METHOD ARTICLE

# Establishment of a method for *Lutzomyia longipalpis* sand fly embryo microinjection: The first step towards potential novel control strategies for leishmaniasis [version 1; peer review: 1 approved, 1 approved with reservations]

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**v1** **First published:** 09 May 2018, **3**:55
https://doi.org/10.12688/wellcomeopenres.14555.1

**Latest published:** 15 Aug 2018, **3**:55
https://doi.org/10.12688/wellcomeopenres.14555.2

## Abstract

Leishmaniasis is a vector-borne parasitic disease transmitted by sand flies that affects 1.3 million people across 98 countries, with limited control strategies due to the lack of an available vaccine and the emergence of insecticide resistance. Novel control strategies that are being explored for mosquito-borne diseases, such as *Wolbachia* bacterial inhibition of pathogens and genetically modified insects (e.g. using CRISPR-Cas9 editing), rely on the ability to consistently inject embryos of the target species. Here we present a novel method to obtain and inject preblastoderm sand fly embryos of the genus *Lutzomyia (Lu.) longipalpis*, the principle vector of zoonotic visceral leishmaniasis in South America. The procedures required to obtain sufficiently young *Lu. longipalpis* colony embryos are described alongside a microinjection technique that permits rapid injection and minimal handling of small sand fly embryos post-injection. Using a strain of *Wolbachia* as a 'marker' for successful injection, our protocol produced early generation *Wolbachia* transinfected *Lu. longipalpis* lines, demonstrating its potential as the first step for use in novel applied strategies for sand fly control.

## Keywords

Sand flies, leishmaniasis, embryo microinjection, Wolbachia

**Open Peer Review**

**Reviewer Status** ✓ ✓

| | Invited Reviewers | |
|---|---|---|
| | **1** | **2** |
| **version 2** (revision) 15 Aug 2018 | | ✓ report |
| **version 1** 09 May 2018 | ✓ report | ? report |

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**Competing interests:** No competing interests were disclosed.

**Grant information:** CLJ and TW were supported by a Wellcome Trust/Royal Society Sir Henry Dale fellowship awarded to TW (101285/Z/13/Z).
MER was supported by a BBSRC David Phillips Fellowship (BB/H022406/1).
*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*



**How to cite this article:** Jeffries CL, Rogers ME and Walker T. **Establishment of a method for *Lutzomyia longipalpis* sand fly embryo microinjection: The first step towards potential novel control strategies for leishmaniasis [version 1; peer review: 1 approved, 1 approved with reservations]** Wellcome Open Research 2018, **3**:55 https://doi.org/10.12688/wellcomeopenres.14555.1

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## Introduction

Leishmaniasis is a vector-borne tropical disease transmitted by phlebotomine sand flies. The causative agent is a kinetoplastid protozoan from the genus *Leishmania,* which can cause a spectrum of diseases, collectively referred to as leishmaniasis. Clinical features range from simple, self-healing or large, chronic skin ulcers (cutaneous and mucocutaneous leishmaniasis) to potentially fatal infection of the liver and spleen (visceral leishmaniasis). The clinical syndrome exhibited is influenced by the species of the infecting parasites, the genetic background of the host and extrinsic factors such as reservoir animal hosts, human migration and control strategies[1]. Leishmaniasis has been reported in 98 countries worldwide, putting an estimated 350 million people at risk of infection. Annually, leishmaniasis affects 1.3 million people, resulting in 20,000–40,000 deaths and an estimated 2.4 million Disability-Adjusted Life Years, where the highest burden on human health is amongst the poorest populations of society[2]. Currently there is no human vaccine available and the choice of effective drugs is limited.

Globally, vector control represents the major arm for leishmaniasis elimination, mainly through indoor residual spraying (IRS). In South America, zoonotic visceral leishmaniasis, caused by *Leishmania* (*Leishmania*) *infantum* (syn. *Leishmania chagasi*), is primarily transmitted by the neotropical sand fly *Lutzomyia (Lu.) longipalpis*. Although sand fly vector control strategies have historically been limited to small trials that have not reached large operational scale[3], recent trials have shown promise using a concentrated formulation containing the pyrethroid permethrin (an adulticide) and the larvicide pyriproxyfen. Although regular spraying can offer some protection to human populations[4], these programmes are often difficult to sustain, particularly in rural areas, where there are many potential resting sites requiring regular spraying. In Brazil, where over 90% of visceral leishmaniasis cases in South America occur, insecticide is applied only after a human case has been identified because of the logistics associated with spraying[5]. Consequently, insecticide-treated bed nets (ITNs) or long-lasting insecticidal nets (LLINs) offer a suitable, cost-effective alternative to IRS. Deltamethrin-impregnated bednets were shown to reduce the human landing rates of *Lu. longipalpis* and the application of permethrin-impregnated netting (Olyset®) showed good efficacy in the first hour, however, the effectiveness diminished over time[6]. A recent study using an adulticide-larvicide mixture of permethrin and pyriproxyfen (Dragon Max®) in neighbouring Argentina was effective at significantly reducing the number of *Lu. longipalpis*[7]. This formulation was effective for at least two weeks but further studies are required to determine if this formulation can have longer-term efficacy. However, the protection offered by treated nets in preventing human biting, and therefore *Leishmania* transmission, may be limited as *Lu. longipalpis* prefers to feed in the early part of the evening, before householders sleep under bed nets.

With the exception of *Phlebotomus argentipes*, the sand fly vector of anthroponotic visceral leishmaniasis in the Indian subcontinent[4], leishmaniasis vectors are highly susceptible to insecticides. However, the long-term feasibility of insecticide-treated materials is debatable due to logistical constraints (e.g., re-impregnation of materials), the potential for insecticide resistance[8] and the economic cost of these interventions[9]. In addition, methods of environmental management to reduce wild reservoir host numbers, e.g. destruction of rodent burrows[10], have been limited. In endemic areas where dogs are domestic reservoirs of cutaneous leishmaniasis, insecticide-impregnated dog collars could be an effective and feasible strategy[11]. The control of visceral leishmaniasis in the Americas has been further complicated by the urbanisation of *Lu. longipalpis*[5].

Research into novel non-insecticide based control strategies has been limited. The entomopathogenic fungus *Metarhizium anisopliae* was shown to have significant effects on egg hatching, survival of larvae and longevity of adult *Lu. longipalpis*[12]. Attractive toxic sugar baits have shown efficacy against other leishmaniasis vectors, including *Phlebotomus papatasi* in Iran[13] and Morocco[14]. Other potential control strategies that are yet to be explored include the use of the endosymbiotic bacterium *Wolbachia*, currently being used for mosquito biocontrol strategies given the ability of this naturally occurring bacterium to significantly reduce the vector competence of *Aedes (Ae.)* mosquitoes for arboviruses[15–20]. Alternative genetic strategies for mosquito control that could be applied to sand flies include the generation of sterile males that are then released to supress target populations[21] and the generation of transgenic lines that are refractory to pathogens using new genome editing tools such as CRISPR/Cas9[22].

Mosquito embryo microinjection has played an integral role as the first step in the development of novel control strategies that are undergoing preliminary field trials in arbovirus endemic countries (https://www.worldmosquitoprogram.org/, http://www.oxitec.com). *Wolbachia*-infected *Aedes* lines, including a super-infected line with two *Wolbachia* strains, have all been successfully generated using mosquito embryo microinjection[15,16,23–26]. Injection of young mosquito embryos has also been required for the successful genetic transformation of disease vectors[27–29]. The application of these novel vector control strategies for leishmaniasis requires the development of a protocol that would allow collection and injection of preblastoderm sand fly embryos. A key component of successful insect embryo injection is obtaining sufficient preblastoderm embryos that have not fully melanised as microinjection needles either are unable to penetrate or break upon contact with the hardened chorion of melanised embryos. Here we describe a method to obtain and microinject sand fly embryos of the genus *Lu. longipalpis*. We outline the steps required to collect sufficiently young *Lu. longipalpis* colony embryos and a method allowing rapid injection and minimal handling of small sand fly embryos post-injection. In order to determine the effectiveness of our protocol for targeting infection of the sand fly germline, we purified *w*Mel *Wolbachia* from *Drosophila melanogaster* embryos and used this endosymbiotic bacterium as a ‘marker’ for successful injection. Our protocol resulted in early generation *Wolbachia* transinfected *Lu. longipalpis* lines, demonstrating its potential to form the basis for novel control strategies for leishmaniasis sand fly vectors including both *Wolbachia*-based strategies and genetic modification.

## Methods

### *Lu. longipalpis* colony establishment and rearing

A laboratory strain of *Wolbachia*-negative *Lu. longipalpis* at the London School of Hygiene and Tropical Medicine was derived from a 30+ year closed colony, originating from Jacobina state, Brazil. Sand flies were maintained at 26–28°C, 12:12 h light:dark cycle, ~80% relative humidity. Larvae were fed an equal part autoclaved mixture of ground-up laboratory rodent food pellets and rabbit faeces. Adult flies were given access to 25% (w/v) sucrose throughout their life and were fed on defibrinated rabbit or human blood to obtain eggs. Bloodfed female flies were encouraged to lay eggs in plaster of paris-lined oviposition pots for 6–7 days in total darkness. Following removal of adult fly bodies, eggs hatched over 3–4 days. The average life cycle duration from egg to egg was 5–6 weeks.

### Oviposition chambers for embryo collection

Gravid females from 3 days post-bloodfeed were removed from cages using a mechanical aspirator and anaesthetised using carbon dioxide by placing the aspirator chamber directly on a *Drosophila* Flystuff Ultimate Flypad. The flow of carbon dioxide was reduced relative to anaesthetising adult mosquitoes to ensure sand flies were not killed by the anaesthesia. An oviposition chamber was generated by removing the bottom of a 50mL falcon tube (Corning®, CentriStar™, Corning Inc.) and replacing this with mesh netting secured with an elastic band (Figure 1a). A fine paintbrush was used to carefully transfer gravid anaesthetised females to the inside of an oviposition chamber laid on its side to avoid damage. Multiple oviposition substrates were made up in falcon tube lids allowing rapid change-over of substrate plates. During preparation, carefully pouring the substrate into the inner raised ring on the inside of the falcon tube lid, to form a substrate platform with a small gap around the edge, before allowing it to set, prior to use, enabled the falcon tube lids to be screwed easily and securely into the oviposition chambers.

As sand fly females typically lay their eggs in humid soil, rich in organic matter, and moisture can increase fecundity in laboratory colonies[30], we trialled three different substrates - including plaster of paris, the standard *Lu. longipalpis* colony larvae rearing substrate[31], and modified *Drosophila* embryo oviposition agarose-based substrates to determine if *Lu. longipalpis* would oviposit on either 2% apple juice agarose gel-based medium or 2% agarose gel prepared with water. For the plaster of paris substrate, a hole was punched through the falcon tube lid, prior to the plaster of paris being poured in to set, to enable humidity to be applied to the plates by placing them on wet paper towels. When appropriate, additional humidity was also applied to the

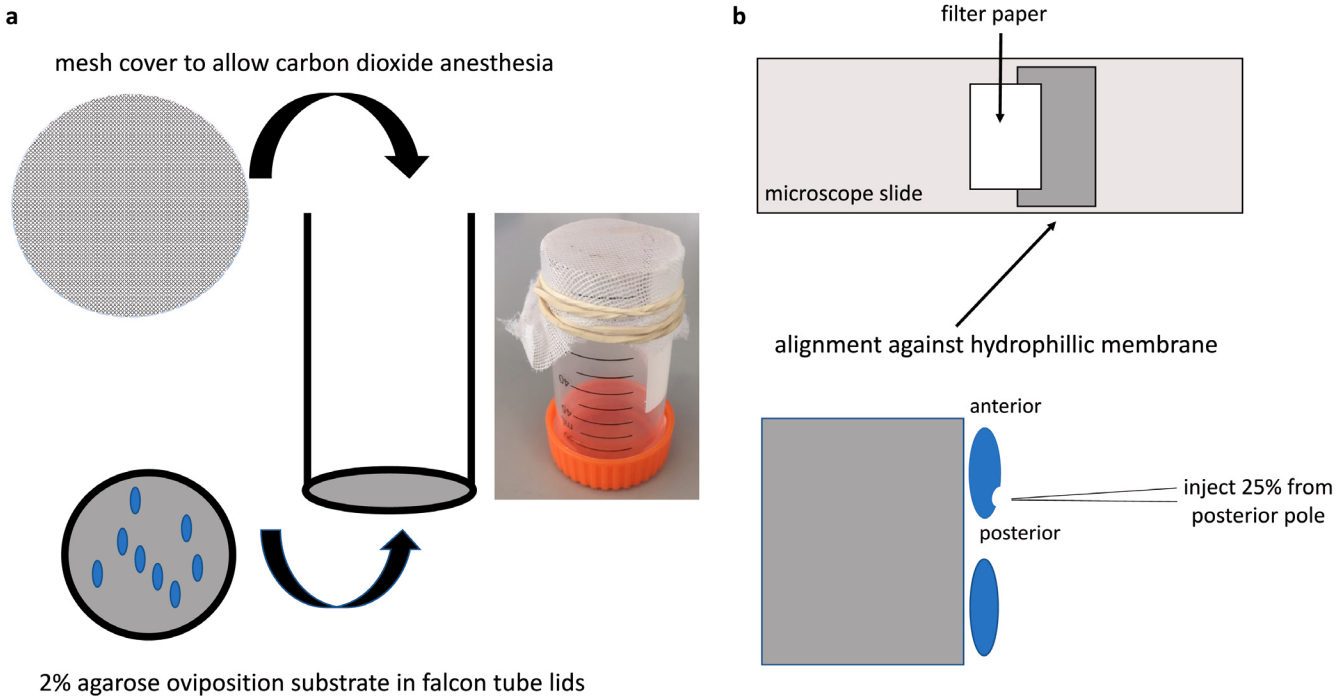


**Figure 1. Schematic representation of the key steps in the embryo microinjection protocol.** (**a**) *Lu. longipalpis* gravid females are removed from rearing cages using a mechanical aspirator and anaesthetised using carbon dioxide before placing on the side of an oviposition chamber. A mesh lid is secured and a falcon tube lid containing 2% agarose medium is screwed in to provide an oviposition substrate. After 45 minutes for oviposition, the mesh end of the chamber is placed on a carbon dioxide anaesthetising pad to allow exchange of oviposition substrate plates and removal of adult females prior to harvesting embryos from oviposition plates. (**b**) *Lu. longipalpis* young embryos are aligned against a hydrophilic membrane in contact with moist filter paper to prevent eggs from desiccating. Embryos are injected with microcapillary needles ~25% of the length from the posterior pole and slides containing injected embryos are transferred to humidified chambers.

plaster of paris lids through gently dropping small quantities of water on to the top of the plaster of paris, and allowing it to soak in at regular intervals to avoid it drying out.

Once transferred to the oviposition chambers, flies were left for 5 minutes or until there was evidence that they were actively walking or flying, before standing up the falcon tube on the lid. The chambers were then left for 45 minutes in the dark in a humidified box at 25°C to encourage oviposition. At the end of the oviposition period, sand flies were anaesthetised quickly using carbon dioxide for the shortest possible time and the oviposition substrate plates quickly exchanged to allow continued oviposition as required, and avoid mortality due to prolonged anaesthesia. Embryos were then harvested from the oviposition plates using very fine paintbrushes (Da Vinci Cosmotop-Spin, 10/0) to minimise damage and enable careful manipulation due to the small size of the eggs. The mortality of adult sand flies was recorded (dead flies were removed during oviposition plate exchanges) and the degree of embryo melanisation (light grey, medium grey, dark grey/black) was scored under a dissecting microscope. Selected females were maintained within oviposition chambers overnight by the addition of sugar soaked cotton wool to the mesh, and with replacement of oviposition substrate plates with empty falcon tube lids if it was desirable to prevent additional oviposition overnight. The flies were maintained between oviposition plate exchanges and overnight at 25°C within humidified incubators.

### Effects of larval rearing substrates on embryo hatch rates

During the oviposition experiment, embryos collected on agarose oviposition plates from each group, at each time point, on days 3–7 post-bloodfeed were transferred, during egg counting and melanisation stage recording to either plaster of paris plates (2 replicates per time point as this is the standard larval rearing medium[31]) or 2% water agarose gel plates (1 replicate per time point). The plates used for larval rearing substrate were prepared in the same way as the oviposition plates, (i.e. with substrate placed in falcon tube lids), and then each plate was screwed into complete falcon tubes, with humidity applied to the plaster of paris plates prior to use and maintained with damp paper towel placed on the bottom of the plates to prevent the plaster drying out. All hatching tubes were then placed in a falcon tube rack on its side and covered with a plastic bag within an incubator at 25°C, with tubes regularly inspected to avoid insufficient or excess humidity.

### *Wolbachia* purification and embryo injection

The *w*Mel strain of *Wolbachia* was purified from *Drosophila (D.) melanogaster* using modification of a method of *Wolbachia* purification described in 32 by gently crushing x10 pairs of dissected ovaries using a plastic pestle in 100 µL of SPG buffer (218 mM sucrose, 3.8 mM $KH_2PO_4$, 7.2 mM $K_2HPO_4$, and 4.9 mM L-glutamate). Centrifugation of the homogenate at 500 x *g* removed cellular debris that would likely clog the microinjection needles. Purified *Wolbachia* in SPG buffer was kept on ice until injection, with subsequent DNA extraction and qPCR analysis performed on a sub-sample of the homogenate to confirm the presence of significant levels of *Wolbachia* bacteria. Embryonic microinjection was undertaken after alignment of young *Lu. longipalpis* embryos against a Hybond hydrophilic membrane as described in 15 and shown in Figure 1b. A very fine paintbrush (size 10/0) was required for alignment of embryos against the membrane. Hairs that fall off the adult sand flies during oviposition can stick to the eggs, making alignment and microinjection more difficult, and needle breakage more likely. Therefore, during alignment the paintbrush was kept wet and rinsed frequently in water to help adhere to the hairs and avoid them building up on the aligned embryos. Injection was carried out at x40 magnification under an Olympus IX73 microscope using an Eppendorf TransferMan® 4r micromanipulator, Eppendorf FemtoJet® 4x programmable microinjector and Eppendorf Femtotip II injection capillaries. After injection, microscope slides with embryos were immediately transferred to humidified boxes, prior to transfer of the eggs to dampened plaster of paris larval rearing medium.

### Isofemale line selection

Colony *Lu. longipalpis* females were screened for *Wolbachia* using universal *wsp* primers[33] prior to starting embryo injection experiments to confirm no evidence of natural resident *Wolbachia* strains. Isofemale lines were generated with modification of the colony rearing method. Emergent G0 females from microinjected embryos were housed with wild type colony males at a ratio of 10 males:1 female overnight to ensure insemination. The next day, G0 females were bloodfed and carefully transferred, individually, to oviposition chambers made from sterile polystyrene 7 mL bijou collection tubes (Costar) with a 1 cm thick moist plaster of paris base and netting top. Inside the tube a 1 cm x 2 cm strip of Whatman grade 4 filter paper was rested at a 45 degree angle perpendicular to the plaster base to allow the fly to defecate their digested bloodmeal. A small cotton wool pellet soaked in sucrose solution was placed on top. When filled, the tubes were sealed inside a plastic box with moistened paper towel to maintain a high humidity and incubated in total darkness to encourage egg-laying. Sugarmeals were replaced every second day and excess moisture on the netting was blotted away. Following egg-laying, fly bodies and filter papers were removed and the emergent G1 larvae fed by depositing small amounts of larval food with sterile fine forceps next to the larvae.

Fly bodies were stored at –80°C until processing and DNA was extracted from G0 females that laid fertile egg batches using DNeasy Blood and Tissue Kits (QIAGEN) per manufacturer's instructions. DNA extracts were eluted in a final volume of 100 µL and stored at –20°C. DNA extracts were screened using real-time PCR with primers specific for the *w*Mel strain of *Wolbachia* (forward primer: 5'-CAAATTGCTCTTGTCCTGTGG-3', reverse primer: 5'-GGGTGTTAAGCAGAGTTACGG-3') and with primers for a *Lu. longipalpis* VATPase gene (forward primer: 5'- ACGTGACGAGCAAGCAGGGG, reverse primer 5' – GCCGAGATCGTCCGACAGGC) to confirm successful DNA extraction. PCR reactions were prepared using 5µl of FastStart SYBR Green Master mix (Roche Diagnostics), a final concentration of 1µM of each primer, 1µl of PCR grade water and 2µl template DNA, to a final reaction volume of 10µl. Prepared

reactions were run on a Roche LightCycler® 96 System for 15 minutes at 95°C, followed by 50 cycles of 95°C for 15 seconds and 55°C for 30 seconds. Amplification was followed by a dissociation curve (95°C for 10 seconds, 65°C for 60 seconds and 97°C for 1 second) to ensure the correct target sequence was being amplified. PCR results were analysed using the LightCycler® 96 software (Roche Diagnostics). The female progeny from infected females were mated to uninfected colony males for 6 generations ($G_0$–$G_5$).

### Statistics

GraphPad Prism 7 was used to generate column bar graphs, Box and whisker plots and pie charts. Microsoft Excel for Mac (version 16.12) was used to generate adult survival curves.

## Results

### Oviposition substrate and embryo melanisation

Preliminary tests were carried out to investigate the optimal methods to obtain large numbers of embryos suitable for microinjection. Initially three substrates - 2% apple juice agarose gel, 2% water agarose gel and moist plaster of paris - were compared, with oviposition chambers kept in humidified boxes, either in the light or placed in the dark. Trials included the addition of food colouring to the plaster of paris to better visualise un-melanised eggs (translucent to light grey in colour). Variations in the number of adult sand flies per oviposition chamber were also tested. Observations were made on the oviposited eggs and the survival of adult females. Like mosquitoes, *Lu. longipalpis* eggs melanised over a period of approximately 4 hours going from a translucent light colour to dark black (Figure 2). However, a significant proportion of fully melanised mature eggs (black in colour) laid within a 45-minute oviposition period were also observed. Fully melanised embryos were also present in the abdomens of gravid sand flies (Figure 2) highlighting that sand fly eggs can fully melanise prior to oviposition, and that at oviposition, there can be variability in the stage of melanisation, and therefore development, of eggs from the same female – an observation not seen in mosquitoes. Larvae hatching on oviposition substrates were also observed shortly after collection from females that laid fully melanised embryos (Figure 2), confirming that gravid sand flies can retain viable mature embryos until an appropriate substrate is available. These initial tests indicated that the optimal conditions for oviposition and adult longevity were obtained when using 2% water agarose gel as the oviposition substrate, with approximately 15 adult sand flies per oviposition chamber and when the flies were kept in the dark between oviposition plate exchanges. This combination of conditions was therefore used for further embryo collections.

### Duration of the embryo collection period and timing of injectable egg collection

The temporal variation in the ability to obtain sufficient embryos to undertake microinjection experiments from one bloodfed cage of *Lu. longipalpis* (approximately 200 bloodfed females) was

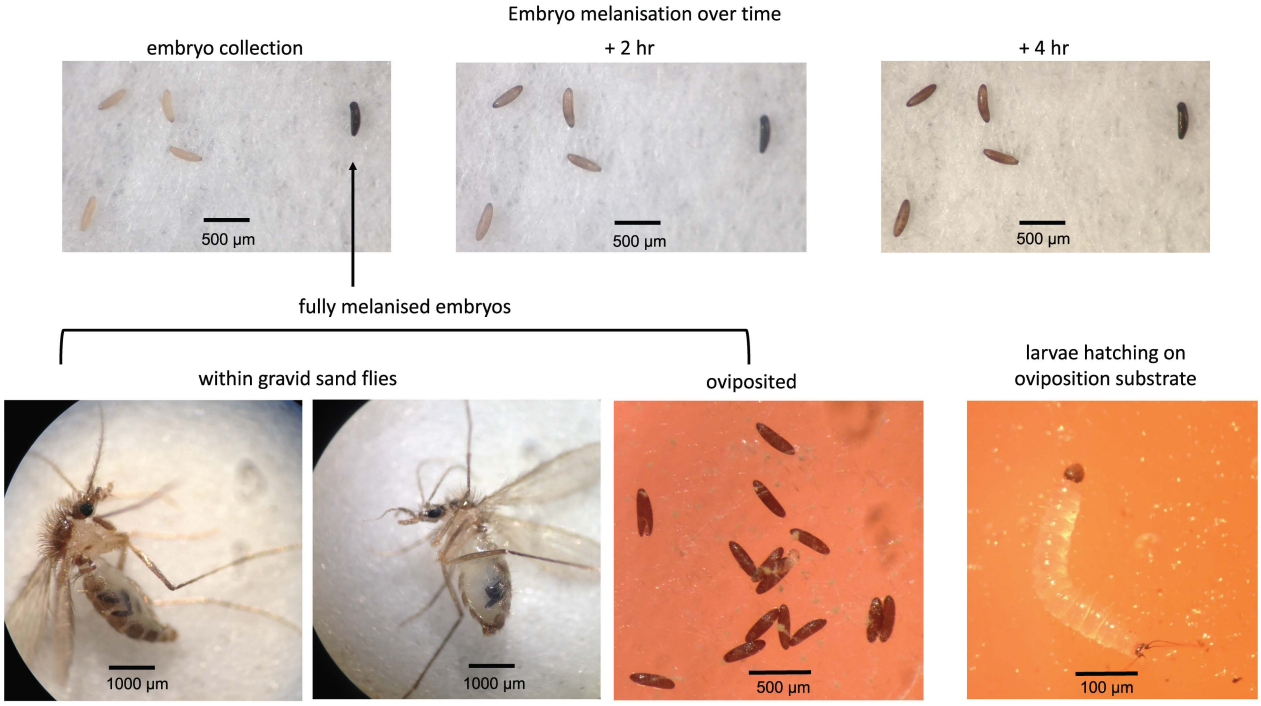


**Figure 2. *Lu. longipalpis* embryo melanisation over time.** Different levels of melanised embryos were laid within oviposition chambers ranging from light grey (low melanisation) through to black embryos (fully melanised). Fully melanised embryos were also observed within the ovaries of gravid females and larvae were seen to hatch on oviposition substrates.

investigated. This involved setting up three replicate groups of females (15 females per chamber) on day 3 post-bloodfeed, 3 replicate groups for the first time on day 4, and 3 groups for the first time on day 5 post-bloodfeed. Each group was initially setup at 9am on the respective day of first oviposition, with agarose oviposition plate exchanges made at 1pm, 5pm and 9am the following morning, and continuing each day with these time intervals until all adult flies had died. At each plate exchange, both the total number of embryos collected per oviposition time period and the number of young embryos that would be suitable for injection (light to medium grey stage of melanisation) was recorded (Figure 3a), as well as the survivorship of gravid *Lu. longipalpis* adult females during oviposition. The majority of injectable eggs was laid on the first exposure to oviposition substrate across all groups (223, 153 and 94 injectable embryos for Day 3, Day 4 and Day 5 groups, respectively) and the greatest proportion of injectable eggs obtained in a day was provided by those flies setup on day 3 post-bloodfeed (Figure 3b). As fully melanised embryos cannot be used for microinjection, and the ability to obtain a large number of light to medium colour embryos within a day increases the efficiency of the injection process, collection of eggs on day 3 post-bloodfeed was considered optimal for both injection and survivability post-injection.

### Female survival after oviposition

Embryo microinjection experiments that either attempt to transinfect *Wolbachia* or to create transgenic lines require the successful generation of isofemale lines. This is dependent on females bloodfeeding and surviving (at the very least) long enough through a single gonotrophic cycle to oviposit the next generation of embryos. In mosquitoes, multiple gonotrophic cycles allows for the collection of progeny from older female mosquitoes, providing multiple chances and greater security that the next generation can be obtained, even if no embryos are produced from the first bloodfeed. To assess this for sand flies, the mortality of gravid females of varying ages was recorded (3–5 days post-bloodfeed) during embryo collection. High rates of mortality were found regardless of the time post-bloodfeeding at which the flies were transferred to oviposition chambers. As shown in Figure 4, rapid mortality within 24 hours was observed for replicate groups of flies removed from colony cages and exposed to oviposition chambers. Although these survival results could suggest that significant mortality occurred from

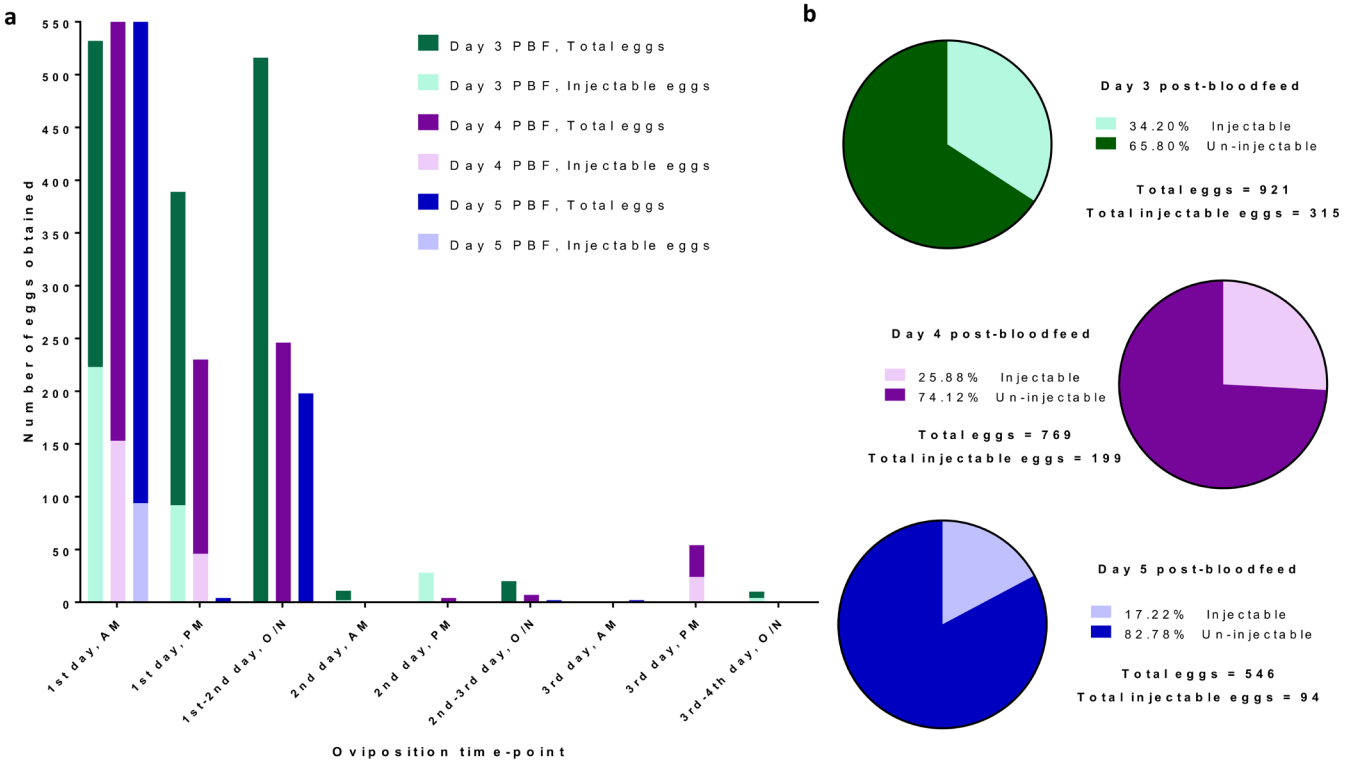


**Figure 3. Timing of gravid *Lu. longipalpis* females oviposition and collection of injectable eggs.** (**a**) The total number of eggs oviposited, overlaid with the number of injectable (light to medium grey) eggs obtained, per oviposition time point, per group setup for first oviposition on either day 3, 4 or 5 post-bloodfeed (PBF). (**b**) The total numbers of embryos collected on the first oviposition day per group (first oviposition on day 3–5 post-bloodfeed comparing injectable (light to medium grey) vs. uninjectable (dark grey/black) embryos.

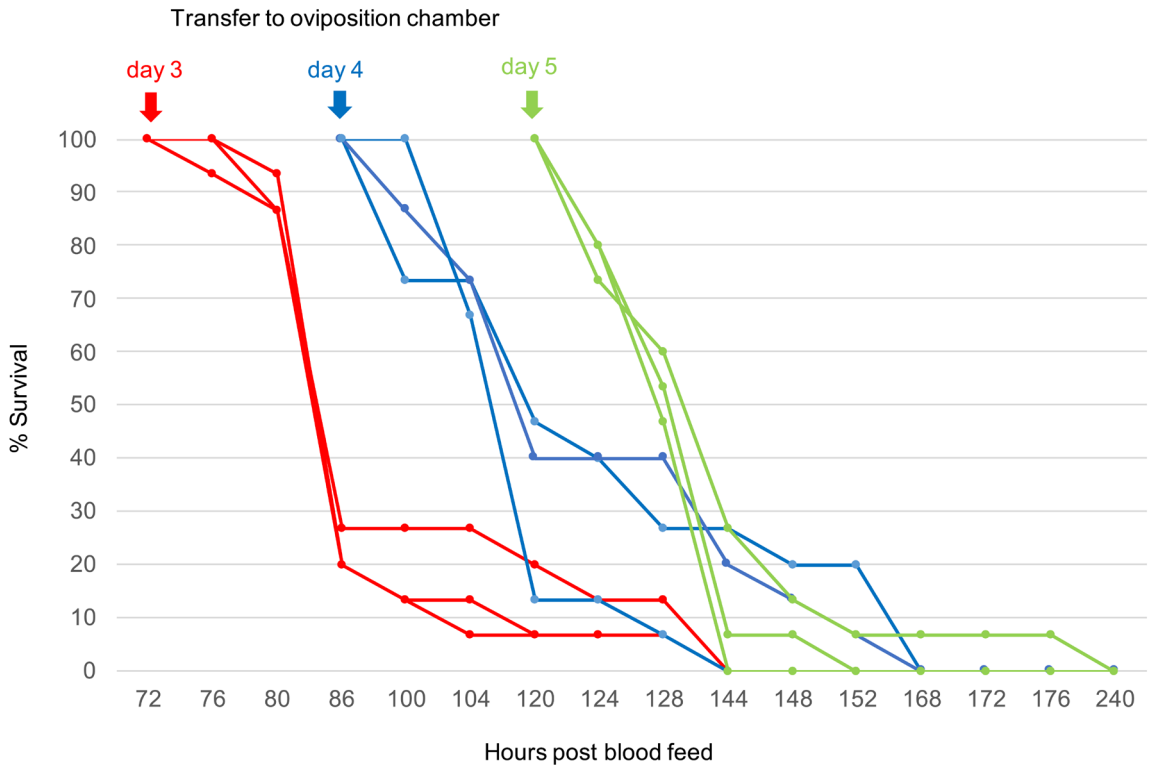


**Figure 4. Survival rates of gravid *Lu. longipalpis* females during oviposition.** 3 replicate groups of 15 flies were setup in oviposition chambers at day 3 (red), day 4 (blue) and day 5 (green) post-bloodfeed and subsequent mortality was recorded over time.

manipulation and exposure to oviposition chambers, high adult female mortality in colony *Lu. longipalpis* shortly after egg-laying was also observed.

### Hatch rates on larval rearing substrates

In order to optimise conditions for successful embryo survival and larval hatching post-injection, the effect of larval rearing substrate on hatching was also investigated. Hatch rates were determined 14 days post-oviposition (Figures 5a and 5b). An overall hatch rate of 57.5% of embryos maintained on agarose, across all oviposition days, compared to only 21.7% of embryos placed on plaster of paris, with a minimum of 37.4% for agarose and a maximum of 25.3% for plaster of paris demonstrates there is a clear improvement in hatch rates when agarose is used as the larval rearing medium over plaster of paris. This improvement may be as a result of the more constant humid environment provided by the agarose gel, providing an optimum environment for the embryos.

### Microinjection of *Wolbachia* purified from *D. melanogaster*

No evidence for natural *Wolbachia* strain infections was seen using PCR screening of the *Lu. longipalpis* colony prior to embryo injection experiments. The *w*Mel strain of *Wolbachia* purified from the ovaries of *D. melanogaster* flies was then injected into the posterior poles of young *Lu. longipalpis* embryos. The injection volume and pressure was determined empirically during injections due to the variable physiology of *Lu. longipalpis* embryos. Slightly desiccated embryos, achieved by blotting of the hydrophilic membrane using filter paper, were re-inflated upon injection without significant flow of cytoplasm back up the injection needle. As *Lu. longipalpis* embryos are 300- 500 µm in length[31] (approximately 50% of the length of *Culex* or *Aedes* mosquito embryos) care was taken to identify the optimal location for injection. For infection of the germline, injection was carried out as near to the posterior pole as possible without significant damage. The posterior pole regions of embryos were not clearly defined so injection was carried out at approximately 25% of the embryo length from the posterior pole. As with mosquito and *Drosophila* embryos, significant variation was found in the injection volumes and pressures required for individual *Lu. longipalpis* embryos. This was expected given the asynchronous oviposition of sand fly embryos at varying stages of melanisation.

A total of 1815 embryos were injected with an average of ~300 eggs injected per day. As *Lu. longipalpis* larvae have previously been shown to have variable larval hatching times[31], with an average of around 16 days[34], a more optimal measure of survivability post-injection was the number of surviving fertile adult G0 females that resulted from the cumulative set of injection experiments. A total of 6 fertile females were produced. This low rate of survival to fertile G0 females is lower but comparable to the 13 fertile females generated from the injection of 2541 *Ae. aegypti* eggs with the *w*Mel strain of *Wolbachia*[15].

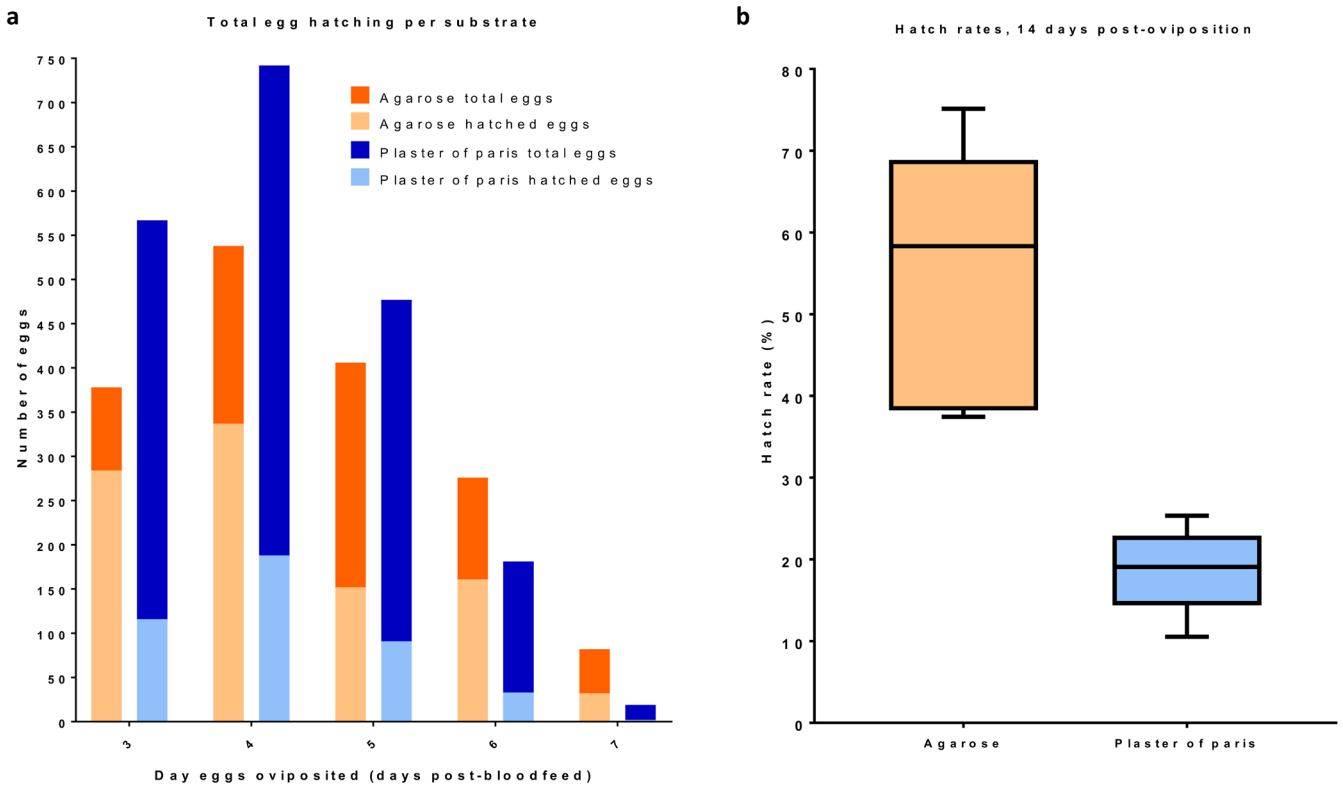


**Figure 5. Hatch rates of *Lu. longipalpis* embryos transferred to either 2% agarose or plaster of paris as the larval rearing substrate.** (**a**) The total numbers of embryos, overlaid with the number of hatched eggs per substrate for each oviposition day. (**b**) Box and whisker plot of hatch rates at 14 days post-oviposition across all days per substrate.

PCR analysis revealed *Wolbachia* infections in 3/6 fertile G0 sand fly females. Although screening of G1 progeny from infected females revealed maternal transmission in 2 lines, qPCR cycle threshold values (>32 cycles) indicated low levels present. Selection based on infection status was continued for generation 3 and 4, but no *Wolbachia* infections were detected in the 5$^{th}$ generation post-injection.

## Discussion

Insect embryo microinjection techniques are dependent on the size and physiology of embryos and additional factors that influence the success rate of experiments, such as preventing excessive embryo desiccation, the injection volume and pressure and the use of a buffer to obtain the optimal pH. The developmental stage of the embryo is also critical given embryos that are too young will burst upon injection but fully melanised embryos have a hardened chorion that prevents needle penetration. Ultimately an embryo microinjection protocol requires 1) the ability to obtain significant numbers of preblastoderm insect embryos within a short period and 2) a method to rapidly inject embryos with survival of fertile G0 females. The protocol developed here has overcome the first hurdle for embryo microinjection in which sufficiently young *Lu. longipalpis* embryos can be harvested for microinjection. Using *Wolbachia* as a 'marker' for successful injection, we were able to generate transient *Wolbachia* infections using our embryo injection protocol although on this occasion it was not possible to successfully generate a stably infected line. Despite our optimised protocol producing early generation *Wolbachia* infections, there are several aspects of sand fly biology that are limiting factors for embryonic injection experiments. Firstly, the observation that gravid *Lu. longipalpis* can oviposit fully melanised embryos (and beyond 3 days post-bloodfeed this can be the majority of embryos) would reduce the efficiency of injection due to the necessity for sorting and exclusion of fully melanised embryos. Secondly, the rapid mortality of females shortly after exposure to oviposition substrates and oviposition itself, suggests there is a low probability of obtaining multiple embryo batches from any given female. This would reduce the generation of progeny obtained from isofemales in the event no embryos were laid during the first gonotrophic cycle. Finally, the long and asynchronous development of sand fly larvae has implications for the successful mating and bloodfeeding of isofemales. However, it should be possible to overcome these difficulties with a sustained effort to inject large numbers of embryos and the ability to maintain a sand fly colony at high densities.

These preliminary trials to develop an embryo microinjection protocol using *Wolbachia* as a 'marker' for successful injection resulted in the detection of the *w*Mel strain in G1-G4 generations indicating infection of the ovaries and maternal transmission between generations. The injection of a larger number of

sand fly embryos may lead to the successful establishment of transinfected *Wolbachia* lines as has been the case for mosquito embryos[15,16,23,24]. Resident *Wolbachia* strains are found in some species of sand flies in both field-caught and laboratory colonies[35–37] indicating stable infections could be achievable. *Wolbachia* strains in *Phlebotomus* sand fly colonies have been shown to induce both the reproductive phenotype cytoplasmic incompatibility[38] and maternal transmission[36], allowing for the invasion of *Wolbachia* into populations. Resident *Wolbachia* strains in mosquitoes have none or only minimal effects on vector competence (reviewed in 39) but transinfection of *Wolbachia* strains from *D. melanogaster* that grow to high densities in mosquito tissues that influence pathogen transmission (e.g. salivary glands) have the greatest inhibitory effects[15–17,40]. Would a high-density strain of *Wolbachia* inhibit *Leishmania* parasites in sand flies? This could only be confirmed through successful generation of a stable line using an efficient embryo microinjection protocol as described here given that recent comparative experiments in *Ae. aegypti* mosquitoes have shown that the magnitude of arboviral inhibition is significantly lower in mosquitoes transiently infected with *Wolbachia* using intrathoracic injection into adults[41]. *Wolbachia* strains have been found to inhibit parasite development in mosquitoes, conferring resistance to *Plasmodium falciparum* malaria infection in *Anopheles stephensi* mosquitoes[42,43] and inhibiting filarial nematode parasite development in *Ae. aegypti*[44]. The tissue tropism of introduced *Wolbachia* strains in sand flies would be crucial to determine if *Leishmania* parasite development would be inhibited within sand flies. As reviewed in 45, *Leishmania* development is confined to the digestive tract with the production of filamentous proteophosphoglycan in the anterior midgut which creates a gel-like plug. Attachment to the stomodeal valve results in damage to the chitin lining and results in reflux of *Leishmania* parasites from the midgut. Therefore, high density *Wolbachia* infections in the sand fly midgut, as occurs for *Drosophila Wolbachia* strains in *Ae. aegypti* mosquitoes[15], would be predicted to result in parasite inhibition.

The ability to inject preblastoderm embryos also provides the possibility of genetic transformation of sand fly species. The widespread success of site-specific nucleases such as transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats CRISPR-Cas9 in model organisms such as *D. melanogaster*[46] has resulted in research into using reprogrammable gene drive systems based on these nucleases spreading beneficial phenotypes in wild insect populations. This genetic engineering using CRISPR-Cas9 has been used to target all major genera of mosquitoes that transmit human diseases. For example, CRISPR-Cas9 based editing has now been used for the principle vector of dengue and Zika viruses, *Ae. aegypti*[47] and has been shown to have the ability to convert female mosquitoes into harmless (non-biting) males[48]. CRISPR-Cas9 has also been used to explore the potential for the use of transgene drive systems in malaria mosquito vectors. The ability to generate sterile female *Anopheles gambiae* mosquitoes with high transmission rates (>90%) to progeny[49] could play a role in modifying wild mosquito populations. In conclusion, this study details an optimised methodology to manipulate bloodfed sand flies to obtain large numbers of *Lu. longipalpis* embryos that are suitable for embryo microinjection. Using this method, we showed successful microinjection using *Wolbachia* as a 'marker' in the first four generations post infection and provide evidence that that this endosymbiotic bacteria can replicate and be maternally transmitted in *Lu. longipalpis*. As such, this method offers a platform to assess the potential of *Wolbachia* as a novel leishmaniasis biocontrol agent but could also assist in the genetic manipulation of this important vector of leishmaniasis.

## Data availability

Raw data is available at Open Science Framework: http://doi.org/10.17605/OSF.IO/S7CZP[50]



### Competing interests

No competing interests were disclosed.

### Grant information

CLJ and TW were supported by a Wellcome Trust/Royal Society Sir Henry Dale fellowship awarded to TW (101285/Z/13/Z). MER was supported by a BBSRC David Phillips Fellowship (BB/H022406/1).

*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

### Acknowledgements

We would like to thank Shahida Begum and Patricia Aiyenuro for assistance in rearing sand fly colonies at LSHTM.

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# Open Peer Review

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Reviewer Report 13 July 2018

https://doi.org/10.21956/wellcomeopenres.15842.r33395



?

**Marcelo Ramalho-Ortigao**
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Review of manuscript "Establishment of a method for *Lutzomyia longipalpis* sand fly embryo microinjection: The first step towards potential novel control strategies for leishmaniasis", by Jeffries CL, Rogers ME and Walker T

The study by Jeffries et al described a methodology for sorting and microinjecting sand fly eggs. The authors use *Wolbachia* wMel as a tracer as this bacterium is not found in natural sand fly populations. Though the authors manage to inject a large number of eggs, the overall rate of survival, estimated from the number of fertile females obtained (six), is very low. The study is a step in the right direction, providing a road map to investigators interested in sand fly biology to adopt techniques currently shown to be successful for mosquitoes and mosquito-borne pathogens, and I congratulate the authors on their efforts. However, there are a number of statements made throughout the manuscript which are misleading or inaccurate, and need to be corrected. Moreover, the results described to some extent "miss the mark" with regards to a number of critical details.

Comments

**Major issues**

- There is a back and forth between melanized eggs and embryos when in actuality the authors should be referring to melanized eggs or chorion, not embryos. "Melanized embryo" is not used correctly and sand fly embryo melanization process was not observed by the authors. Here, egg shell darkening was used as reference of suitability of embryo for microinjection. Whereas melanization of the chorion hardens the egg making it difficult to pierce through the egg shell during microinjection and likely leading to additional damage, such as desiccation of the embryo following piercing or injection, melanization of the embryo itself may occur as an innate immune response.
- The authors use "degree of chorion melanization" as an index to score injectable eggs but this was never paired with any observation of embryo development.

- The rate of survival following injection of the eggs is still extremely low, with a reported 6 fertile females after 1815 eggs injected (0.33% survival). Any idea on the hatching rate [it was not indicated]? It appears as though the authors have compared their 6 fertile sand fly females obtained here with the results obtained by Walker et al. 2011 with *Aedes aegypti* transfected with wMel. In that study, there were a total of 69 G0 females, 39 of which were fertile. However, only 13 were wMel infected. As indicated on page 8, only 6 fertile females were obtained. The ratio of fertile females in the Walker et al study is 1.5%, with a survival rate (total number of females) of 2.71%. The assumption that rate of survival of fertile females is comparable (5 fold difference) might be a bit misleading.
- As authors mentioned, melanized eggs are seldom observed before oviposition in *L. longipalpis*. This phenomenon has not been well understood. Although, a decreasing percentage of suitable eggs for injection obtained longer after blood meal might suggest chorion melanization process is loosely related to oviposition in this sand fly species. Hence, it represents a challenge for the proposed procedure for egg microinjection.
- Authors should be able to present results from screening of wMel strain of *Wolbachia* in G1-G5 for a better understanding of this transient colonization of the bacteria in the isofemale lines.
- Were flies assessed for survival following oviposition while provided with sugar solution during the experiments (i.e., during all the days they were in the ovipots)? This is in reference to results presented in Figure 4. It is not clear from the text whether flies were or were not offered sugar solution.
- Figure 3 legend: (b) the total number of eggs collected, not embryos.
- Figure 2: picture of larvae referred to as “larvae hatching on oviposition substrate” shows what appears to be a L3 (with 2 sets of caudal setae). Either clarify this in the figure legend or replace it with and actual figure of a “hatching larva”. As indicated elsewhere in this review, the melanization takes place in the chorion of the egg and not the embryo. This should be corrected for this figure.
- In the results, the authors indicate that “…gravid sand flies can retain viable mature embryos [in actuality it should be eggs] until an appropriate substrate is available…” Though I agree with this statement, what is lacking here are details of “substrate”. Part of this study was focused on comparing the effects of 2% agarose vs. plaster on egg laying and it appears as though the focus of this statement is on such difference. However, it has been demonstrated that bacteria present in the substrate (typically decaying organic matter) are key for oviposition by sand flies, and this is not mentioned by the authors. In my view, the effect of bacteria on sand fly oviposition also needs to be discussed if trying to interpret the effects of “appropriate substrate”.

**Other issues needing correction**

**Abstract**

“…L. longiplais is the principal vector…”

“…handling of small sand fly eggs…”

It needs to indicate that the transfection with Wolbachia was transient (per the results until the 5$^{th}$ generation).

**Introduction**

- Typically, the spectrum of diseases is referred to as leishmaniases (with an “e”).

- The sentence explaining aspects of the clinical symptoms (or as written “syndrome”) should be re-written for accuracy. Human migration is related to transmission of disease and not “extrinsic” factors associated with the clinical symptoms.

- For the statement of clinical symptoms: “The clinical syndrome exhibited is influenced by the species of the infecting parasites, the genetic background of the host and associated immunity.”

- On paragraph 3 of the Intro: In endemic areas where dogs are domestic reservoirs of **visceral** leishmaniasis (not cutaneous!)
- It seems to me that the paragraph starting with “Research into novel non-insecticide based control strategies…” should be part of the Discussion.

**Methods**

L. longipalpis rearing
Jacobina is not a state in Brazil. The state is actually Bahia; Jacobina is a city. The coordinated for Jacobina, though not necessarily where the flies were originally collected is 11° 10′ 51″ S, 40° 31′ 4″ W. Under laboratory conditions, hatching of *Lutzomyia longipalpis* varies widely and is dependent on temperature and relative humidity. However, this is one of the most malleable species for colonization. Perhaps the authors want to add comments as to whether rearing methodology and subsequent mating procedure may have contributed to a low yield of fertile adult G0 females.

Wolbachia purification and embryo injection

There are two references cited in this section, numbers 32 and 15. Following the journal’s style, they should be superscript, perhaps by citing “author et al” (e.g., Klasson et al[32]; Walket et al[15])

“…by crushing x10 pairs…”. Did you mean crushing ~10 pairs? (“~” as in approximately?)

Isofemale line selection

“…45 degree angle perpendicular to the plaster base…” If lines are perpendicular they are by definition at a 90° degree (right) angle.

In the section where the PCR details are described, please be consistent in the use of µl: there is µl, µL. Also, separate µl and the number digit, as in 1 µl, not 1µl.

**Discussion**
Though the techniques described in this study are a step in the right direction, to “predict” that using Wolbachia will lead to inhibition of Leishmania growth in sand flies based on details of its biology seems a bit of a stretch (although I would like to agree with the authors!). In addition, the authors go on to suggest that CRISPR-Cas9 will be the next natural approach to be tested and that the “optimized” methodology described will allow for the manipulation of large numbers of Lu. longipalpis embryos. The authors are correct with regards to CRISPR, as a handful of labs are already applying this approach to their study of sand flies. However, the success rate is still dismal to say the least. Until the survival rate and the rate of fertile females are higher that what was reported here, it is difficult to see this moving rapidly to be applied in any control, strategies for sand fly-transmitted diseases. In that regard, I do not think the picture is as rosy.

**Is the rationale for developing the new method (or application) clearly explained?**
Yes

**Is the description of the method technically sound?**
Yes

**Are sufficient details provided to allow replication of the method development and its use by others?**
Yes

**If any results are presented, are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions about the method and its performance adequately supported by the findings presented in the article?**
Yes

***Competing Interests:*** No competing interests were disclosed.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 07 Aug 2018

**Thomas Walker**, London School of Hygiene and Tropical Medicine, London, UK

Many thanks Marcelo for your insightful review. We have addressed your comments as follows (in bold):

There is a back and forth between melanized eggs and embryos when in actuality the authors should be referring to melanized eggs or chorion, not embryos.
**We have gone through the manuscript and corrected our terminology on eggs vs embryos as we agree that these are distinct terms.**

The authors use “degree of chorion melanization” as an index to score injectable eggs but this was never paired with any observation of embryo development.
**We did not do this as the use of melanisation to determine ‘injectability’ is commonly used for mosquito injection methodology and provides a ‘usable’ guide as to when to inject embryos independent of embryo development (please see Jasinskiene, N., Juhn, J. & James, A. A. Microinjection of A. aegypti embryos to Obtain Transgenic Mosquitoes. *J. Vis. Exp.* (2007). To determine embryo development for any individual in the time frame required to inject would not be feasible.**

The rate of survival following injection of the eggs is still extremely low, with a reported 6 fertile females after 1815 eggs injected (0.33% survival). Any idea on the hatching rate [it was not indicated]? It appears as though the authors have compared their 6 fertile sand fly females

obtained here with the results obtained by Walker et al. 2011 with *Aedes aegypti* transfected with wMel. In that study, there were a total of 69 G0 females, 39 of which were fertile. However, only 13 were wMel infected. As indicated on page 8, only 6 fertile females were obtained. The ratio of fertile females in the Walker et al study is 1.5%, with a survival rate (total number of females) of 2.71%. The assumption that rate of survival of fertile females is comparable (5 fold difference) might be a bit misleading.
**We agree and have modified our text to reflect this slightly misleading comparison to Walker et al. study. "A total of 6 fertile females were produced, which is low compared to the 39 fertile females generated from the injection of 2541 *Ae. aegypti*eggs with the *w*Mel strain of *Wolbachia* [15]." We also have addressed the idea that rearing methodology can contribute to low yield of fertile adult G0 females "The low number of fertile adult females is likely a result of the combination of both the egg injection procedure but also the variability of survival rates observed in sandfly colonies due to factors including environmental conditions (temperature and humidity) and parasites and pathogens such as fungi [31]."**

As authors mentioned, melanized eggs are seldom observed before oviposition in *L. longipalpis*. This phenomenon has not been well understood. Although, a decreasing percentage of suitable eggs for injection obtained longer after blood meal might suggest chorion melanization process is loosely related to oviposition in this sand fly species. Hence, it represents a challenge for the proposed procedure for egg microinjection.
**We agree and we do mention this in the discussion with the following sentences "Firstly, the observation that gravid *Lu. longipalpis* can oviposit fully melanised eggs (and beyond 3 days post-bloodfeed this can be the majority of eggs) would reduce the efficiency of injection due to the necessity for sorting and exclusion of fully melanised eggs. Secondly, the rapid mortality of females shortly after exposure to oviposition substrates and oviposition itself, suggests there is a low probability of obtaining multiple egg batches from any given female."**

Authors should be able to present results from screening of wMel strain of *Wolbachia* in G1-G5 for a better understanding of this transient colonization of the bacteria in the isofemale lines.
**As the manuscript is a 'methods' paper that is presenting *Wolbachia* as a 'marker' for successful germline infection (rather than establishing a stable line), this data was omitted as it has limited relevance to the injection protocol. The asynchronous development of sandfly immature stages in combination with published studies establishing *Wolbachia*-infected mosquito lines showing huge variation in maternal transmission rates in early generations (Walker et al. Nature 2011, Joubert et al Plos Pathogens 2016) suggests this data is not very informative.**

Were flies assessed for survival following oviposition while provided with sugar solution during the experiments (i.e., during all the days they were in the ovipots)? This is in reference to results presented in Figure 4. It is not clear from the text whether flies were or were not offered sugar solution.
**During the short oviposition collections (45 minutes) the flies were not offered a sugar solution but were maintained on sugar before and after in cages. In our manuscript we have " Selected females were maintained within oviposition chambers overnight by the addition of sugar-soaked cotton wool to the mesh, and with replacement of oviposition substrate plates with empty falcon tube lids if it was desirable to prevent additional oviposition overnight" We also explain that 'high adult female mortality in colony *Lu.***

***longipalpis*** **shortly after egg-laying was also observed' suggesting the absence of sugar for only the 'oviposition' period is unlikely to be the cause of this mortality.**

Figure 3 legend: (b) the total number of eggs collected, not embryos
**We have changed the terminology here.**

picture of larvae referred to as "larvae hatching on oviposition substrate" shows what appears to be a L3 (with 2 sets of caudal setae). Either clarify this in the figure legend or replace it with and actual figure of a "hatching larva". As indicated elsewhere in this review, the melanization takes place in the chorion of the egg and not the embryo. This should be corrected for this figure
**This larvae was from the hatching experiment and was erroneously included in this figure. We have removed this image and modified figure 2 which does actually have evidence of a larvae hatching in the revised figure in panel F (this was also in the original figure). We have also adjusted our terminology of egg rather than embryo for melanization.**

In the results, the authors indicate that "…gravid sand flies can retain viable mature embryos [in actuality it should be eggs] until an appropriate substrate is available…" Though I agree with this statement, what is lacking here are details of "substrate". Part of this study was focused on comparing the effects of 2% agarose vs. plaster on egg laying and it appears as though the focus of this statement is on such difference. However, it has been demonstrated that bacteria present in the substrate (typically decaying organic matter) are key for oviposition by sand flies, and this is not mentioned by the authors. In my view, the effect of bacteria on sand fly oviposition also needs to be discussed if trying to interpret the effects of "appropriate substrate".
**Our oviposition substrates were freshly made and kept at 4C so there was unlikely to be large amounts of bacteria present on the substrates. We fully agree that decaying organic matter is important for oviposition so it's possible that our method can circumvent this need through 'forced oviposition' rather than attracting sandflies to oviposit through bacterial volaties. We have added a sentence to the manuscript to address this point.**

"…L. longiplais is the principal vector…"
**Changed in ms**

"…handling of small sand fly eggs…"
**Changed in ms**

It needs to indicate that the transfection with Wolbachia was transient (per the results until the 5$^{th}$ generation).
**'Transient' is used for Wolbachia transinfection work as meaning no maternal transmission (ie. through adult intrathoracic injection). Here Wolbachia was maternally transmitted through four generations so we feel 'transient' does not represent our results but early generation indicates the absence of a stable line.**

· Typically, the spectrum of diseases is referred to as leishmaniases (with an "e").
**Changed in ms**

· The sentence explaining aspects of the clinical symptoms (or as written "syndrome") should be re-written for accuracy. Human migration is related to transmission of disease and not "extrinsic" factors associated with the clinical symptoms.
· **We have re-written this sentence**

For the statement of clinical symptoms: “The clinical syndrome exhibited is influenced by the species of the infecting parasites, the genetic background of the host and associated immunity
· **We have re-written this sentence**

· On paragraph 3 of the Intro: In endemic areas where dogs are domestic reservoirs of visceral leishmaniasis (not cutaneous!)
· **This was a mistake and we have changed this!**

· It seems to me that the paragraph starting with “Research into novel non-insecticide based control strategies…” should be part of the Discussion.
**We respectfully disagree as this introduces the idea of needing novel control strategies in sandflies and the requirement for egg injection for various strategies currently being used for mosquito control**

Jacobina is not a state in Brazil. The state is actually Bahia; Jacobina is a city. The coordinated for Jacobina, though not necessarily where the flies were originally collected is
11° 10′ 51″ S, 40° 31′ 4″ W.
**We have modified this in the ms**

Under laboratory conditions, hatching of *Lutzomyia longipalpis* varies widely and is dependent on temperature and relative humidity. However, this is one of the most malleable species for colonization. Perhaps the authors want to add comments as to whether rearing methodology and subsequent mating procedure may have contributed to a low yield of fertile adult G0 females.
**We feel this is a good point to raise and have addressed this in the manuscript**

There are two references cited in this section, numbers 32 and 15. Following the journal’s style, they should be superscript, perhaps by citing “author et al” (e.g., Klasson et al[32]; Walket et al[15])
**We have modified this in the ms**

“…by crushing x10 pairs…”. Did you mean crushing ~10 pairs? (“~” as in approximately?)
**We have clarified this – 10 pairs of ovaries**

“…45 degree angle perpendicular to the plaster base…” If lines are perpendicular they are by definition at a 90° degree (right) angle.
**We have removed ‘perpendicular’ to address this mistake**

In the section where the PCR details are described, please be consistent in the use of µl: there is µl, µL. Also, separate µl and the number digit, as in 1 µl, not 1µl.
**We have corrected this in the ms**

Though the techniques described in this study are a step in the right direction, to “predict” that using Wolbachia will lead to inhibition of Leishmania growth in sand flies based on details of its biology seems a bit of a stretch (although I would like to agree with the authors!). In addition, the authors go on to suggest that CRISPR-Cas9 will be the next natural approach to be tested and that

the “optimized” methodology described will allow for the manipulation of large numbers of Lu. longipalpis embryos. The authors are correct with regards to CRISPR, as a handful of labs are already applying this approach to their study of sand flies. However, the success rate is still dismal to say the least. Until the survival rate and the rate of fertile females are higher that what was reported here, it is difficult to see this moving rapidly to be applied in any control, strategies for sand fly-transmitted diseases. In that regard, I do not think the picture is as rosy.
**We agree and have sentences in the discussion such as ‘The injection of a larger number of sand fly embryos may lead to the successful establishment of transinfected *Wolbachia* lines as has been the case for mosquito embryos’ where ‘’may’’ is used to provide balance. We also have ‘Would a high-density strain of *Wolbachia* inhibit *Leishmania* parasites in sand flies? This could only be confirmed through successful generation of a stable line…’ and ‘The tissue tropism of introduced *Wolbachia* strains in sand flies would be crucial to determine if *Leishmania* parasite development would be inhibited within sand flies’ so feel we do have some balance in our discussion. We are unable to comment on the success of ongoing CRISPR studies that are currently unpublished but agree that many technical hurdles may still have to be overcome and have added a further sentence to the end of the discussion.**

***Competing Interests:*** No competing interests were disclosed.

Reviewer Report 22 June 2018

https://doi.org/10.21956/wellcomeopenres.15842.r33300



**Fernando Ariel Genta**
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This article by Jeffries *et al.* describes a new methodology for obtaining embryos of the phlebotomine sand fly *Lutzomyia longipalpis*, an important vector of Visceral Leishmaniasis in South America. The method described allowed the authors to do injections in these embryos at a very large scale. This method will be useful for genetic manipulations of this insect, and it was applied to inoculation of Wolbachia in a strain not previously associated with this bacterium species. Despite the fact that the authors failed in obtaining a stable Wolbachia-sand fly association, the technical achievement described is very relevant to the sand fly community. I have only very few remarks to do about this manuscript, listed below:

1. Abstract line 9 - "principal" instead of "principle"
2. Results page 6, sentence "These initial tests..." I understand that they are initial tests, but I missed a more detailed description and comparison of the data. How much better was the chosen condition in comparison to the others? How many eggs were laid in the three media tested? How strong was the effect of light and sand fly number? These considerations would be important for developers and to have a better understanding of the sand fly biology involved.

3. Figure 3. The legend of the figure does not describe all charts in part b. Maybe it is just a problem in the colors described, they do not match all the pizza slices in the figure.
4. Discussion page 9. Sentence "Secondly, the rapid mortality..." We have just published a report showing second blood feeding and oviposition in sand flies (*Lutzomyia longipalpis* of the very same strain used in this article) in large-scale experiments with the same efficiency when compared to the first blood meal/oviposition cycle[1]. I think our finding might be useful for the authors in the future and it would be interesting to mention them in this context.

I would like to congratulate the authors for this manuscript, it is a quite interesting and relevant work.

**References**

1. Moraes CS, Aguiar-Martins K, Costa SG, Bates PA, et al.: Second Blood Meal by Female Lutzomyia longipalpis: Enhancement by Oviposition and Its Effects on Digestion, Longevity, and Leishmania Infection.*Biomed Res Int*. 2018; **2018**: 2472508 PubMed Abstract | Publisher Full Text

**Is the rationale for developing the new method (or application) clearly explained?**
Yes

**Is the description of the method technically sound?**
Yes

**Are sufficient details provided to allow replication of the method development and its use by others?**
Yes

**If any results are presented, are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions about the method and its performance adequately supported by the findings presented in the article?**
Yes

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Insect Biochemistry and Physiology, Vector Biology, Parasite-Vector Interactions, Sandfly, Leishmania

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Author Response 07 Aug 2018

**Thomas Walker**, London School of Hygiene and Tropical Medicine, London, UK

Many thanks Fernando for your review and we have addressed your comments as follows (in bold):

Abstract line 9 - "principal" instead of "principle"

**We have changed this in the manuscript**

Results page 6, sentence "These initial tests..." I understand that they are initial tests, but I missed a more detailed description and comparison of the data. How much better was the chosen condition in comparison to the others? How many eggs were laid in the three media tested? How strong was the effect of light and sand fly number? These considerations would be important for developers and to have a better understanding of the sand fly biology involved.
**This was indeed more observational during initial tests to indicate the suitability of using agar as a substrate for oviposition (plaster of paris used for colony maintenance). As we found that sandflies laid a significant proportion of melanised eggs during the initial trials, we went on to determine the proportion of 'injectable' eggs on days 3-5 post-bloodfeed (Figure 3, raw data file) as that would have a greater impact on the ability to undertake this work rather than overall numbers of eggs laid.**

Figure 3. The legend of the figure does not describe all charts in part b. Maybe it is just a problem in the colors described, they do not match all the pizza slices in the figure.
**We agree and have modified the figure legend to clarify the difference between egg colour from melanisation and colours on the charts!**

Discussion page 9. Sentence "Secondly, the rapid mortality..." We have just published a report showing second blood feeding and oviposition in sand flies (*Lutzomyia longipalpis* of the very same strain used in this article) in large-scale experiments with the same efficiency when compared to the first blood meal/oviposition cycle[1]. I think our finding might be useful for the authors in the future and it would be interesting to mention them in this context.
**We have now included this result and reference in our discussion.**

***Competing Interests:*** No competing interests were disclosed.