The Mosquito Melanization Response Is Implicated in Defense against the Entomopathogenic Fungus 
Beauveria bassiana

Hassan Yassine*, Layla Kamareddine*, Mike A. Osta*
Department of Biology, American University of Beirut, Beirut, Lebanon

Abstract

Mosquito immunity studies have focused mainly on characterizing immune effector mechanisms elicited against parasites, bacteria and more recently, viruses. However, those elicited against entomopathogenic fungi remain poorly understood, despite the ubiquitous nature of these microorganisms and their unique invasion route that bypasses the midgut epithelium, an important immune tissue and physical barrier. Here, we used the malaria vector Anopheles gambiae as a model to investigate the role of melanization, a potent immune effector mechanism of arthropods, in mosquito defense against the entomopathogenic fungus Beauveria bassiana, using in vivo functional genetic analysis and confocal microscopy. The temporal monitoring of fungal growth in mosquitoes injected with B. bassiana conidia showed that melanin eventually formed on all stages, including conidia, germ tubes and hyphae, except the single cell hyphal bodies. Nevertheless, melanin rarely aborted the growth of any of these stages and the mycelium continued growing despite being melanized. Silencing TEP1 and CLIPA8, key positive regulators of Plasmodium and bacterial melanization in A. gambiae, abolished completely melanin formation on hyphae but not on germinating conidia or germ tubes. The detection of a layer of hemocytes surrounding germinating conidia but not hyphae suggested that melanization of early fungal stages is cell-mediated while that of late stages is a humoral response dependent on TEP1 and CLIPA8. Microscopic analysis revealed specific association of TEP1 with surfaces of hyphae and the requirement of both, TEP1 and CLIPA8, for recruiting phenoloxidase to these surfaces. Finally, fungal proliferation was more rapid in TEP1 and CLIPA8 knockdown mosquitoes which exhibited increased sensitivity to natural B. bassiana infections than controls. In sum, the mosquito melanization response retards significantly B. bassiana growth and dissemination, a finding that may be exploited to design transgenic fungi with more potent bio-control activities against mosquitoes.

Introduction

Melanization is an immediate immune response in arthropods leading to the physical encapsulation of pathogens in a dense melanin coat, and to the generation of toxic metabolites that can harm certain pathogens. It is also a prominent wound healing process manifested by the blackening of the wound area in arthropods. Melanization is triggered by pattern recognition receptors (PRRs) that upon binding pathogen associated molecular patterns (PAMPs) activate a cascade of serine proteases culminating in the proteolytic cleavage and conversion of the prophenoloxidase to phenoloxidase (PPO), the rate limiting enzyme in melanogenesis [1]. The protease cascade acting upstream of PPO involves often a modular protease and several downstream clip-domain serine proteases (CLIPs) [2,3]. This cascade is under tight temporal regulation by serine protease inhibitors (SPRNs). In the dipterans Drosophila and Anopheles gambiae, the absence of SPN43Ac, also called necrotic, [4] and SPRN2 [5], respectively, resulted in the appearance of spontaneous melanotic pseudotumors in adult tissues and a reduced life span, suggesting that aberrant control of melanization imposes a fitness cost on the host. Further, this process is regulated spatially which ensures that melanin formation occurs exclusively on microbial surfaces minimizing collateral damage to the host.

Biochemical studies in Manduca sexta [6] and Tenebrio molitor [7] revealed that PPO activation is further controlled by the requirement of non-catalytic CLIPs [also known as serine protease homologs (SPHs)] as co-factors for prophenoloxidase activating enzymes (PPAE) to trigger proper processing of PPO into PO. SPHs have substitutions at one or more of the critical His/Asp/Ser triad that renders them non-catalytic. Functional genetic studies in the malaria vector A. gambiae revealed that a clip domain-containing SPH termed CLIPA8 is required for the melanization of Plasmodium berghei ookinetes in the mosquito midgut [8] and bacteria in the hemocoel [9]. While there is no evidence yet for the direct involvement of CLIPA8 in the processing of PPO, these studies provided a strong genetic evidence for the central role of SPHs in the melanization response in vivo.
Several reports have linked melanization to insect defense. In the crustacean Pacifastacus leniusculus, PO activity is required for defense against the bacterial pathogen Aeromonas hydrophila. RNAi-mediated silencing of PO was associated with increased susceptibility to A. hydrophila while silencing pacifastin, an inhibitor of the crayfish PO cascade, resulted in increased resistance to the bacterium [10]. The fact that Photobacterium bacteria pathogenic to M. sexta [11], and polydnaviruses carried by female parasitoid wasps [12], evolved independent specific strategies to counteract the host melanization response is a further indication of the importance of this response in insect defense.

Previous genetic studies in the model dipteran Drosophila revealed that the melanization response does not seem to be critical for survival of flies after bacterial or fungal infections [13,14]; rather, melanization seems to enhance the effectiveness of subsequent immune reactions in the fly by weakening a microbial infection at an early stage [14]. However, a more recent study, employing a larger panel of bacterial species, demonstrated an important role for this immune process in modulating tolerance as well as resistance of the fly to specific bacterial infections [15]. Abolishing PO activity in the malaria vector A. gambiae, by silencing CLIPA8, did not affect mosquito survival after infections with Escherichia coli or Staphylococcus aureus [9]. Both bacterial species were cleared from CLIPA8-silenced mosquitoes as efficiently as from controls suggesting that melanization is not critical for antibacterial defense in the mosquito. In A. gambiae, the melanization response to P. berghei is also controlled by CLIPA8 [8], in addition to the complement-like protein TEP1 [16] and two leucine-rich immune proteins, LRIM1 [17] and APL1C [18,19]. The latter two proteins form an obligate disulfide-linked heterodimer in the mosquito hemolymph that interacts with and stabilizes a cleaved form of TEP1 [20,21]. In addition to triggering ookinete lysis in the basal labyrinth of the midgut epithelium [16–18], the TEP1/LRIM1/APL1C complex (henceforth TEP1 complex) is also required for the melanotic response to ookinete in refractory mosquito genotypes [16,17] as well as to bacteria injected directly into the hemolymph (unpublished data). Nevertheless, wildtype laboratory and field caught A. gambiae mosquitoes rarely melanize malaria parasites [22] and melanization does not seem to be important for A. gambiae anti-bacterial defense [9], questioning the role of this response in mosquito immunity.

Research on mosquito immunity has focused mainly on parasites, bacteria (reviewed in [23]) and lately viruses [24–26], whereas entomopathogenic fungi received little attention despite their ubiquitous nature and their route of infection which unlike other microbial classes does not require ingestion by the host. Rather, these fungi infect by direct penetration through the mosquito cuticle into the hemolymph. This mode of infection is particularly attractive for immunity studies because it does not require artificial injection of the microbe into the hemolymph. It also bypasses the midgut epithelium which was shown recently to engage in promoting complement-mediated ookinete lysis in the basal labyrinth of the midgut epithelium, by triggering intracellular nitrilation of ookinete surface proteins [27]. Here, we investigate the role of melanization in defense against natural infections with the entomopathogenic fungus B. bassiana and provide novel insights into the cellular and molecular mechanisms triggering fungal melanization in vivo.

**Results**

**Beauveria bassiana** infection triggers the melanization response of *Anopheles gambiae*

Mosquito immune responses to entomopathogenic fungi such as *B. bassiana* remain poorly understood. We carried a meticulous microscopic analysis of *B. bassiana* development in adult *A. gambiae* mosquitoes to determine whether melanization is also triggered in this model infection system and against which stages. To facilitate detection of the fungus in dissected mosquito abdomens we utilized a GFP-expressing strain of *B. bassiana* [28]. Individual mosquitoes were injected intrathoracically with 200 freshly prepared conidia (spores) and fungal development was monitored in dissected abdomens at 1, 6, 12, 24 and 48 h post-infection (pi). In these assays, mosquitoes were infected by injecting conidia rather than by the natural route (tarsal contact with spores), because in the former mycelial growth was frequently observed in dissected abdomens which was the not case in natural infections. Most conidia were rapidly melanized at 1 hr pi; these appeared black since GFP was masked by the melanotic capsule (Figure 1A). Only rare non-melanized conidia were observed at that time point. At 6 h pi all conidia were melanized (Figure 1B), however, at later time points, some melanized conidia exhibited an enlarged size indicating that germination was taking place within the melanotic capsules (Figure 1C). Indeed, at 24 h pi, germ tubes started emerging from melanized conidia concomitant with melanin formation around their walls (Figure 1D). Two days pi, melanin was also detected on several hyphae that emerged from germ tubes, while few hyphae remained non-melanized (Figures 1E and 1F). Altogether, our data revealed that conidia, germ tubes and hyphae were efficiently melanized in *A. gambiae* mosquitoes, yet this immune reaction was not sufficient to abort completely the development of the mycelium. However, we noticed that hyphal bodies, yeast-like single-cells that differentiate from growing hyphae, were rare and sometimes absent in abdomens at 48 h post conidia injection, whereas these stages were commonly present in the hemolymph of other insect species at the same time point [29,30]. This suggests that melanization might be retarding the growth of the fungus in the mosquito.
Cellular and humoral melanotic responses elicited against *B. bassiana* in adult mosquitoes

The melanization of *P. berghei* in certain refractory *A. gambiae* mosquito genotypes is a humoral response dependent on CLIPA8 [8], and TEP1 complex [16,17,20,21]. In this model system, the midgut basal lamina constitutes a physical barrier that inhibits direct contact between hemocytes and ookinetes residing in the basal labyrinth. Bacteria injected into the hemolymph also elicit a humoral melanotic response dependent on CLIPA8 [9] and TEP1 complex (unpublished data), suggesting that these are core proteins in the mosquito melanization response. To address whether they exhibit similar roles in infections with *B. bassiana*, TEP1 and CLIPA8 were silenced in adult female mosquitoes by RNAi knockdown (kd) [31], then individual mosquitoes were injected with 200 conidia of GFP-expressing *B. bassiana*. Mosquito abdomens were dissected two days after spore injection in order to score fungal melanization. Western blot analysis of hemolymph extracts confirmed that TEP1 and CLIPA8 were efficiently silenced four days after injection of their corresponding double-stranded RNAs (Figure 2A). In *LacZ* kd control mosquitoes, a thick melanin coat covered the majority of the growing mycelium as expected (Figure 2B). In contrast, hyphal melanization was completely abolished in CLIPA8 and TEP1 kd mosquitoes, suggesting that these proteins are indeed core regulators of the melanization response (Figures 2C and 2D, respectively). Intriguingly, in these two genotypes, only the base of the growing mycelium from which hyphae emerged was still melanized as efficiently as in *LacZ* kd controls. These findings were unexpected since the same gene knockdowns completely abolished the melanotic response to *P. berghei* [8,16] and bacterial infections ([9] and unpublished data).

We hypothesized that two distinct mechanisms are driving the melanotic response to the fungus. The first is hemocyte-mediated and targets the early stages of fungal development in the mosquito, including the germinating spores and germ tubes. The second is humoral, dependent on TEP1 and CLIPA8 functions, and targets the hyphae that develop later. To address this point, abdomens dissected from wildtype mosquitoes, at 12 and 48 h after conidia injection were immunostained with polyclonal antibody against PPO6, which is known to be expressed in hemocytes [32,33]. Abdomens dissected at the earlier time point clearly showed a circular arrangement of hemocytes around enlarged conidia that were apparently germinating within the melanotic capsule (Figure 3A). Most of these hemocytes showed absence of, or a faint PPO signal possibly because they have exhausted their PPO

**Figure 1. Mosquito melanization response to *B. bassiana* developmental stages.** Merged fluorescent and bright field images of abdomens dissected from mosquitoes at (A) 1 hr, (B) 6 h, (C) 12 h, (D) 24 h and (E and F) 48 h following the injection of each with 200 conidia of GFP-expressing *B. bassiana*. (A) Conidia were rapidly melanized (arrow) 1 h post-injection (pi); few non-melanized conidia were detected at that time point (arrowhead). (B) All conidia were melanized at 6 h pi. (C) An enlarged conidium germinating within the melanotic capsule (arrowhead) at 12 h pi. (D) A germ tube breaking through the melanin coat at 24 h pi (arrowhead) and another elongating with concomitant melanin formation around its wall (arrow). (E) Partially melanized hypha in a mycelium at 48 h pi showing absence of melanin at the apical part (arrowhead) and presence of a thick melanin coat around the basal part (arrow) (F) A low magnification image showing extensive melanization of hyphae (arrowheads) in the growing mycelium at 48 h pi, with few GFP-expressing, non-melanized hyphae detected (arrow). h, hour; GFP-expressing *B. bassiana* (Green). Scale bars are 10 μm in A–E and 50 μm in F.

doi:10.1371/journal.ppat.1003029.g001
content in the struggle against the germinating conidium. Alternatively, some of these hemocyte may not express PPO. A hemocyte strongly expressing PPO was resting on top of two other hemocytes that are in direct contact with the conidium (Figures 3A), suggesting that hemocytes recruited to the germinating spore may form more than one layer around it attempting to abort its growth, pretty much similar to nodule formation in larger insects. At 48 h after infection, no hemocytes were detected in close proximity to melanized hyphae supporting the humoral nature of this response (Figure 3B). The hyphal tips from where growth occurs exhibited a thin, often barely detectable layer of melanin, but a strong PPO signal (Figure 3B), suggesting that melanin biosynthetic reactions were still particularly active at these foci. Yet, the whole was taking place in the absence of hemocytes from the hyphal tips.

The microscopic analysis described above indicates that the early melanotic response to conidium injection requires the direct participation of hemocytes while that triggered against growing hyphae is humoral and dependent on TEP1 and CLIPA8. To investigate further this point, we measured the temporal dynamics of hemolymph PO activity in CLIPA8, TEP1 and LacZ kd mosquitoes at 24, 48 and 72 h after spraying with a suspension of 1 x 10⁶ conidia/ml. PO activities in both CLIPA8 (Figure 4A) and TEP1 kd mosquitoes (Figure 4B) were similar to that in LacZ kd controls at 24 h post-challenge, when the fungus has just invaded the cuticle. However, at later time points, the activity dropped significantly in both CLIPA8 and TEP1 kd mosquitoes while it remained relatively unchanged in controls, which further supports the humoral nature of the melanotic response to late fungal stages.

PPO recruitment to hyphae is dependent on both TEP1 and CLIPA8

Initiation of the melanization reaction requires limited proteolytic cleavage of zymogen PPO into active PO, the rate limiting enzyme in melanogenesis. The mechanisms which trigger PPO recruitment to microbial surfaces remain unclear. Here, we analyzed PPO localization to hyphae in TEP1, CLIPA8 and LacZ kd (control) mosquitoes at 48 h after conidia injection, using confocal microscopy. In the control group, PPO staining was observed on mycelial structures coated with a thick melanin capsule (data not shown) as previously reported in Figure 3B. Additionally, PPO was also detected along the length of hyphae on which melanin deposition was barely detectable or even absent (Figure 5A), as if a lag phase existed between PPO recruitment and melanogenesis on these hyphal surfaces. PPO staining was often detected around the branching points of established hyphae (Figure 5A). Interestingly, silencing CLIPA8 or TEP1 completely abolished PPO localization to hyphae, and consequently none of these structures was melanized (Figures 5B and 5C, respectively). However, in these genotypes, PO was still detected on the
melanized base of the mycelium from which hyphae emerged, corroborating our previous conclusion that the melanotic response against the early fungal stages is independent of TEP1 and CLIPA8 functions. Interestingly, the rare hyphal bodies detected in control mosquitoes at that time point, were not labelled with PPO (Figure 5A) suggesting that these stages might escape melanization.

TEP1 binds to bacteria enhancing their phagocytosis by a hemocyte-like cell line [34] and to Plasmodium ookinetes, as they egress from midgut epithelial cells into the basal labyrinth, leading to their lysis. The fact that TEP1 binds to evolutionary distant microbial surfaces and that PPO localization to hyphae is TEP1-dependent, prompted us to study whether TEP1 associates with hyphal surfaces to trigger downstream events culminating in PPO activation and subsequent melanin formation. Abdomens dissected from control (LacZ kd) mosquitoes at 48 h after conidia injections revealed strong TEP1 localization on melanin-free hyphal surfaces (Figure 6A); where melanin had previously formed, TEP1 signal was either faint or absent, possibly because it was masked by the thick melanotic capsule. Also, the rare hyphal bodies detected in these abdomens were labelled with TEP1. Thus, TEP1 localization to hyphal surfaces clearly precedes melanin formation; the tips from where hyphae grew were always TEP1 positive but melanin negative (Figure 6A). In TEP1 kd mosquitoes, the melanization of hyphae was completely abolished (Figure 6B). Interestingly, hyphal bodies were more common in these mosquitoes relative to controls, suggesting rapid fungal growth. In summary, our data revealed that TEP1 association with hyphal surfaces is a prerequisite for the initiation of a local melanotic reaction against B. bassiana.

The mosquito melanization response protects against B. bassiana infection

The microscopic observation of melanotic capsules around entomopathogenic fungi has been reported earlier in several insect species including Chironomus [35], the leafhopper Empoasca fabae [36] and the grasshopper Melanoplus sanguinipes [37]. However, the relative contribution of this immune response to anti-fungal defense remains poorly understood. In A. gambiae, melanization is dispensable for defense against bacterial infections, despite the fact...
that bacteria trigger PPO activation in the hemolymph [9]. Additionally, field caught A. gambiae mosquitoes [22] as well as most laboratory strains rarely melanize Plasmodium ookinetes suggesting that melanization is dispensable for defense against these parasite stages. The fact that mosquitoes mounted a strong melanotic response to B. bassiana prompted us to test the relevance of this response to anti-fungal immunity. To address this point, TEP1, CLIPA8 and LacZ kd adult female mosquitoes were naturally infected with a wildtype B. bassiana strain (80.2) either by spraying with a suspension of 1 × 10^8 conidia/ml or by gentle dragging over a lawn of spores on a potato dextrose agar plate. Mosquitoes were then incubated at 27°C at 90% humidity and their survival scored on a daily basis. Survival assays revealed a significant increase in susceptibility of CLIPA8 and TEP1 kd mosquitoes to B. bassiana over controls, whether gentle dragging (Figure 7A) or spraying (Figure 7B) was used to establish an infection. Interestingly, the TEP1 kd group succumbed more quickly to infection than the CLIPA8 kd, suggesting that TEP1 might be controlling more than one anti-fungal effector mechanism.

We then scored hyphal body colony forming units in CLIPA8, TEP1 and LacZ kd mosquitoes four days after spraying with 5 × 10^7 conidia/ml, to determine whether the compromised survival in the two former genotypes is due to increased fungal proliferation. Our data revealed that CLIPA8 and TEP1 kd mosquitoes contained indeed significantly higher numbers of hyphal bodies than controls which contained none at that time point; the median values were 15, 50 and 0, respectively (Figure 7C). Here, it is worth mentioning that, in general, hyphal bodies appeared more quickly in wildtype mosquitoes when fungal infection was established through spraying rather than the natural route. This explains why these stages were sometimes detected in whole mounts of abdomens at 48 h post conidia injection (Figures 5A and 6B), but were absent from mosquitoes even at day four after natural infection (Figure 7C). Hyphal bodies were significantly more abundant in TEP1 (P = 0.0017) than in CLIPA8 kd mosquitoes, which explains the increased sensitivity of the former genotype to B. bassiana challenge.

Discussion

Melanization is an important immune response in insects that is triggered against diverse microbial classes including parasites [38], bacteria [9,15,39] and fungi [35–37]. Functional genetic studies in several insect species revealed an important role for this response in insect immunity to bacterial infections [10,11,15]. Here, we investigated the role of melanization in A. gambiae anti-fungal defense using B. bassiana as a model. Our work has been prompted by early electron microscopy studies showing the formation of melanotic capsules around pathogenic fungi invading the hemocoel of other insect species [36,37], and by the fact that entomopathogenic fungi employ a different route for mosquito invasion compared to bacteria and Plasmodium parasites. The latter two, naturally infect through the oral route and traverse the midgut epithelium in order to gain access into the hemocoel, whereas pathogenic fungi breach the cuticle reaching directly into the hemocoel using a combination of mechanical pressure and an array of cuticle-degrading enzymes [40].

Results obtained from temporal analysis of the melanotic response to B. bassiana developmental stages in adult mosquitoes, using fluorescent microscopy, are in line with early reports showing that this response did not prevent the germination of B. bassiana conidia in the hemolymph of other insect species [37,41]. Melanization occurred rapidly on injected conidia, then progressed over the germ tubes as well as hyphae that constitute the bulk of the mycelium (Figure 1). Only in rare cases was the mycelium completely melanized, rather hyphae always succeeded to break through the melanotic capsule. Our results revealed that the mosquito mounts a potent melanotic response against the fungus, with melanized hyphae sometimes measuring more than one millimeter in length (data not shown). This response, however, is not sufficient to kill the fungus. A possible explanation could be the depletion of hemolymph PPO later during infection, due to the continuous triggering of the response by the rapidly growing fungus; however, western blot analysis excluded such possibility since PPO levels remained relatively unchanged in the hemolymph up to five days post-infection.
Nevertheless, we provided, for the first time, tangible evidence that melanization retards significantly the growth of the fungus in the mosquito. This is reflected in the absence of hyphal bodies in control (LacZ kd) mosquitoes four days after spraying with a conidial suspension, compared to their presence in CLIPA8 and TEP1 kd mosquitoes processed at the same time (Figure 7C).

Figure 5. PPO recruitment to hyphae requires TEP1 and CLIPA8. Mosquito abdomens were dissected from (A) LacZ, (B) CLIPA8 and (C) TEP1 kd mosquitoes at 48 h post-injection of each with 200 conidia of GFP-expressing B. bassiana and stained with PPO6 antibody. Shown are Bright field (Bf) and fluorescence (Fl) images of confocal sections, and Z projections (Z-proj) of whole stacks. (A) An abdomen from LacZ kd mosquitoes at 100× magnification showed uniform PPO staining along an established hypha (Ai, filled arrowhead) elaborating new hyphae and hyphal bodies. Note that melanin was barely detectable on this hyphal surface (Ai, open arrowhead) despite its intense PPO staining. A strong PPO signal was also observed at the branching points of the established hypha (Ai and Aii, arrows with filled heads). The hyphal bodies detected were not labelled with PPO (Aii and Aiii, arrows with open heads). Shown also are PPO-expressing hemocytes (Aii and Aiii, open arrowheads). (B) CLIPA8 and (C) TEP1 kd mosquito abdomens at 40× magnification showing the absence of PPO and hence melanin from hyphal surfaces despite the extensive mycelial growth in these genotypes; melanin formation (Bi and Ci, open arrowheads) and PPO staining (Bii–iii and Cii–iii, open arrowheads) were restricted only to the base of the mycelium from which hyphae emerged. PPO6 (red), B. bassiana-GFP (green) and nuclei (blue). All scale bars are 50 μm. doi:10.1371/journal.ppat.1003029.g005

(Figure S1).
Figure 6. TEP1 localizes to hyphae and hyphal bodies. (A) LacZ (control) and (B) TEP1 kd mosquitoes were injected each with 200 conidia of GFP-expressing B. bassiana. Abdomens were dissected 48 h later and immunostained with TEP1 antibody. Shown are bright field (Bf) and merged bright field and red channel (Bf+red) confocal sections and Z projections (Z-proj) of whole stacks. (A) In control mosquitoes TEP1 staining was observed on established hyphae (Aii, filled arrowheads), young branching hyphae (Aii and Aiii, open arrowheads) and hyphal bodies (Aiii, inset and filled arrowhead). Most TEP1-stained hyphae did not exhibit melanin formation at that time point (compare Ai and Aii), except in rare cases where...
The delay in the differentiation of hyphal bodies in control mosquitoes is probably imposed by the strong melanotic response triggered against hyphae. This is supported by the detection of PPO and TEP1 staining not only on hyphae but also around the branching points where new hyphae and possibly hyphal bodies emerged (Figures 5A and 6A, respectively). Delaying or inhibiting hyphal body differentiation may limit fungal dissemination, since these single cell stages proliferate in the hemolymph ultimately establishing their own mycelia. It was previously reported that melanin exhibits anti-fungal properties in vitro against Aphanomyces astaci [42] and Metarhizium anisopliae [43], however, the mechanism by which it interferes with fungal growth is still not clear. A plausible explanation is the ability of melanin to bind and inhibit the activity of a wide range of proteins [44] including lytic enzymes produced by microbes, such as chitinases, which are involved in fungal cell wall remodeling during cell division [45]. Hence, melanin might slow down fungal growth by interfering with the synthesis of new cell wall material during that process.

**Figure 7.** CLIPA8 and TEP1 kd mosquitoes are more sensitive to *B. bassiana* infections. LacZ, TEP1 and CLIPA8 kd adult female *A. gambiae* mosquitoes were challenged with *B. bassiana* (strain 80.2) either by (A) bringing the mosquitoes in contact with a lawn of conidia on fungal PDA plates or (B) by spraying mosquitoes with a suspension of 1 × 10^6 conidia/ml. Dead mosquitoes were counted daily over the indicated period. Graphs represent percent survival as calculated by the Kaplan-Meier method for one representative experiment of each treatment. Statistical significance was calculated by the log rank test. Survival curves were considered to be significantly different if *P* < 0.05. (C) *B. bassiana* infected CLIPA8 and TEP1 kd mosquitoes contained significantly more hyphal body colony forming units (cfu) than infected LacZ kd controls. Here, mosquitoes were challenged by spraying with *B. bassiana* strain 80.2 at a suspension of 5 × 10^7 conidia/ml and batches of two mosquitoes each were collected four days later, surface sterilized, grinded and serial dilutions plated on *B. bassiana* selective medium. Each circle represents mean hyphal body cfu per mosquito per batch. Medians are indicated with horizontal lines and were 0, 15 and 50 for LacZ, CLIPA8 and TEP1 kd mosquitoes, respectively. The numbers of batches (N) processed per genotype are indicated. Statistical significance was calculated using the Mann-Whitney test; medians were considered significant if *P* < 0.05. Results are from two independent biological experiments involving different batches of mosquitoes and fungal conidia.

doi:10.1371/journal.ppat.1003029.g007
The rare hyphal bodies detected in control mosquitoes at 48 h after conidia injection were not melanized nor exhibited a PPO signal (Figure 5A), suggesting that they escape melanization. The evasion of host defense by these in vivo stages has been proposed earlier and was attributed to their minimal cell wall which lacks immuno-stimulatory carbohydrates [29]. A more recent study based on lectin-mapping revealed that B. bassiana developmental stages exhibit differences in the composition of surface carbohydrates, in particular hyphal bodies which seem to shed most carbohydrate epitopes from their surface [30]. This minimal cell wall, however, did not prevent TEP1 association with the surface of hyphal bodies (Figure 6A). TEP1 recruitment to GFP-expressing P. berghei ookinetes triggers parasite lysis as reflected by the loss of cytoplasmic GFP signal and membrane blebbing [16]. TEP1 labelled hyphal bodies were still expressing GFP and did not show an aberrant morphology, however, it is difficult to conclude that at this stage whether they are live or not without detailed electron microscopy analysis. Nevertheless, the fact TEP1 kd mosquitoes exhibited significantly higher numbers of hyphal bodies and increased sensitivity to natural mosquito melanotic response is reminiscent of wound healing and does not represent an immune defense reaction per se [16]. It is difficult to reconcile our findings with those of the above study for the following reasons. First, in our infection model, PPO does not seem to be recruited to the surface of a dying fungus since melanized hyphae were not killed and were still growing at their tips, often elaborating lateral branches. Second, even though we did not assay directly the co-localization of TEP1 and PPO (both antibodies were produced in the same host species), the fact that both were able to bind hyphae exhibiting minimal or no melanin formation, suggests that they might co-localize on hyphal surfaces.

In summary, the interactions between the mosquito melanotic response and B. bassiana are evocative of an “arms race” where the fungus is almost always the winner. This does not mean that melanization is not protective; we have provided evidence that this immune response retards significantly fungal growth, and might severely compromise or even completely abrogate the growth of fungi that are less virulent than B. bassiana. In fact, by successfully adapting to a particularly wide host range, B. bassiana must have evolved strategies to overcome insect immune defenses and enhance its pathogenesis. This is supported by recent insights.
from the *B. bassiana* genome which revealed species-specific expansions of gene families encoding toxins, proteases and putative effector proteins that may be associated with *B. bassiana* host flexibility and pathogenesis [52].

Based on our findings, we propose that transgenic *B. bassiana* strains designed to incapacitate the mosquito melanotic response once in the hemolymph may prove to be more potent biocontrol agents than wildtype strains. Finally, the observed delay between PPO localization to hyphal surfaces and melanogenesis renders *B. bassiana* a tractable model to study the yet poorly understood molecular interactions that culminate in PPO tethersing and activation on microbial surfaces; these studies would be difficult to perform in other infection models where the microbe becomes quickly melanized upon contact with the hemolymph, as in the case of *Plasmodium* oocystes.

### Materials and Methods

**Ethics statement**

This study was carried in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, U.S.A. The Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut approved the animal protocol (permit number 11-09-199). The IACUC functions in compliance with the Public Health Service Policy on the Humane Care and Use of Laboratory Animals (USA), and adopts the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, U.S.A.

**Anopheles gambiae rearing, antibiotic treatment, and *Beauveria bassiana* strains**

All experiments were performed with *Anopheles gambiae* G3 strain which was reared as previously described [53]. Briefly, *A. gambiae* mosquitoes were maintained at 27°C and 80% humidity with a 12 h day-night cycle. Larvae were reared on tropical fish food. Adult mosquitoes were maintained on 10% sucrose and given mice blood (Mice were anaesthetized with ketamine) for egg production. For antibiotic treatment, freshly emerged mosquitoes were maintained on 10% sucrose containing gentamycin (50 μg/ml), penicillin (10 units/ml) and streptomycin (10 μg/ml) for six days prior to infection with *B. bassiana*, and isolated from the rest of the colony in closed plastic containers. Fresh antibiotics solution was provided every 12 h. This antibiotic regimen was continued for an additional 24 h after fungal infection then stopped. Wildtype *B. bassiana* strain 80.2 (a kind gift of D. Ferrandon) and a GFP-expressing strain (242-GFP; a kind gift of M. Bidochka) were cultured on potato dextrose agar (PDA) plates at 25°C and 80% humidity. Conidia (spores) used for mosquito challenges were harvested from 3-4 weeks old cultures by adding 10 ml ddH₂O to each PDA plate and scraping the surface of the mycelia with sterile cell scrapers. Conidia were separated from other mycelial structures over a sterile funnel packed with autoclaved glass wool, washed two times with ddH₂O by centrifugation at 4000 rpm, counted and diluted to the appropriate concentration. Freshly prepared conidia were used for all experiments.

**Gene silencing by RNA interference**

Double-stranded RNAs (dsRNA) for *LacZ* (control), *TEP1* and *CLIPA8* were synthesized as previously described [9,16,21], respectively. *In vivo* gene silencing by RNA interference was performed as previously reported [31] and efficiency of gene silencing was confirmed by immunoblotting.

**Immunoblotting**

Hemolymph proteins were extracted from *LacZ*, *CLIPA8* and *TEP1* kd mosquitoes by proboscis clipping directly into 1 x non reducing Lane Marker Sample Buffer (Pierce), separated on 8% SDS-PAGE, then transferred to Immun-Blot PVDF membrane (BioRad) by semi-dry blotting (BioRad). Membranes were probed with rabbit polyclonal α-TEP1 (1/1000), mouse monoclonal α-CLIPA8 (1/50), rabbit polyclonal α-SRPNP3 (1/1000) and rabbit polyclonal α-PP06 (1/1000) [32]. The latter two antibodies served as loading controls. Horse radish peroxidase-conjugated α-mouse and α-rabbit secondary antibodies were used at 1/5000 and 1/12000, respectively. To determine hemolymph PPO levels after fungal infection, mosquitoes were sprayed with a suspension of *B. bassiana* (strain 80.2) containing 1x10⁹ conidia/ml in 0.05% Tween-80, and hemolymph was collected from 20 mosquitoes at each of the indicated time points using the same procedure describe above. Rabbit polyclonal α-TEP1 and horse radish peroxidase-conjugated α-rabbit secondary antibodies were used at 1(1000) and 1/12000, respectively.

**Mosquito survival and *B. bassiana* proliferation assay**

Freshly emerged adult mosquito females were injected with dsRNAs (3 μg/ml) for *LacZ*, *TEP1* and *CLIPA8*; left four days to recover, then challenged with *B. bassiana* (strain 80.2). For survival assays, fungal challenges were conducted in two ways. A batch of fifty cold-anaesthetized mosquitoes per genotype were either sprayed with a suspension of 1x10⁶ conidia/ml in 0.05% Tween-80, using glass atomizers purchased from sally@AccessoriesforFragrances.com, or dropped gently over a lawn of conidia in PDA cultures. Mosquito survival was scored on daily basis over a week. Three biological experiments were performed for each treatment. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test.

For the fungal proliferation assay, *LacZ*, *TEP1* and *CLIPA8*-silenced mosquitoes were sprayed with a suspension of *B. bassiana* (strain 80.2) containing 5x10⁶ conidia/ml in 0.05% Tween-80. Four days post-infection, approximately 15 batches of 2 mosquitoes each per genotype were grinded in 400 μl ddH₂O containing 0.05% Tween-80, then 50 μl of the homogenate was spread on *B. bassiana* selective medium [PDA containing 1 mg/ml yeast, 50 μg/ml Gentamicin, 50 μg/ml Penicillin, 50 μg/ml Streptomycin, 5 μg/ml Crystal violet and 250 μg/ml Dodeine (Sigma)]. Plates were incubated at 25°C at 80% humidity and hyphal body colony forming units were scored six days later. The experiment was repeated twice. Statistical significance was calculated using the Mann-Whitney test; medians were considered significantly different if P<0.05.

**Immunohistochemistry and confocal microscopy**

Four days post-gene silencing, individual mosquitoes were injected with approximately 200 conidia of GFP-expressing *B. bassiana* (strain 242-GFP). Abdomens were dissected at the indicated time points, fixed in 4% formaldehyde for 50 minutes, washed 3 times in 1 x phosphate buffered saline (PBS) and blocked for 1 h at room temperature in blocking buffer (1 x PBS containing 2% BSA and 0.05% Triton X-100). Then, abdomens were incubated overnight at 4°C with rabbit polyclonal α-TEP1 (1/350) or rabbit polyclonal α-PP06 (1/500) diluted in blocking buffer. Following incubation, abdomens were washed three times with 1 x PBS containing 0.05% Triton X-100, then incubated with Alexa-546 conjugated α-rabbit secondary antibody (Molecular Probes) diluted 1/800 in blocking buffer. After washing, nuclei were stained with Hoechst (1/10000) and abdomens mounted in...
Mosquito Melanotic Response to Beauveria bassiana

Vectorshield mounting medium (Vector Laboratories). Images were collected using a Zeiss LSM 710 META confocal microscope.

**PPO enzymatic assay**

CLIPAs, TEPI and LacZ kd mosquitoes were sprayed with a suspension of 1×10⁶ conidia/ml of *B. bassiana* strain 80.2. Hemolymph was collected at 24, 48 and 72 h after challenge in ice-cold phosphate buffered saline (PBS) containing protease inhibitors and protein concentration was determined using the Bradford Reagent (Fermentas). PO enzymatic assay was performed as previously described [9] using approximately 5 µg hemolymph proteins per reaction. Absorbance at 492 nm was measured in a Multiskan Ex microplate reader (ThermoLabystems) after incubation with L-DOPA at room temperature for 50 min.

**Supporting Information**

**Figure S1** Hemolymph PPO levels after natural *B. bassiana* infection. Western blot analysis showing hemolymph PPO levels in adult female mosquitoes at the indicated times points after spraying them with a suspension of 1×10⁶ conidia/ml of *B. bassiana* (strain 80.2). Each lane contains hemolymph extracts from 20 mosquitoes. C, control non-infected mosquitoes. Asterisks indicate non-specific bands.

(TIF)

**References**

1. Cerniš̆us I, Lee BI, Soderhall K (2008) The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol 29: 263–271.
2. Park JW, Kim CH, Kim JH, Je BR, Roh KB, et al. (2007) Clustering of peptidoglycan recognition protein-SA is required for sensing lysine-type peptidoglycan in insects. Proc Natl Acad Sci U S A 104: 6692–6697.
3. Wang Y, Jiang H (2006) Interaction of beta-1,3-glucan with its recognition protein activates hemolymph proteinase 14, an initiation enzyme of the prophenoloxidase activation system in *Manduca sexta*. J Biol Chem 281: 9271–9278.
4. Levashina EA, Langlely E, Green C, Gubb D, Ashburner M, et al. (1999) Constitutive activation of toll-mediated antifungal defense in serpin-deficient *Drosophila*. Science 285: 1917–1919.
5. Michel K, Budd A, Pinto S, Gibson TJ, Kafatos FC (2005) *Anopheles gambiae* SRPN2 facilitates midgut invasion by the malaria parasite *Plasmodium berghei*. EMBO Rep 6: 891–897.
6. Yu XQ, Jiang H, Wang Y, Kanost MR (2003) Nonproteolytic serine proteinase homologs are involved in prophenoloxidase activation in the tobacco hornworm, *Manduca sexta*. Insect Biochem Mol Biol 33: 197–208.
7. Lee KY, Zhang R, Kim MS, Park JW, Park HY, et al. (2002) A zymogen form of peptidoglycan recognition protein-SA is required for sensing lysine-type peptidoglycan in insects. Proc Natl Acad Sci U S A 99: 2306–2311.
8. Schnitger AK, Kafatos FC, Osta MA (2007) The Melanization Reaction Is not regulated by a single genomic control region. EMBO Rep 7: 231–235.
9. Schnitger AK, Kafatos FC, Osta MA (2007) The Melanization Reaction Is not Required for Survival of *Anopheles gambiae* Mosquitoes after Bacterial Infections. J Biol Chem 282: 21804–21808.
10. Liu H, Jiravanichpaisal P, Cerenius L, Lee BI, Soderhall I, et al. (2007) PhenoLDHase is an important component of the defense against *Arenavirus hydrophila* infection in a crustacean, *Pacifastacus leniusculus*. J Biol Chem 282: 33595–33598.
11. Elefterianos I, Boundy S, Joyce SA, Adam S, Marshall JW, et al. (2007) An antibiotic produced by an insect-pathogenic bacterium suppresses host defenses through phenoLDHase inhibition. Proc Natl Acad Sci U S A 104: 2419–2424.
12. Lu Z, Beck MH, Wang Y, Jiang H, Strand MR (2008) The viral protein Egl1-0 is a dual activity inhibitor of prophenoloxidase-activating proteinases 1 and 3 from *Manduca sexta*. J Biol Chem 283: 21325–21333.
13. Leclerc V, Pelte N, El Chamly L, Martinelli C, Ligoyssagis P, et al. (2006) Prophenoloxidase activation in Drosophila is not required for survival to microbial infections in *Drosophila*. EMBO Rep 7: 231–235.
14. Tang H, Kambriz S, Lemaitre B, Hashimoto C (2006) Two proteases defining a melanization cascade in the immune system of *Drosophila*. J Biol Chem 281: 20070–20078.
15. Ayres JS, Schneider DS (2008) A signaling protease required for melanization in *Drosophila* affects resistance and tolerance of infections. PLoS Biol 6: 2764–2773.
16. Blandin S, Shiao SH, Moita LF, Janse CJ, Waters AP, et al. (2004) Complement-like protein TEPI is a determinant of vectorial capacity in the malaria vector *Anopheles gambiae*. Cell 116: 661–670.
17. Osta MA, Christophides GK, Kafatos FC (2004) Effects of mosquito genes on *Plasmodium* development. Science 303: 2030–2032.
18. Rihele MM, Markianos K, Niaro O, Xu J, Li J, et al. (2006) Natural malaria infection in *Anopheles gambiae* is regulated by a single genomic control region. Science 312: 577–579.
19. Rihele MM, Xu J, Lazzaro BP, Rothschafer SM, Coulbaly B, et al. (2008) Anopheles gambiae APLI is a family of variable LRR proteins required for Rell-mediated protection from the malaria parasite, *Plasmodium berghei*. PLoS One 3: e3672.
20. Fratree M, Baxter RR, Steinert S, Chelliah Y, Frolet C, et al. (2009) Two mosquito LRR proteins function as complement control factors in the TEPI-mediated killing of *Plasmodium*. Cell Host Microbe 5: 273–284.
21. Povelones M, Waterhouse RM, Kafatos FC, Christophides GK (2009) Leucinerich repeat protein complex activates mosquito complement in defense against *Plasmodium* parasites. Science 324: 258–261.
22. Niaire O, Markianos K, Volz J, Ouedraogo F, Toure A, et al. (2002) Genetic loci affecting resistance to human malaria parasites in a West African mosquito vector population. Science 298: 213–216.
23. Yassine H, Osta MA (2010) Anopheles gambiae innate immunity. Cell Microbio 12: 1–9.
24. Souza-Neto JM, Barroso NJ, Souza-Neto JA, Robinson TJ, Hershley CL, et al. (2009) Discovery of insect and human dengue virus host factors. Nature 458: 1047–1050.
25. Souza-Neto JA, Sim S, Dimopoulos G (2009) An evolutionary conserved function of the JAK-STAMP pathway in anti-dengue defense. Proc Natl Acad Sci U S A 106: 17841–17846.
26. Waldock J, Olson KE, Christophides GK (2012) STAMPs activate mosquito antimicrobial immunity to Plasmodium infection. Proc Natl Acad Sci U S A 109: 2417–2422.
27. Sessions OM, Barrows NJ, Souza-Neto JA, Robinson TJ, Hershey CL, et al. (2009) Toward a comprehensive understanding of insect antiviral immune mechanisms. *Cell* 137: 3121–3133.
28. Blandin S, Moita LF, Kocher T, Wilm M, Kafatos FC, et al. (2002) Reverse genetics in the mosquito *Anopheles gambiae*: targeted disruption of the *Defensin* gene. EMBO Rep 3: 852–856.

**Figure S2** TEPI-silenced aseptic mosquitoes are still sensitive to *B. bassiana* infection. LacZ and TEPI kd female *A. gambiae* mosquitoes treated with antibiotics, to eliminate or at least reduce substantially their microbial flora, were challenged with *B. bassiana* (strain 80.2) by spraying mosquitoes with a suspension of 1×10⁶ conidia/ml. Dead mosquitoes were counted daily over the indicated period. (A) and (B) Two independent experiments performed with different batches of mosquitoes and fungal conidia. Graphs represent percent survival as calculated by the Kaplan-Meier method. Statistical significance was calculated by the log rank test. Survival curves were considered to be significantly different if *P*<0.05. (TIF)

**Acknowledgments**

We thank AUB Imaging Core Facility for the use of the confocal microscope. We also want to express our acknowledgments to Dr. George Christophides at Imperial College London, UK, for providing the anti-TEPI antibody and Dr. Michael Bidochka at Brock University, Canada, for providing the *B. bassiana* 242-GFP strain.

**Author Contributions**

Conceived and designed the experiments: HY LK MAO. Performed the experiments: HY LK. Analyzed the data: HY LK MAO. Wrote the paper: MAO.

10.1371/journal.ppat.1003029
32. Muller HM, Dimopoulos G, Blass C, Kafatos FC (1999) A hemocyte-like cell line established from the malaria vector Anopheles gambiae expresses six prophenoloxidase genes. J Biol Chem 274: 11727–11735.

33. Pinto SB, Lombardo F, Koutsos AC, Waterhouse RM, McKay K, et al. (2009) Discovery of Plasmodium modulators by genome-wide analysis of circulating hemocytes in Anopheles gambiae. Proc Natl Acad Sci U S A 106: 21270–21275.

34. Levashina EA, Moita LF, Blandin S, Vriend G, Lagueux M, et al. (2001) Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, Anopheles gambiae. Cell 104: 709–718.

35. Gotz P, Enderlein G, Roettgen I (1987) Immune reactions of Chironomus larvae (Insecta: Diptera) against bacteria. J Insect Physiol 33: 993–1004.

36. Butt TM, Wraight SF, Galaini-Wraight S, Hamber RA, Roberts DW, et al. (1988) Humoral encapsulation of the fungus Erynia radicans (Entomophthorales) by the potato leafhopper, Empoasca fabae (Homoptera: Cicadellidae). J Invertebr Pathol 52: 49–56.

37. Bidochka MJ, Khachatourians GG (1987) Hemocytic defense response to the entomopathogenic fungus Beauveria bassiana in the migratory grasshopper Melanoplus sanguinipes. Entomol Exp Appl 45: 151–156.

38. Li J, Tracy JW, Christensen BM (1992) Phenol oxidase activity in hemolymph compartments of Aedes aegypti during melanotic encapsulation reactions against microfilariae. Dev Comp Immunol 16: 41–48.

39. Hillyer JF, Schmidt SL, Christensen BM (2004) The antibacterial innate immune response by the mosquito Aedes aegypti is mediated by hemocytes and independent of Gram type and pathogenicity. Microbes Infect 6: 448–459.

40. St. Leger RJ, Cooper RM, Charnley AK (1988) Cuticle degrading enzymes of entomopathogenic fungi; mechanisms of interaction between pathogen enzymes and insect cuticle. J Invertebr Pathol 47: 295–302.

41. Goetz P, Roettgen I, Lingg W (1977) Encapsulment humoral in tant que reaction de defense chez les Dipteres. Ann Parasitol 32: 95–97.

42. Hung SY, Boucas DG, Vey AJ (1993) Effect of Beauveria bassiana and Candida albicans on the cellular defense response of Spodoptera exigua. J Invertebr Pathol 61: 179–187.

43. Xiao G, Ying SH, Zheng P, Wang ZL, Zhang S, et al. (2012) Genomic perspectives on the evolution of fungal entomopathogenicity in Beauveria bassiana. Sci Rep 2: 483.

44. Danielli A, Loukeris TG, Lagueux M, Muller HM, Richman A, et al. (2000) A modular chitin-binding protease associated with hemocytes and hemolymph in the mosquito Anopheles gambiae. Proc Natl Acad Sci U S A 97: 7136–7141.