

Infection of *Anopheles gambiae* mosquitoes with entomopathogenic fungi: effect of host age and blood-feeding status

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Abstract Physiological characteristics of insects can influence their susceptibility to fungal infection of which age and nutritional status are among the most important. An understanding of host–pathogen interaction with respect to these physiological characteristics of the host is essential if we are to develop fungal formulations capable of reducing malaria transmission under field conditions. Here, two independent bioassays were conducted to study the effect of age and blood-feeding status on fungal infection and

survival of *Anopheles gambiae* s.s. Giles. Mosquitoes were exposed to 2×10^{10} conidia m^{-2} of oil-formulated *Metarhizium anisopliae* ICIPE-30 and of *Beauveria bassiana* I93-825, respectively, and their survival was monitored daily. Three age groups of mosquitoes were exposed, 2–4, 5–8, and 9–12 days since emergence. Five groups of different feeding status were exposed: non-blood-fed, 3, 12, 36, and 72 h post-blood feeding. Fungal infection reduced the survival of mosquitoes regardless of their age and blood-feeding status. Although older mosquitoes died relatively earlier than younger ones, age did not tend to affect mosquito susceptibility to fungal infection. Non-blood-fed mosquitoes were more susceptible to fungus infection compared to all categories of blood-fed mosquitoes, except for those exposed to *B. bassiana* 72 h post-blood feeding. In conclusion, formulations of *M. anisopliae* and *B. bassiana* can equally affect mosquitoes of different age classes, with them being relatively more susceptible to fungus infection when non-blood-fed.

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Introduction

Recent studies increasingly demonstrate the potential for controlling malaria vectors by using entomopathogenic fungi (Scholte et al. 2005; Blanford et al. 2005; Farenhorst et al. 2008; Mnyone et al. 2009). These fungi do not cause instant mortality but cause sublethal and late-life lethal effects on different stages of the mosquito life cycle. Due to such properties, fungi can potentially be used as “evolution-proof” agents and overcome mosquito resistance unlike the currently deployed fast-acting chemical insecticides (Ffrench-Constant 2005; Koella et al. 2009; Read et al. 2009). As part of an integrated strategy, fungi could

respond to, or avert emergence of serious levels of insecticide resistance. For instance, recent laboratory studies by Farenhorst et al. (2009) recorded similar susceptibility to fungal infection between insecticide-resistant and non-resistant colonies of *Anopheles* mosquitoes. Furthermore, theoretical modeling suggests the existence of a synergistic interaction between fungal biopesticides and insecticide treated bed nets even in scenarios of high malaria intensity and widespread insecticide resistance (Hancock 2009). This suggestion was validated by a recent study in Benin, where there was a strong interaction of the insecticide permethrin and the fungi *Metarhizium anisopliae* or *Beauveria bassiana* (Farenhorst et al. personal communication) on mosquito survival.

Under field conditions, entomopathogenic fungi target mosquitoes of different physiological characteristics, which may influence their overall effectiveness as biocontrol agents. Host species, age, developmental stage, sex, and nutritional status have frequently been reported to affect insect susceptibility to fungal infection (Feng et al. 1985; Maniania and Odulaja 1998; Dimbi et al. 2003). Host age and feeding status are among the most important physiological characteristics. A critical understanding of the host–pathogen interaction with respect to these host physiological characteristics, therefore, is essential for the development of formulations capable of suppressing malaria transmission under real life conditions.

Mosquito populations are known to experience senescence (Harrington et al. 2001; Okech et al. 2003; Styer et al. 2007). As mosquitoes age, adverse changes to major life-history parameters have been recorded, including differences in mortality, flight capacity, immune function, salivary gland function, and cuticular hydrocarbon composition (Rowley and Graham 1968; Nayar and Sauerman 1973; Beckett 1990; Chen et al. 1990; Brei et al. 2004; Hugo et al. 2006). The susceptibility of mosquitoes to fungal infection could be affected when (1) the insect cuticle weakens with age, thereby facilitating more successful fungal penetration; and (2) a lowered immune response allows the fungus to rapidly propagate inside the host. Recent research has demonstrated greater susceptibility of older (17–21 days old) than younger (3–7 days old) *Anopheles stephensi* mosquitoes to three species of entomopathogenic fungi (Stevenson J., unpublished data). This age composition, however, may not be representative of that of mosquitoes under realistic field conditions, where only a small proportion of mosquitoes may survive for 21 days or longer (Gillies and Wilkes 1965). More importantly, the wild vector community is normally composed of various species of mosquitoes, and this may dictate their survival and aging effect trends. As such, knowledge about species-specific aging effects in younger mosquitoes compared between a relatively narrow age

range will be of added value. Higher susceptibility in older than younger individuals has also been reported in studies of other insects. The susceptibility of tsetse flies to *M. anisopliae* was greater among older than younger individuals (Maniania and Odulaja 1998). Similar phenomena have been observed with regard to insecticides. The mean knockdown times of *A. stephensi* and *Anopheles gambiae* mosquitoes exposed to pyrethroids were significantly shorter if they were 10 days old as compared to those of newly emerged ones (Hodjati and Curtis 1999). Nevertheless, contrasting reports, where greater susceptibility has been recorded in younger than older insects, also exist (Mullens 1985; Dimbi et al. 2003).

Nutritional differences between non-blood-fed and blood-fed mosquitoes may also affect the efficacy of a fungal-based intervention. Various studies have recorded contradicting effects of blood feeding on mosquito longevity. One hypothesis is that mosquitoes which are fed on blood alone, without access to sugar, will have lower survival, possibly because nutrients are diverted for egg production (Okech et al. 2003). Alternatively, other experiments have recorded that blood feeding can actually increase mosquito survival when compared with mosquitoes fed only on sugar (Scott et al. 1993; Scott et al. 1997). The nutritional benefits of blood feeding could alter the efficacy of fungal infections due to changes in the host's ability to activate an immune response or, conversely, alter the level of nutrients available for pathogen propagation. *Rhodnius prolixus* bugs that were fed on blood showed a higher immune response to *Enterobacter cloacae* infection compared to *R. prolixus* fed on plasma (Feder et al. 1997). A similar susceptibility trend was observed between freshly blood-fed and non-blood-fed *A. gambiae* (G3 strain, originating from The Gambia) mosquitoes (J. Stevenson, unpublished data). In that study mosquitoes were fed directly on rats.

Under realistic field situations, mosquito vectors of different age and blood-feeding status may be encountered. An understanding of host–pathogen interaction with respect to mosquito age and blood-feeding status, therefore, is essential if we are to develop fungal formulations capable of reducing malaria transmission. This study was designed to examine if efficacy of *M. anisopliae* ICIP-30 and *B. bassiana* 193-825 varied with mosquito age and human blood feeding. This information will be essential for designing delivery mechanisms to expose the most susceptible portion of the mosquito population to fungal infection.

Materials and methods

Mosquitoes

Mosquitoes originated from a colony of *A. gambiae* s.s. established from Njage village, Tanzania in 1996 and

maintained at the Ifakara Health Institute. Larvae and adults were reared using procedures described by Huho et al. (2007). Bioassays were conducted using fed and unfed adult female mosquitoes.

Fungal isolates, formulation, and application

Two fungal isolates were used in this study: *M. anisopliae* var. *anisopliae* ICIP-30, isolated in 1989 from the maize stalk borer, *Busseola fusca* (Lepidoptera, Noctuidae) in Western Kenya, and *B. bassiana* I93-825 (IMI 391510), isolated from a chrysomelid beetle (Coleoptera) in the USA. Before each experiment, viability of conidia (i.e., percent germination on Sabouraud Dextrose Agar) was confirmed to be >85%.

Conidia were formulated in Enerpar, a highly refined mineral oil (Enerpar M002®, BP Southern Africa Ltd.). Stock and working conidia suspensions were prepared using procedures described by Mnyone et al. (2009). In all bioassays, a concentration of 2×10^{10} conidia m^{-2} was used. Mosquitoes were exposed to conidia applied to sheets of A4 printing paper within plastic exposure tubes, closed with netting also treated with conidia. The paper and netting were treated using a hand-held pressure sprayer (Minijet®, SATA, Germany) operating at a constant pressure of two bars. The nozzle of the spray gun was held 50 cm away from, and at a right angle to, the application surface. A working solution of 23 ml containing 2×10^{10} conidia was applied to a 1-m^2 area. Treated surfaces were left to dry for 24 h.

Bioassay procedures

Thirty to forty adult *A. gambiae* s.s. mosquitoes were introduced into the fungal exposure tube (8.2 cm diameter \times 12.5 cm height) and held for 6 h after which they were transferred to separate untreated cages (9 \times 9 \times 9 cm) and maintained at 26–27°C and 85–95% relative humidity (RH) with access to 9% glucose solution ad libitum. Based on earlier observations, 6 h was considered an appropriate exposure period (Mnyone et al. 2009). Mosquito survival was determined by recording mortality in each holding cage for a maximum of 28 days. Cadavers were collected daily, put onto moist filter paper in Petri dishes, sealed with Parafilm and incubated at 26–27°C and 85–95% RH for 3–4 days. After that, they were then examined for evidence of fungal sporulation. Similar bioassay procedures were followed for 30–40 mosquitoes in control groups, except that they were exposed to untreated surfaces. During all of the bioassays, six independent replicates were used for each experimental factor. Two different bioassays were conducted to study the effect of age and blood feeding on infection and survival of *A. gambiae* s.s.

Bioassay 1: effect of mosquito age on infection and survival

Three different age groups of female mosquitoes were exposed to both fungal isolates: (1) 2–4 days, (2) 5–8 days, and (3) 9–12 days postemergence.

Bioassay 2: effect of mosquito blood feeding on infection and survival

Five groups with differing blood-feeding status were exposed to both fungal isolates: (1) non-fed, (2–5) 3, 12, 36, or 72 h post-blood feeding. Mosquitoes were starved of glucose for 6 h before blood feeding. For blood feeding, a volunteer placed a bare forearm inside the cage and allowed mosquitoes to feed for 10 min. After blood feeding, all unfed and partially fed mosquitoes were aspirated out of the cage leaving only fully engorged mosquitoes. Blood feeding was scheduled so that when the mosquito groups were exposed to the fungus they were in the age range of 3–6 days.

Data analysis

Mosquito survival data were analyzed using Cox regression to determine the relative risk of dying [hazard ratios (HR)] for the specific treatment group compared with the baseline group. The explanatory factors included in the analysis were fungal treatment, mosquito age, and blood feeding status, as required. Hazard ratios of each mosquito category and its respective control were generated in independent models to observe if confidence intervals (CI) were suggestive of any interaction between fungus-related mortality and age or blood meal. Non-blood-fed mosquito data was run against each of the four categories of blood-fed mosquitoes generating four independent models to confirm interaction between fungus-related mortality and a blood meal. Kaplan–Meier pairwise method was used to obtain median survival times (MST) for treated and untreated group of mosquitoes. The Bonferroni method was employed to compensate for multiple comparisons. SPSS version 17 was used.

Results

Bioassay 1: effect of mosquito age on infection and survival

Overall, the survival of all three different age groups of mosquitoes was reduced relative to their control counterparts when exposed to both *M. anisopliae* and *B. bassiana* (Table 1). The daily risk of dying was over twofold greater in mosquitoes exposed to *M. anisopliae* relative to controls [HR=2.26 (95% CI=1.86–2.74), $p < 0.001$], whereas the

Table 1 Median survival times (MST ± SE) of different age groups (2–4, 5–8, and 9–12 days old) of *A. gambiae* s.s. exposed to *M. anisopliae* ICIPE-30 and *B. bassiana* I93-825

Fungus species	MST ± SE (days)			
	Age (days)	Control	Treatment	<i>P</i> value
<i>M. anisopliae</i> ICIPE-30	2–4	15±0.51	8±0.51	<0.001
	5–8	14±0.39	7±0.39	<0.001
	9–12	11±0.25	5±0.25	<0.001
<i>B. bassiana</i> I93-825	2–4	15±0.51	7±0.28	<0.001
	5–8	14±0.39	6±0.22	<0.001
	9–12	11±0.25	6±0.28	<0.001

daily risk of dying was over threefold greater in mosquitoes exposed to *B. bassiana* relative to controls [HR=3.46 (95% CI=2.85–4.19), $p<0.001$].

Irrespective of fungus infection, the age of mosquitoes affected the rate at which they were dying, with older mosquitoes dying relatively earlier than younger ones (*M. anisopliae*: Wald=75.54; df=2; $p<0.001$, *B. bassiana*: Wald=76.16; df=2; $p<0.001$). For *M. anisopliae*, the risk of dying was greater in 9–12-day-old mosquitoes relative to 2–4 days old [RH=1.52 (95% CI=1.27–1.82), $p<0.001$] and 5–8-day-old [RH=1.25 (95% CI=1.04–1.50), $p<0.001$] mosquitoes. Similarly, for *B. bassiana*, the risk of dying was greater in 9–12-day-old mosquitoes relative to 2–4-day-old [RH=1.51 (95% CI=1.26–1.81), $p<0.001$], and 5–8-day-old [RH=1.51 (95% CI=1.26–1.83), $p<0.001$] mosquitoes. For both fungal species, the latter two age groups of mosquitoes had a similar risk of dying [RH=1.22 (95% CI=1.01–1.47), $p=0.06$]. There was no overall significant interaction, however, between age and rate at which fungus-infected mosquitoes were dying (*M. anisopliae*: Wald=1.76; df=2; $p=0.42$, *B. bassiana*: Wald=1.60; df=2; $p=0.21$).

Bioassay 2: Effect of mosquito blood feeding on infection and survival

The survival of all fungus-infected mosquitoes was reduced compared to their control counterparts when mosquitoes were non-fed or 3, 12, 36, or 72 h post-blood feeding (Table 2). The daily risk of dying was over twofold greater in mosquitoes exposed to *M. anisopliae* [HR=2.75 (95% CI=2.27–3.33), $p<0.001$] and *B. bassiana* [HR=2.28 (95% CI=1.87–2.78), $p<0.001$] relative to controls.

A blood meal significantly affected the rate at which mosquitoes died, and overall, blood-fed mosquitoes had a lower risk of dying relative to unfed ones (*M. anisopliae*: Wald=95.39; df=4; $p<0.001$, *B. bassiana*: Wald=80.16; df=4; $p<0.001$). With independent models where non-

Table 2 Median survival times (MST ± SE) of *A. gambiae* s.s. exposed to *M. anisopliae* ICIPE-30 and *B. bassiana* I93-825 when non-blood-fed and 3, 12, 38, and 72 h since blood feeding

Fungus species	Blood-feeding status before exposure (no. of hours)	MST ± SE (days)		<i>P</i> value
		Control	Treatment	
<i>M. anisopliae</i> ICIPE-30	Unfed	14±0.56	7±0.41	<0.001
	3	14±0.70	7±0.46	<0.001
	12	18±0.06	9±0.50	<0.001
	36	18±0.76	9±0.46	<0.001
	72	16±0.81	9±0.70	<0.001
<i>B. bassiana</i> I93-825	Unfed	14±0.56	7±0.22	<0.001
	3	14±0.70	8±0.41	<0.001
	12	18±0.06	10±0.57	<0.001
	36	18±0.76	10±0.46	<0.001
	72	16±0.81	10±0.32	<0.001

blood-fed mosquitoes were independently compared with blood-fed categories, there was an interaction between blood feeding and fungus-induced mortality except for mosquitoes exposed to *B. bassiana* 72 h since blood feeding (*M. anisopliae*: Wald=23.25–52.56; df=1; $p<0.001$, *B. bassiana*: Wald=16.63–50.42; df=1; $p<0.001$).

Discussion

When mosquitoes were exposed to either *M. anisopliae* or *B. bassiana* their survival, in all bioassays, was significantly reduced relative to their control counter parts. This supports previous laboratory trials that have demonstrated the potential of entomopathogenic fungi for adult mosquito control (Scholte et al. 2005; Blanford et al. 2005; Farenhorst et al. 2008). Our bioassays investigated the interaction between the pathogen, and host age and blood-feeding status, which are key factors affecting initiation and development of fungal infection (Dimbi et al. 2003).

Although older mosquitoes died relatively earlier than younger individuals, age did not affect mosquito susceptibility to fungal infection. Increased risk of death in older than younger individuals has also been reported elsewhere (Harrington et al. 2001; Styer et al. 2007). Finally, no interaction between age and fungus-induced mortality has been reported in housefly *Musca domestica*, onion fly *Delia antiqua*, and blackfly *Phormia regina* exposed to *M. anisopliae* and *B. bassiana* (Rizzo 1977). Contrarily, age-dependent fungus-induced mortality has been observed, for example, in scarabeid larvae (Ferron 1967), *Heliothis zea* larvae (Mohamed et al. 1977), and adult tsetse flies (Maniania and Odulaja 1998). In these three cases, the fungus-induced mortality was relatively higher in older than younger individuals. Fungus-induced mortality, however,

has been shown to decrease with age in noctuid larvae (Getsin 1961; Fargues and Rodriguez-Rueda 1980; Boucias et al. 1984).

Increased mortality rate among older individuals could be explained by various physiological properties that change with the age of mosquitoes and possibly other insect species. Older mosquitoes show reduced immune function. Melanization responses in laboratory reared *A. gambiae* declined within a short period of time (Chun et al. 1995; Schwartz and Koella 2002), and older individuals were only able to mount strong immune response after they were supplemented with blood (Schwartz and Koella 2002). Enzymes such as phenol oxidases, which are suggested to be important for melanization and sclerotization in the hemolymph (Chun et al. 1995), and numbers of immune competent hemocytes (Li et al. 1992) decrease with age. A change in the number and morphology of circulating hemocytes was attributed to increased mortality in older *Aedes aegypti* (Hillyer et al. 2005). Moreover, glycogen and total soluble proteins deteriorate (Nayar and Sauerman 1973; Mourya et al. 1993) with age.

Non-blood-fed mosquitoes were more susceptible to fungus infection than blood-fed ones. A digested blood meal increases the nutrient reserve of the host, thus more time may be required for the fungus to deplete nutrients and kill the insect. In addition, insects with a digested blood meal can also mount a stronger immune response. This could have been the reason for the lower mortality in blood-fed relative to unfed mosquitoes, especially considering that histopathological studies of tissues infected by fungus suggest that the insect dies due to the combination of nutrient depletion, mechanical damage, and toxicosis (Ferron 1981; Gillespie and Claydon 1989). The fact that blood-fed mosquitoes are less susceptible to fungal infection could be beneficial in terms of evolution proofing against resistance development. Although fungal infection reduces the fecundity of female mosquitoes (Scholte et al. 2006), they are still able to pass their genes to the subsequent generation reducing selection pressure on resistance against fungi (Thomas and Read 2007). Furthermore, fungal infections suppress the successful development of *Plasmodium* parasites in the vectors (Blanford et al. 2005), and hence both effects (i.e., fungus-induced mortality and parasite resistance) lead to a significantly reduced parasite transmission risk.

Conclusions

Formulations of *M. anisopliae* and *B. bassiana* can equally infect mosquitoes of different age classes, more so when non-blood-fed. Therefore, delivery techniques that target host-seeking or house entering mosquitoes may be more

effective than techniques targeting resting (and often blood-fed) mosquitoes. To expect more in terms of reducing wild mosquito populations, however, more efforts should gear towards producing more virulent and persistent fungal products.

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Competing interests Authors declare that they have no competing interests.

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