Mosquito Transcriptome Profiles and Filarial Worm Susceptibility in Armigeres subalbatus

Matthew T. Aliota¹, Jeremy F. Fuchs¹, Thomas A. Rocheleau¹, Amanda K. Clark², Julián F. Hillyer², Cheng-Chen Chen³, Bruce M. Christensen¹*

¹Department of Pathobiological Sciences, University of Wisconsin-Madison, Madison, Wisconsin, United States of America, ²Department of Biological Sciences and Institute for Global Health, Vanderbilt University, Nashville, Tennessee, United States of America, ³Department of Microbiology and Immunology, National Yang-Ming University, Taipei, Taiwan Authority

Abstract

Background: Armigeres subalbatus is a natural vector of the filarial worm Brugia pahangi, but it kills Brugia malayi microfilariae by melanotic encapsulation. Because B. malayi and B. pahangi are morphologically and biologically similar, comparing Ar. subalbatus-B. pahangi susceptibility and Ar. subalbatus-B. malayi refractoriness could provide significant insight into recognition mechanisms required to mount an effective anti-filarial worm immune response in the mosquito, as well as provide considerable detail into the molecular components involved in vector competence. Previously, we assessed the transcriptional response of Ar. subalbatus to B. malayi, and now we report transcriptome profiling studies of Ar. subalbatus in relation to filarial worm infection to provide information on the molecular components involved in B. pahangi susceptibility.

Methodology/Principal Findings: Utilizing microarrays, comparisons were made between mosquitoes exposed to B. pahangi, B. malayi, and uninfected bloodmeals. The time course chosen facilitated an examination of key events in the development of the parasite, beginning with the very start of filarial worm infection and spanning to well after parasites had developed to the infective stage in the mosquito. At 1, 3, 6, 12, 24 h post infection and 2–3, 5–6, 8–9, and 13–14 days post challenge there were 31, 75, 113, 76, 54, 5, 3, 13, and 2 detectable transcripts, respectively, with significant differences in transcript abundance (increase or decrease) as a result of parasite development.

Conclusions/Significance: Herein, we demonstrate that filarial worm susceptibility in a laboratory strain of the natural vector Ar. subalbatus involves many factors of both known and unknown function that most likely are associated with filarial worm penetration through the midgut, invasion into thoracic muscle cells, and maintenance of homeostasis in the hemolymph environment. The data show that there are distinct and separate transcriptional patterns associated with filarial worm susceptibility as compared to refractoriness, and that an infection response in Ar. subalbatus can differ significantly from that observed in Ae. aegypti, a common laboratory model.

Citation: Aliota MT, Fuchs JF, Rocheleau TA, Clark AK, Hillyer JF, et al. (2010) Mosquito Transcriptome Profiles and Filarial Worm Susceptibility in Armigeres subalbatus. PLoS Negl Trop Dis 4(4): e666. doi:10.1371/journal.pntd.0000666

Editor: Jesus G. Valenzuela, National Institute of Allergy and Infectious Diseases, United States of America

Received January 15, 2010; Accepted March 10, 2010; Published April 20, 2010

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Funding: Financial support for these studies was provided by NIH grant AI019769 (to B.M.C.) and Vanderbilt University Discovery Grant (to J.F.H.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: christensen@svm.vetmed.wisc.edu

Introduction

Human lymphatic filariasis (LF) is caused by several species of mosquito-borne filarial nematodes, including Brugia malayi, Brugia timori, and Wuchereria bancrofti. Lymphatic filariasis is not a disease that causes mortality but it is by no means trivial. It is estimated that 120 million people in the world have LF, with ~1.1 billion at risk of becoming infected. Although LF is rarely fatal, severe morbidity (including adverse economic and psychosexual effects) involves disfigurement of the limbs and male genitalia (elephantiasis and hydrocele, respectively) [1–3]. Mosquitoes belonging to a number of different genera, including Culex, Aedes, Mansonia, Anopheles, and Armigeres, can serve as competent vectors. Following ingestion of an infective bloodmeal, microfilariae (mf) penetrate the mosquito midgut epithelium and migrate to the thoracic musculature. Migration to the head region occurs after a series of larval molts to the infective third stage (L3), which occurs in thoracic muscle cells. The L3 is then passed to a vertebrate host when the infected mosquito takes a bloodmeal. In these mosquito-parasite systems, the coevolutionary history of parasite and vector in one geographic region can differ from vector-parasite relationships in another area [4].

The ability of vector mosquitoes to ingest mf of filarial worm parasites and to support their development after ingestion is an important determinant of filariasis transmission. For a plethora of reasons, often times the number of L3s developing from ingested mf is not constant [5]. Resistance to mf in the mosquito mainly involves melanotic encapsulation (e.g., Ar. subalbatus and B. malayi, Aedes aegypti and Dirofilaria immitis, Anopheles quadrimaculatus and B. pahangi), but permissiveness of the midgut for parasite penetration,
and physiological suitability of the thoracic muscle cells also may be important determinants of vector competence [6–9]. When melanization occurs, mf are rapidly melanized in the hemocoel once they have penetrated the midgut. As soon as 10 minutes following a bloodmeal, melanin deposition is evident on the mf cuticle. At 12–16 hours post feeding, melanization is well underway and pathological effects on the mf are evident. At 24 to 48 hours post feeding mf begin to die, and by 72 hours post feeding, the response is all but complete [7,8].

Armigeres subalbatus is a natural vector of the filarial worm Brugia pahangi, but it kills B. malayi mf by melanotic encapsulation [6]. Because B. malayi and B. pahangi are morphologically and biologically similar, this mosquito-parasite system serves as a valuable model for studying resistance mechanisms in mosquito vectors [10]. Previously, we assessed the transcriptional response of Ar. subalbatus to B. malayi, which revealed the possible involvement of a number of unknown and conserved unknown gene products, cytoskeletal and structural components, and stress and immune responsive factors in the mosquito’s anti-filarial worm response. The data showed that the anti-filarial worm immune response by Ar. subalbatus is a highly complex, tissue-specific process involving varied effector responses working in concert with blood cell-mediated melanization [11]. Therefore, comparing Ar. subalbatus-B. pahangi susceptibility and Ar. subalbatus-B. malayi refractoriness could provide significant insight into recognition mechanisms required to mount an effective anti-filarial worm immune response in the mosquito, as well as provide considerable detail into the molecular components involved in vector competence.

Accordingly, we initiated transcriptome profiling studies of Ar. subalbatus in relation to filarial worm infection to provide information on the molecular components involved in B. pahangi susceptibility for comparison with our earlier studies on B. malayi refractoriness [11]. In addition, these studies also provide information on the infection response of a natural vector, i.e., the overall transcriptional and physiological change that occurs in the mosquito as a result of parasite infection, for comparison with our previous studies that employed a highly susceptible laboratory model, Aedes aegypti [12]. The time course chosen facilitated an examination of key events in the development of the parasite, beginning with the very start of filarial worm infection and spanning to well after infective-stage parasites had completed development in the mosquito. The data presented herein provide us with a cadre of information to design wet lab experiments and select candidates for further study to more fully dissect the nature of the anti-filarial worm immune response in this mosquito-parasite system. And with no genome sequence available for Ar. subalbatus, these data sets, in conjunction with data generated from our previous work (see [11]), represent the most complete set of transcriptomic information available to date for this mosquito species, which should be of interest to numerous laboratories investigating vector biology and innate immunity in general.

Methods

Mosquito maintenance

Ar. subalbatus used in this study were maintained at the University of Wisconsin-Madison as previously described [7]. Ar. subalbatus supports the development of B. pahangi to L5 [6,13]. Three- to four-day-old female mosquitoes were used for blood-feeding and sucrose starved for 14 to 16 hours prior to this event.

Exposure to infective bloodmeal

All animals and animal facilities are under the control of the School of Veterinary Medicine with oversight from the University of Wisconsin Research Animal Resource Center, and their use in experimentation was approved by the University of Wisconsin Animal Care and Use Committee. Mosquitoes were exposed to B. pahangi and B. malayi (originally obtained from the University of Georgia NIH/NIAD Filaria Research Reagent Repository Center) by feeding on ketamine/xylazine anesthetized gerbils, Meriones unguiculatus. The same animals were used for all three biological replicates. Microfilaremias were determined, using blood from orbital punctures, immediately before each feeding and ranged from 15–60 mf/20 μl blood. Control mosquitoes were exposed to anesthetized, uninfected gerbils. Mosquitoes that fed to repletion were separated into cartons and maintained on 0.3 M sucrose in an environmental chamber at 26.5±1°C, 75±10% RH, and with a 16 hr photoperiod with a 90 minute crepuscular period.

Mosquito collection and verification of parasite infection

Nine sample groups were created from thirteen timepoints to study mosquito transcriptome changes in response to B. pahangi development. In each sample group, comparisons were made between mosquitoes exposed to an infective bloodmeal containing B. pahangi mf and those exposed to a bloodmeal without parasites. In addition, a separate set of microarray analyses directly compared transcriptome profiles between mosquitoes in which filarial worms develop to infective stage larvae (B. pahangi) and mosquitoes in which an anti-filarial worm immune response had been initiated (B. malayi). This direct comparison contained groups of mosquitoes exposed to an infective bloodmeal containing B. pahangi mf or exposed to a bloodmeal containing B. malayi mf. Sample collection followed the same time course chosen for the DNA microarray experiments investigating B. malayi refractoriness previously published [11], but included three new biological replicates of mosquitoes exposed to B. malayi, done concurrently with the B. pahangi exposures. This direct comparison was advantageous because indirect comparisons of separate experimental data sets that employ a control that is assumed to be the same treatment for each could yield false information. For example, indirect comparisons made between DNA microarrays that compared B. pahangi vs. blood in this study compared to B.
**Coupling and purification of CyDye labeled cDNA**

Purified cDNA from each synthesis reaction was coupled to Cy3 or Cy5 according to manufacturers' instructions. The CyDye (GE Healthcare) probes were purified using the ChipShot™ Membrane Clean-Up system (TM261, Promega). Purified, dye-coupled cDNA was measured at 260 nm (Cy5 @ 650 nm and Cy3 @ 550 nm) to calculate yield. Probes (10 pmol/dye/slide) were dried down using a speedvac, resuspended at room temperature in 45 μl Pronotol™ hybridization buffer, incubated at 95°C for 5 minutes, and applied to the DNA microarrays. DNA microarrays were hybridized overnight at 42°C. Hybridized DNA microarrays were processed using the Pronto! microarray hybridization kit (Corning) according to manufacturer's specifications.

**Microarray analysis**

The microarray data were prepared according to “minimum information about a microarray experiment” (MIAME) recommendations, deposited in the Gene Expression Omnibus (GEO) database, and can be accessed via the web (accession number GSE20205). All transcript and EST data for this project are publicly accessible in ASAP (A Systematic Annotation Package for community analysis of genomes) [20] as the complete collapsed set (ARALL v2) or through NCBI's GeneBank database (accession numbers EU204979-EU212998) via the web. Microarray scanning and analysis was conducted as described previously [11]. Briefly, signal intensities were normalized using GeneSpring GX 7.3.1 software. All slides were normalized using a global linear regression (Lowess) curve fit to the log-intensity vs. log-ratio plot, and 20% of the data were used to calculate the Lowess fit at each point. All data were averaged for replicate spots upon a slide, and then further averaged across slides. Minimum and maximum values were recorded and t-test p-values generated for all replicate sets. Genes showing differential expression over controls were isolated using volcano plots at a 95% confidence interval over 2-fold values. Tests were parametric, but all variances were considered equal.

**Quantitative PCR**

Transcript levels of seven selected genes were measured using SYBR dye technology and quantitative polymerase chain reaction (qPCR) to validate microarray results as previously described [11]. Group 1 included three transcripts at 1 hour post challenge: a glycine-rich secreted salivary gland protein (Genbank:EU207085) and two unknowns (Genbank:EU211627) and (Genbank:EU209694). Group 2 included one transcript at 3 hours post challenge: a potassium: amino acid symporter (Genbank:EU210503). Group 3 included four transcripts at 6 hours post challenge: a mucin-like peritrophin (Genbank:EU206650), a glycine-rich secreted salivary gland protein (Genbank:EU207085), a potassium: amino acid symporter (Genbank:EU210503), and a serine protease (Genbank:EU205658). Finally, Group 4 included one transcript at 12 hours post challenge: a chitinase (Genbank:EU205713). All primer sequences used in microarray validation are presented in Table S1.

**Embedding, sectioning, and visualization of aldehyde-fixed mosquito thoraces**

Thoraces from mosquitoes exposed to B. pahangi-infected and uninfected bloodmeals were separated from whole bodies at 6, 9, and 14 days after blood feeding by making transverse cuts along the cervical membrane and the first abdominal segment. The legs and thoracic ventral cuticle were partially removed by making a coronal cut along the mesosternum and tissues were fixed by immersion in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.0). Thoraces were then prepared for light microscopy as described [21]. Briefly, aldehyde-fixed thoraces were dehydrated through a graded ethanol.
series, infiltrated in JB4-Plus resin (Electron Microscopy Sciences, Hatfield, PA), and anaerobically embedded in polyethylene molding trays. Coronal and transverse sections of 2.5 μm thickness were cut, stained with Gill's hematoxylin and Eosin Y, and mounted on glass slides using Poly-Mount (Polysciences Inc., Warrington, PA). Tissues were then imaged using differential interference contrast (DIC) optics on a Nikon Eclipse 90i compound microscope connected to a Nikon DS-Fi1 high-definition color CCD camera (Nikon Corp., Tokyo, Japan).

Results

B. pahangi development in Armigeres subalbatus

The development of B. pahangi was observed microscopically in Armigeres subalbatus at 1 h to 14 d (a total of 11 time points) post ingestion (PI) of a microfilaremic bloodmeal for the first biological replicate, and at 1 h and 14 d PI for the subsequent two biological replicates. The results are summarized in Table 1. Parasites were recovered from 70 of the 75 mosquitoes examined for an overall infection prevalence of 93.3%. Microfilariae were recovered from 1 h to 24 h PI, and constituted the majority of the total parasites through this time period. From 24 h to 3 d PI, almost all parasites had differentiated into intracellular L1s. L1s molted to L2s in the thoracic musculature by 5 d PI, and L2s were the only developmental stage identified between 5 and 6 d PI. The transformation from L2 to L3 occurred at 8–9 d PI. At 8 d PI, L2s and L3s were recovered from the thoracic musculature with only 2% of the total worms located in the head and proboscis. By 9 d PI all worms had molted to the L3 stage. At this time 43% of the L3s were observed in the thorax and 57% were located in the head and proboscis. By 13–14 d PI, all L3s were observed in the head and proboscis. The prevalence of L3s (for all three biological replicates on 14 d PI) was 87% (n = 15) and the mean intensity was 11.3 ± 9.6 L3s.

Transcriptome changes in B. pahangi-infected vs. uninfected mosquitoes

Direct comparisons were made at each time point within DNA microarrays hybridized with probes made from RNA of whole female Armigeres subalbatus exposed to either a B. pahangi-infected or uninfected bloodmeal. Volcano plots were used to create working gene lists to identify differentially expressed genes at each time point. At 1, 3, 6, 12, 24 h PI and 2–3, 5–6, 8–9, and 13–14 d PI there were 31, 75, 103, 76, 54, 5, 3, 13, and 2 detectable transcripts of the 6,143 features on the DNA microarray, respectively, with significantly different transcriptional patterns (increased or decreased transcript abundance at a 95% confidence interval over two-fold values) as a direct or indirect result of parasite development (Figure 1). Between each time point there was very little overlap (i.e., less than 27 transcripts shared between any two timepoints) in the transcripts that showed significantly different transcriptional patterns (Table 2).

The vast majority of changes in the mosquito transcriptome occurred within the first 24 h of infection, when mf were penetrating through the midgut and invading thoracic muscle cells. In contrast, relatively minor changes in the mosquito’s transcriptome were noted at later times during parasite development. This is somewhat surprising, considering that at 5–6 d PI parasites were actively feeding on mosquito tissue, and by 13–14 d PI parasites had grown considerably in size and migrated to the mosquito’s head and proboscis. Of the transcripts that showed significantly different transcriptional patterns over this experiment, only 10% had putative immune functions (Table 3). In addition, of the 364 transcripts that showed significantly different transcriptional patterns over the time course of the B. pahangi vs. blood experiment, 193 (53%) of those transcripts were unknowns or conserved unknowns, i.e., they have no previously described function (Figure 2). This suggests that the function of many factors involved in the infection response to developing filarial worms (i.e., the factors involved in helping the mosquito maintain homeostasis during a persistent infection) are not known. Table S2 provides a full representation of all transcripts showing a detectable increase or decrease in abundance at all time points.

Transcriptome changes in B. pahangi- vs. B. malayi-exposed mosquitoes

Direct comparisons were made at each time point within DNA microarrays hybridized with probes made from RNA of whole female Armigeres subalbatus exposed to either a B. pahangi- or B. malayi-infected mosquito bloodmeal. Volcano plots were used to create working gene lists to identify differentially expressed genes at each time point. At 1, 3, 6, 12, 24 h PI and 2–3, 5–6, 8–9, and 13–14 d PI there were 31, 75, 103, 76, 54, 5, 3, 13, and 2 detectable transcripts of the 6,143 features on the DNA microarray, respectively, with significantly different transcriptional patterns (increased or decreased transcript abundance at a 95% confidence interval over two-fold values) as a direct or indirect result of parasite development (Figure 1). Between each time point there was very little overlap (i.e., less than 27 transcripts shared between any two timepoints) in the transcripts that showed significantly different transcriptional patterns (Table 2).

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**Table 1.** The development of Brugia pahangi in Armigeres subalbatus was recorded each time mosquitoes were collected for microarray analysis.

| Time post feeding | Percentage of mosq. harboring worms (total dissected) | Developmental stage of Brugia pahangi | Total worms |
|-------------------|-----------------------------------------------|---------------------------------------|------------|
|                   | Microfilariae  | L1         | L2         | L3         |           |
| 1 hr              | 100% (15)     | 100% (152) |            |            | 152       |
| 12 hr             | 100% (5)      | 42.2% (35) | 57.8% (48) |            | 83        |
| 24 hr             | 80% (5)       | 4.5% (1)   | 94.5% (21) |            | 22        |
| 2 d               | 100% (5)      | 0% (0)     | 100% (38)  |            | 38        |
| 3 d               | 100% (5)      | 0% (0)     | 100% (28)  |            | 28        |
| 5 d               | 60% (5)       |            |            | 100% (7)   | 7         |
| 6 d               | 100% (5)      |            |            | 100% (55)  | 55        |
| 8 d               | 80% (5)       |            | 19.3% (11) | 80.7% (46) | 57        |
| 9 d               | 100% (5)      |            | 0% (0)     | 100% (49)  | 49        |
| 13 d              | 100% (5)      |            |            | 100% (61)  | 61        |
| 14 d              | 87% (15)      |            |            | 100% (150) | 150       |

doi:10.1371/journal.pntd.0000666.t001
infective bloodmeal. Volcano plots were used to create working gene lists to identify genes with a significant fold difference to begin to better understand the transcriptional profiles associated with exposure to each species of parasite. At 1, 6, 12, 24 h PI and 2–3 d post challenge there were 10, 14, 15, 27, and 4 detectable transcripts, respectively, more associated with B. malayi infection relative to B. pahangi resistance. Following the same time course, there were 63, 20, 57, 81, and 6 detectable transcripts, respectively, more associated with B. pahangi resistance relative to B. pahangi infection (Figure 3).

Microarray validation

Microarray data were confirmed using both in silico analyses of known transcriptional information in the literature and laboratory-based analyses via qPCR [18,23,24]. The transcriptional activity

| Time          | # of transcripts |
|---------------|------------------|
| 1 h           | 33               |
| 3 h           | 75               |
| 6 h           | 103              |
| 12 h          | 76               |
| 24 h          | 54               |
| 2/3 d         | 5                |
| 5/6 d         | 0                |
| 8/9 d         | 0                |
| 13/14 d       | 0                |

Figure 1. Transcriptional changes in Armigeres subalbatus following a Brugia pahangi-infected bloodmeal. The bar graph represents the number of significantly changed transcripts over the course of experimentation. Volcano plots were used to create working gene lists to identify differentially expressed transcripts at each time point. At 1, 3, 6, 12, 24 h post infection and 2–3, 5–6, 8–9, and 13–14 d post challenge there were 29, 75, 103, 76, 5, 3, 13, and 2 detectable transcripts, respectively, with significantly different transcriptional behavior (increased or decreased transcript abundance at a 95% confidence interval over two-fold values) as a result of parasite development. The sample groups chosen are defined by the time post ingestion of the bloodmeal and represent significantly different stages of parasite development. The bar graph represents groups of mosquitoes that included those exposed to an infective bloodmeal containing B. pahangi mf (~15–60 mf/20 μl blood) and those exposed to a bloodmeal without parasites. doi:10.1371/journal.pntd.0000666.g001

Table 2. Few Armigeres subalbatus genes are differentially transcribed in multiple stages of the infection response to Brugia pahangi.

| Time          | 1 h | 3 h | 6 h | 12 h | 24 h | 2/3 d | 5/6 d | 8/9 d | 13/14 d |
|---------------|-----|-----|-----|------|------|-------|-------|-------|---------|
| 1 h           | 33  | 4   | 5   | 5    | 4    | 0     | 1     | 1     | 1       |
| 3 h           | 75  | 27  | 14  | 12   | 0    | 0     | 0     | 0     | 0       |
| 6 h           | 103 | 16  | 14  | 0    | 1    | 1     | 1     | 1     | 1       |
| 12 h          | 0   | 0   | 0   | 76   | 12   | 0     | 0     | 2     | 0       |
| 24 h          | 0   | 0   | 0   | 0    | 54   | 0     | 1     | 2     | 1       |
| 2/3 d         | 0   | 0   | 0   | 0    | 5    | 0     | 0     | 0     | 0       |
| 5/6 d         | 0   | 0   | 0   | 0    | 0    | 3     | 1     | 1     | 1       |
| 8/9 d         | 0   | 0   | 0   | 0    | 0    | 0     | 0     | 13    | 1       |
| 13/14 d       | 0   | 0   | 0   | 0    | 0    | 0     | 0     | 0     | 2       |

Between each time point there was very little overlap in the transcripts that showed significantly different transcriptional behavior. This incongruity between the time points suggests that there is a great deal of informative and continual change in the transcriptome of Armigeres subalbatus in response to infection with Brugia pahangi. doi:10.1371/journal.pntd.0000666.t002

The majority of changes that occurred in the mosquito transcriptome were associated with B. malayi resistance (76%); however, there may be some bias in these data because there was no B. pahangi-exposed mosquito cDNA included in the EST libraries used to create our Armigeres microarray [17]. Of the transcripts that showed significantly different transcriptional patterns as a result of B. pahangi infection, only 14% had putative immune functions (Table 4) and 49% were unknowns or conserved unknowns. Of the transcripts that showed significantly different transcriptional patterns as a result of B. malayi resistance, only 12% had putative immune functions (Table 4) and 67% were unknowns or conserved unknowns. These results suggest that although the biosynthetic pathway of melanization is well understood, many factors involved in the anti-filarial worm immune response are not known. Filarial worm susceptibility in Armigeres subalbatus is much more complicated than merely an absence of the melanization immune response (Figure 4); likewise, filarial worm resistance in Armigeres subalbatus is much more complicated than the presence of melanization and likely includes factors involved in recovery and maintenance of homeostasis. For example, there were few transcripts involved in the metabolism of reactive intermediates that showed significantly different transcriptional patterns as a result of B. pahangi infection vs. uninfected blood (Table 3). This is in contrast to what was observed in Armigeres subalbatus in response to B. malayi [11], and probably has to do with the fact that mosquitoes infected with B. pahangi do not have to protect themselves from the damaging effects of the oxidative stressors produced during melanization reactions [22]. Table S3 provides a full representation of all transcripts showing a detectable difference in abundance between B. pahangi- and B. malayi-infected mosquitoes at all time points.

Microarray validation

Microarray data were confirmed using both in silico analyses of known transcriptional information in the literature and laboratory-based analyses via qPCR [18,23,24]. The transcriptional activity
of a number of different response genes, induced by bacteria or filarial worms, has been characterized previously in several mosquito species. Based on this information, and the fact that the RNA used to screen the DNA microarrays in these studies result from the exposure to filarial worms, it was expected that a number of parasite responsive genes on the DNA microarrays would show significant transcriptional patterns [11,25–30]. Comparisons made between our data and parasite responsive genes in the literature corroborated our findings and provided validation of our DNA microarray results. In addition, a number of “house-keeping” genes (ex. Ribosomal genes, actin, cytochrome C oxidase, etc.) included on the DNA microarray showed no

### Table 3. Analysis of *Armigeres subalbatus* immunity genes transcribed by *Brugia pahangi*-infected mosquitoes.

| Genbank ID | Description | Category | Relative Transcript Abundance* |
|------------|-------------|----------|-------------------------------|
| EU211663   | peptidoglycan recognition protein LE | PAT RECG | 2.51 |
| EU205423   | C-type lectin | PAT RECG | 2.82 |
| EU206838   | phenylalanine hydroxylase | MELN | −2.15 |
| EU209622   | serine protease | MELN | 2.65 3.51 6.17 |
| EU205154   | serine protease | MELN | 4.32 4.44 3.89 2.41 |
| EU208339   | serine protease | PROT | 3.62 3.40 2.09 |
| EU208787   | serine protease | PROT | 2.47 |
| EU206217   | serine protease | PROT | 2.05 |
| EU205815   | serine protease | PROT | 3.12 2.29 |
| EU207063   | serine protease | PROT | 2.84 |
| EU205658   | serine protease | PROT | 4.39 5.91 3.21 2.06 |
| EU208659   | cecropin C | AMP | 3.43 3.66 |
| EU210693   | cecropin A | AMP | 4.61 |
| EU205505   | lysozyme | IMMUN | 3.13 |
| EU21110    | holotricin 3 | IMMUN | 2.57 |
| EU205824   | defensin | AMP | 2.24 |
| EU205803   | oxidoreductase | ANTIOX | 2.17 |
| EU208063   | thioredoxin | ANTIOX | −8.70 |
| EU211799   | oxidoreductase | ANTIOX | −2.62 |
| EU206910   | thioredoxin peroxidase | ANTIOX | −11.45 |
| EU206583   | transferrin 1 | IRON | 2.58 2.28 |
| EU212056   | serpin | PHAG | 2.19 |
| EU206038   | serine protease | PHAG | 4.55 3.67 |
| EU209050   | monophenol monooxygenase activator | PHAG | 3.65 |
| EU206136   | G protein-coupled receptor kinase | PHAG | −7.09 |
| EU207422   | protein phosphatase regulator | PHAG | −29.24 |
| EU205197   | gelsolin | PHAG | 2.06 |
| EU212174   | serine protease | SIGNL | 2.03 |
| EU205809   | protein kinase C inhibitor | SIGNL | 2.14 |
| EU205109   | receptor signaling protein | SIGNL | −2.15 |
| EU205867   | juvenile hormone epoxide hydrolase | SIGNL | −2.23 |
| EU205966   | transmembrane protein serine/threonine kinase | SIGNL | 2.27 |
| EU209623   | protein kinase | SIGNL | −2.56 |
| EU210294   | calcium-independent phospholipase | APOPT | −9.17 |
| EU205045   | protein transporter | APOPT | −2.06 |
| EU210658   | protein transporter | APOPT | −2.50 |
| EU206601   | cathepsin | APOPT | −2.11 |
| EU206650   | mucin-like peritrophin | IMMUN | 2.06 2.58 |

*Fold change (p < 0.05), by time post infection.

PAT RECG = Pattern Recognition, MELN = Melanization, PROT = Proteolysis, AMP = Antimicrobial peptide, IMMUN = Immune Response, ANTIOX = Antioxidant, IRON = Iron Metabolism, PHAG = Phagocytosis, SIGNL = Signal Transduction, APOPT = Apoptosis.

doi:10.1371/journal.pntd.0000666.t003
detectable change in transcript abundance throughout experimentation (data not shown), thereby providing further validation of the expression patterns detected.

In conjunction with in silico validation of DNA microarray results, qPCR provided independent, experimental verification of transcript abundance from the same total RNA used in the initial DNA microarray experiment. Because the corroboration of all microarray data was impractical, a subset of seven genes was chosen at random from our lists of significant genes for confirmatory studies. Transcriptional activity of three selected genes was verified at 1 h, one selected gene at 3 h, four selected genes at 6 h, and one selected gene at 12 h post challenge, and eight of the nine conditions tested corroborated with transcriptional patterns detected on the DNA microarray (Text S1). The elimination of one gene from our dataset may reflect the differential sensitivities of the techniques used or sample variation. This still validated that the DNA microarray was working as expected, showing all three conditions (increase, decrease, and no detectable change in transcript abundance). These results, in combination with in silico and laboratory-based validation, provided confidence that the transcriptional profiles are an accurate depiction of the biological phenomena under study.

**Histology**

Histological analyses of thoraces from infected mosquitoes confirmed the infection timeline observed using whole body dissections. At 6 d PI worms were developing in the thoracic musculature and, with rare exceptions, were oriented parallel to the muscle fibrils. At 9 d PI the worms were in the process of exiting the muscle fibers and migrating to the head, where they generally remain for the lifetime of the mosquito (Figure 5).

Because the microarray data showed remarkably few mosquito transcriptional changes associated with parasite maturation and migration in the hemocoel, we undertook a comparative histological analysis of the thoracic musculature from *B. pahangi*-infected and uninfected mosquitoes, specifically surveying for obvious morphological changes associated with worm infection. As indicators of tissue damage, particular attention was paid to signs of muscle fiber degradation, the structure of myofibrils that were in direct contact with the worms, the morphology of host muscle

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**Figure 2. Functional composition of transcripts significantly affected by parasite infection.** The categories are based on abundant and immunity-related EST clusters observed from *Armigeres subalbatus* cDNA libraries. Transcripts with a detectable increase in abundance (top) and with a detectable decrease in abundance (bottom) 1, 3, 6, 12, and 24 hours after exposure to a *Brugia pahangi* infective blood meal. doi:10.1371/journal.pntd.0000666.g002
The bar graph represents the number of genes with a significant fold difference between two conditions. Volcano plots were used to create working gene lists to identify transcripts associated with a specific condition. The bar graph represents groups of mosquitoes that included those exposed to blood containing filarial worms. Following the same time course, there were 63, 20, 57, 81, and 6 detectable transcripts, respectively, more associated with filarial worms in infected mosquitoes. The bar graph represents groups of mosquitoes that included those exposed to blood containing filarial worms and those exposed to blood containing filarial worms and infected normal control bloodmeals. Significant increases or decreases in transcript abundance cannot be delineated.

doi:10.1371/journal.pntd.0000666.g003

Discussion

Interactions between mosquitoes and filarial worms can range from a benign symbiotic relationship to a fatal competition resulting in the death of the host, or to a fatal competition resulting in the death of the parasite. In A. subalbatus, both tolerance and resistance strategies can occur depending on the species of filarial worm infecting the mosquito, but high mosquito mortality, caused by ingestion of too many mf, can occur with either scenario. It is important to distinguish between tolerance and resistance because their relative importance will have substantial consequences for the ecology and evolution of host-parasite interactions [31]. Traditionally, most studies have examined the anti-filarial worm immune response in A. subalbatus (e.g., [11,25,29]), or explored innate immune responses to other pathogens in other mosquito species (e.g., [4,32,33]), but few have examined pathogen susceptibility in a natural mosquito vector (in this case, a laboratory strain). Studies like this one, aimed at understanding the molecular basis of parasite-host interactions in a compatible system, will help identify the components involved in host resistance vs. parasite tolerance [4].

It is important to note that resistance and tolerance can be mutually exclusive, interchangeable, or complementary compo-
Table 4. Analysis of *Armigeres subalbatus* immunity related genes transcribed by either *Brugia pahangi*- or *Brugia malayi*-exposed mosquitoes.

| Genbank ID | Description                          | Category       | Relative Transcript Abundance<sup>a</sup> |
|------------|--------------------------------------|----------------|------------------------------------------|
|            |                                      | 1 h  | 6 h  | 12 h | 24 h |
| **B. pahangi** |                                      |     |     |      |      |
| EU206366   | G-protein coupled receptor           | APOPT  | 2.10 |
| EU208958   | cathepsin                            | APOPT  | 2.97 | 5.80 |
| EU205277   | defensin A                           | AMP    | 2.93 |
| EU206217   | serine protease                      | PROT   | 2.23 | 2.03 |
| EU206528   | serine protease                      | PROT   | 2.84 |
| EU208879   | serine carboxypeptidase              | PROT   | 2.18 | 2.43 |
| EU207696   | serine protease                      | IMMUN  | 2.22 |
| EU206649   | calreticulin                         | PAT RECG | 2.13 |
| EU205878   | *Ae. aegypti* putative protein G12   | SIGNL  | 2.07 |
| **B. malayi** |                                      |     |     |      |      |
| EU212480   | serine protease                      | PROT   | 2.20 |
| EU212003   | serine protease                      | PROT   | 2.75 |
| EU208214   | serine protease                      | PROT   | 2.03 |
| EU208787   | serine protease                      | PROT   | 2.28 |
| EU207063   | serine protease                      | PROT   | 2.29 |
| EU205658   | serine protease                      | PROT   | 2.75 |
| EU205450   | serine protease                      | PROT   | 3.42 |
| EU205154   | serine protease                      | MELN   | 2.95 |
| EU209622   | serine carboxypeptidase              | PROT   | 3.95 |
| EU206047   | serpin 27A                           | MELN   | 2.91 |
| EU206540   | mannos-β-binding lectin assoc. ser. prot. | SIGNL  | 2.35 |
| EU209082   | salivary secreted serpin             | PROT   | 2.50 |
| EU211889   | immune reactive protease inhibitor   | IMMUN  | 6.58 |
| EU208239   | toll-7                               | SIGNL  | 2.11 |
| EU211110   | holotrocin 3                         | IMMUN  | 2.98 |
| EU211628   | diptericin B                         | IMMUN  | 3.16 | 2.09 |
| EU208970   | CD36-protein                         | SIGNL  | 2.44 |
| EU208273   | scavenger receptor                   | SIGNL  | 7.81 |
| EU207965   | defensin A                           | AMP    | 2.47 |
| EU205465   | defensin C2                          | AMP    | 3.64 |
| EU206070   | cecropin                             | AMP    | 4.93 |
| EU205409   | cecropin B1                          | AMP    | 2.84 | 3.24 |
| EU206422   | gambicin                             | AMP    | 2.16 |
| EU206257   | c-type lectin                        | PAT RECG | 2.29 |
| EU208542   | c-type lectin                        | PAT RECG | 2.56 | 2.53 |
| EU208009   | glutathione transferase              | ANTIOX | 2.10 |
| EU209050   | monophenol monoxygenase activator    | PHAG   | 2.18 |
| EU206650   | mucin-like peritrophin               | IMMUN  | 2.39 |

<sup>a</sup>Fold difference between *B. pahangi* and *B. malayi* (*p*<0.05), by time post infection.

PAT RECG = Pattern Recognition, MELN = Melanization, PROT = Proteolysis, AMP = Antimicrobial peptide, IMMUN = Immune Response, ANTIOX = Antioxidant, IRON = Iron Metabolism, PHAG = Phagocytosis, SIGNL = Signal Transduction, APOPT = Apoptosis.

doi:10.1371/journal.pntd.0000666.t004

Additional text:
recognition molecules, etc) in the current study, and this may be an example of these transcripts being directed at damage or other harmful substances resulting from infection with the parasite.

Apoptosis also could explain the activity of a number of the immune responsive transcripts present in this study, because cell death in vertebrates has been shown to trigger both innate and adaptive immune responses [37,38]. It also could explain the activity of those transcripts implicated in phagocytosis, because phagocytosis could be functioning to clean-up apoptotic cells [37–39] that were destroyed by mf penetrating the midgut. This is consistent with previous reports of apoptosis in the midgut of parasite and/or virus infected mosquitoes [40–46], and it has been shown previously that basal and apical plasma membranes are destroyed by mf penetrating the midgut, likely resulting in cell death [47].

A number of antimicrobial peptides (AMPs) showed significantly different transcriptional behavior in the current study as well. Anti-microbial peptides are small, immune-related molecules named for their in vitro activity against bacteria and are detectable in the fat body, hemocytes, midgut, and epithelial tissues of mosquitoes. Although considered a primary defense mechanism against bacteria in mosquitoes, AMP transcription has been associated with responses to B. malayi infection in Armigeres and Aedes, Plasmodium infection in Anopheles, and fungal infection in other mosquito species [11,32,48,49]. Despite these associations, our knowledge of the molecular mechanisms and the true role of these peptides in mosquito innate immunity remain limited, and perhaps, the anti-microbial activity of AMPs might be an ancillary property. Recently, it has been shown that antimicrobial compounds function primarily to protect insects against bacteria that persist within the body, rather than to clear the infection. It has been proposed that AMPs may act as response elements helping insects deal with infection when pathogens in the hemolymph exceed the phagocytic or melanotic capacity of the hemocytes [50,51]. The results of the current study are consistent with this hypothesis, i.e., the fact that AMP induction is evident as a result of B. pahangi infection in addition to B. malayi resistance seems to support the hypothesis that AMPs may play an alternative role, perhaps helping to maintain homeostasis of the hemolymph environment during a persistent infection or to clean up after a successful immune response, but are transcribed regardless of the type of pathogen present.

In the vertebrate host, filarial nematodes apply successful strategies to evade the host’s immune response [52,53]. Their
strategy has evolved to be assimilation, defusing aggressive immune reactions, and inducing forms of immunological tolerance to permit their long-term survival [53]. In the *Armigeres-Brugia* system, *B. pahangi* may employ similar strategies; whereas, *B. malayi* is not successful in evading the mosquito’s immune response. The specificity of resistance in this vector-parasite relationship warrants further exploration, and we postulate it to occur at the level of recognition, possibly involving well defined motifs (e.g., β 1,3-glucan [27]) recognized by receptors that could be the products of resistance genes. Immune defenses (e.g., melanization) would then be triggered only if recognition of the parasite occurs [4]. Therefore, the general hypothesis is that *Ar. subalbatus* recognizes a surface component(s) of *B. malayi* mf but not *B. pahangi* mf, i.e., there is a fundamental biologic difference in the surface components of the two filarial worm species. Consistent with this hypothesis is the fact that a number of transcripts associated with targeting/initiating an immune response changed as a result of *B. malayi* infection relative to *B. pahangi* infection: e.g., CD-36-protein (GenBank: EU208970), scavenger receptor (GenBank: EU208273), toll-like receptor 7 (GenBank: EU208239), 2 C-type lectins (GenBank: EU206257 and EU208542), and a mannose-binding lectin associated serine protease (GenBank: EU206540).

If vector-parasite specificity is not achieved at the recognition level, we postulate that specificity could be explained by direct or indirect interactions between the products of immune suppressive genes of the parasite and the products of host resistance genes [4], i.e., *B. pahangi* mf can actively suppress the anti-filarial worm response in *Ar. subalbatus* but *B. malayi* mf cannot. Consistent with this hypothesis is the fact that the majority of changes that occurred in the mosquito’s transcriptome were associated with *B. malayi* resistance (76%) relative to *B. pahangi* susceptibility. In addition, indirect comparisons between *B. malayi* resistance [11] and *B. pahangi* susceptibility showed considerably more changes associated with resistance (761 vs. 346) over the course of 72 h.

Recently, we examined the infection response of *Ae. aegypti* to *B. malayi* at the transcriptional level [12]. In this experimental model system, essentially all ingested parasites successfully develop to L3s. This study revealed very few changes in the *Ae. aegypti* transcriptome until L2s were present, and the most profound transcriptional changes were observed in mosquitoes that harbored infective-stage parasites. In comparison, the vast majority of changes in the *Ar. subalbatus* transcriptome as a result of *B. pahangi* infection were observed between 1 and 24 hours PI when mf were penetrating the midgut and invading thoracic muscle cells, and very few transcriptional changes were observed in mosquitoes that harbored L2s or infective-stage parasites. It is important to note that the array platforms used to conduct these two studies were different (*Ar. subalbatus-B. pahangi* = EST-based DNA microarray; *Ae. aegypti-B. malayi* = whole genome DNA microarray) because there is no genome sequence available for *Ar. subalbatus*, and some of the differences observed between the two studies may be the result of the inherent differences in these two approaches. But the different transcriptional changes observed in these two mosquito species that support parasite development also can provide clues to the molecular mechanisms that determine compatibility in natural mosquito-filarial worm associations. By comparing the two infection responses we can begin to better understand the intricacies involved in susceptibility vs. refractoriness, i.e., by identifying transcripts that are shared between both compatible mosquito-parasite systems and from the refractory condition, we can begin to rule out their involvement in anti-filarial worm immunity. Such comparisons have been made previously between other mosquito genera (e.g., [54]), but this study is the first to make a comparison between the susceptible vs. refractory state in the same species of mosquito, and may provide a better representation of the genes required to deter filarial worm infection in an incompatible system. These results also illustrate the fact that not all mosquitoes respond the same way to filarial worm infection and not all filarial worm species will elicit the same response in a host. Host-parasite interactions represent coevolved adaptations of significant complexity, and these relationships depend on the relative capacities of the host and pathogen to adapt to and maintain this unique relationship.

Furthermore, earlier studies assessing *B. malayi* and *B. pahangi* infection of *Ae. aegypti* and *Mansonia uniformis* found that nematode migration and development causes minor and severe damage to the thoracic indirect flight muscles [35,56]. Later, it was proposed that this damage was not pathogen specific because similar damage was observed in mosquitoes physically traumatized by intrathoracic insertion of a metal probe [57]. In contrast to those

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**Figure 5. Development of *Brugia pahangi* in the mosquito thorax.** At 6 days post-infection parasites (e.g., arrows) are developing in the thoracic musculature and are oriented parallel to the myofibers (A). At 9 days post-infection parasites exit the myofibers (B) and migrate to the cervix and head region (C; 14 days post-injection). *, thoracic cuticle; #, cervical cuticle; scale bar, 100 μm.

doi:10.1371/journal.pntd.0000666.g005
studies, the experiments we report here failed to detect significant pathology in the thoracic musculature of *Ar. subalbatus* infected with *B. pahangi*, with the exception of breaks in myofibers that appear to be the direct result of nematode exit from the indirect flight muscles. This is consistent with other studies assessing *B. pahangi* infection in mosquitoes, where infection of the natural vector *Aedes togoi* resulted in undetectable flight muscle damage (suggesting complete myofiber repair following migration to the head), whereas infection of the artificial vector *Ae. aegypti* resulted in severe degeneration of the flight muscles [58,59]. The histological and transcriptomic data reported in this study further verify the need to work with natural mosquito-parasite systems, because it is evident that extrapolating what is learned from a laboratory model of a parasite-vector relationship to the natural model can be problematic.

Aside from myofiber breaks, the primary difference between the thoracic musculature from infected versus normal bloodfed *Ar. subalbatus* was the accumulation of eosinophilic granules between the nematode cuticle and the host myofibers. We presume that these granules are host mitochondria, and if so, these data would be in accord with electron microscopic data showing similar accumulations following *B. pahangi* infection of *Ae. aegypti* [60]. In that study, pooled mitochondria showed no evidence of damage and it is not clear if any deleterious effect is associated with their accumulation. However, because similar structures were observed in the nematode gut [60], it is possible that filarial nematodes subsist by ingesting mitochondria. Our data and that of others [60] have provided no evidence that filarial nematodes ingest the contractile components of the thoracic musculature.

Although our data did not reveal any obvious pathological consequence to infection, pathology may still exist. The techniques used in this study only allowed for the examination of structural damage at the light microscopic level and did not molecularly assess cell death, or explore pathology at the ultrastructural level. Significant mortality has been associated with filarial nematode invasion of and exit from the indirect flight muscles of *Ar. subalbatus*.

**Figure 6. Comparative histological imaging of thoracic indirect flight muscles after *Brugia pahangi*-infected and uninfected bloodmeals.** Aside from occasional host-derived tissue pooling between the worms and the myofibrils (A, A inset), no obvious pathology associated with infection was observed at 6 days (A, B), 9 days (C, D) or 14 days (E, F) after mosquitoes received *Brugia pahangi*-infected (A, C, E) and uninfected (B, D, F) bloodmeals. *B. pahangi*, yellow arrows; host tissue pooling, black arrow; *, fat body; A–F scale bar, 100 µm; A inset scale bar, 25 µm.

doi:10.1371/journal.pntd.0000666.g006
(Aliota et al., unpublished; and [61]) and it is possible that this mortality is a consequence of damage that impairs flight, resulting in decreased feeding and other essential biological processes.

Finally, it is important to consider that host and parasite genotypes share control of epidemiological parameters of their relationship. Most models of the evolutionary processes in host-parasite systems assume that the evolution of attack or defense strategies is governed by the balance of their evolutionary costs and benefits from the point of view of either the parasite or the host and, thus, hold the other partner constant. In other words, they consider that the traits of the relationship are determined by the genotype either of the host or of the parasite, but not an interaction between the two [62]. Future studies that explore the Interactome- the whole set of molecular interactions of both organisms in a symbiotic relationship- of a host-pathogen system should be extremely valuable in determining the evolutionary basis for tolerance vs. resistance and help to elucidate the underlying components of vector competence.

Supporting Information

Table S1  PCR primer sequences.

Found at: doi:10.1371/journal.pntd.0000666.s001 (0.05 MB DOC)

Table S2 Analysis of all Armigeres subalbatus genes transcribed by Brugia pahangi-infected mosquitoes. A tabular representation of all transcripts showing a detectable increase or decrease in abundance at all time points. The table lists the ASAPID number, associated product, GO designation, and relative transcript abundance. All time points are represented.

Found at: doi:10.1371/journal.pntd.0000666.s002 (0.08 MB XLS)

Table S3 Analysis of all Armigeres subalbatus genes transcribed by either Brugia pahangi- or Brugia malayi-infected mosquitoes. A tabular representation of all transcripts showing detectable differences in abundance at all time points. The table lists the ASAPID number, associated product, GO designation, and relative transcript abundance expressed as the fold difference between mosquitoes infected with either parasite. All time points are represented.

Found at: doi:10.1371/journal.pntd.0000666.s003 (0.06 MB XLS)

Text S1 Correlation of log2 ratios from microarray expression data with log2 qPCR expression values. Validation of microarray data with qPCR. The expression values (log2 ratios) for seven genes in four separate time points are plotted against the RT-qPCR expression values. The Pearson’s correlation coefficient of 0.702 and the goodness of the fit ($R^2 = 0.500$) indicates a high degree of correlation.

Found at: doi:10.1371/journal.pntd.0000666.s004 (0.07 MB DOC)

Acknowledgments

The authors thank Erin Reddy for time spent dissecting mosquitoes, Sara Erickson for critical review of the manuscript, and Dr. Michelle Michalski for providing some of the $B. pahangi$ infected gerbils.

Author Contributions

Conceived and designed the experiments: MTA JFH CCC BMC. Performed the experiments: MTA JFF AKC JFH CCC BMC. Analyzed the data: MTA JFF AKC JFH CCC BMC. Contributed reagents/materials/analysis tools: JFH CCC BMC. Wrote the paper: MTA JFH BMC.

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