

METHODOLOGY

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A novel assay to isolate and quantify third-stage *Dirofilaria immitis* and *Brugia malayi* larvae emerging from individual *Aedes aegypti*

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Abstract

Background: Mosquitoes transmit filarial nematodes to both human and animal hosts, with worldwide health and economic consequences. Transmission to a vertebrate host requires that ingested microfilariae develop into infective third-stage larvae capable of emerging from the mosquito proboscis onto the skin of the host during blood-feeding. Determining the number of microfilariae that successfully develop to infective third-stage larvae in the mosquito host is key to understanding parasite transmission potential and to developing new strategies to block these worms in their vector.

Methods: We developed a novel method to efficiently assess the number of infective third-stage filarial larvae that emerge from experimentally infected mosquitoes. Following infection, individual mosquitoes were placed in wells of a multi-well culture plate and warmed to 37 °C to stimulate parasite emergence. *Aedes aegypti* infected with *Dirofilaria immitis* were used to determine infection conditions and assay timing. The assay was also tested with *Brugia malayi*-infected *Ae. aegypti*.

Results: Approximately 30% of *Ae. aegypti* infected with *D. immitis* and 50% of those infected with *B. malayi* produced emerging third-stage larvae. Once *D. immitis* third-stage larvae emerged at 13 days post infection, the proportion of mosquitoes producing them and the number produced per mosquito remained stable until at least day 21. The prevalence and intensity of emerging third-stage *B. malayi* were similar on days 12–14 post infection. Increased uptake of *D. immitis* microfilariae increased the fitness cost to the mosquito but did not increase the number of emerging third-stage larvae.

Conclusions: We provide a new assay with an associated set of infection conditions that will facilitate assessment of the filarial transmission potential of mosquito vectors and promote preparation of uniformly infectious third-stage larvae for functional assays. The ability to quantify infection outcome will facilitate analyses of molecular interactions between vectors and filariae, ultimately allowing for the establishment of novel methods to block disease transmission.

Keywords: *Aedes aegypti*, *Aedes albopictus*, *Dirofilaria immitis*, *Brugia malayi*, Mosquito, Filaria, Transmission

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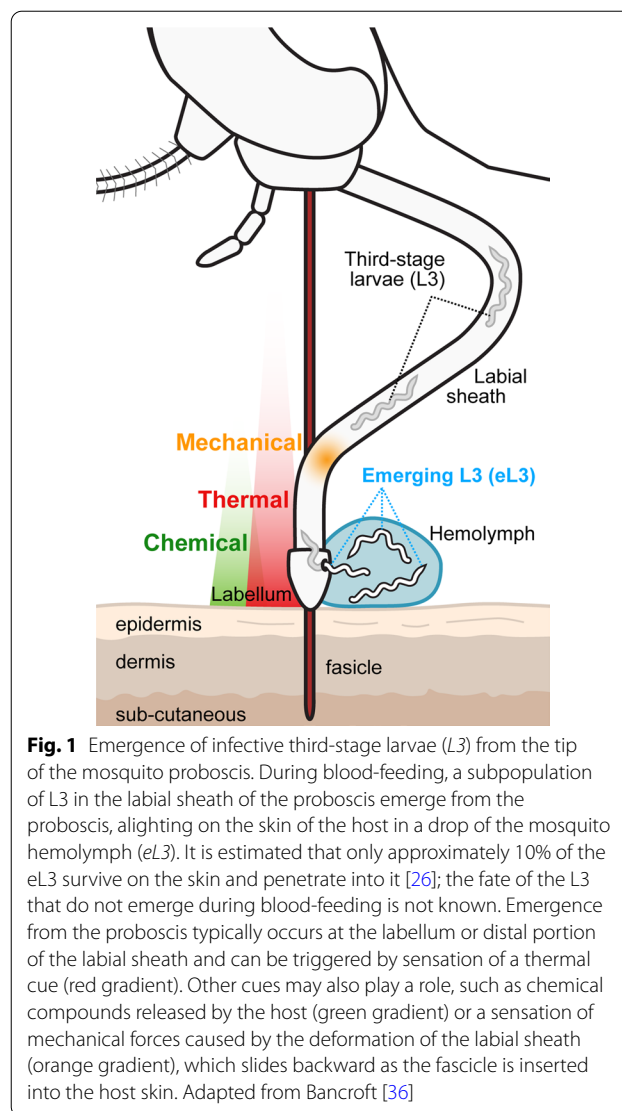
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Background

Arthropods serve as intermediate hosts and vectors of numerous human and animal infective filariae that contribute to a large disease burden worldwide. Indeed, mosquito-transmitted lymphatic filariasis, caused by *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*, affects approximately 120 million people in 83 countries [1]. Mosquitoes are also responsible for transmission of animal infective filariae, the most studied being *Dirofilaria immitis*, the causative agent of canine heartworm disease. Although detailed global numbers are not available, there are an estimated 250,000–500,000 infected dogs in the USA, with some areas, such as the Mississippi River basin, reporting infection prevalence as high as 40% [2, 3]. Humans can also be incidentally infected by *D. immitis*, but they do not support the entire life cycle and typically present with only mild clinical signs [3].

Within the mosquito host, ingested microfilariae (mf) migrate from the midgut to specific tissues specific to the mosquito species. The filarial agents of lymphatic filariasis migrate to the indirect flight muscles in the thorax, whereas *D. immitis* migrates into the Malpighian tubules (MT) [4]. In these tissues, the parasites develop intracellularly, undergoing successive molts to form third-stage larvae (L3). Some L3 migrate to the proboscis where they are poised for transmission (Additional file 1: Movie 1). Infective L3 emerge most typically from the mosquito labellum but they can also emerge from the labial sheath during blood-feeding (Fig. 1) [5]. Larvae emerging from the mosquito are deposited onto the skin in a drop of hemolymph where they can enter the skin through the bite site [5]. The fate of the L3 that do not emerge is not known.

Due to their importance to the disease cycle, much attention is focused on the transmission of infective L3. Different methods have been employed to assay L3 in mosquitoes. A common approach, especially for field isolates, is the detection by PCR of larvae in the head and other body parts of the mosquito following dissection [6–8]. Although this method is not established for all filariae, stage-specific PCR has been used for detection of infectious L3 in *W. bancrofti* infected mosquitoes [9]. PCR is sensitive and species specific and can be used on pools of mosquitoes in cases where infection prevalence is low. Another commonly used approach is to physically examine larvae in the labial sheath of the proboscis or in dissected tissues of the mosquito. This approach is particularly useful in a laboratory setting to determine the number of larvae at each developmental stage and to identify their tissue of residence. However, while each of these assays can reveal the number of L3 that have developed, specific assays to enumerate L3 capable of emerging (eL3) when the mosquito blood-feeds have not been previously described.



In addition to assessing the intensity of transmission in field populations, an ability to enumerate and collect L3 capable of emergence would greatly enhance functional assessment. Currently, studies of host immune responses to L3, as well as *in vitro* and *in vivo* drug efficacy studies, are typically carried out on L3 collected from infected mosquitoes gently disrupted with a mortar and pestle and then separated by filtration through a mesh [10–12]. However, it is unknown whether all L3 harvested by this method are mature and competent to infect the host. Furthermore, obtaining microfilaria for mosquito infections requires access to an infected animal or access to samples from another source, and although *D. immitis* microfilaria have been shown to be capable of cryopreservation [13], this method of storage is not commonly used. More typically, fresh samples are shipped, despite being infective, little is known

about how short-term storage during shipping affects development to L3.

Blocking parasite development in the vector is a novel approach being considered for controlling disease transmission and requires a thorough understanding of the molecular interactions between parasites and their vector. The means to quantify the prevalence of mosquitoes with eL3 and determine the number of emerging L3 per mosquito would greatly facilitate this work. Here we present a new assay to quantify infectious eL3 that works with different vector and filarial species. We then use this assay to study the development and characteristics of *D. immitis* eL3 in mosquitoes. The detailed infection parameters and assay conditions presented here to quantify and produce maximum yields of eL3 could potentially facilitate both studies between filariae and their mosquito and vertebrate hosts.

Methods

Mosquito strains and culture

Aedes aegypti and *Ae. albopictus* strains were provided by the National Institutes of Health/National Institute of Allergy and Infectious Diseases (NIH/NIAID) Filariasis Research Reagent Resource Center (FR3) and the Malaria Research and Reference Reagent Resource Center (MR4) through distribution by the NIH/NIAID BEI Resources Repository. *Aedes aegypti*^S (*Ae. aegypti*, Strain Black Eye Liverpool, Eggs, FR3, NR-48921) is a *Dirofilaria immitis*- and *Brugia malayi*-susceptible strain, and *Ae. aegypti*^R (*Ae. aegypti*, Strain LVP-IB12, Eggs, MR4, MRA-735, contributed by David W. Severson) is a *D. immitis*- and *B. malayi*-refractory strain. Both strains were reared at 27 °C and 80% humidity under a 12/12-h photoperiod. *Aedes albopictus*^{NJ} (*Ae. albopictus* Strain ATM-NJ95, Eggs, MR4, NR-48979) [14] was reared at 24 °C and 70% humidity under a 16/8-h (light/dark) photoperiod. Mosquitoes were housed in 30-cm³ cages (BugDorm, Taichung, Taiwan) at a density of approximately 1000 per cage. Larvae were maintained at a density of 1 larva/3 ml. Larvae were fed a suspension of liver powder in water (MP Biomedicals, Solon, OH, USA), and adults were maintained on 10% sucrose in water, which was changed daily. Heparinized sheep blood (Hemostat, Dixon, CA, USA) was provided using an artificial membrane feeder at 37 °C for egg production.

Mosquito infection with *Dirofilaria immitis* and *Brugia malayi*

The full protocol is described in greater detail by Povolonos and McCrea [15]. Blood containing *D. immitis* microfilariae was obtained from an experimentally infected dog according to Institutional Animal Care and Use Committee-approved protocols and in accordance

with the guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania (IACUC, protocol 805059). The microfilarial load in this dog was approximately 50,000 mf/ml. Blood containing *B. malayi* microfilariae (*B. malayi* microfilariae in cat blood, live, FR3, NR-48887) was obtained from an experimentally infected cat containing with a microfilarial load of approximately 12,000 mf/ml (provided by the NIH/NIAID FR3 for distribution by BEI Resources, NIAID/NIH). In both cases, the blood was diluted with the appropriate volume of heparinized sheep blood to a concentration of 4000 mf/ml. The sample was warmed to 37 °C, and a 3.5-ml aliquot was placed in the indentation on the outside of the bottom of a 300-ml plastic baby bottle (Advent; Phillips, Amsterdam, the Netherlands) and covered with Parafilm. The inside of the bottle was filled with water at 37 °C. This assembly was placed with the membrane side down onto the top mesh of mosquito cages and the insects are allowed to feed for 15 min (Fig. 2a). Immediately following the

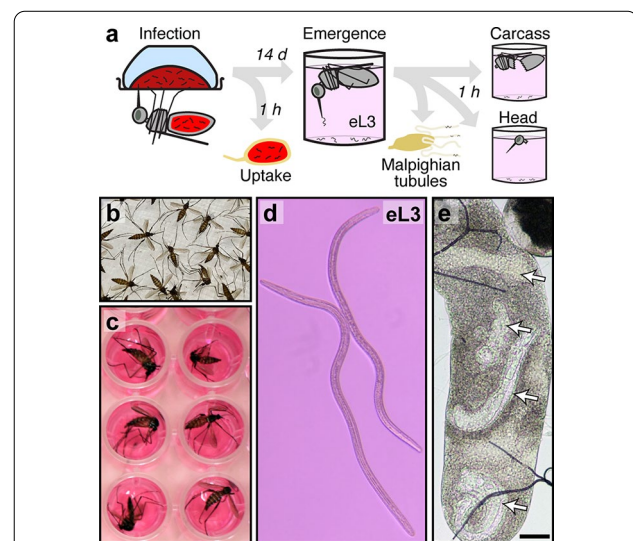


Fig. 2 Assay for eL3. **a** Infection and assay protocol followed in this study. Mosquitoes are fed on blood containing *Dirofilaria immitis* microfilariae. Uptake is measured in a small group, and the remaining mosquitoes are maintained until an emergence assay is performed. Immediately following the emergence assay with whole mosquitoes, the Malpighian tubules are dissected and analyzed. The dissected head and carcass are individually placed into an emergence assay to assay L3 that failed to emerge from intact mosquitoes. Additional file 2: Figure S1 shows images of dissected mosquitoes and Malpighian tubules. **b, c** Mosquitoes are rinsed with 70% ethanol:30% water for wetting, rinsed with water (**b**) and then placed individually into wells of a 96-well plate (**c**). **d** Emerging eL3 from intact mosquitoes or L3 from dissected heads and carcasses at the bottom each well are scored by microscopy. Additional file 3: Movie 2 shows typical movement of eL3. **e** Larvae (white arrows) in live Malpighian tubules are scored by microscopy. Scale bar: 50 μ m

feed, fully engorged blood-fed mosquitoes were separated using CO₂ anesthesia and housed under standard insectary conditions as described above. Any partial or unfed mosquitoes were discarded.

Microfilarial uptake

Within 1 hour of blood-feeding, fed mosquitoes were anesthetized with CO₂ at room temperature. The entire undamaged midgut was dissected in deionized water in a depression slide and immediately transferred to a standard microscope slide containing a 50- μ l drop of deionized water. Damaged midguts leaking blood during dissection were not used. The epithelium of the gut was separated from the blood bolus and moved through the water to remove any residual blood before it was discarded. The blood was roughly dispersed using forceps and then pipetted up-and-down with a 20- μ l pipette. Microfilaria in the entire drop were counted immediately without a coverslip.

Emergence assay for eL3 and other larval stages

The full protocol is described in greater detail by Povelones and McCrea [16], which is modified from a previously published method [17]. Briefly, eL3 were assayed by placing live mosquitoes in 70% ethanol in water for 1 min, following which they were rinsed twice in deionized water and then placed individually into wells of a 96-well plate containing 200 μ l Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-glutamine, a high level of glucose and sodium pyruvate (Corning Mediatech, Manassas, VA, USA). At this point, the mosquitoes are still alive and can be observed moving in the buffer, but they cannot stand on the surface due to the wetting procedure. The plate was then placed in a 37 °C incubator with 5% CO₂ for 60 min, and the number of eL3 were determined without removing the mosquitoes using an inverted microscope with a \times 4 objective. In some experiments, after the eL3 emergence assay, mosquitoes were removed from the plate individually for assaying other larvae. First, the Malpighian tubules were dissected by removing the posterior two abdominal segments. The set of five Malpighian tubules was removed from the midgut and transferred to a microscope slide containing 15 μ l phosphate buffered saline and covered with a 22-mm² coverslip. The larvae were counted, and although different stages were present we did not categorize them (Additional file 4: Figure S2). The head was then separated from the body, and the dissected head and carcass fragment were placed separately into fresh wells of a 96-well plate prepared and incubated as described above. L3 were allowed to migrate out of the dissected

tissue for at least 60 min and scored as described above. Only a portion of the mosquitoes, between 30 and 36 from each replicate, used for the eL3 emergence assay were processed in this manner due to processing time constraints.

Emergence assay time course and microfilaria concentration series

Groups of 432–565 mosquitoes were fed on blood containing *D. immitis* microfilariae at different concentrations and housed as described above. Mosquito mortality was monitored daily, as assessed by the fraction of the population that died each day. This was necessary since groups of 50 mosquitoes were removed for eL3 assays as described above. Since the emergence prevalence was very low on day 12, emergence assays were performed with only 25 mosquitoes per replicate saving more of the population for later days where there would be a higher eL3 prevalence. For the concentration-series experiments, a blood dilution containing 32,000 mf/ml was created and then subjected to twofold serial dilution to create feeding doses of 16,000, 8000 and 4000 mf/mL. At each concentration, five mosquitoes of each strain were used to measure uptake, as described above. For these experiments, mortality was monitored daily until day 17 when an assay for eL3 was performed on the remaining mosquitoes. Kaplan–Meier survival analysis was performed between adjacent doses within each mosquito strain and between the same doses across the mosquito strains.

Results

Novel assay for filarial nematode L3 emerging from individual mosquitoes

In our assay, outlined in Fig. 2a, we infect mosquitoes using an artificial membrane feeder and then determine the number of microfilariae ingested immediately afterwards [15]. The microfilarial uptake represents the theoretical maximum number of parasites in the assay. To assay the L3 capable of emerging from the mosquitoes, we simulate the thermal cue that would be experienced by a mosquito landing on a mammalian vertebrate host by placing mosquitoes in buffer and warming the buffer to 37 °C [16]. Mosquitoes are placed individually into wells of a 96-well plate after the wetting procedure (Fig. 2b, c), and care is taken to minimize damage to the mosquito during transfer. Upon warming, competent L3 emerge from the mosquito and sink to the bottom of the well, where they can be counted (Fig. 2d; Additional file 3: Movie 2). eL3 collected by this method are capable of molting to the fourth larval stage *in vitro* [18] and are infectious to dogs [17], and we believe

they represent the number of L3 that emerge onto the skin of the host. After the emergence assay, the mosquitoes are still viable and can move, but they cannot escape the buffer due to the wetting procedure. Notably, following the emergence assay, further analyses can be carried out to determine the number and location of larvae remaining within the mosquito. In our study, both dissected Malpighian tubules (Fig. 2e), and the head and remaining carcass were assayed separately, as described in the following section (Additional file 2: Figure S1).

Microfilariae robustly migrate into the Malpighian tubules but only a fraction develop to eL3

To determine the efficiency of eL3 development we infected *D. immitis* susceptible (*Ae. aegypti*^S) or refractory (*Ae. aegypti*^R) strains and performed an emergence assay 14 dpi. We found that *Ae. aegypti*^R released no eL3, despite having a median uptake of 12 mf/mosquito (Fig. 3a). This is consistent with previous observations that ingested microfilariae arrest their development soon after invading cells of the Malpighian tubules [19–23]. In the susceptible strain (*Ae. aegypti*^S), 81 of the 252 mosquitoes assayed (32%) had at least one eL3 when given a median uptake of 15 mf/mosquito (Fig. 3b). A portion of the susceptible mosquitoes used for the emergence assay were dissected, and a substantial number had L3 remaining in the dissected heads (26/102; 25%) and carcasses (48/102; 47%) (Fig. 3b). The majority of larvae assayed at 14 dpi were present in the Malpighian tubules, and nearly all of the dissected mosquitoes (93/96; 97%) had at least one larva present in the Malpighian tubules (Fig. 3b). In a few cases, Malpighian tubules were damaged during the dissection and excluded from this and subsequent analyses. Pooling all larvae present across all tissues revealed that the median total number of larvae per mosquito was eight. Therefore, at 14 dpi, 53% of the 15 ingested microfilariae could be accounted for as larvae in the mosquito (Fig. 3b). In the dissected Malpighian tubules, we found larvae in various stages of development, but we did not determine their stage (Additional file 4: Figure S2). We note, however, that larvae found in the Malpighian tubules, even the most stunted sausage forms, are viable, as we observed them to be capable of movement. Previous studies suggest that not all L3 emerge when infected mosquitoes blood-feed on an animal [5]. Since this previous observation was not quantitative, we analyzed the data from the emergence assay (Fig. 3b) to compare the number of eL3 to the total L3 (eL3 + L3 in dissected heads, and dissected carcasses). We did not include larvae found in the Malpighian tubules since we

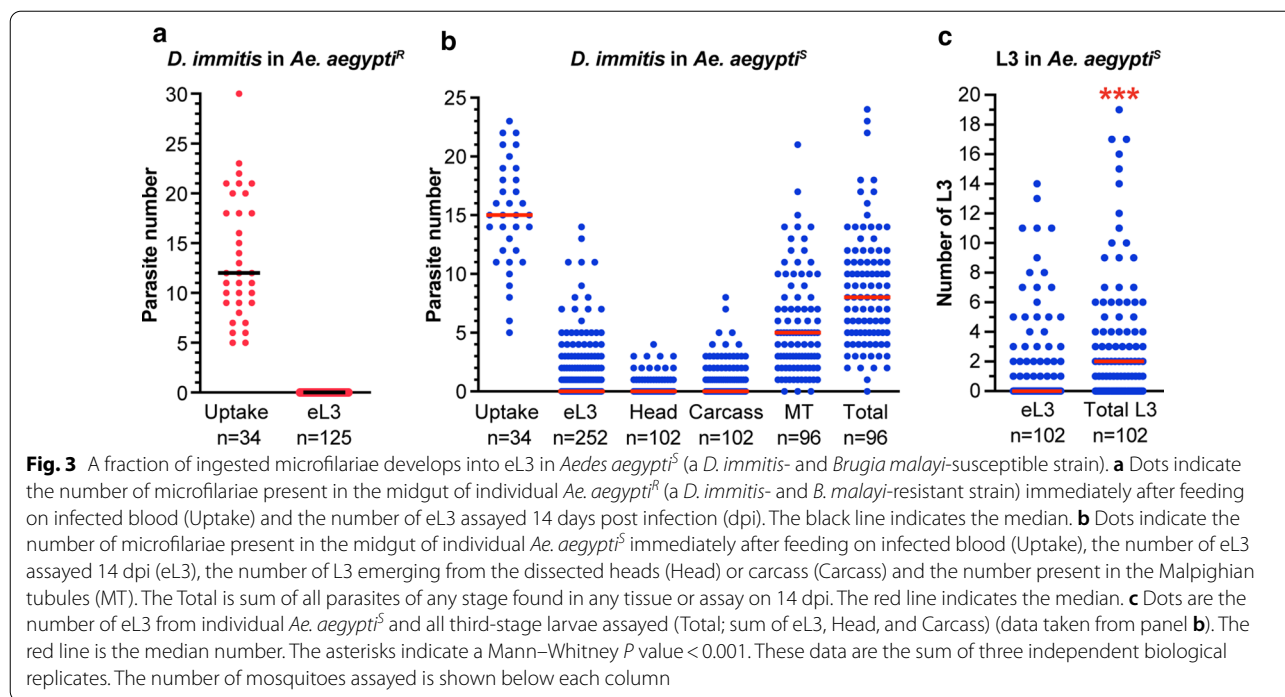
could not unambiguously characterize them as L3. We found that the number of eL3 was significantly lower than the total L3 (Fig. 3c). Therefore, we concluded that only a fraction of the larvae that develop to L3 emerge during our emergence assay, consistent with previous observations [5].

Once eL3 have developed, their intensity and prevalence are constant

To determine the optimal day to perform the emergence assay, we wanted to identify a time point at which eL3 recovery was maximized but mosquito mortality was still minimal. To do this, we infected populations ranging from 432 to 565 individuals, and then removed groups of approximately 50 mosquitoes on consecutive days for an emergence assay. Data from four independent experiments were pooled since it was not possible to obtain all time points from a single infection. We assessed the variation in eL3 number over the 10-day period from 12 to 21 dpi. The main emergence phase started at 13 dpi, as we observed only a single eL3 in 48 mosquitoes at 12 dpi. Once L3 begin to emerge from mosquitoes, both the number that emerge per mosquito as well as the prevalence of mosquitoes with at least one emerging L3 are relatively constant from 13 to 21 dpi (Fig. 4). These data suggest that larvae develop synchronously and that once they reach the L3 stage, their numbers remain relatively stable over time. Beyond 13 dpi, mosquito mortality continues and eL3 prevalence or intensity is not increased. Therefore, to maintain population sizes and maximize eL3 yield we typically assay mosquitoes 14–17 dpi.

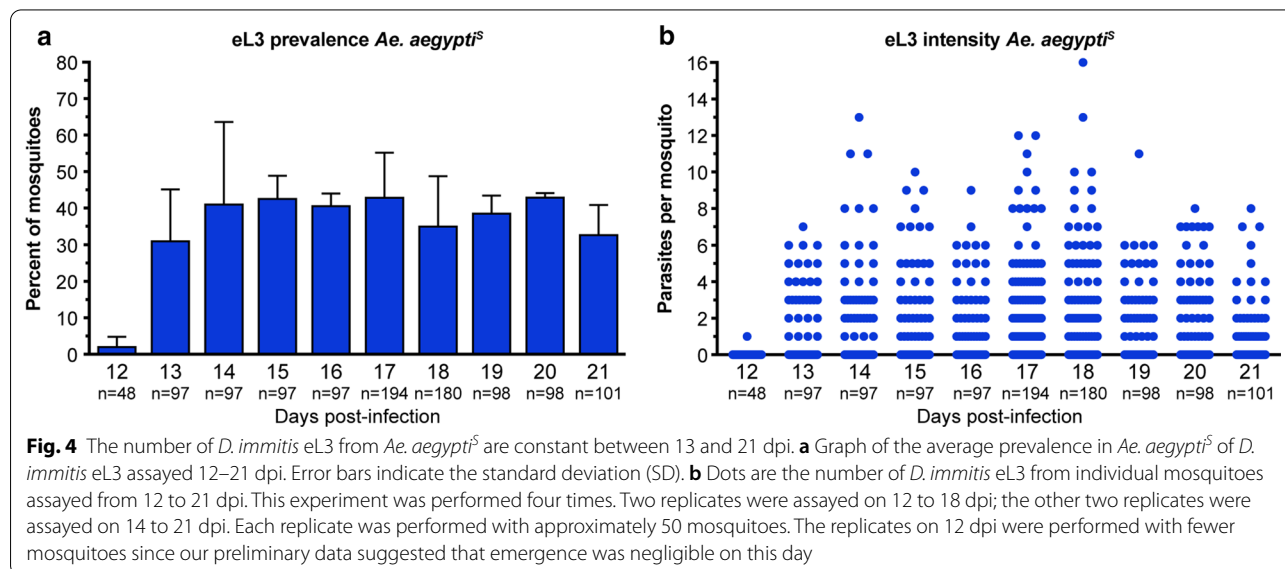
Increasing the number of *Dirofilaria immitis* microfilariae ingested does not increase eL3 prevalence

To optimize the number of eL3 recovered from the mosquito, we next determined if increasing the number of microfilariae in the blood meal improved the recovery of eL3. For these experiments we used the standard concentration of 4000 mf/ml and included three additional concentrations of 8000, 16,000 and 32,000 mf/ml. We found that increased concentrations of microfilaria in the membrane feeder had a consequent impact on the number present in the mosquito midgut immediately after blood-feeding, with no significant difference between *Ae. aegypti*^S and *Ae. aegypti*^R (Fig. 5a). At each successive concentration, microfilariae present in the blood meal increased approximately twofold. We monitored mosquito survival for 17 days, at which time an emergence assay was performed. We chose day 17 to allow more time to monitor mosquito survival. The prevalence of eL3 in *Ae. aegypti*^S was not significantly different, regardless of the concentration of microfilariae fed (Fig. 5b). On a population level, the number of eL3 produced per mosquito was not increased



by feeding concentrations of microfilariae greater than 4000 mf/ml (Fig. 5c). Increasing the concentration of *D. immitis* microfilaria used to infect *Ae. aegypti*^S increased mosquito mortality in a dose-dependent manner (Fig. 5d). Each increase in microfilaria concentration significantly increased mortality from the previous one, including the lowest concentration, which elevated mortality compared to those fed on uninfected blood. A similar dose–response was observed in the refractory strain; however, only the

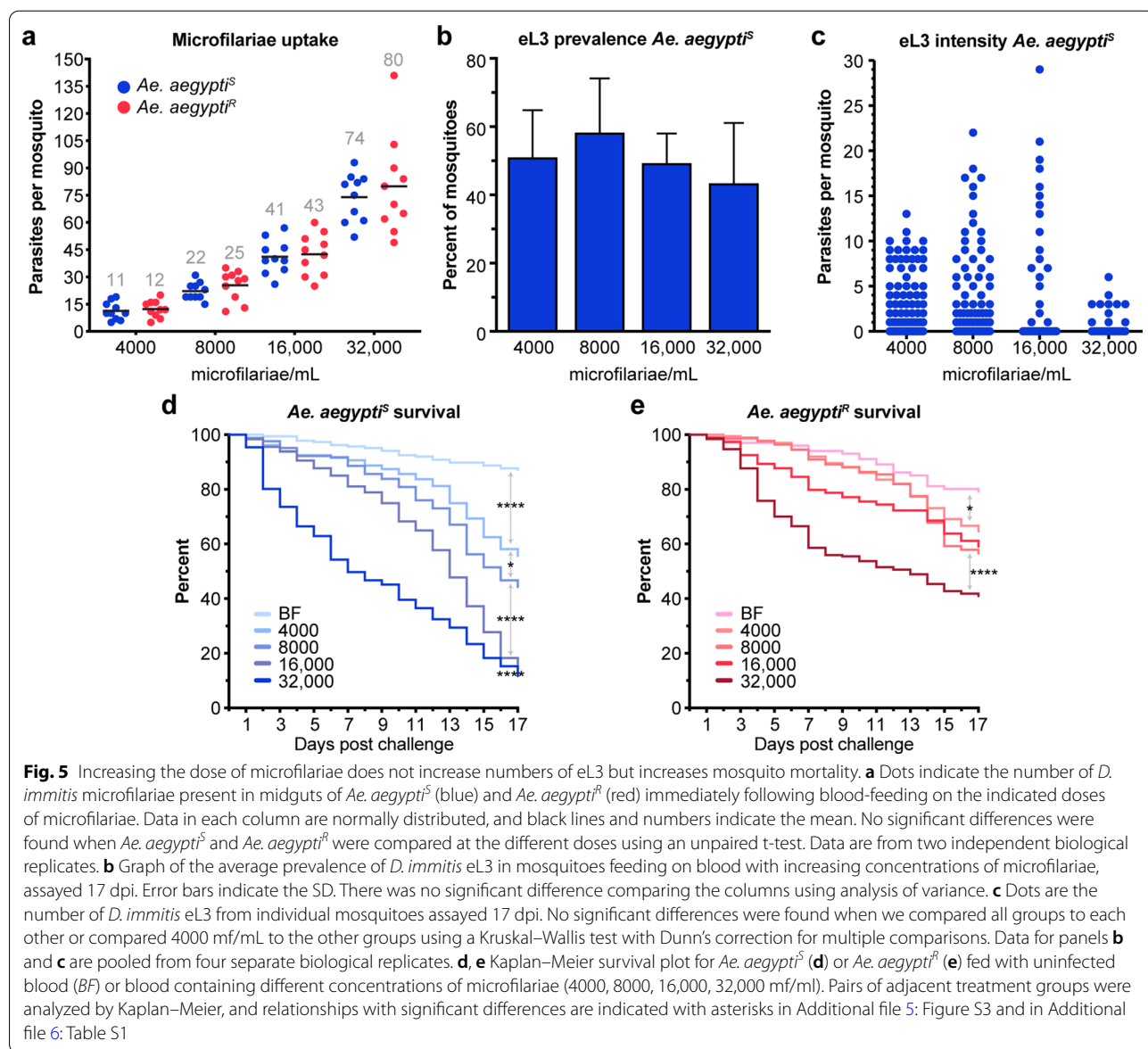
uninfected blood to 4000 mf/ml and 16,000 to 32,000 mf/ml comparisons were significantly different (Fig. 5e). When we compared the survival of *Ae. aegypti*^S and *Ae. aegypti*^R at the different concentrations of microfilariae, there was no difference in survival at the lowest concentration (Additional file 5: Figure S3). There was a modest difference at 8000 mf/ml, with *Ae. aegypti*^S showing greater mortality, and this difference further increased at the two highest concentrations.



Stored blood containing microfilariae retains its ability to infect mosquitoes and produce eL3

All *D. immitis* experiments described above used freshly isolated microfilaric blood. However, there are times when it is not possible to use this material immediately, such as when blood is shipped from another location. We wanted to compare the number of eL3 produced when mosquitoes were fed infected blood stored at 4 °C for 1 or 2 days. For these experiments, we fed mosquitoes with freshly isolated infected blood and on the next 2 consecutive days fed new cohorts with the same sample that had been stored at 4 °C. We performed an emergence assay at day 17 of each infection. There was no significant difference in the prevalence of eL3 between mosquitoes fed

fresh microfilaric blood and ones fed on an aliquot of the same blood sample stored for 1 day at 4 °C (Fig. 6a). However, there were significant decreases in eL3 prevalence in mosquitoes fed on microfilaric blood stored for 2 days at 4 °C compared to mosquitoes fed on blood stored at 4 °C for 1 day and to mosquitoes fed on fresh blood. Similar trends were observed in infection intensity (Fig. 6b). Our data show that although microfilaric blood stored at 4 °C for 2 days can still infect mosquitoes, there is a trend towards lower numbers of eL3 following blood storage, suggesting that refrigerated samples should be used as soon as possible for maximum infection potency.



Emergence assay works in a natural vector of *Dirofilaria immitis*

Since *Ae. aegypti* is not considered to be an important natural vector of *D. immitis*, we wanted to determine whether eL3 could be obtained from a mosquito species associated with *D. immitis* transmission. For these experiments we used a strain of *Ae. albopictus* isolated from Keyport, NJ, USA (*Ae. albopictus*^{NJ}). We found that eL3 were present at 17 dpi, but their prevalence and number were lower than those present in *Ae. aegypti*^S (Fig. 7a, b) when the mosquitoes were reared and housed in our standard insectary conditions (27 °C with 80% relative humidity [RH] and a 12:12-h photoperiod). When we tested *Ae. albopictus*^{NJ} reared and infected under its ideal temperature and humidity conditions (24 °C, 70% RH, and a 16:8-h [light/dark] photoperiod), the prevalence and number of eL3 were increased, although they were still lower than was typical for *Ae. aegypti*^S (Fig. 7c, d), likely because the strain of *Ae. albopictus*^{NJ} used did not feed to repletion from an artificial membrane feeder under any conditions that we tested. Even though we were not able to make quantitative comparisons to *Ae. aegypti*^S, our data show that the emergence assay works with different mosquito species.

Individual mosquito emergence assay for transmission stage *Brugia malayi*

To determine whether the emergence assay will be informative for enumerating eL3 for other filarial nematodes, we infected mosquitoes with *B. malayi*. These parasites have a shorter developmental period in the mosquito [24], so we performed our emergence assay at 12–14 dpi in both *Ae. aegypti*^S and *Ae. aegypti*^R mosquitoes. Despite taking up a similar number of microfilariae in the blood meal (Fig. 8a), no eL3 were produced by *Ae. aegypti*^R, showing that this strain is refractory to both *B. malayi* and *D. immitis* (Fig. 8b). In contrast, a significant proportion of *Ae. aegypti*^S mosquitoes developed eL3, showing that this strain is susceptible to both *B. malayi* and *D. immitis* (Fig. 8b). There was no significant difference in either the prevalence of mosquitoes producing eL3 or in the number of eL3 produced per mosquito between the days we assayed (Fig. 8b-c). These data indicate that despite their different life-cycle in the mosquito, eL3 of human filariae can be measured using our emergence assay.

Discussion

The development of an assay for eL3 may be valuable for studying mosquito–filaria interactions, thereby providing a clearer indication of vector competence and vector transmission intensity. As our data and a previously reported observation [5] indicate, only a portion of L3

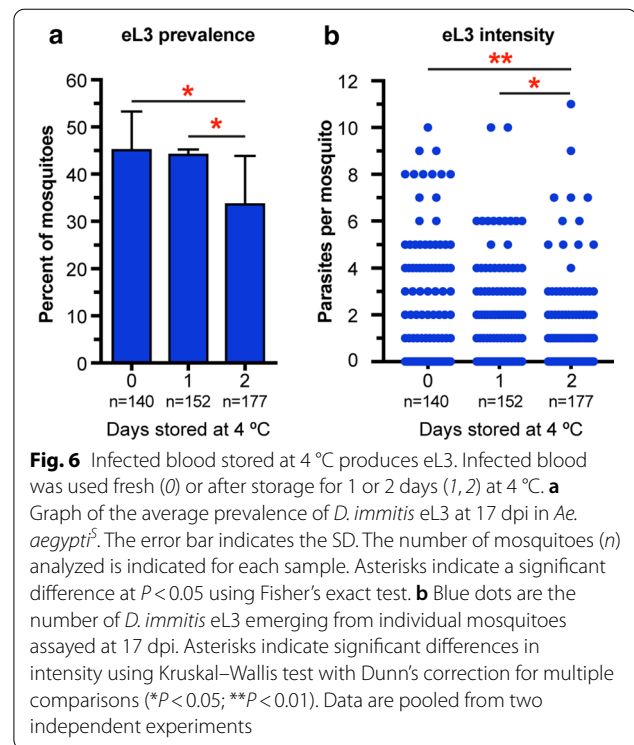
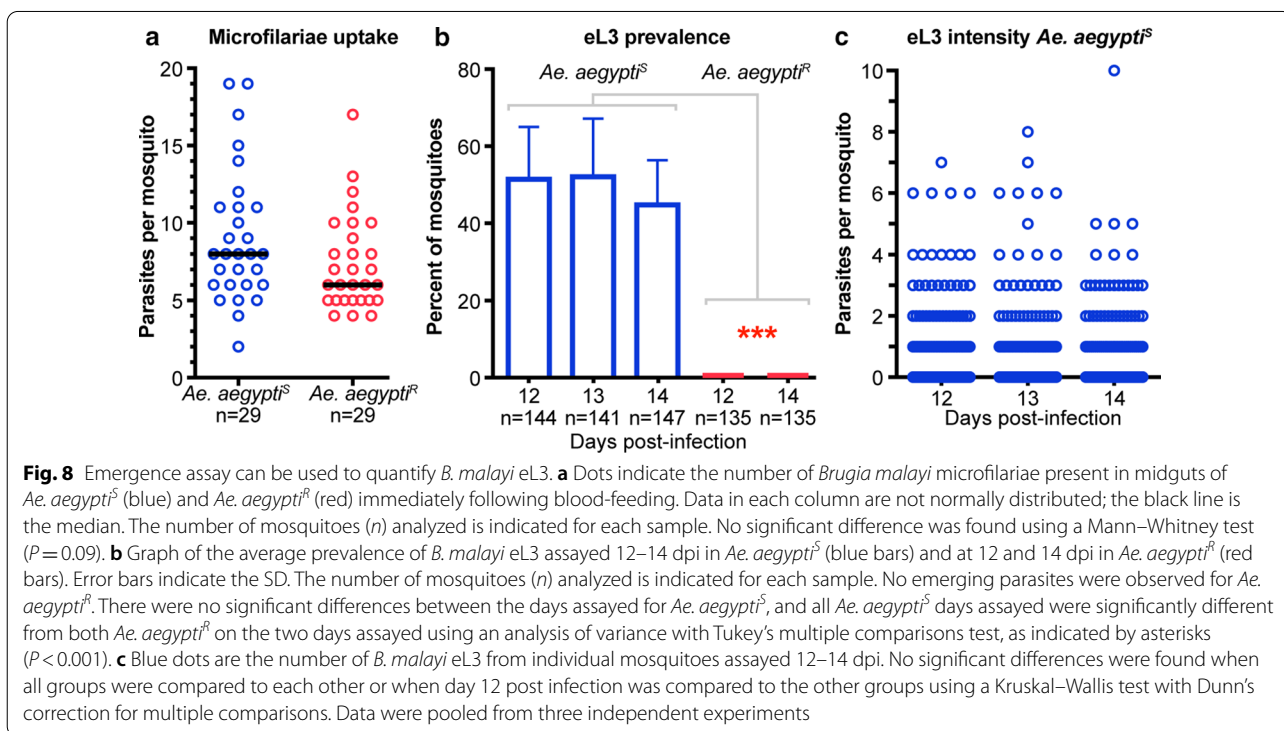
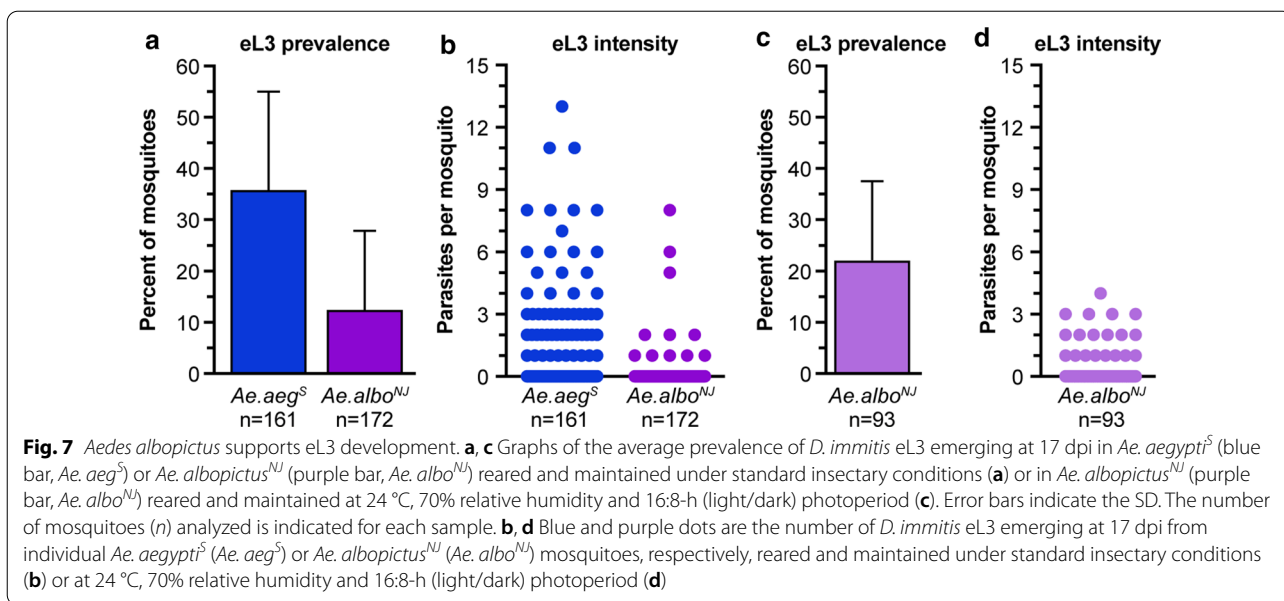


Fig. 6 Infected blood stored at 4 °C produces eL3. Infected blood was used fresh (0) or after storage for 1 or 2 days (1, 2) at 4 °C. **a** Graph of the average prevalence of *D. immitis* eL3 at 17 dpi in *Ae. aegypti*^S. The error bar indicates the SD. The number of mosquitoes (n) analyzed is indicated for each sample. Asterisks indicate a significant difference at *P* < 0.05 using Fisher’s exact test. **b** Blue dots are the number of *D. immitis* eL3 emerging from individual mosquitoes assayed at 17 dpi. Asterisks indicate significant differences in intensity using Kruskal–Wallis test with Dunn’s correction for multiple comparisons (**P* < 0.05; ***P* < 0.01). Data are pooled from two independent experiments

residing in the mosquito emerge when stimulated either by warming the mosquito or by direct contact with a mammalian host, even if already residing in the head. Our method provides a more accurate indication of a particular vector species’ potential for pathogen transmission. For example, our results show that not all strains of *Ae. aegypti* or *Ae. albopictus* support eL3 development, even though microfilariae invade the Malpighian tubules. In other studies, we used this assay to show that activation of the Toll immune signaling pathway restricts development of *D. immitis* and *B. malayi* eL3 [19, 25]. In addition, this assay could also permit assessment of manipulations of the mosquito host or larvae that result in an inability of L3 to emerge in response to thermal stimuli or due to motor defects that could be missed by current methods. Finally, the results of our assays suggest that quantitating all L3 by dissection will likely result in overestimating the number of larvae that are capable of emerging from the mosquito. This is particularly true in the case where the number of L3 is derived by mosquito homogenization, as L3 from the body cavity and those present in the Malpighian tubules are likely to be included in this method.

Overall, we found that 32% of the *D. immitis*-infected and approximately 50% of *B. malayi*-infected mosquitoes develop at least one eL3, which is similar to previous observations measuring emergence of L3 [26–28]. Since mosquitoes typically blood-feed more than once, it



is interesting to speculate that the L3 that do not emerge may enter the proboscis and emerge in a subsequent blood meal, thereby increasing the chance of transmission. On the other hand, it is also possible that the eL3 we observe are the only ones competent to emerge and infect another host. In this case, the failure of the remaining L3 to emerge could be due to competition for limited

resources in the vector or a parasite strategy to control the density of L3 to limit damage to the vector.

Results of our experiments to optimize recovery of eL3 by increasing the dose of *D. immitis* microfilariae given to mosquitoes reveal that microfilarial uptake and eL3 emergence are uncoupled, as we did not observe significant increases in the prevalence of mosquitoes with eL3

or the number of eL3 per mosquito at the doses tested. While it is likely that microfilarial uptake and eL3 emergence are coupled at lower concentrations of microfilariae, we did not test this since our main goal was to establish conditions where eL3 recovery is maximized to support future study of transmission-blocking strategies. Varying the timing of the assay revealed that at 13 dpi there is no significant difference in the prevalence or number of eL3. Taken together, our data suggest that the Malpighian tubules have a limited capacity to support *D. immitis* development and that no additional eL3 are produced once the limit is reached. However, we observed a range of developmental phenotypes in the Malpighian tubules of mosquitoes 14 days after *D. immitis* infection, raising the possibility that larval damage to the tubules prevents further development, as all larvae are viable at this time point, even those displaying delayed development phenotypes. While a substantial number of midgut epithelial cells are invaded during infection by ookinetes of *Plasmodium*, the causative agent of malaria, damaged cells are eliminated and replaced through tissue regeneration [29, 30]. As larval development is blocked and mosquito fitness is negatively impacted over time, this process suggests that damaged cells of the Malpighian tubules persist and are not replaced. In addition, the continuing metabolism of the increased number of larvae or an accumulation of larval waste products may also contribute to the increased mosquito mortality we observed in *Ae. aegypti*^S at later timepoints.

By quantifying microfilarial uptake in individual mosquitoes and comparing it to all parasites that emerge from either the whole mosquito, the head or the carcass or to those present in the Malpighian tubules at 14 dpi, we found that approximately 60% of the ingested microfilariae are accounted for at 14 dpi. Although we are accounting for a majority of the parasites ingested by the mosquitoes, it is nevertheless interesting to speculate about the fate of those that are not assayed. Some L3 remain in the proboscis of dissected heads, even after the primary emergence assay and the subsequent emergence assay after dissection. However, we did not systematically attempt to quantify these, and it remains possible that L3 may also be retained in the dissected carcasses. In addition, because some microfilariae may be damaged by the mosquito immune system or fail to follow sensory cues, they may fail to invade the Malpighian tubules and may be voided into the hindgut and then the feces [4, 31, 32]. Likewise, the activity of microfilariae and larvae in the Malpighian tubule cells may damage these cells, sometimes to the point where microfilariae are released into the tubule lumen, where they also could be eliminated as waste. Alternatively, if the basal side of the Malpighian tubule cell is compromised, parasites could be released

into hemolymph within the mosquito body cavity, where they could be eliminated or sequestered. As the possibilities outlined above are not mutually exclusive, we believe they could collectively account for the differential between microfilarial uptake and total parasites ultimately detected. Interestingly, it was recently shown using field populations of *Ae. albopictus*, a natural vector of *D. immitis*, that in areas of greater infection prevalence, larval damage to the mosquito is better tolerated, benefitting both the vector and the parasite [33].

We used our emergence assay to analyze the effects of storing microfilaric blood at 4 °C and showed that it is possible to produce eL3 after a storage time of at least 2 days, but likely even longer. However, while delays in initiating mosquito infection do not preclude experimentation, the strongest prevalence and intensity of eL3 production was observed in mosquitoes infected through the ingestion of fresh microfilaric blood. Given the loss of eL3 production with blood storage time, it is not recommended to make comparisons within an experiment using a blood sample stored for different lengths of time. However, internal comparisons are reasonable, as is the goal of using the stored blood to produce eL3.

Because L3 obtained in our assay have responded to physiological conditions, L3 produced via an emergence assay may provide a more easily accessible source of infective larvae than L3 obtained from gently crushed and sieved mosquitoes [34]. While we developed this emergence assay for use on single mosquitoes, it is also suitable for use on populations en masse, and eL3 obtained this way are known to be infective [17]. As such, eL3 may be quite valuable for future identification and testing of novel heartworm preventatives, which requires infectious *D. immitis* L3, as well as for studies of the host immune response to infection, which often require infectious L3. Similarly, *in vitro* L3 to L4 molting assays, such as the newly developed genetic transformation protocol for *B. malayi* [35], may benefit from L3 produced using our method. Finally, given the widespread nature of vector-borne filarial diseases and their potential to spread due to changing environmental conditions, new methods like this could help assess transmission potential in the field and to develop methods in the laboratory to block transmission. This easy, efficient and cost-effective method has the potential to be a valuable tool for the quantitative analysis of mosquito transmission of filariae, especially to researchers in countries most affected by these parasites where resources are often constrained.

Conclusions

We have established a novel assay to determine the number of infectious L3 filarial larvae capable of emerging from individual mosquitoes, which we refer

to as eL3. We have shown this assay works with both different mosquito and filarial nematode species. As such, our method is likely to be suitable for assessment eL3 of other mosquito transmitted filariae, such as *Dirofilaria repens*, a canine and human skin-dwelling relative of *D. immitis*, and *Wuchereria bancrofti*, which is responsible for approximately 90% of human lymphatic filariasis and can be transmitted by several genera of mosquitoes (*Culex*, *Aedes* and *Anopheles*). It remains to be determined if our assay will work for filariae transmitted by other arthropod vectors, such as biting flies, mites, fleas and ticks. In summary, the method we describe here will greatly facilitate current efforts to block transmission of arthropod transmitted filariae by enabling quantitative analysis in the vector as well as providing a more reliable way to harvest infectious larvae to evaluate new preventatives, treatments or vaccines.

Supplementary information

The online version contains supplementary material available at <https://doi.org/10.1186/s13071-020-04529-w>.

Additional file 1: Movie 1. Third-stage larvae visible moving in the proboscis of *Ae. aegypti*^S 14 days after infection with *D. immitis*.

Additional file 2: Figure S1. Mosquito dissection for analysis after emergence assay. After the emergence assay from the whole body (a), the head (b) and carcass (c) were placed individually into separate wells. The number of L3 larvae emerging from the dissected head and carcass were assayed after incubation at 37 °C. The Malpighian tubules were removed from the carcass prior to placing it in the well. Mosquito after removing head with fine forceps (d) and carcass after the Malpighian tubules (arrow) are dissected out (e). The scale bars in a–c and d, e are 500 and 1000 μm, respectively.

Additional file 3: Movie 2. Two eL3 in a well of a 96-well plate following an emergence assay performed on *Ae. aegypti*^S 14 days after infection with *D. immitis*. Another eL3 is visible at the top of the frame.

Additional file 4: Figure S2. Example of *D. immitis* larval lengths scored in Malpighian tubules. Malpighian tubules dissected following an emergence assay contain viable larvae of different lengths indicated with asterisks. Different stage larvae are typically present.

Additional file 5: Figure S3. *Ae. aegypti*^S have greater mortality than *Ae. aegypti*^R following *D. immitis* infection. Pairs of adjacent treatment groups from Fig. 5d, e were analyzed by Kaplan–Meier and relationships, with significant differences indicated with asterisks here and in Additional file 6: Table S1. *Aedes aegypti*^S and *Ae. aegypti*^R are indicated in blue and red, respectively.

Additional file 6: Table S1. Pairwise comparisons of mosquito survival following blood feeding on different doses of *D. immitis* microfilariae.

Abbreviations

BE, susceptible, *Ae. aegypti*^S; Blackeye Liverpool strain; LVP, refractory, *Ae. aegypti*^R; *Aedes aegypti* Liverpool strain; mf: Microfilariae; MT: Malpighian tubules; L3: Third-stage larvae; eL3: Emerging third-stage larvae.

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Authors' contributions

MP was responsible for the study conception. ARM, EBE, GTO, FMO and MP were responsible for acquisition of data. ARM, EBE, GTO, FMO, TJN, JBL and MP were responsible for the analysis and interpretation of data. MP and ARM were responsible for drafting and critical revision of the manuscript. EBE, GTO, FMO, TJN and JBL were responsible for critical revision of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

Blood containing *D. immitis* microfilariae was obtained from an experimentally infected dog according to Institutional Animal Care and Use Committee approved protocols and in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania (IACUC, protocol 805059).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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