A novel assay to measure the emergence of third-stage filarial nematodes in individual mosquitoes

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Abbreviations: *Aedes aegypti* Blackeye Liverpool strain (BE, susceptible, *Ae. aegypti*^{S}), *Aedes aegypti* Liverpool strain (LVP, refractory, *Ae. aegypti*^{R}), microfilariae (mf), Malpighian tubules (MT), third-stage larvae (L3), emerging third-stage larvae (eL3).

Abstract

Mosquitoes transmit filarial nematodes to both human and animal hosts, resulting in 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. An inability to determine what proportion of third-stage larvae are capable of emerging from the mosquito has hindered efforts to assess the efficacy of experimental transmission-blocking strategies and to evaluate the transmission competence of field vectors. To overcome this hurdle, we have developed a novel method to assess the infective third-stage larvae of *Dirofilaria immitis* that emerge from individual *Aedes aegypti* and *Ae. albopictus* mosquitoes. This method does not require specialized equipment, making it amenable for field work. Using this assay, we have determined the proportion of microfilariae that successfully develop into emerging third-stage larvae as well as the fate of those that do not. We have found that once *D. immitis* third-stage larvae emerge at 13 days post infection, the proportion of mosquitoes producing them, and the number produced per mosquito remain stable until at least day 21. However, during this time, the fitness cost of the infection to the mosquito increases, suggesting that larvae continue to either damage the vector, compete for essential nutrients, or produce toxic metabolites. Increasing uptake of *D. immitis* microfilariae increases the fitness cost to the mosquito but does not increase the number of emerging third-stage larvae. Notably, we find that our assay is also suitable for assessment of emerging third-stage larvae of *Brugia malayi*, one of the filarial nematodes responsible for human lymphatic filariasis. Thus, it is likely that this assay will be applicable to all mosquito-transmitted filariae and possibly filariae transmitted by other hematophagous arthropods. Together, our results indicate that this new assay will facilitate assessment of the transmission potential of arthropod vectors and promote preparation of uniformly infectious L3 for challenge experiments and other functional assays. It will also facilitate analyses of molecular interactions between vectors and filariae, ultimately allowing for the establishment of novel methods to block disease transmission.

Introduction

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 *Brugia 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 agent of canine and feline heartworm disease. Although detailed global numbers are not available, there are an estimated 250,000-500,000 infected dogs in the United States 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 only mild clinical signs [3].

Within the mosquito host, ingested microfilariae migrate from the midgut to specific tissues particular to the species of the filariid. The filarial agents of lymphatic filariasis migrate to the indirect flight muscles in the thorax whereas *D. immitis* migrates into the Malpighian tubules [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 (supplemental movie 2). Infective third-stage larvae emerge most typically from the mosquito labellum but can also emerge from the labial sheath during blood feeding (Fig. 1) [5]. Thermal, chemical, and mechanical cues that arise during blood feeding trigger larval emergence, though the exact contribution of these particular cues remains unknown. Larvae emerging from the mosquito are deposited on the skin in a drop of hemolymph where they can enter the skin through the bite site [5].

Due to its 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 to use PCR to detect larvae in the head and other body parts following dissection [6-8]. This method is sensitive, species-specific, and can be used on pools of mosquitoes in cases where the 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 for determining the number of larvae at each developmental stage and identifying 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 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 larvae 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 using mesh [9-11]. However, it is unknown whether all L3 harvested by this method are mature and competent to infect the host. As blocking parasite development in the vector is a novel approach being considered for controlling disease transmission, understanding the molecular interactions between parasites and their vector is essential for transmission-blocking strategies. The ability to assay the prevalence of mosquitoes with emerging L3 and to assess the number of emerging L3 per mosquito would greatly facilitate this work. Here we present a newly developed assay that works with different vector and filarial species and reveals the number of L3 that are capable of emergence in individual mosquitoes We then use this assay to study the development and characteristics of emerging L3 *D. immitis* in mosquitoes.

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 determine the number of microfilariae ingested immediately afterwards [12]. 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 landing on a mammalian vertebrate host by placing mosquitoes in buffer and warming to 37°C [13]. 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 and supplemental movie 1). Emerging L3 larvae (hereafter referred to as eL3) collected by this method are capable of molting to the L4 stage in vitro [14] and are infectious to dogs [15]. Notably, following the emergence assay, further analysis can be carried out to determine the number, location, and developmental stage 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 below (Fig. S1).

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

To determine the fate of ingested microfilariae in *D. immitis* susceptible (*Ae. aegypti*^{S}) or refractory (*Ae. aegypti*^{R}) strains, we infected mosquitoes and performed an emergence assay 14 days post-infection. Consistent with their resistant phenotype, we found that *Ae. aegypti*^{R} released no eL3, despite having a median uptake of 12 microfilariae per mosquito (Fig. 3A). However, in the susceptible strain (*Ae. aegypti*^{S}), 32% of the mosquitoes had at least one eL3 when given a median uptake of 15 microfilariae per mosquito (Fig. 3B). However, a substantial number of L3 remained in the dissected heads and carcasses of susceptible mosquitoes following the emergence assay (Fig. 3B). The majority of larvae assayed at day 14 were present in the Malpighian tubules and nearly all of the dissected mosquitoes (93 of 96; 97%) had at least 1 larva present in the Malpighian tubules (Fig. 3B). Pooling all larvae present across all tissues revealed that the median total number of larvae per mosquito was 8. Therefore, at day 14 post infection, 53% of the 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. Larvae present in the tubules were categorized as sausage, intermediate, or L3 form (Fig. 3C, Fig. S2) and subsequent enumeration revealed that the sausage and intermediate forms are the most abundant, at 51% and 30%, respectively. The remaining 19% of larvae are as long and as active as eL3. Thus, our results revealed that greater than 80% of the larvae remaining in the Malpighian tubules of susceptible-strain mosquitoes are arrested or developmentally delayed.

Moreover, all larvae found in the Malpighian tubules, even the most stunted sausage forms, are viable, as they were capable of movement.

Emergence assay does not account for all L3

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 plus 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 is significantly lower than the total L3 (Fig. 3D). Therefore, only a fraction of the larvae that develop to L3 emerge during our emergence assay, consistent with previous observations [5]. These data distinguish eL3 from other L3 and suggests that only a subpopulation of L3 are capable of emerging. This assay will facilitate development of new ways to manipulate mosquitoes to block L3 emergence, as well as increase our understanding of what makes a larva capable of emerging, which is not possible using extant methods.

Once eL3 have developed, their intensity and prevalence are constant

We wanted to determine the optimal day to perform the emergence assay and establish when in the course of infection, L3 are first capable of emerging. To do this, we infected large populations of mosquitoes and then removed groups of approximately 50 mosquitoes on consecutive days for an emergence assay. Four independent experiments were pooled since it was not possible to obtain all time points (day 12 to day 21) from a single infection. The emergence phase clearly started at day 13, as we observed only one larva emerging from one mosquito out of 48 prior to this time (at day 12 post-infection). We next assessed the variation in eL3 number over the 10-day window in which larvae are competent to emerge. Interestingly, we found that 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 days 13-21 post infection (Fig. 4). These data suggest that larvae develop synchronously and that once they reach the L3 stage, their numbers remain relatively stable over time. In these experiments, we also monitored mosquito survival. Since mosquitoes were removed on days 12-21, we expressed mortality as the percentage of the population that died daily. In the first few days of infection, the rate of mosquito death was low. However, there was a moderate increase in death from days 4-12, after which, there was a marked increase in daily mosquito mortality coinciding with the presence of eL3, (Fig. S3). More detailed analysis of how infection burden impacts mosquito mortality will be discussed below.

Increasing the number of D. immitis microfilariae ingested does not increase eL3 prevalence

To optimize the number of eL3 recovered from the mosquito we next tested the effect of increased numbers of microfilariae in the blood meal on the development of transmission stage parasites. 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, roughly twice as many microfilariae were present in the blood meal from the previous concentration. 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 equivalent 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). However, the maximum number of eL3 from an individual mosquito did increase with microfilarial concentration in the bloodmeal, with the maximum for the 4000, 8000, and 16,000 mf/mL groups being 13, 22, and 29, respectively (Fig. 5C). That trend was abolished in the group fed with 32,000 mf/mL, which only had a maximum value of 6.

Increasing the number of D. immitis *microfilariae ingested increases mosquito mortality in both susceptible and resistant Ae. aegypti.*

Even at the lowest concentration of microfilariae, mortality in *Ae. aegypti*^{S} was elevated compared to that in mosquitoes fed on uninfected blood (Fig. 5D). There was also a dose-dependent increase in *Ae. aegypti*^{S} mortality at each increasing concentration of microfilariae in the bloodmeal (Fig. 5D). In general, *Ae. aegypti*^{R} were less likely to die after infection compared to *Ae. aegypti*^{S}; however, there was a significant increase in mortality among *Ae. aegypti*^{R} fed the lowest concentration of microfilariae relative to *Ae. aegypti*^{R} *fed* with uninfected blood (Fig. 5E). The was no difference in mortality in *Ae. aegypti*^{R} between those taking bloodmeals with 4000 and 8000 mf/mL or between those with 8000 and 16,000 mf/mL in their bloodmeals. By contrast there was a significant difference in mortality between groups fed on blood containing 16,000 and 32,000 mf/mL (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 (Fig. 5F). However, there was a modest difference at 8000 mf/mL, with *Ae. aegypti*^{S} showing greater mortality (Fig. 5G), and this difference further increased at the two highest concentrations (Fig. 5H-I). These data suggest that as *D. immitis* continues to develop and increase in size in *Ae. aegypti*^{S}, the parasites can significantly damage the mosquito Malpighian tubules, compromising their function and resulting in even greater damage in susceptible strains.

Alternatively, the continuing metabolism of the larvae in *Ae. aegypti*^{S} may pose an accumulating resource constraint on the mosquito or an accumulation of larval waste products that are deleterious to mosquito fitness. The data from *Ae. aegypti*^{R} also suggest that Malpighian tubules are unable to regenerate and that once damaged, they remain functionally compromised for the remainder of the mosquito's life.

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

All *D. immitis* experiments described above used freshly isolated microfilaremic 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 4ºC for one or two days. For these experiments, we fed mosquitoes with freshly isolated infected blood and on the next two consecutive days with the same sample that had been stored at 4°C. We performed an emergence essay at day 17 of each infection. There was no significant difference in prevalence of eL3 between mosquitoes fed fresh microfilaremic blood and ones fed on an aliquot of the same blood sample stored for one day at 4ºC (Fig. 6A). However, there were significant decreases in eL3 prevalence in mosquitoes fed on microfilaremic blood stored for two days at 4° C compared to mosquitoes fed on blood stored at 4° C for one day and to mosquitoes fed on fresh blood. Similar trends were observed in infection intensity (Fig. 6B). Our data show that although blood stored at 4ºC for 2 days still retains the ability to robustly infect mosquitoes, there is a trend towards lower numbers of eL3 following storage of blood, suggesting that refrigerated samples should be used as soon as possible for maximum infection potency.

Emergence assay works in natural vector of Dirofilaria immitis

Since *Ae. aegypti* is not considered 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, New Jersey (*Ae. albopictus*^{NJ}). We found that eL3 were present at day 17, but the prevalence and number of eL3 were lower than those of *Ae. aegypti*^{S} (Fig. 7A-B) when the mosquitoes were reared and housed in our standard insectary conditions, 27º C with 80% relative humidity and a 12:12 h photoperiod. When we tested *Ae. albopictus*^{NJ} reared and infected at its ideal temperature and humidity (24ºC, 70% relative humidity, and a 16:8 h photoperiod), the prevalence and number of eL3 were increased, although they were still lower than typical for *Ae. aegypti*^{S} (Fig. 7C-D). This is 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 are 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 [16], so we performed our emergence assay on days 12-14 post infection in both *Ae. aegypti*^{S} and *Ae. aegypti*^{R} mosquitoes. We found that 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 develop 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 lifecycle in the mosquito, eL3 of human filariae can be measured using our emergence assay.

Discussion

We have established a novel assay to determine the number of infectious L3 filarial larvae that are capable of emerging from individual mosquitoes, which we refer to as eL3. As we have shown this assay works with both different mosquito and filarial nematode species, we propose that this assay represents a universal method of quantifying eL3 in any mosquito and filarial nematode combination. As such, our assay may be particularly valuable for study of eL3 of *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),* but is understudied since there are no animal infection models to generate microfilariae. Our assay could also be used rapidly assess emerging zoonotic threats, such as *D. repens*, a causative agent of canine heartworm that is becoming increasingly prevalent throughout Europe. Finally, we speculate that our assay could be used to quantify eL3 for other arthropod transmitted filariae such as *Loa loa* and *Onchocerca volvulus*, which are transmitted by the biting flies, *Chrysops* spp. and *Simulium* spp. as well as for other filariae including *Litomosoides carinii*, *Acanthocheilonema reconditum,* and *Acanthocheilonema viteae* that are transmitted through arthropod vectors with different feeding behaviors, such as mites, fleas, and ticks.

Development of an assay for eL3 is particularly exciting since it provides a clearer indication of vector competence and vector transmission intensity. From our data and a previously reported observation [5], not all L3 residing in the mosquito emerge when stimulated either by warming the mosquito or by direct

contact with mammalian host. This is true even for L3 residing in the head. Therefore, this method may provide a more accurate indication of a particular vector species' potential for pathogen transmission. For example, in a field setting, estimates of eL3 prevalence by this assay could reveal whether pathogen transmission is actually possible for a particular vector species. Similarly, estimates of eL3 intensity from the assay would provide more easily acquired and more accurate data for input into calculations of transmission indices than estimates from manual vector dissection. In our own experiments, we have found that not all strains of *Ae. aegypti* or *Ae. albopictus* support eL3 development, even though microfilariae invade the Malpighian tubules. In addition, this assay would permit assessment of important late phenotypes in the laboratory. For example, 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 could be missed by current methods. Finally, results from our assays suggests 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 included.

While we developed this emergence assay for use on single mosquitoes, it is also suitable for use on populations en masse. This will greatly facilitate field studies, as it permits assessment of wild mosquito populations in the field without specialized equipment or sophisticated technical expertise. In this case, pools of mosquitoes could be assayed initially and in areas with higher rates of infected mosquitoes, pool size could decrease, ultimately down to individual mosquitoes if needed. Since eL3 sink to the bottom of the culture dish, this pooling strategy could potentially be performed using a funnel, similar to a Baermann apparatus, to increase screening efficiency. As this assay can be performed on individual mosquitoes and is non-destructive, detailed taxonomic or molecular species identification could be done following individual emergence assays and restricted to the most informative specimens i.e. those producing eL3. The emerging larvae could also be saved for further characterization or molecular analysis.

Results of our experiments to optimize recovery of eL3, in which we manipulated the dose of *D. immitis* microfilariae given to mosquitoes, support 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 microfilariae, we did not test this since our main goal was to establish the conditions to study transmission-blocking strategies where a greater percentage of mosquitoes with eL3 is desired. Varying the timing of the assay supported that after day 13 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, once the limit is reached, no

additional eL3 are produced. 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 [17, 18]. As larval development is blocked and mosquito fitness is negatively impacted over time, it 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 are present in the Malpighian tubules on day 14, we found that approximately 60% of the ingested microfilariae are accounted for at day 14. Although we are accounting for a majority of the parasites ingested by the mosquitoes, it is nevertheless interesting to speculate about the fate of the 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, 19, 20]. 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.

We used our emergence assay to analyze the effects of storing blood microfilaremic blood at 4ºC and showed that it is possible to produce eL3 after storage for at least two 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 by ingestion of fresh microfilaremic 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 eL3 obtained in our assay have responded to physiological conditions and emerged via a natural route of egress, we believe that eL3 may provide an easily accessible source of L3 that more efficiently infect the host than L3 larvae obtained from sieved, gently crushed mosquitoes [21]. In support of this hypothesis, studies using eL3 harvested according to the protocol described in this paper result in more adult heartworms in experimentally infected dogs [15, 22] compared to studies using the same number of L3 isolated by the gentle crush method [9-11]. 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 [23], may benefit from a more physiologically derived population of L3. Even if the L3 selected following the gentle crush can be as efficient for infection studies or other functional assays, the method described here will likely be more consistent as it eliminates nuanced techniques, such as crushing mosquitoes to liberate L3, without damaging the larvae and eliminates potential bias that can be imposed by selection of L3s based appearance and movement.

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. Finally, it will help in field studies to assess filariae transmission potential by providing a low cost, straightforward assay that can be utilized on large groups of mosquitoes en masse to determine quantitative transmission indices.

Materials and Methods

Mosquito strains and culture

Ae. aegypti and *Ae. albopictus* strains were provided by the NIH/NIAID Filariasis Research Reagent Resource Center for distribution by BEI Resources, NIAID, NIH. *Ae. aegypti*^{S} (*Ae. aegypti*, Strain Black Eye Liverpool, Eggs, NR-48921) is a *D. immitis* and *B. malayi* susceptible strain. *Ae. aegypti*^{R} (*Ae. aegypti*, Strain LVP-IB12, Eggs, MRA-735, contributed by David W. Severson) is a *D. immitis* and *B. malayi* refractory strain. Both strains were reared at 27ºC and 80% humidity with a 12-hour photoperiod. *Ae. albopictus*^{NJ} (*Ae. albopictus* Strain ATM-NJ95, Eggs, NR-48979) [24] was reared at 24 ºC and 70% humidity with 16:8-hour photoperiod. Mosquitoes were housed in 30 cm^3 cages (Bugdorm, 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) and adults were maintained with 10% sucrose in water changed daily. Heparinized sheep blood (Hemostat, Dixon, CA) was provided using an artificial membrane feeder at 37ºC for egg production.

Mosquito infection with Dirofilaria immitis and Brugia malayi

A full protocol with greater detail is available online (dx.doi.org/10.17504/protocols.io.bb7birin) [12]. 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 microfilaremia in this dog is approximately 50,000 mf/mL. Blood containing *B. malayi* microfilariae (*B. malayi* microfilariae in cat blood, live, NR-48887) was obtained from an experimentally infected cat containing approximately 12,000 mf/mL provided by the NIH/NIAID Filariasis Research Reagent Resource Center for distribution by BEI Resources, NIAID, NIH. In both cases, blood was diluted with the appropriate volume of heparinized sheep blood to a concentration of 4000 mf/mL. The sample was warmed to 37ºC and 3.5 mL was placed in the indentation on the outside of the bottom of a 300 mL plastic baby bottle (Advent, Phillips, Amsterdam, NL) and covered with Parafilm. The inside of the bottle was filled with water at 37º C. This assembly was placed with the membrane side down on the top mesh of mosquito cages and the insects are allowed to feed for 15 minutes (Fig. 2A). Immediately following the feed, blood fed mosquitoes were separated using CO_2 anesthesia and housed under standard insectary conditions described above.

Microfilarial uptake

Within an hour of blood feeding, fed mosquitoes were anesthetized with carbon dioxide 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 µL 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 µL pipette. Microfilaria in the entire drop were counted immediately without a coverslip.

Emergence assay for eL3 and other larval stages

A full protocol with greater detail is available online (dx.doi.org/10.17504/protocols.io.bb7airie) [13], which is a modified from a previously published method [15]. Briefly, eL3 were assayed by placing live mosquitoes in 70% ethanol in water for 1 min. Afterward the mosquitoes were rinsed twice in deionized water and then placed individually into wells of a 96-well plate containing 200 µL Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine, high glucose and sodium pyruvate (Corning Mediatech, Manassas, VA). The plate was placed in a 37ºC incubator with 5% CO2 for 60 minutes and the number

of eL3 was determined without removing the mosquitoes using an inverted microscope with a 4x 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 µL PBS and covered with a 22 mm^2 coverslip. The larvae were scored by size and shape as “sausage”, “intermediate”, and “L3” (Fig. S2). The head was separated from the body. The dissected head and carcass fragment were placed separately into fresh wells of a 96-well plate prepared and incubated as described above. L3 larvae were allowed to migrate out of the dissected tissue for at least 60 minutes and scored as described above. Only a portion of the mosquitoes used for the eL3 emergence assay were processed in this manner.

Emergence assay time course and microfilaria concentration series

Groups of 300-500 mosquitoes were fed on blood containing *D. immitis* microfilariae at different concentrations and housed as described above. Mosquito mortality was monitored daily. Mortality was assessed by determining 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 is a higher eL3 prevalence. For concentration series experiments, a blood dilution containing 32,000 mf/mL was created and then subjected to two-fold serial dilution to create feeding doses of 16,000 mf/mL, 8000 mf/mL, and 4000 mf/mL. For 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.

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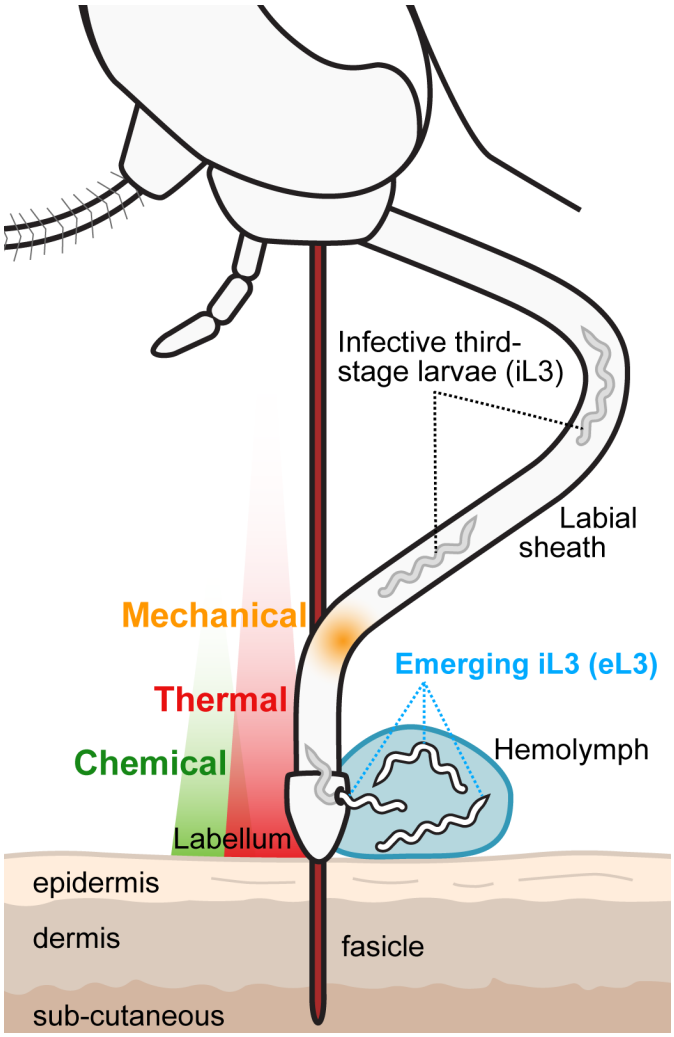

Fig. 1. **Emergence of iL3 from tip of the mosquito proboscis.** During blood feeding, a subpopulation -residing of iL3 in the labial sheath of the proboscis, emerge from the proboscis, alighting on the skin of the host in a drop of the mosquito hemolymph (eL3). Emergence from the proboscis typically occurs at the labellum or distal portion of the labial sheath and can be triggered by sensation of a thermal cue (red gradient). Other cues may also play a role, such as chemical compounds released by the host (green gradient), or by sensation of mechanical forces caused by the deformation of the labial sheath (orange gradient), which slides backward as the fascicle is inserted into the host skin. This figure is adapted from Bancroft B Med J, 1904 [25].

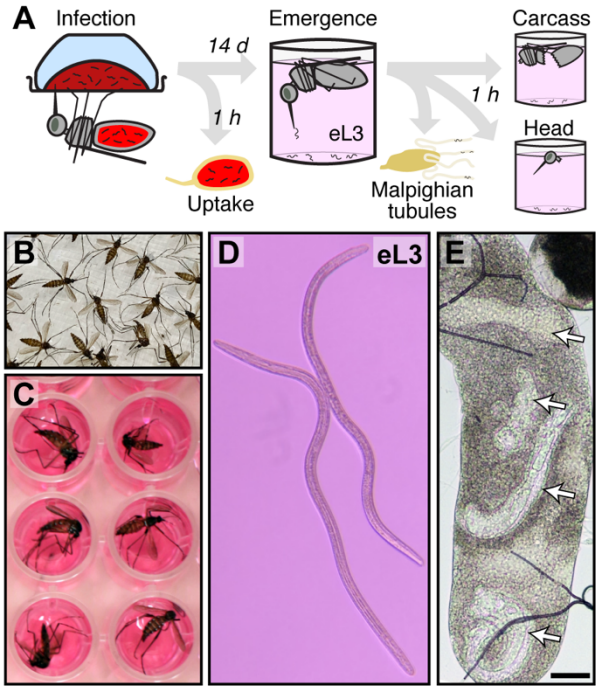

Fig. 2. **Assay for eL3.** (**A**) Infection and assay protocol followed in this study. Mosquitoes are fed on blood containing *D. immitis* microfilariae. Uptake is measured in a small group, and the remaining mosquitoes are maintained until an emergence assay is performed. Immediately following the 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. Figure S1 shows images of dissected mosquitoes and Malpighian tubules. (**B**) Mosquitoes are rinsed with 70% ethanol to wet, rinsed with water, and (**C**) placed individually into wells of a 96-well plate. (**D**) Emerging third-stage larvae (eL3) from intact mosquitoes or L3 from dissected heads and carcasses at the bottom each well are scored by microscopy. Supplemental movie 1 shows typical movement of eL3. (**E**) Larvae (white arrows) in live Malpighian tubules are scored by microscopy. Scale bar is 50 μm.

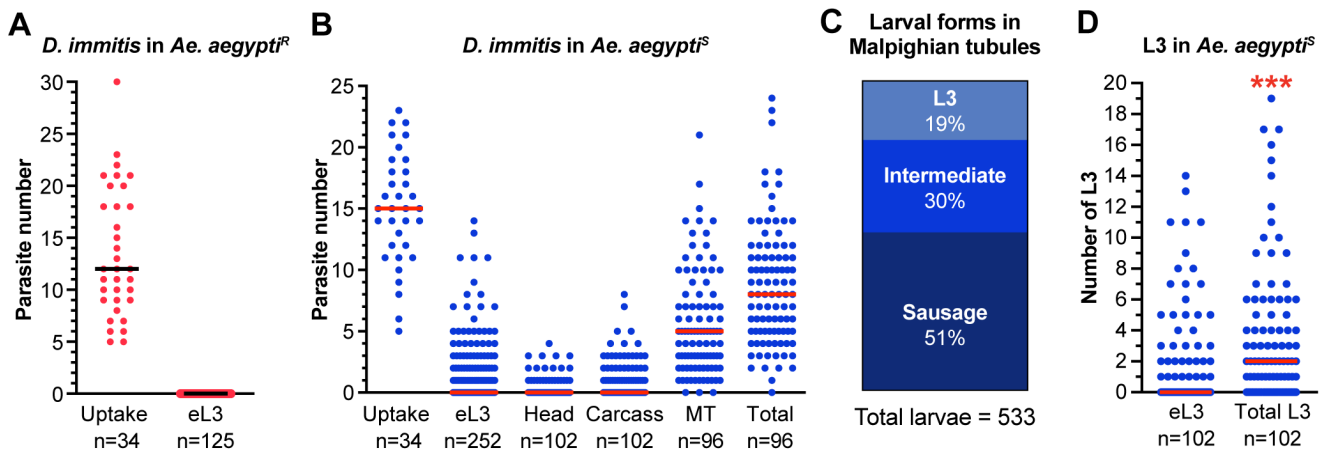

Fig. 3. **A fraction of ingested microfilariae develops into eL3 in *Ae. aegypti*^{S}.** (**A**) Dots indicate the number of microfilariae present in the midgut of individual *Ae. aegypti*^{R} immediately after feeding on infected blood (Uptake) and the number of eL3 assayed 14 days post infection. The black line indicates the median. (**B**) Dots indicate the number of microfilariae present in the midgut of individual *Ae. aegypti*^{S} immediately after feeding on infected blood (Uptake), the number of eL3 assayed 14 days post infection, the number of L3 emerging from the dissected heads (Head) or carcass (Carcass), and the number present in the Malpighian tubules (MT). The Total is sum of all parasites of any stage found in any tissue or assay on day 14. The red line indicates the median. (**C**) Graph indicates the percentage of larvae in the three developmental stages we scored (Fig. S2). Total larvae scored from all three is indicated. (**D**) Dots are the number of eL3 from individual *Ae. aegypti*^{S} and all third-stage larvae assayed (Total; sum of eL3, Head, and Carcass) (data taken from panel B). The red line is the median number. The asterisks indicate a Mann-Whitney P-value < 0.001. These data are the sum of three independent biological replicates. The number of mosquitoes assayed is shown below each column.

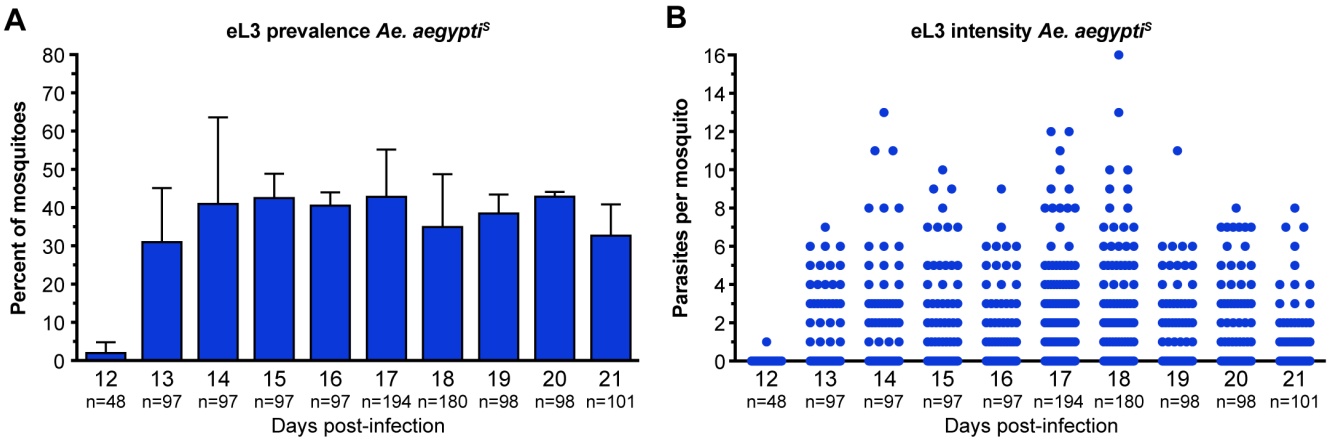

Fig. 4. The number of eL3 *D. immitis* from *Ae. aegypti*^{S} are constant 13-21 post infection. (**A**) Graph of the average prevalence in *Ae. aegypti*^{S} of *D. immitis* eL3 assayed 12-21 days post-infection. Error bars indicate the standard deviation. (**B**) Dots are the number of eL3 *D. immitis* from individual mosquitoes assayed 12-21 days post-infection. We performed this experiment four times. Two replicates were assayed on days 12-18. The other two replicates were assayed on days 14-21. Each replicate was performed with approximately 50 mosquitoes. The replicates on day 12 were performed with fewer mosquitoes since our preliminary data suggested that emergence was negligible on this day.

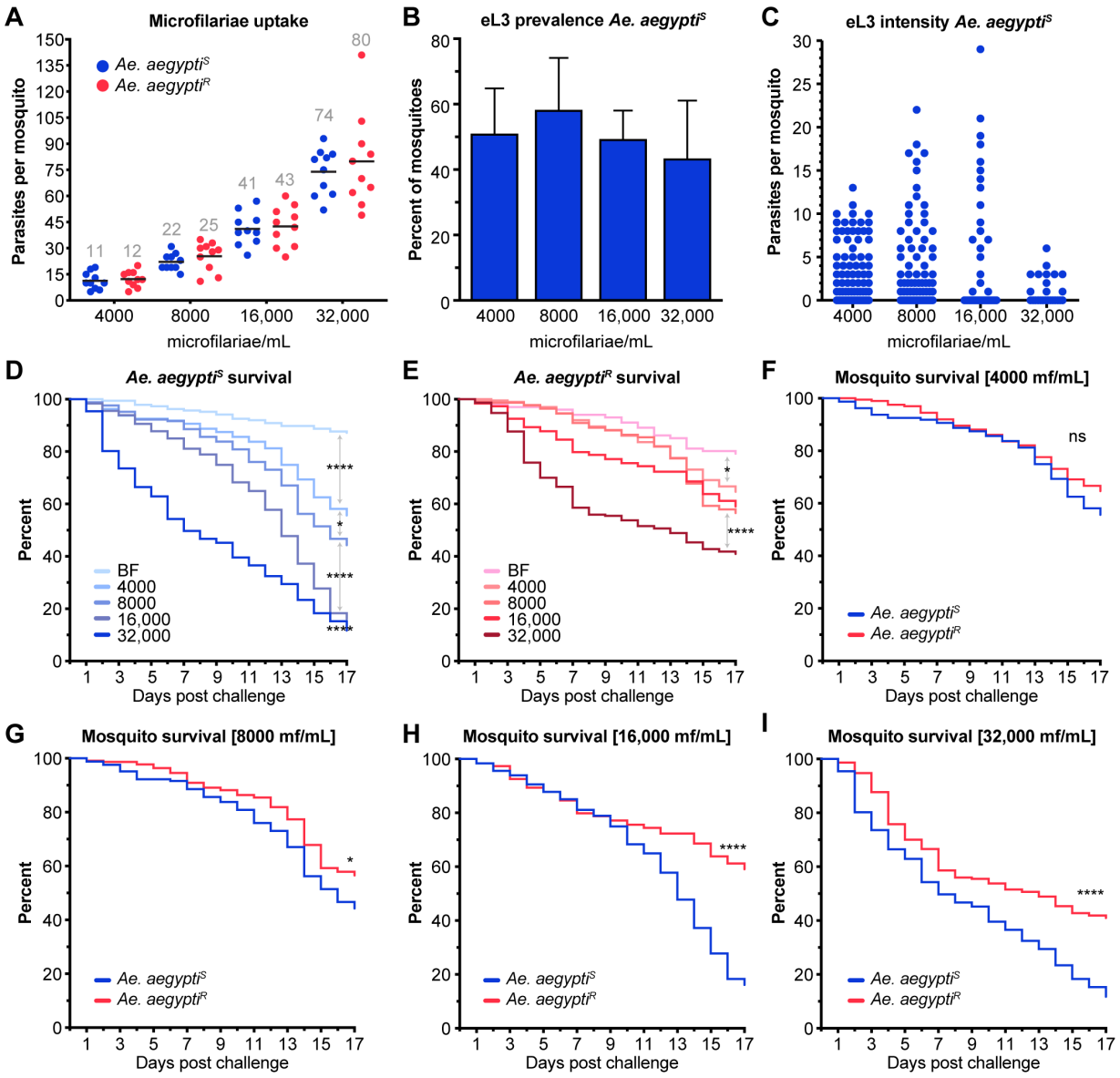

Fig. 5. Increasing the dose of microfilariae increases mosquito mortality but does not increase numbers of eL3. (**A**) Dots indicate the number of *D. immitis* microfilariae present in midguts of *Ae. aegypti*^{S} (blue) and *Ae. aegypti*^{R} (red) immediately following blood feeding on the indicated doses of microfilariae. Data in each column are normally distributed and black lines and numbers indicate the mean. No significant differences were found when *Ae. aegypti*^{S} and *Ae. aegypti*^{R} were compared at the different doses using an unpaired t test. Data are from two independent biological replicates. (**B**) Graph of the average prevalence of *D. immitis* eL3 in mosquitoes feeding on blood with increasing concentrations of microfilariae, assayed 17 days post-infection. Error bars indicate the standard deviation. There was no significant difference comparing the columns using and ANOVA. (**C**) Dots are the number of *D. immitis* eL3 from individual mosquitoes assayed 17 days post-infection. No significant differences were found when we compared all groups to each other or comparing 4000 mf/mL to the other groups using a Kruskal-Wallis test with Dunn's correction for multiple comparisons. Data for panels B and C are pooled from four separate biological replicates. (**D**) Kaplan Meier survival plot for *Ae. aegypti*^{S} or (**E**) *Ae. aegypti*^{R} fed with uninfected blood (BF) or blood containing different concentrations of microfilariae. Pairs of adjacent treatment groups were analyzed, and relationships with significant differences indicated with asterisks here and in Table S1. (**F-I**) Kaplan Meier analysis of data in panels D and E to compare survival of *Ae. aegypti*^{S} (blue) and *Ae. aegypti*^{R} (red) at different concentrations of microfilariae.

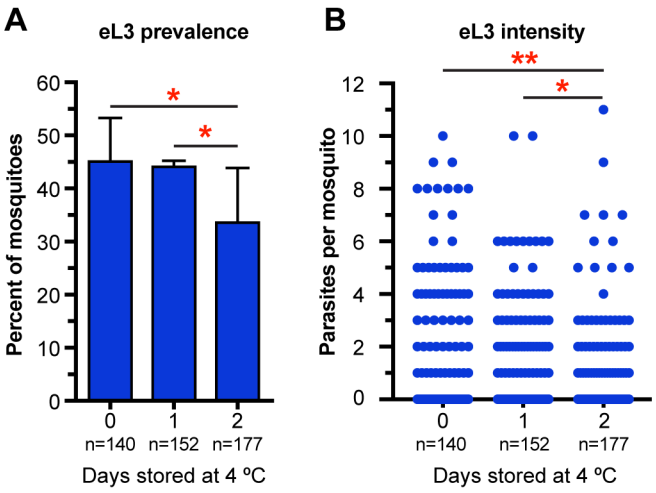

Fig. 6. **Infected blood stored at 4 ºC produces eL3.** Infected blood was used fresh (0) or after storage for 1 or 2 days at 4ºC. (**A**) Graph of the average prevalence of *D. immitis* eL3 17 days post-infection in *Ae. aegypti*^{S}. The error bar indicates the standard deviation. The number of mosquitoes (n) analyzed indicated for each sample. The asterisks indicate P<0.05 using a Fisher's exact test. (**B**) Blue dots are the number of *D. immitis* eL3 emerging from individual mosquitoes assayed 17 days post-infection. Significant differences in intensity using Kruskal-Wallis test with Dunn's correction for multiple comparisons are indicated with one and two asterisks for P<0.05 and <0.01, respectively. Data pooled from two independent experiments.

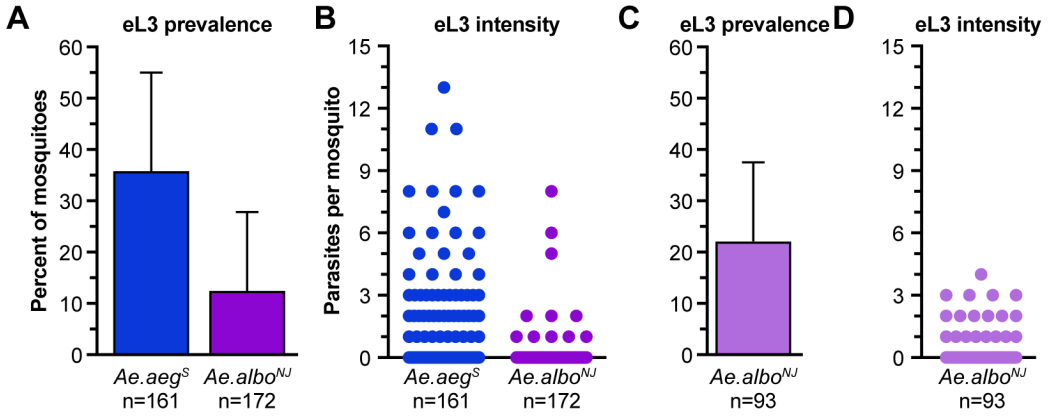

Fig. 7. ***Aedes albopictus* supports eL3 development.** (**A**) Graph of the average prevalence of *D. immitis* eL3 17 days post-infection in *Ae. aegypti*^{S} (blue, *Ae. aeg*^{S}) or *Ae. albopictus*^{NJ} (purple, *Ae. albo*^{NJ}). Error bars indicate the standard deviation. The number of mosquitoes (n) analyzed indicated for each sample. (**B**) Blue and purple dots are the number of eL3 *D. immitis* larvae emerging from individual *Ae. aegypti*^{S} (*Ae. aeg*^{S}) or *Ae. albopictus*^{NJ} (*Ae. albo*^{NJ}) mosquitoes assayed 17 days post-infection, respectively.

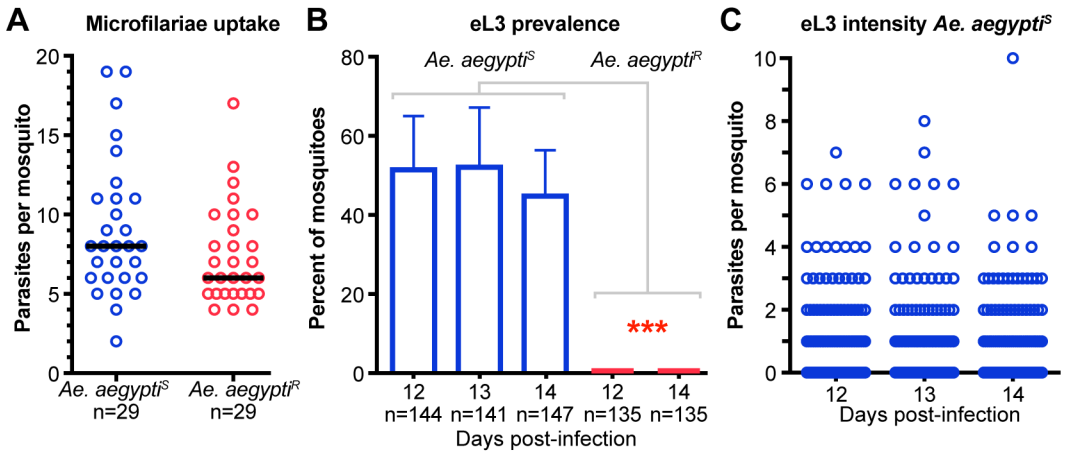

Fig. 8. Emergence assay can be used to quantify *B. malayi* eL3. (**A**) Dots indicate the number of *B. malayi* microfilariae present in midguts of *Ae. aegypti*^{S} (blue) and *Ae. aegypti*^{R} (red) immediately following blood feeding. Data in each column are not normally distributed and black line is the median. The number of mosquitoes (n) analyzed indicated for each sample. No significant difference was using a Mann Whitney test (P=0.09). (**B**) Graph of the average prevalence of *B. malayi* eL3 assayed 12-14 days post-infection in *Ae. aegypti*^{S} (blue bars) and at days 12 and 14 in *Ae. aegypti*^{R} (red bars). Error bars indicate the standard deviation. The number of mosquitoes (n) analyzed indicated for each sample. No emerging parasites were observed for *Ae. aegypti*^{R}. There were no significant differences between the days assayed for *Ae. aegypti*^{S}, and all *Ae. aegypti*^{S} days assayed were significantly different from both *Ae. aegypti*^{R} on the two days assayed using an ANOVA with Tukey's multiple comparisons test as indicated by asterisks (P<0.001). (**C**) Blue dots are the number of *B. malayi* eL3 from individual mosquitoes assayed 12-14 days post-infection. No significant differences were found when we compared all groups to each other or comparing day 12 to the other groups using a Kruskal-Wallis test with Dunn's correction for multiple comparisons. Data were pooled from three independent experiments.