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Erickson, Sara M.; Fischer, Kerstin; Weil, Gary J.; Christensen, Bruce M.; and Fischer, Peter U., "Distribution of Brugia malayi larvae and DNA in vector and non-vector mosquitoes: Implications for molecular diagnostics." *Parasites & Vectors*. 2:. 56. (2009).  
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Distribution of *Brugia malayi* larvae and DNA in vector and non-vector mosquitoes: implications for molecular diagnostics
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Abstract

**Background:** The purpose of this study was to extend prior studies of molecular detection of *Brugia malayi* DNA in vector (*Aedes aegypti*-Liverpool) and non-vector (*Culex pipiens*) mosquitoes at different times after ingestion of infected blood.

**Results:** Parasite DNA was detected over a two week time course in 96% of pooled thoraces of vector mosquitoes. In contrast, parasite DNA was detected in only 24% of thorax pools from non-vectors; parasite DNA was detected in 56% of midgut pools and 47% of abdomen pools from non-vectors. Parasite DNA was detected in vectors in the head immediately after the blood meal and after 14 days. Parasite DNA was also detected in feces and excreta of the vector and non-vector mosquitoes which could potentially confound results obtained with field samples. However, co-housing experiments failed to demonstrate transfer of parasite DNA from infected to non-infected mosquitoes. Parasites were also visualized in mosquito tissues by immunohistochemistry using an antibody to the recombinant filarial antigen Bm14. Parasite larvae were detected consistently after mf ingestion in *Ae. aegypti*-Liverpool. Infectious L3s were seen in the head, thorax and abdomen of vector mosquitoes 14 days after Mf ingestion. In contrast, parasites were only detected by histology shortly after the blood meal in *Cx. pipiens*, and these were not labeled by the antibody.

**Conclusion:** This study provides new information on the distribution of filarial parasites and parasite DNA in vector and non-vector mosquitoes. This information should be useful for those involved in designing and interpreting molecular xenomonitoring studies.

**Background**
Human lymphatic filariasis (LF) is caused by the mosquito-borne filarial nematodes *Wuchereria bancrofti*, *Brugia malayi*, and *B. timori*. These parasites are currently targeted for elimination by the Global Program for the Elimination of Lymphatic Filariasis (GPELF), and workers in this program have reported both achievements and future challenges to eliminating parasite transmission in endemic areas [1-3]. One important component of the elimination program is the ability to estimate infection prevalence and transmission rates, especially during mass drug administration (MDA), in order to accurately evalu-
ate the progress towards the goal of LF transmission interruption [4]. Molecular detection assays provide sensitive and specific tools for identifying and distinguishing parasites in host populations. Molecular techniques commonly used to study LF infection, or exposure, in humans include the detection of parasite DNA, circulating filarial antigen, and filarial antibodies in blood samples [5]. Molecular techniques also have been applied to the detection of filarial worms in mosquitoes, and these primarily target parasite DNA [6-9].

The detection of parasite DNA in mosquito samples is a valuable tool for molecular xenomonitoring (MX), but this does not differentiate parasite developmental stages or distinguish whether the DNA is from living or dead parasites [10-12]. Recently, RNA-based assays have been developed to detect *B. malayi* and *W. bancrofti* in mosquitoes [13,14], including the distinction of *B. malayi* infected (a constitutive parasite transcript) and infective mosquitoes (a L3-specific transcript) [14]. However, RNA-based detection assays have not yet been tested in the field or incorporated into LF surveillance programs. Vector-parasite interactions influence the applicability and interpretation of molecular detection assays used in vector surveillance studies. There are several factors that should be carefully considered when using molecular techniques to investigate parasites within the mosquito intermediate host, including the (1) various life cycle stages and their tissue locations, (2) likelihood of parasite development to the infective stage, i.e., vector competence, and (3) limitations of the particular detection assay, i.e., ability to distinguish infection stages and living from dead parasites. The separation of mosquitoes into body regions has been used to circumvent the inability of some assays to distinguish infective-stage parasites. For example, *Anopheles* spp. have been divided into two body regions (head/thorax and abdomen) to provide better estimates of mosquitoes infected with *Plasmodium* sporozoites and/or pre-sporozoite stages [15-17] and the heads of blackflies have been removed (by mass dissection techniques) for the restricted, head-only, PCR assays targeting *Onchocerca* DNA, which is more likely to provide a better estimate of infective-stage parasites because other developmental stages generally reside outside of the head [18,19].

The studies conducted herein follow our previous work, which demonstrated that DNA-based diagnostics are unable to distinguish the developmental stage of LF parasites or whether parasites are living or dead in the mosquito [10]. Despite these limitations, there are benefits to using DNA-based assays over dissection to assess the persistence of filariasis in populations. Because filarial DNA is detectable for two weeks or longer following a microfilaricnic blood meal in both vector and non-vector mosquitoes, all anthropophilic mosquitoes can be included in the screening of mosquitoes for parasite DNA to provide MX data [10]. Herein, we have further examined the persistence of filarial parasites and parasite DNA in mosquitoes; we used a combination of mosquito dissection, immunohistology and PCR assays to determine the location(s) of filarial worms and DNA in mosquitoes that are susceptible or refractory to filarial parasite development. These studies allowed us to assess the potential value of tissue specific assays (e.g., mosquito heads only) to estimate the prevalence of infective-stage larvae in mosquitoes; we also investigated the issue of direct mosquito to mosquito transfer of parasite DNA that could confound MX studies.

### Results

**Development of *B. malayi* in *Ae. aegypti* and *Cx. pipiens***

Table 1 summarizes the recovery of parasites from dissected mosquitoes. In *Ae. aegypti*-LVP, 73.6% of the recovered *B. malayi* mf at 2 h post ingestion (PI) had successfully penetrated the midgut, with 49.4% located in the thorax. At 14 days post ingestion of microfilaricnic blood (DPI), 80% of *Ae. aegypti* harbored L3s. In contrast,

| Time post ingestion | Number of parasites recovered<sup>a</sup> | Total worms<sup>a</sup> | Percentage of mosquitoes harboring parasites |
|---------------------|------------------------------------------|-------------------------|--------------------------------------------|
|                     | Midgut | Abdomen | Thorax | Head |                                   |
| *Ae. aegypti*-LVP   | 2 h    | 23      | 21     | 43   | n.d. | 87                       | 93% (6.2 ± 6.1)<sup>b</sup> |
|                    | 7 d    | 0       | 0      | 65   | 0    | 65                       | 73% (5.9 ± 5.9)             |
|                    | 14 d   | n.d.    | 7      | 30   | 22   | 59 (20)                  | 80% (3.9 ± 3.2)             |
| *Cx. pipiens*      | 2 h    | 100     | 4      | 0    | n.d. | 104                      | 93% (7.5 ± 7.1)             |
|                    | 7 d    | 0       | 4      | 0    | 0    | 4                        | 13% (2.0 ± 1.4)             |
|                    | 14 d   | 0 (10)  | 0      | 0    | 0    | 0                        | 0%                          |

<sup>a</sup>Parasites were observed in mosquito dissections by microscopy at various times after ingestion of microfilaricnic blood. Fifteen mosquitoes were dissected at each time point, except as noted (n.d., 10, or 20).

<sup>b</sup>Mosquito infection rate (mean intensity ± SD)
from Cx. pipiens only mf were recovered and 93% of them were found in the midgut lumen.

**B. malayi DNA detection in pooled mosquito body regions**

PCR results are summarized in Figure 1. Parasite DNA was detected in 74% of Ae. aegypti-LVP pooled body region samples (n = 300) and 36% of Cx. pipiens (n = 300) pooled body regions tested. These differences were highly significant (P < 0.0001). In Ae. aegypti-LVP, parasite DNA was detected in all four body regions with 43, 71, 88, and 96% of heads, midguts, abdomens, and thoraces (n = 75 for each) positive by qPCR, respectively. Parasite DNA also was detected in all Cx. pipiens body regions, with 17, 24, 47, and 56% of heads, thoraces, abdomens, and midguts (n = 75 for each) positive, respectively. The differences in the percentage of B. malayi DNA positive samples were significant between mosquito species in all body regions (heads, P = 0.001; thoraces, P < 0.0001; abdomens, P < 0.0001) except the midguts (P = 0.09). The detection of parasite DNA within certain mosquito body regions was positively or negatively correlated with time. Specifically, the detection of B. malayi DNA was negatively correlated with time in ‘whole body’ Cx. pipiens (r² = 0.93, P = 0.0075) and Cx. pipiens midguts (r² = 0.82, P = 0.034), and positively correlated with time in Ae. aegypti-LVP heads (r² = 0.77, P = 0.05).

**B. malayi DNA detection in individual mosquito body regions**

Individual mosquitoes that were separated into body regions for DNA detection assays were compared to results of the pooled mosquito body regions (Fig. 2). Following a microfilaremic blood meal of 191 mf/20 μl blood, there was no difference in DNA detection between pooled and individual Ae. aegypti-LVP body regions (P-values = 0.35-0.61) or between pooled and individual Cx. pipiens body regions (P-values = 0.78-1.0).

**Detection of parasite DNA in mosquito excreta and feces**

Figure 3 summarizes the detection of B. malayi DNA in individual housed mosquitoes and their voided excreta and feces. All mosquitoes were positive for parasite DNA immediately (2 hr) after ingesting microfilaremic blood. From 1-4 DPI all Ae. aegypti-LVP tested positive for parasite DNA, and three of these (15%) mosquitoes had detectible B. malayi DNA in their feces. In contrast, 60% of Cx. pipiens were DNA negative at 4 DPI, but B. malayi DNA was detected in 100% of Cx. pipiens feces tested at 3-4 DPI. Of the twenty samples of feces collected over the entire observation period of 1-4 DPI from each species, B. malayi DNA was detected in 15 and 65% of Ae. aegypti-LVP and Cx. pipiens fecal samples, respectively (P = 0.003).

**Parasite DNA contamination of B. malayi positive and negative mosquitoes**

In order to test the possibility that infected mosquitoes contaminate uninfected mosquitoes while they are together in the same trap or sampling tube, we housed uninfected Ae. aegypti-LVP together with Cx. pipiens that had fed on a microfilaremic gerbil. After 7 days, mosquitoes were collected and pooled by species. None of the 17 Ae. aegypti-LVP pools (with 10 mosquitoes per pool) were positive by real-time PCR. In contrast, 17 of the 21 Cx. pipiens pools (with 5 mosquitoes each) were positive. Most of these samples had relatively high Ct values indicating small amounts of B. malayi DNA, but 5 pools had higher Ct values ranging between 29 and 35. Although B. malayi DNA can be detected in feces of infected mosquitoes, feces did not cause false positive DNA signals from uninfected mosquitoes after co-housing.

**Detection of Bm14 in mosquito-stage parasites by immunohistology**

Immunohistology studies were performed to confirm the dissection results and to better document the fate of B.
In *Ae. aegypti-LVP*, unlabeled mf were detected within the midgut directly after the bloodmeal (Fig. 4A). Strong-labeling was observed with the Bm14 antibody after the larvae reached the thoracic muscles. Thus this antibody can be used to sensitively detect developing filarial larvae in vectors (Fig. 4B-E). Strongly labeled L3s were observed in all body parts of *Ae. aegypti-LVP* at 14 DPI (Fig. 4F-G). L3s were not confined to the head or the thoracic musculature; they were also seen in the abdomen, outside of the midgut (Fig. 4J).

Stretched, intrauterine mf in adult female *B. malayi* are usually labeled by the Bm14 antibody (Fig. 5A), but mf were not labeled in the midgut of *Cx. pipiens* directly after the blood meal. No larvae were detected in histological sections of *Cx. pipiens* at later times points (Fig. 5C, D). Dead and/or dying larvae in the thorax of *Brugia* refractory *Ae. aegypti-RKF* were not labeled by Bm14 (Fig. 5G, I, J). In contrast, developing larvae in *Ae. aegypti-LVP* were always strongly labeled at the same time points (Fig. 5F, H).

These results suggest that the anti-Bm14 antibody specifically detects viable and developing *B. malayi* larvae in vectors.

**Discussion**

The application of molecular assays to detect parasites within vectors is influenced by the vector-parasite interaction. In these studies, we analyzed the detection of filarial worms in susceptible and refractory mosquitoes by targeting parasite DNA or protein in molecular assays. The three mosquito strains examined have very different interactions with *B. malayi* that were documented in our previous paper on detection of parasite DNA from living and dead parasites within mosquitoes [10]. The follow-up studies discussed herein were designed to investigate: (1) the mosquito body region(s) containing the persistently detected parasite DNA within *Cx. pipiens* (in this mosquito species mf are seldom able to migrate out of the midgut lumen), and (2) the detection of parasite proteins as larvae develop in susceptible and refractory mosquitoes.
studies [20] have shown that L3s are not restricted to the toes contain L3s in other body parts. Our results and prior provide a false negative infectivity signal when mosqui-
approach. Because PCR can detect DNA from live or dead
infectivity rates than DNA detection in whole mosquitoes
(see Fig. 2), we do not advocate separating heads for this pur-
DNA also was not restricted to the midgut in Cx. p. midgut lumen, mf are sometimes detected outside of the midgut by dissection (Table 1 and Erickson and Chris-
tensen, unpublished data). Although these few mf that do
penetrate the midgut epithelium could be the source of parasite DNA outside of the Cx. p. midgut, an alternate-
tion in mosquitoes; several studies have used this
Immunohistochemistry provides an alternative approach
to detecting and studying parasite migration and develop-
ment in mosquitoes; several studies have used this
approach with antibodies to Plasmodium circumsporo-
zoite protein (CSP) [16,29-31]. Most immunodiagnostic
research on filariasis has focused on the parasite stages
that occur within the vertebrate host [32,33], and this
work has produced sensitive and specific diagnostic tests
for Bancroftian and brugian filariasis [5,34,35]. In con-

Separation of mosquitoes into body regions (head, tho-
remnants of ingested mf (especially in mosquitoes with
fectivity rates at that time point. Although DNA detec-
tion in mosquito heads might provide a better estimate of infectivity rates than DNA detection in whole mosquitoes
Fig. 2), we do not advocate separating heads for this pur-
purpose in field studies, because false positive and false neg-
active infectivity signals are likely to be high with this approach. Because PCR can detect DNA from live or dead parasites from any developmental stage, heads could be falsely positive (without L3s being present) because of remnants of ingested mf (especially in mosquitoes with armed cibarial and/or pharyngeal pumps). Heads could provide a false negative infectivity signal when mosqui-
to contain L3s in other body parts. Our results and prior studies [20] have shown that L3s are not restricted to the head.

However, to prevent cross-contamination, the following steps are recommended for processing wild-caught mos-
quitoes: (1) Immediately kill mosquitoes to limit mos-
quitos that cross-contamination is probably uncommon.

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Figure 3
Detection of B. malayi DNA in mosquito feces. A
Prevalence of parasite DNA in individually housed Ae. aegypti-LVP and their feces. B Prevalence of parasite DNA in individ-
ually housed Cx. p. and their feces. Sample number is indicated above each bar.

B. malayi DNA also was not restricted to the midgut in Cx. p. midgut lumen, mf are sometimes detected outside of the midgut by dissection (Table 1 and Erickson and Chris-
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for Bancroftian and brugian filariasis [5,34,35]. In con-
trast, few studies have examined antigen detection as a method for identifying and distinguishing LF parasites in mosquitoes. A monoclonal antibody raised against *B. malayi* L3s (NEB-D1E5) is specific to a *B. malayi* L3 surface antigen and distinguishes *B. malayi* L3s from infective-stage larvae of other filarial worms apart from *B. timori* [36,37]. In the current study, antibodies to recombinant *B. malayi* antigen Bm14 [38,39] were used to detect this protein in filarial larvae in mosquitoes. Bm14 was detected in mf and all other developmental stages of parasites in the competent vector, *Ae. aegypti*-LVP. In contrast, the protein was not detected in parasites present in non-vector *Cx. pipiens* (harboring mf in the midgut), or *Ae. aegypti*-RKF (harboring parasites that developmentally arrest as L1s which then die within mosquito muscle cells). These results suggest that Bm14 may be a specific biomarker for viable filarial parasites in mosquitoes.

**Conclusion**

Improved methods are needed for assessing changes in mosquito infection and infectivity rates in the context of LF control/elimination programs. As infection rates in humans and vectors decrease following MDA, increased numbers of mosquitoes must be tested to accurately estimate parasite prevalence [40]. This makes dissection impractical, and favors use of molecular detection assays with pooled mosquitoes. This study provides new information on the persistence of filarial worm DNA in non-vectors that has practical implications for MX studies regarding methods for processing field-caught mosquitoes and for interpreting MX data. Additional studies are needed to determine whether the presence of Bm14 antigen is a reliable marker for viable filarial worms in pooled mosquito samples. Although this study focused on *B. malayi*, the findings may be of interest to scientists and
programs that use molecular techniques to detect other pathogens (helminths, viruses, or protozoa) in vectors.

Materials and methods
Mosquito maintenance and parasite exposures
Mosquitoes used for these studies were obtained from colonies of *Aedes aegypti* (black-eyed, Liverpool strain; LVP), *Ae. aegypti* (Rockefeller strain; RKF) and *Culex pipiens pipiens* (Iowa strain) maintained at the University of Wisconsin-Madison, as previously described [21,41]. These mosquitoes differ in their vector competence for *B. malayi*. *Ae. aegypti*-LVP support the development of *B. malayi* from mf to L3s, but parasites do not develop in *Cx. pipiens*, because mf do not penetrate the midgut epithelium. In *Ae. aegypti*-RKF, mf penetrate the mosquito midgut and migrate into thoracic muscles where they fail to develop to L2s. This mosquito strain was only used for comparison in the immunohistology experiments. Four- to seven-day-old mosquitoes were sucrose starved ~14 h prior to blood feeding. Mosquitoes were exposed to *B. malayi* by blood feeding on microfilaremic cat blood in a water-jacketed membrane feeder fitted with a parafilm membrane [42]. Mosquitoes also were blood fed on uninfected gerbils (*Meriones unguiculatus*) to serve as parasite-negative, blood-fed controls. Engorged mosquitoes were sorted and maintained in the laboratory.

Laboratory animals were handled according to guidelines approved by the Animal Care Committee at the University of Wisconsin-Madison. The mf densities of *B. malayi*-infected cat blood obtained from the NIAID Filariasis Research Reagent Repository Center [http://www.filariasiscenter.org](http://www.filariasiscenter.org) used in these studies ranged from 24-191 mf/20 μl blood.

Mosquito dissection
Five mosquitoes were dissected at 2 hr, 7 d, and 14 d post ingestion of microfilaremic blood (PI) to estimate the mean intensity of infection, and to record the stage of *B. malayi* development. Individual mosquitoes were sepa-
rated into head, thorax, midgut, and abdomen, and each body region was teased apart and individually examined for parasites by microscopy as previously described [10].

*Ae. aegypti*-LVP and *Cx. pipiens* were separated into body regions (head, thorax, midgut, and abdomen) and placed, separately or in pools of four, into 2.0 ml microcentrifuge tubes for parasite DNA detection. To create a pooled sample, four mosquitoes were separated into body regions, and the body regions were combined by type into tubes. For example, four mosquitoes were used to produce one pool of four heads, one pool of four thoraces, one pool of four abdomens, and one pool of four midguts. Five pooled samples were prepared at 2 h, 1, 3, 7, and 14 d PI; thus, a total of 20 mosquitoes were collected at each time point for pooled samples. In addition to creating pooled samples, individual mosquitoes were dissected into body regions as described above and then placed individually into tubes. Five individuals were dissected at each time point to create twenty samples: five tubes contained individual heads, five contained a single thorax, five contained an abdomen, and five contained a midgut. These samples were screened for *B. malayi* DNA to compare detection results between individuals and pooled samples. All samples were cataloged to track a given body region samples. All samples were used for negative control, and 100 pg of DNA was extracted using a commercial column method as described previously [10]. Quantitative real-time PCR was performed using an MGB probe to detect a 120 bp fragment of the *Brugia Hha* (page number not for citation purposes) repeat [44]. In all real-time PCR assays, water was used as no-template negative control; DNA extracted from a pool of non-infected mosquitoes acted as extraction negative control, and 100 pg of DNA isolated from adult *B. malayi* was used as positive control.

**Immunohistology**

Five mosquitoes of each species were collected at 2 h, 1, 3, 7, and 14 d PI (i.e., the same time points as DNA detection assays) and stored in 80% ethanol at room temperature until embedding. Mosquitoes were embedded in paraffin, and *B. malayi* larvae were stained using the alkaline phosphatase anti-alkaline phosphatase method as described previously [45]. A polyclonal mouse antibody raised against recombinant Bm14 protein was used as the primary antibody for these studies [38].

**Statistical Analysis**

Data were graphed and analyzed with GraphPad Prism 5.0 [http://www.graphpad.com](http://www.graphpad.com). Fisher's exact tests, with two-tailed P-values, were used to compare parasite development and DNA detection between *Ae. aegypti*-LVP and *Cx. pipiens*. Pearson correlation tests were used to test for trends in parasite DNA detection over time. Statistical results were considered significant at *P* ≤ 0.05.

**List of abbreviations**

MF: microfilariae; L1, L2, L3: first- to third-stage larva; PI: post ingestion of microfilaremic blood; DPI: days post infection.
ingestion of microfilaremia blood; MX: molecular xenomonitoring.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
SE participated in the conception of the study including its design and organization, performed mosquito exposures and sample collection, data interpretation, statistical analysis, and drafted the manuscript. LF participated in data interpretation and critical manuscript revisions. BC participated in study design, data interpretation, and critical manuscript revisions. PF participated in the conception of the study including its design and organization, data interpretation, and helped draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements
We thank the NIAID/NIH Filarialis Research Reagent Repository Center (FR3) for supplying Brugia malayi-infected cat blood. This research was supported in part by the National Institutes of Health grants AI 007414-17 (graduate fellowship to SM Erickson), AI-19769 (BM Christensen), AI-065715 (GJ Weil), and by a Grant from The Barnes Jewish Hospital Foundation (GJ Weil).

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