Cryo-SEM showed that the hydrophobic conidia and hydrophilic blastospores of M. brunneum adhered to the surface of the cuticle of T. brevipalpis. Blastospores adhered strongly to the head and mouthparts as well as abdominal setae and siphon (Fig. 3A–F). Blastospores were often observed in clumps with individual cells being connected by sheets or strands of mucilage (Fig. 3B–F). Isolated blastospores producing penetration hyphae were observed (Fig. 4A, B). Conidia of M. brunneum appeared to adhere through hydrophobic forces, often in clusters on or near the base of setae (Fig. 5A–C). There was no evidence of conidia germinating and producing germ tubes or appressoria beyond the first 24 h post-inoculation (pi). Conidia were clearly visible in the gut lumen of T. brevipalpis but none of these germinated or infected through the
midgut epithelium (Fig. 6A–C).

Blastospores adhered to the *A. aegypti* cuticle surface but were also concentrated in the gut lumen at 24 h pi. They would penetrate through the gut lumen and invade the haemocoel (Fig. 7A–D). In contrast, conidia of *M. brunneum* did not adhere to the cuticle surface of *A. aegypti* larvae but were ingested and concentrated in the gut lumen. They did not germinate in the gut lumen.

Cross sections of the *T. brevipalpis* gut lumen showed ingested *A. aegypti* larvae at different stages of digestion. Recently ingested *A. aegypti* larvae had intact gut structure and content, with conidia or blastospores clearly visible in the gut lumen (Figs. 8 and 6). Few spores were observed in the gut lumen of *T. brevipalpis* larvae; some may have been ingested while others were probably released from the prey during the digestive process. Fluorescence microscopy showed that both conidia and blastospores are expelled relatively intact in faecal pellets of *T. brevipalpis* larvae (Fig. 9A, B). Spores which expressed the GFP were clearly viable and active while the non-fluorescing GFP spores were probably quiescent or damaged and, therefore, non-viable (Fig. 9A and B).

### 3.3. Interaction between *M. Brunneum* and *T. Brevipalpis*

In the absence of *M. brunneum* ARSEF4556, all *A. aegypti* larvae survived 5 days incubation (Figs. 10 and 11). However, when incubated
with a single *T. brevipalpis* larva, ca. 67% were consumed (Fig. 4), with the differences between these controls being statistically significant ($\chi^2 = 30.150$, df = 3, $P < .001$; Table 4). Irrespective of fungal formulation (conidia or blastospores), survival of *A. aegypti* larvae was significantly lower when using combinations of *M. brunneum* and *T. brevipalpis* than with *T. brevipalpis* alone (Table 3 and 4; Figs. 10 and 11).

The interactions between these two biocontrol agents, as seen in Fig. 12, were antagonistic at the low concentrations ($10^5$ and $10^6$ spores ml$^{-1}$) for both the blastospore and conidia formulations. Antagonism increased with blastospore concentration (Fig. 12), where *A. aegypti* larvae survival was similar in the presence or absence of the predator at $10^7$ and $10^8$ spores ml$^{-1}$ (Table 3). However, with conidial treatment, the combined effect of fungus and predator increased at higher fungal concentrations, to the point where the interaction was additive at $10^7$ spores ml$^{-1}$ and synergistic at $10^8$ spores ml$^{-1}$ (Fig. 12).

4. Discussion

Mycoinsecticides based on strains of EPF belonging to the genera *Metarhizium*, *Beauveria*, *Isaria* and *Lecanicillium* are either formulated as conidia or blastospores (de Faria and Wraight, 2007; Ravensberg, 2011). The latter is the preferred choice since it is comparatively cheaper to produce and is generally more virulent (Alkhaibari et al.,...
enthesis. brevipalpis have accelerated mortality of T. brevipalpis to facilitate penetration of the cuticle (Butt et al., 2016). Conidia fail to determine whether this was the route the fungus killed this predator. However, when a combination of M. brunneum conidia or blastospores, used at low concentrations, and T. brevipalpis together resulted in significantly higher control of A. aegypti than using either agent alone.

Differences in pathogenesis could not entirely explain the differential susceptibility of these mosquito species. For example, conidia adhered to the surface of T. brevipalpis but not A. aegypti; this should have accelerated mortality of T. brevipalpis but no obvious infection structures (i.e. appressoria, penetrating hyphae) were observed questioning whether this was the route the fungus killed this predator. Presumably, conidia adhered but did not perceive the right cues to facilitate penetration of the cuticle (Butt et al., 2016). Conidia fail to adhere to the surface of A. aegypti due to weak adhesion forces (Greenfield et al., 2014). In contrast, the sticky, mucilage-producing blastospores firmly adhered to the surfaces of both mosquito species and appeared to have the capacity to penetrate the host cuticle and could account for the high mortality of this particular formulation (Alkhairi et al., 2016).

Conidia and blastospores were readily ingested by A. aegypti but not in T. brevipalpis, reflecting differences in feeding mechanisms of these two species. The latter grab and chews on its prey while Aedes species browse and filter food. Some propagules may enter the digestive tract when the predator starts to feed on mosquito prey but the majority of propagules are probably released during the digestion process. The fact that viable propagules were present in faecal pellets suggests that they are not digested.

The current study suggests that blastospores infect T. brevipalpis via the cuticle but not midgut epithelium. In contrast, blastospores can infect through both the cuticle and midgut epithelium of A. aegypti larvae, resulting in accelerated mortality (Alkhairi et al., 2016). It is unclear if ingested conidia cause stress-induced mortality in T. brevipalpis as reported for A. aegypti larvae (Butt et al., 2013). In the latter case, conidia do not germinate in the gut lumen but the spore bound protease, Pr1, triggers stress induced apoptosis ultimately leading to death (Butt et al., 2013). The fact that T. brevipalpis mortality increased with concentration suggests that the conidia may have contributed to the mortality via this mechanism albeit with the conidia mostly being derived from the prey during the digestion process.

This study shows that the potential exists for the combined use of M. brunneum ARSEF 4556 and T. brevipalpis to control A. aegypti larvae. Combinations of these two biocontrol agents can potentially be antagonistic (weaker than additive), additive, or synergistic (stronger than additive) (Koppenhöfer and Kaya, 1997). The current study shows that significant reductions in lethal times were achieved by combining M. brunneum conidia with T. brevipalpis over a wide range of fungal concentrations, compared to fungal treatment alone. While beneficial, this interaction proved to be antagonistic at lower fungal conidia concentrations, but becoming at least additive at higher concentrations. However, when blastospores were used, addition of T. brevipalpis was only advantageous (but antagonistic) over fungus treatment alone at lower fungal concentrations, with no additional effects of the predator.
Fig. 12. The interaction between *Metarhizium brunneum* treatments (blastospores – left-hand panels, and conidial – right-hand panels) and *Toxorhynchites brevipalpis* on survival of *Aedes aegypti* larvae. Survival proportion (mean with 95% confidence intervals) of *A. aegypti* treated with: 1) four concentrations of the fungus ("F"), *M. brunneum* (10⁵, 10⁶, 10⁷, 10⁸ spore ml⁻¹), alone; 2) the fungus combined with one larva of the predator ("F + P"), *Tx. brevipalpis*; and 3) one larva of the predator ("P"), *Tx. brevipalpis* alone. The dotted line represents the expected level of the survival when the combination of fungus and predator are simply additive.
over fungus alone at the highest concentrations. The increasing antagonism between predator and blastospores may have been simply due to the fast action of the fungus in killing A. aegypti larvae before the predators had any additional effect, or due the fungus directly affecting the predators. In contrast, the combined effects of the conidia and predator were stronger with increasing fungal dosage. Many interacting factors can influence the combined effects of fungus and predator. For example, if the predator bites but does not kill its larval prey, then the fungus may find a way in through the wound and accelerate death (Wu et al., 2015). However, injury will activate phenoloxidase leading to production of melanin and precursors which are toxic to fungi (Tanada and Kaya, 2012; Butt et al., 2016). Furthermore, fungal infection may reduce larval mobility, so increasing their susceptibility to predation (Gehman and Byers, 2017).

Clearly the potential exists to develop IVM strategies targeting mosquito larvae through careful selection of the optimal concentration and formulation of M. brunneum. The laboratory findings may not always reflect what happens in the field due to a range of environmental factors. However, they do illustrate the sort of scenarios that likely take place in the field. Thus the fungus could be applied alone at low concentrations to work in concert with natural populations of Toxorhynchites with little risk to the latter. Alternatively, synergy between M. brunneum and Toxorhynchites could be exploited by using low concentrations of the fungus with concomitant introduction of the predator. The approaches outlined above will reduce costs, accelerate control, and concomitantly reduce risks to beneficial mosquito predators such as Toxorhynchites. Indeed, reduced application rates have been shown to reduce risks to several aquatic non-target aquatic invertebrates (Garrido-Jurado et al., 2015). In urban areas where rapid “knockdown” of a mosquito population is often necessary then high concentrations of M. brunneum blastospores would be required. However, there are many other situations where regular application of EPF would be required for example: to prevent mosquito establishment, eradication of invasive species or suppression of mosquito populations (cryptic habitats, remote rural habitats) to pre-empt sudden outbreaks following rainfall or flooding. IVM programmes could be improved through a thorough understanding of interactions between EPF and mosquito predators whether natural or introduced.

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