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Establishment of *in vivo* and *in vitro* platforms for investigation of the effects of *Wolbachia* on the vector competence of *Culex pipiens*

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Abstract

Wolbachia is an intracellular bacterial symbiont found in nearly half of all arthropod species. In some mosquito vector species, it is known to exhibit repressive effects on viral replication and transmission. Sindbis virus (SINV) is an arthritogenic alphavirus that causes yearly human infections in Northern Europe. Its transmission cycle involves passerine birds and two main mosquito vectors, *Culex pipiens* and *Cx. torrentium*. *Cx. pipiens* is almost always infected with *Wolbachia*, yet the impact on its vector competence for SINV has never been investigated. The purpose of this study was to establish *in vivo* (Aim 1) and *in vitro* (Aims 2 and 3) platforms for the investigation of the effect of *Wolbachia* on vector competence and viral replication in mosquitoes of the *Cx. pipiens* complex. For Aim 1, a colony of lab-reared *Cx. pipiens molestus* was cleared of its natural *Wolbachia* infection through tetracycline treatment. For Aim 2, a proliferating primary cell culture was initiated from the eggs of *Cx. pipiens molestus*. For Aim 3, attempts were made to infect Hsu cells with *Wolbachia* from *Cx. pipiens molestus* ovaries with varying success. Protocols for all three primary aims have been established. Preliminary experiments suggest that Hsu cells do not support SINV replication. Additionally, the vector competence of *Wolbachia*-infected *Cx. pipiens molestus* for SINV has been investigated, however with inconclusive results. Continued maintenance of the *Wolbachia*-free mosquito line and primary cell culture is projected to allow future studies of *Wolbachia*-mediated phenotypes and its effect on virus transmission by *Cx. pipiens*.

Popular scientific summary

Learning how mosquitoes use friendly bacteria to fight viruses

In the mosquito, three organisms meet that could not be more distantly related – viruses, bacteria, and the mosquito itself. The way this meeting goes has far-reaching consequences for human health, and scientists are now close to answering the question of how wild bacteria could be protecting Swedish nature enthusiasts from years of joint pain.

Every year, an estimated 600-1200 people in Sweden are infected with a virus that causes arthritis, the majority of which don't even notice it. The only sign of potentially being infected, other than the rarely seen rash and fever, is a mosquito bite. The culprit is the Sindbis virus – a bird virus transmitted by mosquitoes that sometimes spills over into the human population. Most cases appear in late summer, when bird fledglings are born and the amount of virus in nature explodes. Although people rarely meet them, the most important species driving this explosion are the two bird-biting mosquitoes *Culex pipiens*, the northern house mosquito, and *Culex torrentium*, a closely related mosquito that lacks a common name.

Even though they look identical and behave similarly, there is one key difference when it comes to their impact on your joints – their ability to transmit Sindbis virus. *Culex torrentium* is a monstrous Sindbis-replicating and spitting factory, while *Culex pipiens* is more modest in its ability to amplify and transmit the virus. Additionally, *Culex pipiens* carries a friendly bacterium inside its cells called *Wolbachia*, but *Culex torrentium* does not. Research from the past 20 years suggests that these two facts might be connected.

Until recently, *Wolbachia* was considered to be nothing more than a parasite, inhabiting countless arthropod species and changing the reproductive mechanisms of its host to ensure its own propagation. However, studies of tropical mosquitoes show that it might be giving its host resistance to viral infection. Daring Australian scientists even released mosquitoes artificially infected with *Wolbachia* into the wild and their risk paid off – cases of mosquito-borne viruses plummeted. Could *Wolbachia* be an unknown guardian angel, protecting hundreds of Swedes from lasting joint pain? No one has asked until now.

Significant progress has been made on answering that question. A laboratory colony of a subspecies of *Culex pipiens* has been treated with antibiotics to cure it of its *Wolbachia*.

Soon, once the mosquitoes have recovered from the treatment, experiments will be performed to test if the absence of *Wolbachia* allows *Culex pipiens* to ramp up its virus production.

To answer even deeper questions, cells were extracted from the eggs of the mosquito and allowed to grow in a dish. Although they don't quite feel at home yet, they will have soon grown enough to be used for experiments and reveal the intricate and fascinating mechanisms through which *Wolbachia* interacts with its mosquito host and the virus trying to infect it. And perhaps, one day, we will learn how the relentless hail of viruses can be deflected by the tiniest of shields.

Introduction

Wolbachia are intracellular alpha-proteobacteria found in an estimated 40% of all terrestrial arthropods and some filarial nematodes of medical importance, such as *Onchocerca volvulus* and *Brugia malayi* (1). The type species, *Wolbachia pipientis*, was first discovered in the northern house mosquito, *Culex pipiens*, in 1924 (2). Since then, the genus *Wolbachia* has been subdivided into 18 supergroups, A-R, based on phylogeny. The first discovered supergroups, A and B, are mostly found in arthropods, while C and D are found in filarial nematodes. Supergroups, in turn, contain strains, e.g. *w*Pip, discovered in *Culex pipiens*, supergroup B, and *w*Mel, discovered in *Drosophila melanogaster*, supergroup A (3).

In insects, *Wolbachia* spreads through populations as a vertically inherited reproductive parasite, manipulating host reproduction to promote its own propagation. Reproductive effects induced by *Wolbachia* include feminization of embryos, death of male progeny, and, most commonly, sperm-egg incompatibility, also known as cytoplasmic incompatibility (CI) (4). The link between CI and *Wolbachia* was proposed in 1971 by Yen & Barr (5). CI is the only effect on reproduction found in mosquitoes and occurs due to a toxin-antitoxin system, the mechanism of which was discovered only recently (6–8). In brief, *Wolbachia* secretes toxins in spermatid progenitor cells that disrupt the first mitosis unless deactivated by antitoxins supplied by the *Wolbachia* in the oocyte, meaning that infected males can only fertilize infected females. This gives *Wolbachia*-infected females a reproductive advantage, leading to a spread of the endosymbiont in the local population (9–11). Furthermore, the toxin and antitoxin variants must be compatible with each other. *Cx. pipiens*, despite being almost always infected with *Wolbachia*, therefore has a very high number of crossing types that are incompatible with each other due to a diversification of the toxin-antitoxin genes (8).

Long before the mechanism behind CI was understood, *Wolbachia* was used to control arboviral disease through the incompatible insect technique (IIT) which suppresses vector populations (12). More recently, experimental *Wolbachia* infection was found to have major direct effects on arbovirus transmission by its mosquito host. To be successfully transmitted, an arbovirus must be able to overcome three primary challenges: [1] infecting the midgut, measured by infection rate of whole bodies, [2] escaping from the midgut, measured by dissemination of the virus to the legs, and [3] infecting the salivary glands, measured by presence of virus in the saliva (13). *Wolbachia* is known to impede the virus from overcoming these obstacles, as has been shown for flaviviruses such as Dengue virus (DENV) and Zika virus (ZIKV), and the alphavirus Chikungunya virus (CHIKV) in the most

important arboviral vectors with regard to public health globally – the yellow fever mosquito, *Aedes aegypti*, and the Asian tiger mosquito, *Ae. albopictus* (14–18). Release of *Ae. aegypti* artificially infected with *w*Mel (it is naturally *Wolbachia*-free) has resulted in successful infiltration of the local population and a reduction in the number of DENV infections in Australia (19).

For mosquito vectors that naturally carry *Wolbachia*, the effects of their native endosymbiont on vector competence are not as evident, possibly due to the adaptation of the virus to the presence of the bacterium (20). In *Ae. albopictus*, which is naturally superinfected by the strains *w*AlbA and *w*AlbB, its native *Wolbachia* has minor and possibly beneficial effects on CHIKV replication (21,22). Regarding DENV replication, there are reports of both a reduction in vector competence due to *Wolbachia* (23,24) and no effect (25). In the southern house mosquito, *Cx. quinquefasciatus*, a vector of West Nile virus (WNV), the native *w*Pip infection limits virus transmission by a laboratory strain, although the *Wolbachia* density in wild mosquitoes might not be sufficiently high to give a similar effect in a natural setting (26,27).

In cell culture, *Wolbachia* often has suppressing effects on viral replication (28–32). Although the exact mechanism through which *Wolbachia* exerts its effects remains undiscovered, current hypotheses include alterations of lipid metabolism, priming of the immune response, and direct effects on viral RNA (33). In-depth investigation of these potential mechanisms has often benefitted from *in vitro* platforms due to the relative ease with which cells can be manipulated, as opposed to whole mosquitoes (33). Most *in vitro* studies, even those involving viruses transmitted by *Culex*, have been performed in *Ae. aegypti* or *Ae. albopictus* cells, partly due to the widespread use and availability of *Aedes* cell lines (34). Currently, only one *Culex* cell line has found significant use in research – Hsu, derived from the ovarian tissues of *Cx. quinquefasciatus*, a vector of several arboviruses and filarial nematodes and a close relative of *Cx. pipiens* (35). This stems from the relatively few cell lines initiated from *Culex* and the poor availability of *Culex* cell lines, where direct contact with researchers is often the only way to obtain one (34). Although a handful of other cell lines have been established from *Culex*, their use in virus-related research is very sparse (36–41). So far, no studies on the effects of *Wolbachia* on viral replication have been done in *Culex* cells.

In Sweden, Sindbis virus (SINV) is so far the only detected mosquito-borne virus (42). It is an enveloped, (+)ssRNA virus that belongs to the genus *Alphavirus*, family Togaviridae (43). Birds, passerines (e.g. thrushes) in particular, serve as the natural hosts of the virus (44). Humans are sometimes infected after being bit by promiscuous mosquitoes that feed on a variety of animal hosts, of which *Ae. cinereus* has been suggested as a likely candidate (45). Cases of SINV infection are reported each year in Northern Europe, primarily in Finland, but also in central and northern Sweden (45–47). The infection is usually mild with most commonly reported symptoms being a rash and a fever, although up to a quarter of the symptomatic patients suffer lasting joint pain (43). Human cases are most common in July and August, possibly due to the emergence of bird hatchlings, which serve as potent amplifying hosts (48).

SINV circulates in bird populations through transmission primarily by the two bird-biting mosquitoes *Cx. pipiens* and *Cx. torrentium* (49). Of the two species, *Cx. torrentium* is the more important vector based on field infection rates (45,50) and is also a more competent vector as shown by experimental infection (51,52). Intriguingly, these closely related vector species differ in their *Wolbachia* infection status since *Cx. torrentium*, as opposed to *Cx. pipiens*, very rarely carries *Wolbachia*, both in continental Europe (53–57) and in SINV-endemic regions of Sweden (unpublished data). Potentially, the lower vector competence of *Cx. pipiens* for SINV is in part due to its *Wolbachia* infection, but this has never been tested experimentally.

The main aim of this project was to create *in vivo* and *in vitro* platforms for investigations of *Wolbachia*'s effect on viral replication kinetics. This was accomplished by:

1. Creating a *Wolbachia*-free line of *Cx. pipiens molestus*, a subspecies of *Cx. pipiens*, and to investigate the impact of the endosymbiont on SINV transmission. *Cx. pipiens molestus* is part of the *Cx. pipiens* complex but has advantageous characteristics for maintenance in a laboratory environment, namely the ability to breed in confined spaces, the ability to lay eggs without requiring a blood meal and not hibernating during the winter (58).
2. Starting a primary cell culture derived from *Cx. pipiens molestus* that retains its natural infection by *w*Pip.
3. Infecting a *Wolbachia*-free cell line of the closely related species *Cx. quinquefasciatus*, Hsu, with its native *w*Pip strain.

Materials and methods

Generation of a *Wolbachia*-free line of *Cx. pipiens molestus*

A colony of *Cx. pipiens molestus* (58) that are naturally infected with *Wolbachia* have been reared in our lab for over thirty generations. The larvae were maintained on Brewer's yeast and fish feed, while the adults were maintained on a 10% sucrose solution. Approximately 400 pupae from three consecutive generations (colony-F30, colony-F31, and colony-F32) were collected over two weeks and designated generation F0, of which approximately 350 emerged successfully and survived until the start of the treatment. The emerged adults were orally treated with tetracycline hydrochloride (Sigma-Aldrich, St Louis, MO, USA) for two weeks. Oral tetracycline treatment is a method commonly used for generations of *Wolbachia*-free lines of mosquitoes (59–61).

During the first week, tetracycline hydrochloride was administered at a concentration of 1 mg/mL in a 10% sugar solution buffered with Tris to pH 7.0. The effect of the treatment in F0 was measured by sampling 4-5 individuals, preferentially males, every day during the treatment. During the second week, the concentration of tetracycline was increased to 1.5 mg/mL since no effect was seen on the *Wolbachia* infection status.

After two weeks of treatment, the adults were allowed to lay eggs. F1 adults were orally treated with tetracycline at a concentration of 1.5 mg/mL in 10% sugar solution, buffered with Tris to pH 7.0 for one week, after which they were allowed to lay eggs. Consecutive generations starting from F2 were not treated with antibiotics. The effect of the treatment on F1 was measured by sampling mosquitoes that died within the last 24 hours since the low number of individuals did not allow for sampling healthy mosquitoes. The overall effect of the treatment was determined by testing F3 mosquitoes for *Wolbachia* using PCR as described below.

Detection of *Wolbachia* using conventional PCR

Individual adult mosquitoes were homogenized in 500 µL of homogenizing buffer, containing PBS supplemented with 20% Foetal Bovine Serum (FBS), 1% PenStrep (Thermo Fisher Scientific, Waltham, MA, USA), and 1% Amphotericin B (Sigma-Aldrich) using TissueLyser II (Qiagen, Hilden, Germany) and two steel beads at 20 shakes per second for 2 min. 5 µL of the homogenate was diluted in 20 µL of dilution buffer supplemented with 0.5 µL of DNA Release additive, part of the TissueDirect PCR Master Mix kit (Thermo Fisher Scientific, Vilnius, Lithuania). The homogenate was then heated to 98 °C for 2 min, according to the manufacturer's instructions, and stored at -20 °C. 1 µL of the diluted

homogenate was used as a template for a 20 µL PCR reaction using TissueDirect Master Mix (Thermo Fisher Scientific) and general *Wolbachia* primers, targeting a 610 bp region of the *Wolbachia* surface protein (*wsp*) gene: 81F (5'- TGGTCCAATAAGTGATGAAGAAAC-3') and 691R (5'- AAAAATTAAACGCTACTCCA -3') (62) at a concentration of 0.5 µM. The cycling conditions were as follows: initial denaturation at 98 °C for 5 min followed by 40 cycles of 98 °C for 5s, 58 °C for 5 seconds and 72 °C for 20s, with a final extension at 72 °C for 1 min. The PCR products were visualized on a 1.5% agarose gel stained with GelRed® Nucleic Acid Gel stain (Biotium, Fremont, CA, USA).

Initiation of a primary cell culture from eggs of *Cx. pipiens molestus*

Six attempts were made to initiate a primary cell culture from eggs of colony *Cx. pipiens molestus*, largely following the protocol by Segura et al. (37). The procedures are summarized in detail in Table 1. In brief, freshly laid egg rafts were collected from an adult cage and placed in a petri dish that was then incubated at two different temperatures to promote cell division and placed in a LAF-bench. The egg rafts were then transferred into a 1.5 mL Eppendorf tube using a spatula and the individual eggs were separated using a soft paintbrush. The eggs were washed and homogenized in 100 µL of cell culture medium using a sterile pestle and transferred into a vessel for growth – either a well on a 96-well cell culture plate or a 25 cm^2 tissue culture flask. The medium was changed once every week.

In the first five attempts, the cells were passaged 1:1 into a 24-well plate after three days but the old 96-well plate with the few remaining cells was retained. In the 24-well plate, the cells were grown in 0.7 mL of medium that was changed once every week. As the 24-well plate was later abandoned due to contamination, the cells that proliferated in the original 96-well plate were passaged 1:1 again, 49 days after the original seeding, into a neighbouring well on the 96-well plate. Simultaneously, the temperature at which they were incubated was increased to 28 °C and the concentration of TPB reduced to 5%.

In addition, dense cell clusters in the original well were disrupted with a 200 µL pipette tip due to persistent lack of growth in these areas, 58 days after the original seeding, and passaged into a neighbouring well.

Table 1. Stepwise procedure for the two methods used for starting a primary cell culture in this study. The first five attempts were performed using method 1 and the sixth attempt was performed using method 2.

	Method 1	Method 2
Starting material	1 egg raft	10 egg rafts
Incubation	24h at 4 °C 6h at room temperature	24h at 4 °C 3h at 37 °C
Disinfection through submersion	3 x 70% ethanol for 2 min	1 x 0.5% NaClO for 5 min 1 x 95% ethanol for 1 min
Washes	3 x sterile distilled water 1 x cell culture medium	3 x sterile distilled water 1 x cell culture medium
Medium	Equal parts L-15[1]/Grace's[2] 20 % FBS[1] 10% TPB[1] 1% PenStrep[1]	Equal parts L-15[1]/Grace's[2] 20 % FBS[1] 1% PenStrep[1] 1% Amphotericin B[2]
Growing vessel	One well in 96-well cell culture plate	25 cm^2 tissue culture flask
Incubation for growth	25 °C without CO_2	28 °C without CO_2
Total attempts	5	1

[1] – Thermo Fisher Scientific; [2] – Sigma-Aldrich. Abbreviations: FBS – Foetal Bovine Serum, TPB – Tryptose Phosphate Broth.

For the sixth attempt, cells were never passaged due to the large amount of space available in the T25 flask. Instead, cell culture medium was changed every week. Cells that had not attached were rescued by centrifugation of the old medium at 200 x G for 5 minutes and subsequent resuspension in fresh medium.

Infection of Hsu cells with *Wolbachia*

Hsu cells were maintained at 28 °C without CO_2 in Schenider's Drosophila medium (Thermo Fisher Scientific) supplemented with 10% FBS, 5% TPB (Thermo Fisher Scientific), and 1% PenStrep (Thermo Fisher Scientific). Although *Cx. quinquefasciatus* is naturally infected with *Wolbachia*, Hsu cells are not (data not shown). Therefore, Hsu cells were inoculated with *Wolbachia* derived from *Cx. pipiens molestus* ovaries using the shell vial technique, modified from Dobson et al. (63).

Four attempts were made to infect Hsu cells with *Wolbachia*, the details of which are shown in Table 2. The extraction of donor material was done largely following the protocol by Lynn (64). In brief, female mosquitoes were stunned by freezing in -20 °C for 10 min, after which they were disinfected by soaking in 70% ethanol. The ovaries were dissected in a drop of

dissection medium (see Table 2) and homogenized in a 1.5 mL Eppendorf tube containing 300 µL of cell medium using a pestle. The homogenate was overlaid onto Hsu cells grown to approximately 50-80% confluency in a well on a 24-well cell culture plate, which was then centrifuged to destabilize the membrane of recipient cells and allow *Wolbachia* to infect them (63). As a negative control, a similar number of cells were grown in a neighbouring well and treated in the same manner but without the inclusion of ovaries in the overlaid medium. In the first attempt, the plate was directly incubated at 28 °C, which resulted in massive contamination in the well after 24 hours of growth. In the following attempts, cells were washed once with medium after centrifugation and passaged 1:1 into a well on a 6-well tissue culture plate. Upon reaching confluency, the cells were passaged into larger vessels until being continuously passaged in 75 cm^2 tissue culture plates. Over the passages, PenStrep supplementation was gradually reduced to 1%. The presence of *Wolbachia* was monitored through quantitative PCR (qPCR) as described in the next section.

Table 2. Detailed stepwise procedures for the four attempts to infect Hsu cells with *Wolbachia* derived from *Cx. pipiens molestus* ovaries.

	Attempt 1	Attempt 2	Attempt 3	Attempt 4
Disinfection of mosquitoes	1. Soaking in 70% ethanol 2. Drying 3. Soaking in 70% ethanol 4. Washing in sterile water	1. Soaking in 70% ethanol 2. Drying 3. Soaking in 70% ethanol 4. Drying	1. Soaking in 70% ethanol 2. Drying 3. Soaking in 70% ethanol 4. Drying	1. Soaking in 70% ethanol 2. Washing in PBS 3. Drying
Dissection medium	Cell culture medium[1]	Sterile water	Sterile water	PBS
Dissection yield	19 ovaries	15 ovaries	27 ovaries	19 ovaries
Washes of ovaries	1 x sterile water	1 x 70% ethanol	1 x sterile water	1 x PBS
Decontamination[2]	Submersion in cell culture medium[1] for 2h at room temperature	Not done	Not done	Not done
Medium for homogenization, overlay, and initial growth	Schneider's[3] 10% FBS[3] 5% TPB[3] 1% PenStrep[3]	Schneider's[3] 10% FBS[3] 5% TPB[3] 10% PenStrep[3] 1% Amphotericin B[4]	Schneider's[3] 10% FBS[3] 5% TPB[3] 10% PenStrep[3] 1% Amphotericin B[4]	Schneider's[3] 10% FBS[3] 5% TPB[3] 10% PenStrep[3] 1% Amphotericin B[4]
Centrifugation	1000 x G for 1h at 15 °C	800 x G for 1h at RT	800 x G for 1h at RT	800 x G for 1h at 26 °C

Passaging from 24-well plate	Not passaged	Passaged 1:1 into a well on a 6-well cell culture plate directly after centrifugation	Passaged 1:1 into a well on a 6-well cell culture plate directly after centrifugation	Passaged 1:1 into a well on a 6-well cell culture plate directly after centrifugation
Further passaging	Not done due to contamination	1. 1:1 into a 25 cm^2 tissue culture flask 2. 1:1 into a 75 cm^2 tissue culture flask (PenStrep reduced to 5%) Further passaging not done due to low and declining presence of *Wolbachia*	1. 1:1 into a 25 cm^2 tissue culture flask (PenStrep reduced to 5%) 2. 1:1 into a 75 cm^2 tissue culture flask (PenStrep reduced to 1%) 3. Continuous passaging 1:4 into a 75 cm^2 tissue culture flask	1. 1:1 into a 25 cm^2 tissue culture flask Further passaging not done due to contamination

[1] – same medium as for ovary homogenization and cell overlay; [2] – allows contaminating cells to diffuse away from the tissue; [3] – Thermo Fisher Scientific; [4] – Sigma-Aldrich.

Quantification of *Wolbachia* using qPCR

Suspensions of *Wolbachia*-inoculated Hsu cells were collected on each passage. The DNA from these was extracted using E.Z.N.A.® Tissue DNA Kit (Omega Bio-Tek; Norfolk, GA, USA) according to the manufacturer's instructions and used as a template for qPCR.

qPCR was run in triplicates using QuantiTect® SYBR® Green RT-PCR kit (Qiagen) in 10 µL reactions, of which 1 µL was the template. For detecting *Wolbachia*, the forward primer wspFQALL (5'-GCATTTGGTTAYAAAATGGACGA-3') and the reverse primer wspRQALL (5'- GGAGTGATAGGCATATCTTCAAT-3') (65) were used to target the gene for *Wolbachia* surface protein (*wsp*). For normalization against a mosquito gene, primers targeting the gene for ribosomal protein L32 (*RpL32*) were used, namely RpL32-F2 (5'-AAGCCGAAAGGTATCGACAA-3') and RpL32-R2 (5'-CAGTAGACGCGGTTCTGCAT-3') (27). Both primer pairs were used at a final concentration of 1 µM. The following cycling conditions were used: initial activation at 95 °C for 15 min followed by 40 cycles of denaturation at 95 °C for 15s, annealing at 60 °C for 30s and extension at 72 °C for 30s. A melt curve analysis was done in the range 60-95 °C. The qPCR was carried out on a CFX96™ Real-Time PCR detection system (BioRad, Hercules, CA, USA).

DAPI staining of *Wolbachia*-infected Hsu cells

As *Wolbachia*'s 800kbp genome is large enough to be stained by nucleic acid dyes and be visible under a fluorescence microscope (66,67), DAPI staining was used in an attempt to verify the presence of *Wolbachia* inside individual cells. $2.5E^5$ Hsu cells were seeded onto glass cover slips at the bottom of a 24-well plate and allowed to grow overnight. The cells were fixed with methanol at -20 °C for 20 min, washed three times with PBS and permeabilised with 0.1% Triton X-100 (Merck Millipore, Burlington, MA, USA) for 15 minutes at room temperature. After another three washes with PBS, ProLong™ Diamond Antifade Mountant with DAPI (Life Technologies, Eugene, OR, USA) was added onto the slips that were allowed to dry and imaged under a Nikon Eclipse 90i fluorescence microscope. Image processing was done using the software Fiji/ImageJ2 (68).

Virus strain

SINV genotype I, strain 09M-358-5, was used in all experiments involving live virus. The virus strain was originally isolated from one *Cx. torrentium* collected in Mehedeby, Sweden, in 2009. The virus stock that was used in infection of live mosquitoes and in the 7-day experiment involving Hsu cells had been passaged two times in Vero cells. For the 12-hour experiment involving Hsu cells, virus stock that had been passaged three times in Vero cells was used.

Infection of Hsu cells with SINV

For a 7-day experiment, approximately $2.5E^5$ cells were seeded into each well on two 24-well plates, one surface-treated and one non-treated, and allowed to grow overnight. The number of cells in each well on the day of infection was determined by counting the cells in two wells on each plate, after which the cells were infected with MOI 0.1 (non-treated plate) and MOI 0.5 (surface-treated plate) of virus in 0.2 mL of cell medium (Scheider's Drosophila medium supplemented with 10% FBS, 5% TPB, 1% PenStrep and 1% amphotericin B). The virus was allowed to infect the cells over 1 hour at 28 °C. The virus dilution was then removed, and the cells were washed once with 0.5 mL of cell medium. Secretion of viral particles into the supernatant was measured over 168 hours (7 days) by sampling and refilling all of the supernatant in wells designated specific time points up until time point 72h. After 72h, wells designated time points 0h, 24h, 48h, and 72h, were re-sampled for time points 96h, 120h, 144h, and 168h, respectively. The experiment was performed in duplicate for each MOI.

To obtain a higher resolution of SINV replication kinetics during the first 12 hours after inoculation, $5.3E^6$ Hsu cells were seeded into three 25 cm^2 tissue culture flasks and allowed

to grow overnight. After removal of the medium, 1.5 mL of virus suspension (in Dulbecco's Modified Eagle Medium [Thermo Fisher Scientific] supplemented with 15% FBS and 1% PenStrep) containing $4.5E^6$ PFU of virus, which corresponds to an estimated MOI 0.4 based on the cell doubling time, was added to the flasks and allowed to infect the cells for 1h at 28 °C. The cells were washed with medium once and thereafter incubated at 28 °C for 12 hours with 5 mL of medium. Medium was sampled without replacement every two hours. After 12 hours, the medium was refilled to 5 mL and the cells allowed to grow for an additional 18 hours, after which the medium was removed, the cells washed three times with PBS and resuspended in 5 mL of medium. The cell suspension was sampled to investigate possible attachment and entry of SINV into Hsu cells. As a negative control, $1.5E^6$ PFU was seeded into a 25 cm^2 tissue culture flask containing 5 mL of medium but no cells.

To assess the degradation of SINV RNA over one week (i.e. the total time of the cell infection experiment), a dilution of SINV was kept under the same conditions, but without cells. 0.7 mL of cell medium containing approximately 10^5 PFU/mL of SINV was seeded into 21 wells on a 24-well plate and triplicate samples were taken every day over the course of 7 days to measure the level of SINV RNA in the well. Negative control wells containing no virus were sampled on days 2, 4, and 6.

The amount of viral RNA, both for viral replication and RNA degradation experiments, was determined through RT-qPCR using a standard curve, obtained from serial dilutions of a virus stock with a known number of PFU per mL. To account for evaporation during the 7-day experiments, the copy number of viral RNA was normalized to the retrieved amount of supernatant from the well.

RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Presence of virus was detected using Reverse Transcription qPCR (RT-qPCR) with the qScript™ One Step qRT PCR-kit (Quantabio, Beverly, MA, USA). RT-qPCR was performed in 25 µL reactions using the forward primer 5'-GGTTCCTACCACAGCGACGAT-3' and the reverse primer 5'-TGATACTGGTGCTCGGAAAACA-3' at concentrations of 0.8 µM and the TaqMan probe FAM-TTGGACATAGGCAGCGCA-MGBNFQ at a concentration of 0.1 µM, which target a 74 base region of the nsp1 gene (69). The template was the extracted RNA and composed 5 µL of the total reaction volume. The RT-qPCR was carried out on a CFX96™ Real-Time PCR detection system (BioRad) with the following cycling conditions: reverse transcription

at 50 °C for 10 min, initial activation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 15s and annealing/extension at 60 °C for 1 min.

To infer statistical significance of differences in RNA titer in the viral replication experiment, multiple paired *t*-tests with Bonferroni correction were used to compare each time point to all other time points.

Infection of *Cx. pipiens molestus* with SINV

Female *Cx. pipiens molestus* between one and two weeks old were sorted out and starved for 24h before being allowed to feed on horse blood spiked with 10^6 PFU/mL of virus. The blood was administered through an odorized cotton pad over two hours; cotton pad samples were taken before and after feeding to ensure virus stability. Blood-fed females were sorted out and maintained at 21 °C for three weeks before being used to measure infection, dissemination, and transmission rates. Mosquitoes that had not fed on blood after the first attempt were saved in a separate jar and another attempt on blood feeding was performed one week later.

Infection rate was measured by detecting virus in whole bodies of blood-fed mosquitoes. Dissemination rate was measured by detecting virus in the legs of infected mosquitoes, removed from the bodies using forceps. Transmission rate was measured by detecting virus in the saliva of infected mosquitoes, collected through forced salivation. Briefly, mosquitoes were stunned by freezing for 10 min in -20 °C and saliva was extracted through the intubation of the proboscis into a capillary tube containing 1 µL of immersion oil for 30 min. Bodies, legs, and saliva were placed into Eppendorf tubes containing 300 µL of homogenizing buffer and stored at -80 °C.

Mosquito legs and bodies were homogenized using TissueLyser II (Qiagen), as described earlier (see Detection of *Wolbachia* using conventional PCR). RNA extraction and RT-qPCR were performed as described in the previous section (see Infection of Hsu cells with SINV). Confidence intervals for the infection, transmission and dissemination rates were calculated using the Wilson score interval.

Data analysis

All data storage and statistical analysis was done in Microsoft Excel 2016 (Microsoft, Redmond, WA, USA) and RStudio (RStudio team, Boston, MA, USA).

Results

Elimination of *Wolbachia* in adult mosquitoes by tetracycline treatment over two generations

To create a *Wolbachia*-free line of *Cx. pipiens molestus*, colony mosquitoes were treated with oral tetracycline over two generations. From the first generation of mosquitoes treated with tetracycline, 5 males were tested for *Wolbachia* after the first week of treatment and an additional 7 males and 5 females were tested near the end of the two weeks treatment (Figure 1A). All tested F0-mosquitoes were found to still carry *Wolbachia*, and five F0-females that were collected 25 days after completed treatment also remained positive for *Wolbachia*.

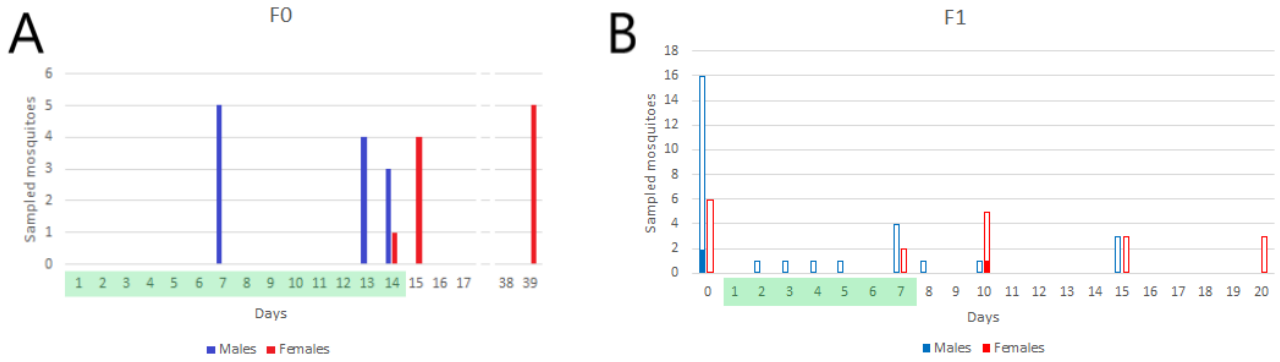

Figure 1. Number of tested F0 (A) and F1 (B) mosquitoes for the presence of *Wolbachia*. The tetracycline treatment period is highlighted in green. Bar height shows the total number of tested mosquitoes and their day of collection relative to the treatment period. Filled portions of bars indicate mosquitoes positive for *Wolbachia* and outlined bars indicate mosquitoes negative for *Wolbachia*. Day 0 for F1 mosquitoes includes all mosquitoes sampled before the start of the treatment.

The effect of the treatment became noticeable when sampling F1-mosquitoes, the second treated generation. 16 males and 6 females were sampled before the start of the treatment, of which 2 males and no females were positive for *Wolbachia*. In total, 48 mosquitoes, 29 males and 19 females, were sampled from the F1 generation, of which a total of 2 males and 1 female contained traces of *Wolbachia* (Figure 1B).

The treatment resulted in a fatality rate of 69% for the F0 generation, adjusted for sampling. Most deaths occurred during the second week of treatment, coinciding with the increase in tetracycline dosage. A similar pattern can be seen for the F1 generation. However, fewer deaths occurred during the treatment, which lasted only one week (Figure 2). No comparative assessment was made to elucidate the confounding effects of aging; thus, despite the high number of deaths among the treated group no conclusion can be drawn about the health effects of tetracycline.

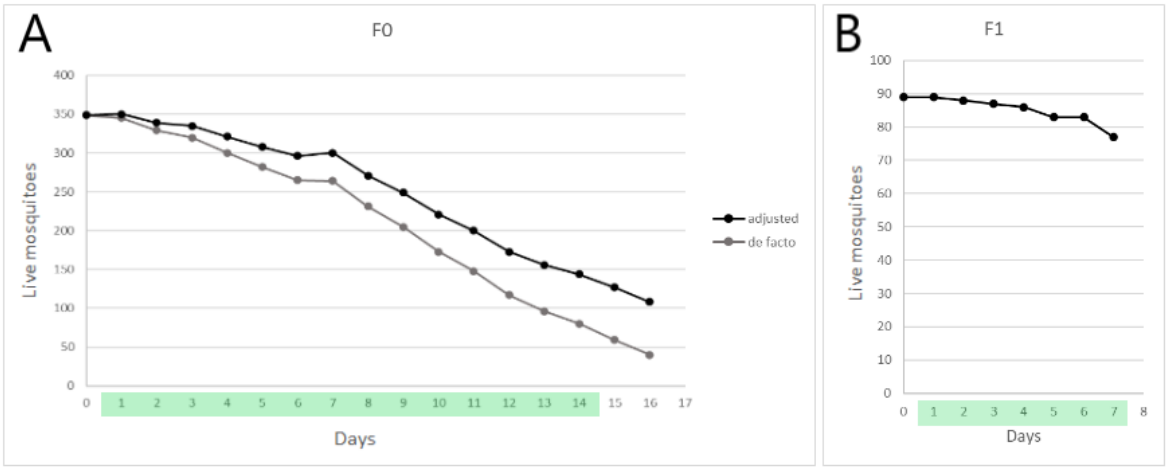

Figure 2. Survival curves for F0 (A) and F1 (B) mosquitoes during the tetracycline treatment. The black line in graph A shows survival adjusted for daily sampling of live mosquitoes while the de facto mosquito numbers are shown as a grey line. Since no sampling of live mosquitoes was done for the F1 generation, no adjustment is needed in graph B. The treatment period is highlighted in green.

The fecundity of the F0 mosquitoes was reduced, with few egg rafts laid and a low hatch rate. The 40 mosquitoes that were alive at the end of the experiment, of which the majority were female, produced only 111 adult offspring in total. The reduced reproductive capacity persisted into the F2 generation, albeit to a smaller extent. The F3 generation could reproduce at levels not conspicuously different to that of the non-treated mosquitoes maintained in the colony.

35 live adult males and 15 live adult females were sampled from the F3 generation. Of these, none were found to carry *Wolbachia*. A *Wolbachia*-free line of *Cx. pipiens molestus* was thus successfully generated although at least two generations were needed for recovery from the tetracycline treatment.

Isolation of embryonic cells from *Cx. pipiens molestus*

To establish a platform for *in vitro* studies of the impact of *Wolbachia* on viral replication in *Cx. pipiens*, a primary cell culture was initiated from homogenized embryos of *Cx. pipiens molestus*. Out of the six attempts, two resulted in failure to isolate cells and three resulted in failure to obtain consistent cell growth, where the isolated cells were few in number and did not increase over time. The five attempts that failed did so due to two main reasons: (1) the eggs were homogenized overzealously, leading to extensive cell damage, and (2) the egg rafts were laid too long before the homogenization, leading to premature hatching of larvae during the disinfection process and subsequent death of exposed cells. The number of cells recovered was thus too low for stable proliferation. The results described in this section are

for the one successful attempt, which also happened to be the first one, and originates from following the protocol for method 1 (see Materials and Methods).

Directly after homogenization, lumps and individual cells could be seen (Figure 3). Within two days after seeding, some cells had attached, and numerous lumps were seen twitching. On the third day, the cells were passaged into a 24-well plate, upon which most of the lumps were disrupted. The cells remaining in the original well kept proliferating, albeit very slowly. Lumps were seen twitching for up to three weeks after the original seeding.

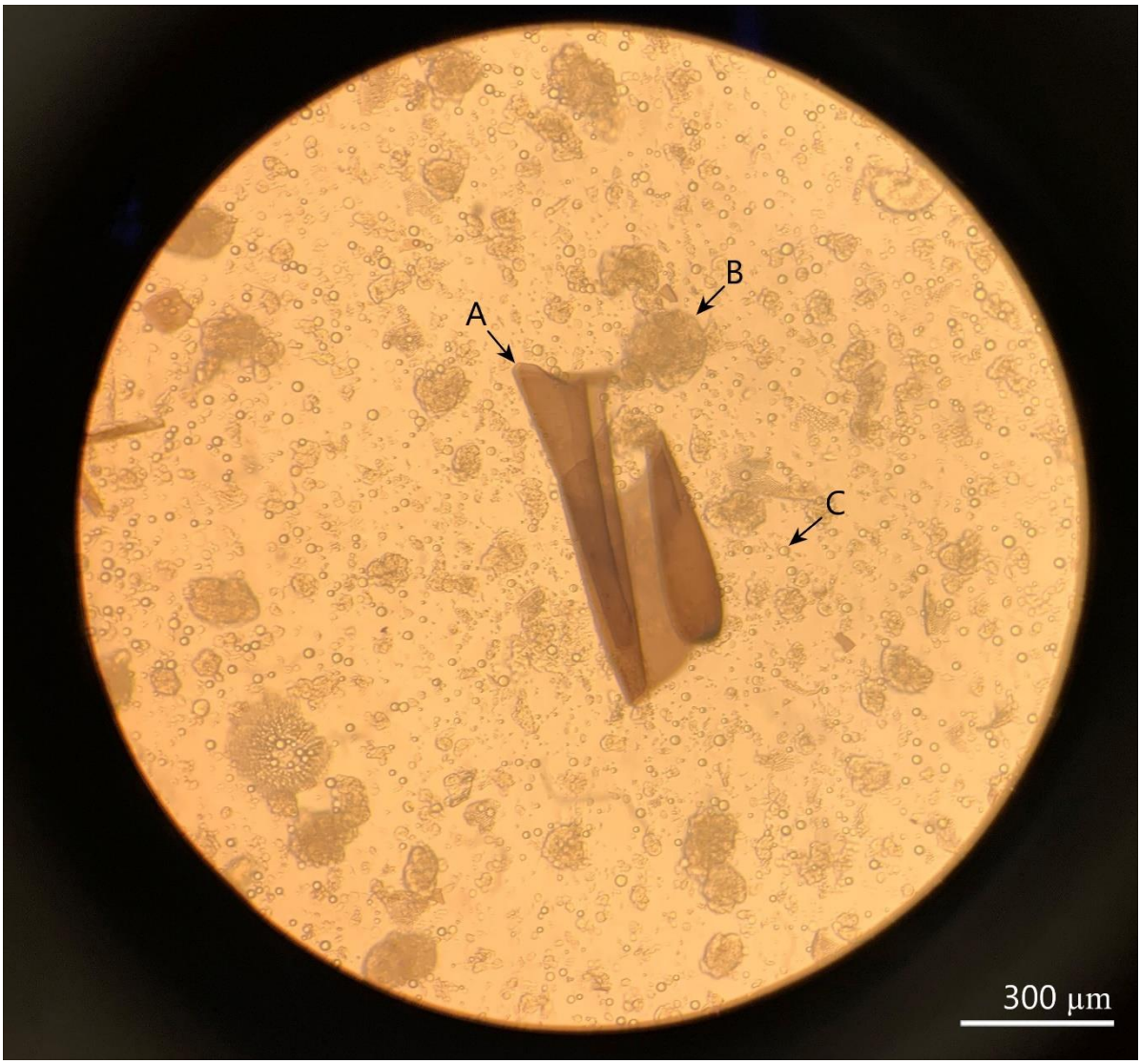

Figure 3. Embryo homogenate in a 96-well plate viewed through an inverted microscope. Remains of an eggshell (A) are visible, as well as both lumps of cells (B) and individual cells (C).

In the 24-well plate, cells had differentiated and grew in clusters. Certain cells formed tissue-like structures on the bottom of the well, including pulsating muscle cell constellations (Figure 4A). These, however, proliferated very sparingly, if at all. Following the first change

of cell medium, non-attached cells were resuspended following centrifugation and placed in a separate well, where they attached to the surface. These were mostly round cells and grew in tight clusters (Figure 4B) with a doubling time of approximately three days, after which proliferation slowed. Due to contamination issues occurring one month after the original seeding, the cells in the wells on 24-well plates were transferred to a new 24-well plate but failed to re-attach to the surface. Instead, they attached to each other and grew in suspended lumps. After three weeks of growing in suspension, they were passaged back into a well on a 96-well plate in an attempt to concentrate them in sufficient numbers for attachment to the surface. A small fraction of the cells was recovered and successfully attached to the surface, but their growth was too slow at the time of writing to assess progress.

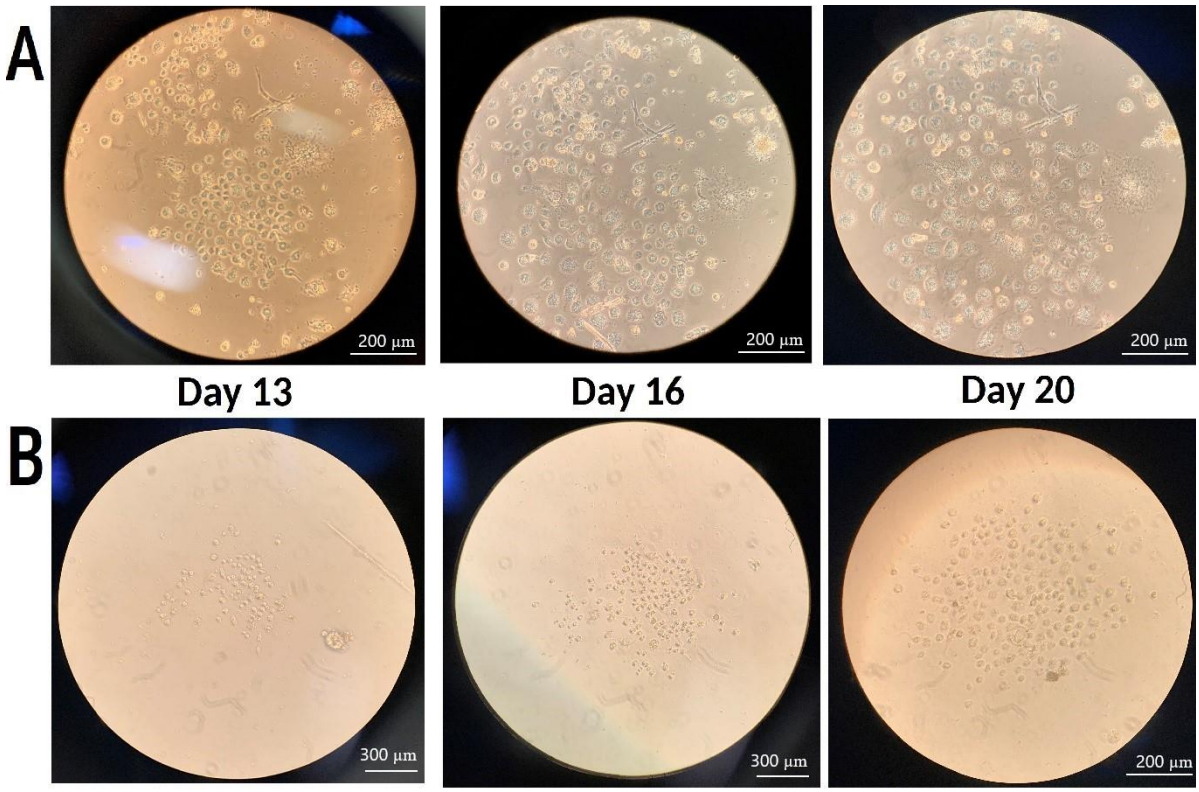

Figure 4. Images from an inverted microscope showing growth of two different cell clusters in two different wells on a 24-well plate over 7 days, day 13 to day 20 after the homogenization of embryos. Row A shows progression of a cluster with morphologically diverse cells, including an actively pulsating muscle cell constellation that does not increase in size or number of cells. These have been passaged directly from the original well and have grown considerably in size but very little in number over the course of one week. Row B shows a cluster of attached cells, originating from suspended cells taken from the well harbouring cells in row A. These cells have nearly doubled in number between days 13 and 16 but have thereafter slowed in their proliferation.

Back in the original well, cells were growing in great numbers near the edge of the well (i.e. from the area where most cells remained after the first passage) but eventually stopped, with no significant growth over one month, upon which the cells from this original well were passaged once again, this time into a neighbouring well. After passaging, most cells attached

to the surface, although some formed lumps. Movement was once again seen in the lumps and intensified over the course of two weeks in one of the lumps. Although movement partially subsided thereafter, large spherical structures composed of cells appeared on the lump (Figure 5). In general, cell growth was subtle, with little increase in cell numbers over time (Figure 6).

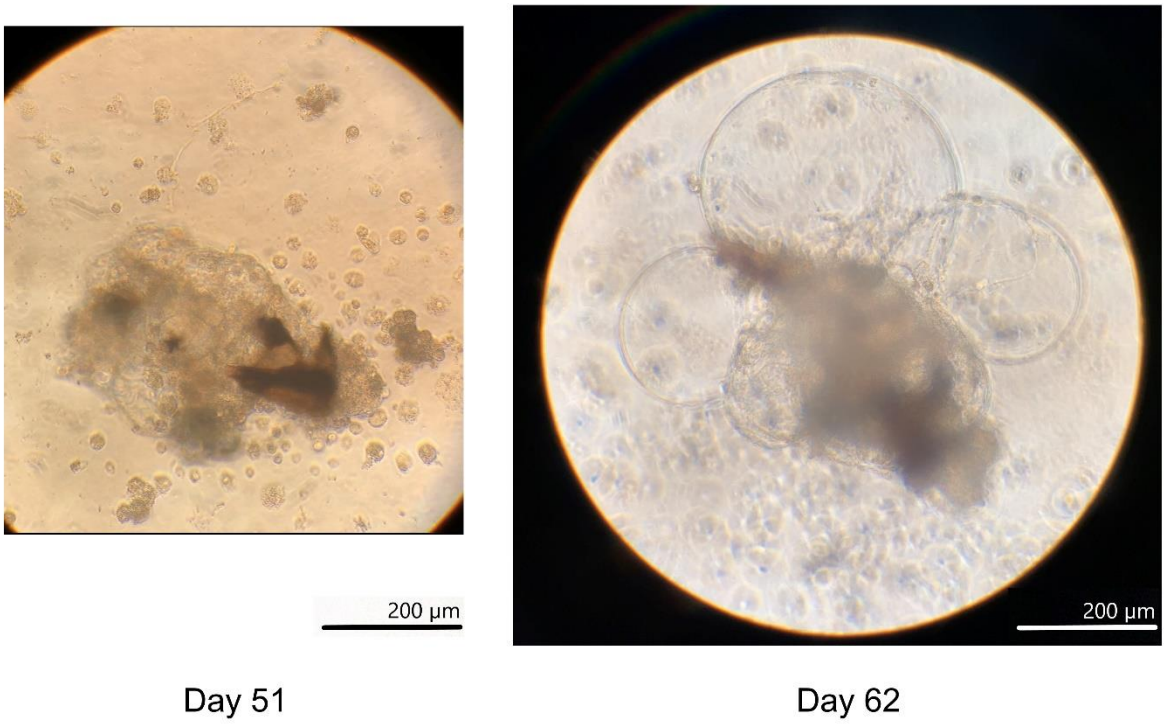

Figure 5. A lump of cells that emerged following the second passage of cells from the original 96-well plate well has over 11 days gained large growths of epithelioid cells encapsulating empty space. Image taken through the lens of an inverted microscope.

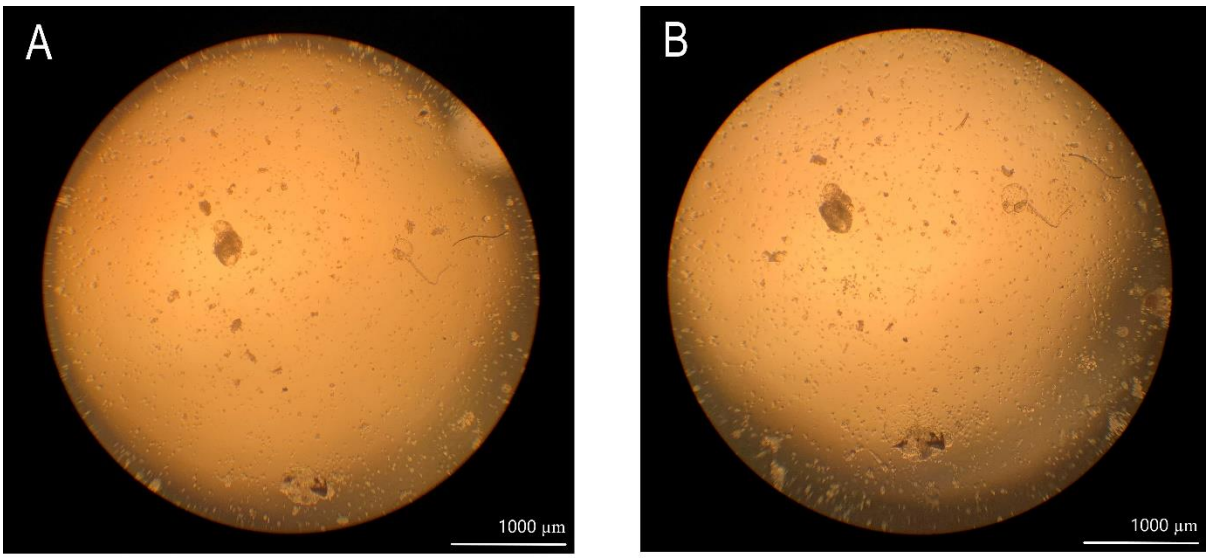

Figure 6. Passaged cells in a 96-well plate well one week apart (day 50 – A, and day 57 – B), viewed through an inverted microscope. Little increase in cell numbers is seen. The clearest sign of growth is the increase of the size of the cell lump attached to a contaminating fibre in the top right quadrant of the images.

As the second passage failed to displace the numerous cells on the edges, the cell scraper was abandoned in favour of a pipette tip and a third passage from the original well was performed. Edge-living cells were successfully moved into a neighbouring well and were seen to attach and form clusters within a day of passaging. These, however, grew very little in number and scattered over time (Figure 7). In the well as a whole, however, cells persisted albeit little change was seen in their numbers.

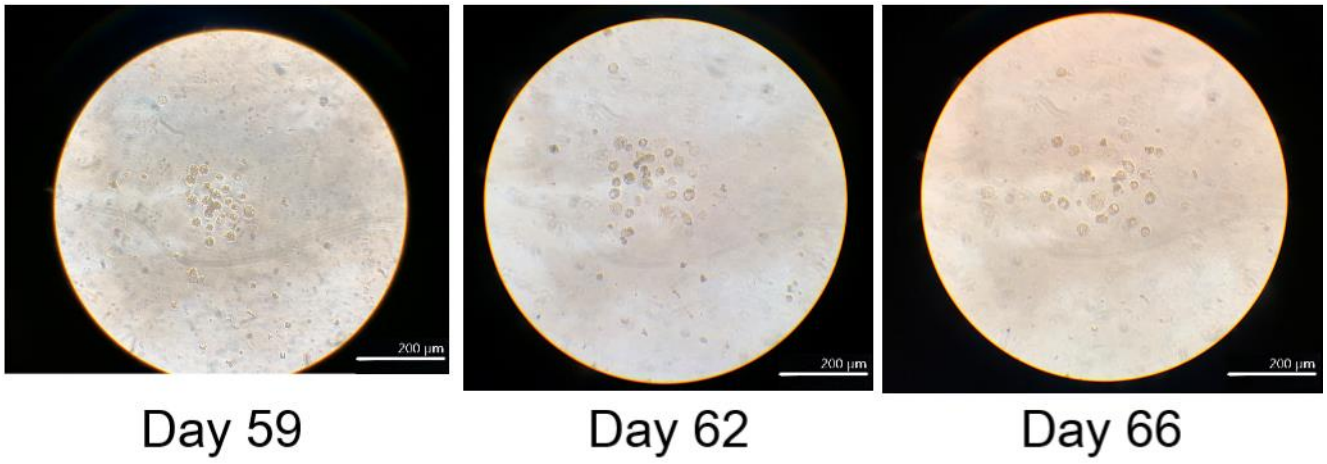

Figure 7. Cell clusters formed one day after the passaging of edge-living cells with the help of a pipette tip. Over one week, the cells did not increase in number and dispersed from their cluster.

In summary, embryonic cells were successfully isolated from the eggs of *Cx. pipiens molestus*. These differentiated into morphologically different cell types, but the variation in cell type diminished over time as some cells grew more than others. At the time of writing, 89 days after first plating the egg homogenate, the primary cell culture was alive and propagating but very slowly. A functioning protocol for cell isolation and growth has thus been established.

Wolbachia **infection in Hsu cells**

Four attempts were made to infect Hsu cells with *Wolbachia* using the shell vial technique and *Cx. pipiens molestus* ovaries as donor material as an alternative method of creating an *in vitro* platform for investigation of the effect of *Wolbachia* on viral replication. Two attempts, attempt 1 and 4, failed due to contamination while two attempts, attempts 2 and 3, resulted in successful cell recovery. Both *Wolbachia*-inoculated cells and the similarly treated negative control replicated substantially slower, with a 2-3-fold increase in doubling time seen after centrifugation. Although the cells' rate of proliferation gradually recovered over five passages, a stable infection by *Wolbachia* could not be demonstrated. qPCR results suggested a consistent reduction in *Wolbachia* amount of approximately 60-90% on every passage after

the first, where *Wolbachia* was reduced by 36% in attempt 2 and by 57% in attempt 3 (Figure 8). Although a wsp:RpL32 ratio of 2.2 was seen in attempt 3 right after the infection procedure, the ratio had declined to 0.0005 after the fifth passage.

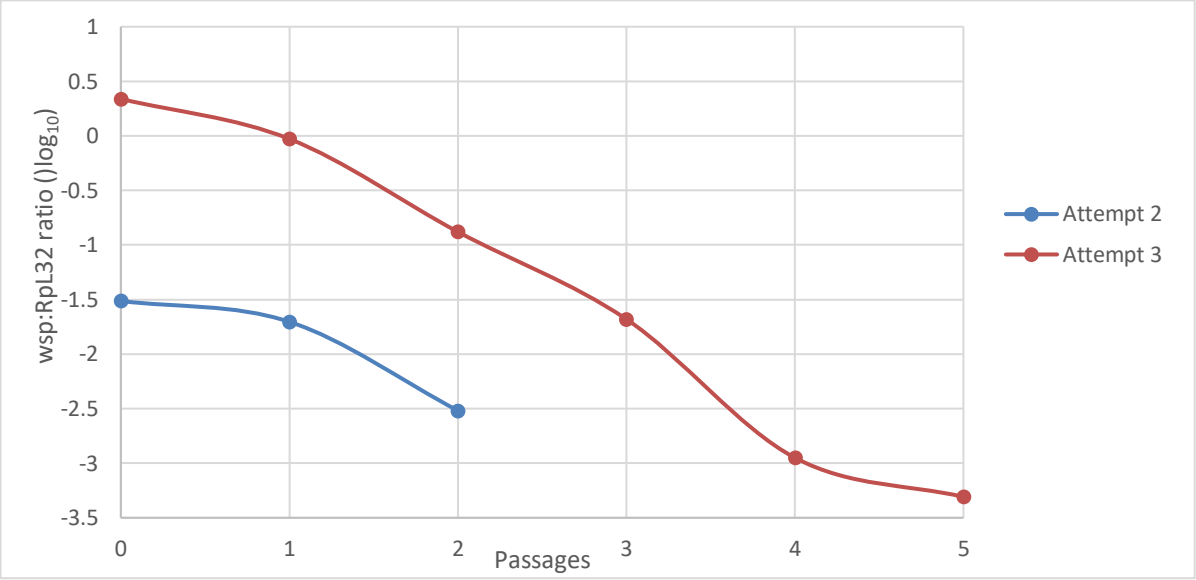

Figure 8. The development of the ratio of the copy number of *wsp* to *RpL32* over sampled passaged for the two attempts to infect Hsu cells with *Wolbachia* that resulted in in successful cell recovery. Only the original inoculated cells and the first two passages have been sampled for attempt 2 due to declining *Wolbachia* rates and forfeiting of the cell line. Every point represents the mean of a technical triplicate of one sample.

DAPI staining was performed on cells from both attempts 2 and 3 to visualize *Wolbachia* as fluorescent specks in the cytoplasm. For the cells from attempt 2, the cells used for DAPI staining were from passage 3 (for which qPCR was not performed) and for attempt 3, the cells were from passage 4, where the wsp:RpL32 ratio of 0.001 is equivalent to 2 *Wolbachia* per 1000 cells as *wsp* is present in only one copy in the bacterium while *RpL32* is present in two due to the diploid nature of Hsu cells. Representative images are shown in Figure 9.

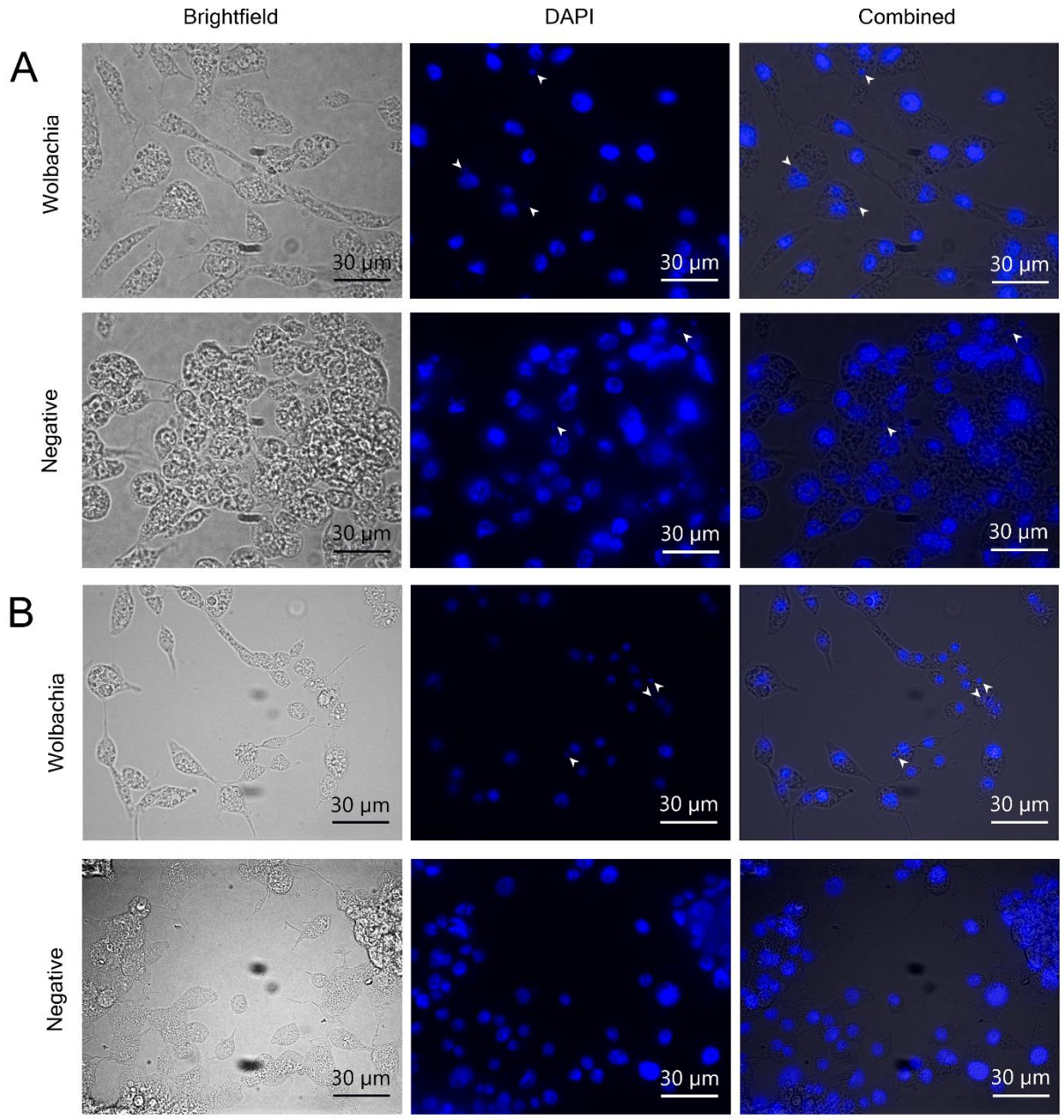

Figure 9. Brightfield and DAPI images of *Wolbachia*-inoculated cells and a corresponding negative control from attempts 2 (A) and 3 (B). Examples of small specks of DAPI stain, seen in *Wolbachia*-inoculated cells from both attempts and also in the negative control from attempt 2, are highlighted with arrowheads. No DAPI-stained specks of DNA were seen in the negative control for attempt 3.

Minor specks of DAPI-stained DNA can be seen both in the *Wolbachia*-inoculated cells and in the negative control cells from attempt 2, discrediting them as a reliable indication of *Wolbachia* (Figure 9A). The specks were present in the *Wolbachia*-inoculated cells from attempt 3 but seemed absent in the negative control (Figure 9B). The specks of DNA were very rare, and their low abundance is in accordance with the expected low frequency of *Wolbachia*. Nonetheless, the non-specific staining by DAPI, combined with the presence of

stained specks in the negative control following attempt 2, prevents the aggregations of DNA seen in attempt 3 from being unambiguously attributed to *Wolbachia*.

SINV replicates poorly in Hsu cells

To verify the possibility of comparing SINV replication in *Wolbachia*-infected and non-infected Hsu cells, a pilot experiment to measure SINV replication in healthy, non-centrifuged Hsu cells was performed. All of the supernatant was sampled from separate wells on a 24-well plate for the timepoints 0-72hpi, after which the wells were refilled and re-sampled again for timepoints 96-168hpi. Cell-free controls, containing a higher starting amount of virus, were sampled every 24 hours from wells specifically dedicated to each time point. A minor increase in SINV RNA amounts is seen after 6 hours post infection (hpi) for both MOI 0.1 and MOI 0.5, albeit no time points in the two replicates differed significantly in their RNA content (Figure 10). SINV did not seem to replicate effectively over time as the RNA content tended to decrease to a greater extent in the presence of cells than without cells, both before and after 96hpi (the breakpoint for well re-sampling). After 48 hpi, cells began to detach from the bottom of the wells in the plate containing cells infected with MOI 0.1. The detachment was minimal at first but increased at later timepoints. After 144 hpi, we tended to detect more SINV RNA in the samples from cells infected with MOI 0.1 than with MOI 0.5.

The observed increase in viral RNA at 6 hpi prompted a repeat experiment to obtain a higher resolution of viral replication kinetics during the first 12 hpi. No sign of secretion of viral particles into the medium was seen as the RNA copy number remained constant across all measurements (Figure 11A). However, samples of the cells at 30 hpi, taken after they were washed and resuspended in medium, contained substantially more SINV RNA copies than the cell free-negative control, subject to the same washes and resuspension to show the expected reduction in RNA copy number due to washing (Figure 11B). Furthermore, the RNA copy number was significantly greater in cells resuspended at 30 hpi than in the supernatant at 12hpi (t-test: $p = 0.014$). On average, there was 7 times more viral RNA detected in the resuspended cells at 30 hpi than in the supernatant at 12 hpi.

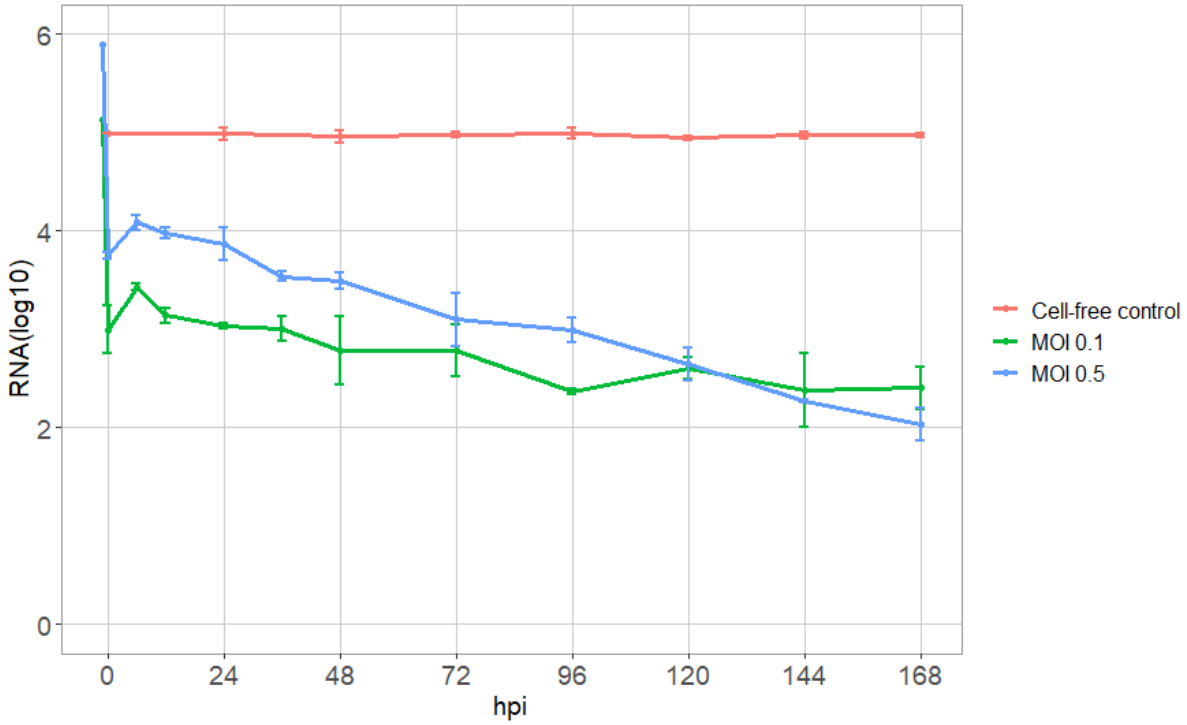

Figure 10. SINV RNA copy numbers per well in a 24-well plate following infection of Hsu cells with MOI 0.1 and MOI 0.5 measured over one week, as well as in the cell-free control. Time point -1 hpi corresponds to the RNA copy number in the medium used to inoculate the cells. Error bars show one standard deviation. MOI – multiplicity of infection, hpi – hours post infection.

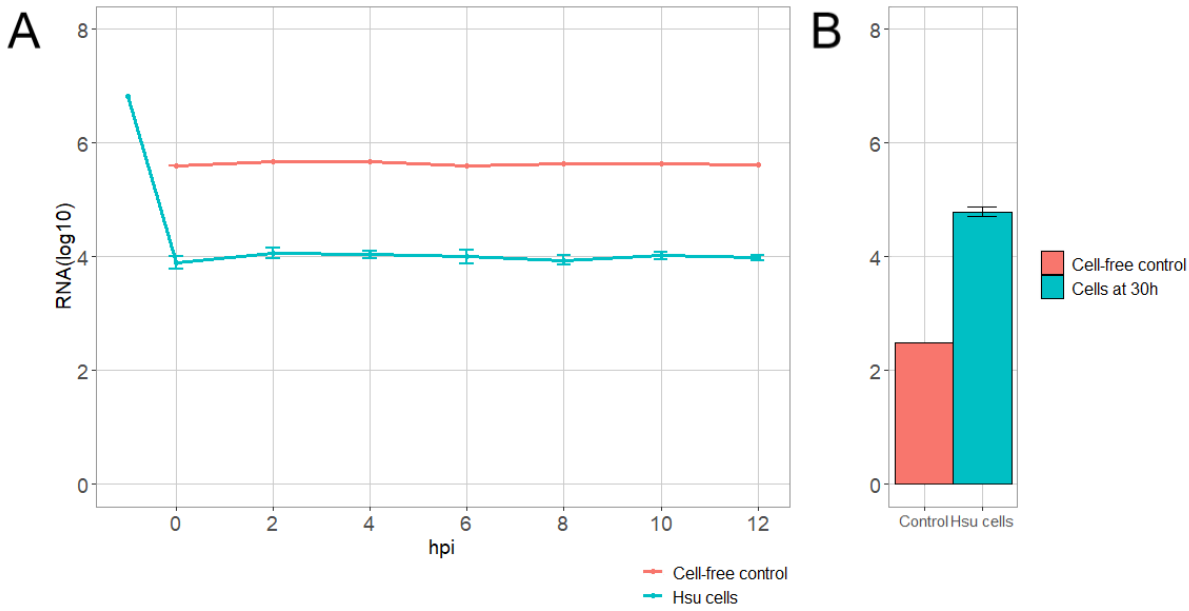

Figure 11. (A) SINV RNA copy number per mL in the supernatant during the first 12 hours after inoculation of Hsu cells and a cell-free control. (B) SINV RNA copy number per mL in washed and resuspended Hsu cells at 30 hours after inoculation as well as in the cell-free control flask subject to the same washing and resuspension. Error bars show one standard deviation. hpi – hours post infection.

SINV infection of *Cx. pipiens molestus*

Three rounds of mosquito infections, with two attempted blood-feedings each, were performed to determine the vector competence of *Wolbachia*-infected *Cx. pipiens molestus* for SINV transmission. Mosquitoes with clear presence of blood in the abdomen were considered blood-fed, regardless of the amount. It must be noted that the majority of the blood-fed mosquitoes only had minor amounts of blood in the abdomen, with only a few being fully engorged. In total, 25 blood-fed mosquitoes were tested for SINV infection after 3 weeks of incubation. Of these, three were positive for SINV in whole-body samples and were subject to testing for dissemination and transmission through RT-qPCR on leg and saliva samples, respectively. All three had presence of SINV in their legs and two of three, those with the highest amounts of SINV RNA in the bodies (data not shown), had SINV present in their saliva. The results are summarized in Table 3.

Table 3. Summary of the results from blood-feeding, survival and vector competence of *Cx. pipiens molestus*.

		Start (dead between attempts)	Fed	*Feeding rate*	Live at 21d	*Survival rate*	Body	Legs	Saliva
Round 1	Attempt 1	24	15	*63%*	9	*60%*	0/9	ND	ND
		(2)							
	Attempt 2	7	1	*14%*	0	*0%*	-	-	-
Round 2	Attempt 1	26	4	*15%*	3	*75%*	0/3	ND	ND
		(2)							
	Attempt 2	20	7	*35%*	3	*43%*	0/3	ND	ND
Round 3	Attempt 1	41	7	*17%*	7	*100%*	3/7	3/3	2/3
		(7)							
	Attempt 2	27	3	*11%*	3	*100%*	0/3	ND	ND
	Total:	91*	37	*41%*	25	*68%*	3/25	3/3	2/3

*- the sum of all mosquitoes present in the first attempts, i.e. all mosquitoes offered a blood meal at least once. ND – not done.

Based on these observations, the infection rate was determined to be 12% (95% CI: 4.2-30%), the dissemination rate 100% (95% CI: 44-100%), and the transmission rate 67% (95% CI: 21-98%).

Discussion

Wolbachia are intracellular symbiotic bacteria known to impact the ability of viruses to replicate inside mosquito cells and the vector competence of certain mosquito species as a whole (70,71). In this project, we attempted to create both *in vivo* and *in vitro* platforms for the study of *Wolbachia*'s effect on the replication of arboviruses in *Culex*, the most important mosquito vector genus in Sweden and northern Europe.

Tetracycline treatment of mosquitoes

The native *Wolbachia* infection was successfully eradicated from *Cx. pipiens molestus* after treating two consecutive generations of adults. Although no F0 adults had successfully cleared their *Wolbachia* infection, almost all of their tested progeny was free of *Wolbachia* already before the second round of treatment. Similarly, *Wolbachia* was detected in an F1 female after the completion of the treatment, despite all 50 tested F3 individuals being free of *Wolbachia*. This observation would suggest that the effect of tetracycline treatment on adult *Cx. pipiens molestus* is best assessed in their offspring, and not in the treated generation itself.

Unfortunately, the tetracycline treatment had a negative effect on the health of the mosquitoes, both during and after the treatment. However, these observations were not compared to a control group and must thus be interpreted with caution. Tetracycline treatment of adult *Ae. albopictus* has been shown to be the most effective way of removing *Wolbachia* with the highest survival rates, around 80% after one week of 1.0 mg/ml in sucrose at worst, but the hatch rates of the offspring were as low as 10% (59,61). It must be noted that the *Ae. albopictus* likely responds differently to tetracycline treatment, as Joanne et al. (61) successfully removed *Wolbachia* from the all tested F0 females after two weeks of 1.25 mg/ml treatment with tetracycline, and from 72% of F0 females after two weeks of 1.0 mg/ml, which was not seen in this study. Similar to our observations, the egg hatch rate in *Ae. albopictus* was reduced, with approximately 60% of the eggs hatching.

In *Culex*, tetracycline treatment of consecutive generations of adults is a commonly seen approach, although treatment effects on health have not been assessed (60,72). An early study where only one adult generation of *Cx. pipiens fatigans* was treated with 1.0 mg/ml over four days also report a reduced egg hatch rate despite incomplete removal of *Wolbachia* (73). Combining treatment of adults with treatment of larvae can be used to reduce the dose administered to adults, although survival rates of larvae are low for *Ae. albopictus* and are not reported for *Culex* (59,61,72). Treatment of larvae has historically been the primary approach

for *Wolbachia* elimination but, according to the few early studies that report the effects on survival and fecundity in *Culex*, larval mortality is high and hatch rates of eggs laid by F0 are low (73,74).

Due to the negative health effects, the mosquitoes should be allowed to recover over several generations to regain their strength and natural flora to validate comparisons of vector competence to their non-treated counterparts. In studies involving *Aedes* mosquitoes, convention suggests a recovery time of between 2 and 4 generations following tetracycline treatment (14,25,75). In the study by Glaser & Meola (26), investigating West Nile virus (WNV) in *Cx. quinquefasciatus*, *Wolbachia* was cleared over one generation through oral tetracycline treatment of adults. A similar difference in vector competence was observed in both the 5th and the 14th generation of *Wolbachia*-free mosquitoes. Since our study involved an additional generation of treatment, vector competence comparisons are presumed valid after the F6 generation.

As the mosquitoes recover from the treatment over successive generations, their fecundity may recover as well, or even surpass that of *Wolbachia*-infected mosquitoes, as is reported for *Cx. quinquefasciatus* (60). Intriguingly, the opposite has been shown for *Ae. albopictus*, where the females were less fecund and shorter-lived without their *Wolbachia* infection (23,76). The adaptation of the host to the endosymbiont and the cost of infection are two forces that affect mosquito viability in opposite directions once *Wolbachia* is removed (76,77). As concluded by Duron et al. (77), the cost of infection correlates with *Wolbachia* density. Combined with the observations of higher *Wolbachia* density in lab reared mosquitoes by Micieli & Glaser (27), the increase in fecundity in *Cx. quinquefasciatus* could be an artifact of numerous lab-reared generations. Potentially, the disruption of mosquito microbiota could also have an impact that needs to be separated from the removal of *Wolbachia*, although the microbiota of *Ae. aegypti* does not seem to have an effect on longevity and fecundity of adults, despite being essential for larval development (78). Further and more structured studies are needed on the tetracycline-treated *Cx. pipiens molestus* to determine the effects of *Wolbachia* on the health of this species.

Primary cell culture

A primary cell culture was successfully initiated from the embryos of *Cx. pipiens molestus*, although cell growth is slow at the current stage. Our experiences were very similar to those previously published for other species of *Culex*. Kuwata et al. (38) likewise report slow

growth in the early stages of a cell line established from *Cx. tritaeniorhynchus*, with 73 days from seeding embryo fragments into a 35 mm tissue culture dish to the first subculture into a tissue culture flask. The cells did not reach levels of proliferation comparable to conventional cell cultures until after the 7th passage, nearly 400 days after the original seeding.

Segura et al. (37) started a primary cell culture from the eggs of *Cx. quinquefasciatus* and achieved a confluent monolayer in a T25 culture flask after 40-60 days of growth. Their comparatively fast growth of cells was perhaps due to the 600 eggs used as starting material, instead of the single egg raft used in this study and by Kuvata et al. (38). The structures reported are very similar to what has been shown here, including twitching lumps of cells in the early stages of the culture and spheres of epithelioid cells encapsulating empty space.

A smaller volume for the first seeding was chosen in this study because of the small amount of seeding material, which facilitates both the appropriate timing for raft collection, best done at 16-20 hours after it was laid (34), and the disinfection procedure. The high density of cells in a well on a 96-well plate is beneficial for their proliferation due to cell-to-cell proximity, but in turn poses problems for upscaling and passaging into larger wells. Nonetheless, the similarity of the observed cell behaviour to previous successful attempts of starting cell lines from embryonic tissue strengthens the potential of this primary culture. Passaging into a larger vessel for increased future growth should be done once sufficient cell numbers are achieved in the small 96-well plate wells.

The different cell morphologies that were seen have not been properly characterized, with only one cell type clearly distinguishable – the muscle cell, in part due to the morphology but primarily due to the twitching motion. Since the future applications of this cell line will require fast proliferation, characterizing the cell types at this stage is redundant and should instead be done once splitting and subculturing can be done on a weekly basis. Even widely used mosquito cell lines, such as *Ae. aegypti* Aag2 cells, have still not been properly characterized as to what type of cells they are composed of (79). Quite possibly, the most abundant cells seen in our cell culture are granulocytes, the most common subtype of insect haemocytes – phagocytic immune cells with diverse functions and high proliferation rates during embryonic development, as well as a tendency to migrate and readily attach to surfaces (80,81).

Wolbachia infection in Hsu cells

Four attempts were made to infect Hsu cells with *Wolbachia* derived from *Cx. pipiens molestus* ovaries, of which none proved successful in establishing a stable infection. Traditionally, eggs or infected cell lines are used as donor material due to the relative ease of disinfection and inherent sterility (29,63,82). However, no cell lines that are infected with *w*Pip are currently available and using eggs entails the necessity of obtaining large amounts of donor material, approx. 20 mg, due to the relatively low *Wolbachia* density in embryos (83). Since *Wolbachia* is a reproductive parasite, the highest densities are seen in the gonads, with over 99% of the *Wolbachia* in female *Cx. pipiens* residing in the ovaries (84). To reduce the necessary amount of donor material, ovaries from elderly mosquitoes were used since *Wolbachia* density increases with age, both in male and female *Cx. pipiens* (83,85). A similar approach has been applied before when using ovaries from the small brown planthopper, *Laodelphax striatellus*, to infect cell lines derived from *Ae. albopictus* and the corn earworm, *Helicoverpa zea*, with *Wolbachia* (86).

Using ovaries, in turn, carries the risk of contaminating the donor material with bacteria from the exoskeleton and the digestive tract during dissection, which caused two attempts to fail. The first attempt failed likely due to a standard amount of PenStrep supplementation when higher amounts were shown to be needed. The fourth attempt failed likely due to excessive contamination by intestinal flora. For future experiments, cautiousness should prevail over condonation when deciding whether to include ovaries that have possibly been contaminated by nearby tissues.

Monitoring *Wolbachia* infection through qPCR showed that *Wolbachia*-infected cells, if present at all, were not proliferating to the same extent as non-infected cells. Alternatively, no cells were successfully infected and the decline of the wsp:RpL32 ratio over the passages can be explained by the gradual degradation of *Wolbachia* DNA, as can be seen when *Wolbachia*-infected cells are treated with tetracycline to cure them of *Wolbachia*, where the cells remain positive for *Wolbachia* through PCR for several passages after the treatment (63).

Partly due to the pattern of growth displayed by Hsu cells, DAPI staining and subsequent fluorescent imaging of cells to detect *Wolbachia* showed not to be a reliable method. Hsu cells attach to each other in nodes, connected by thin stretches of cells (Figure 12). The vast

majority of the cells are thus not visible as a monolayer and conclusions have to be drawn based on the few cells between the nodes.

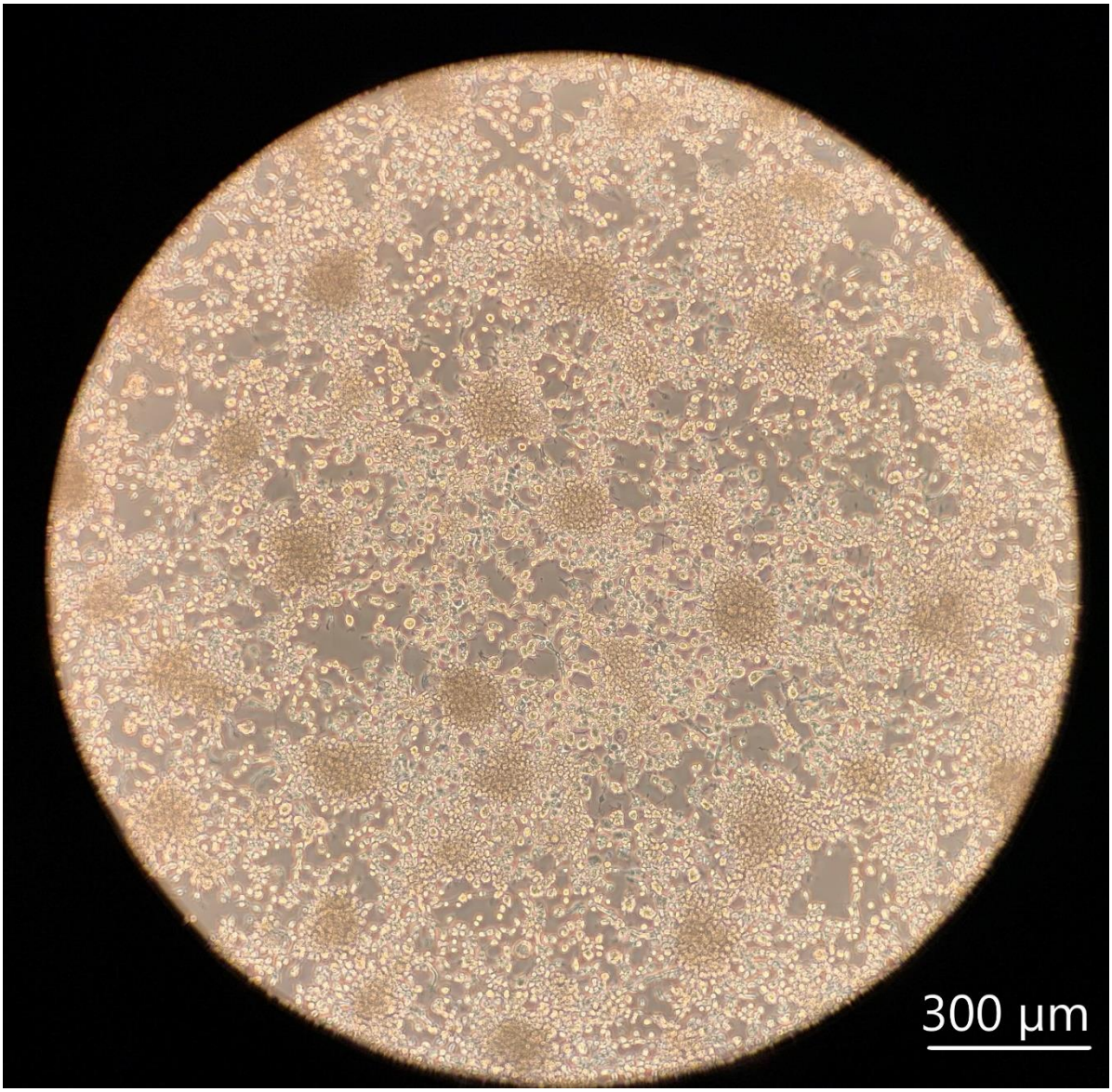

Figure 12. Growth pattern of Hsu cells. Image taken through the lens of an inverted microscope.

Due to the presence of DNA aggregates in the negative control following infection attempt 2 and the relatively low numbers of *Wolbachia* present in the infected cell culture, approx. 2 bacteria per 1000 cells for attempt 3 at the time of imaging, specks of DAPI-stained DNA cannot be certainly attributed to the presence of *Wolbachia*. Alternative sources of stained DNA could be mycoplasma contamination (87) or cellular debris. Indeed, since more specks of DAPI were seen on the first staining attempt, which was done on cells that had been passaged fewer times after centrifugation, endocytosed cell fragments seem the most likely explanation for the stained DNA aggregates. A more precise method of fluorescence-based

detection is needed to demonstrate the presence of *Wolbachia* inside cells, such as fluorescent in-situ hybridisation, targeting 16S rRNA or immunofluorescence, targeting wsp (14,29,88).

Replication of SINV in Hsu cells

This study showed that SINV replicates poorly in Hsu cells and no persistent or lytic infection could be established, even though *Cx. quinquefasciatus*, from which Hsu cells are derived, is able to transmit SINV (89). This is in agreement with an early study that also showed that SINV is unable to replicate in Hsu cells (90), although the study had several differences compared to ours. Firstly, viral titers were determined by determining the LD_{50} titer for mice sucklings, and, secondly, the earliest time point was 24 hours. Our replicate of the study by Hsu (90) showed similar results, with no apparent difference between SINV replication in cell culture and a cell-free control. If anything, the SINV RNA tended to degrade to a larger extent in the presence of cells than in sterile medium, where it appears to be stable for at least 168h.

Some loss of virus is expected after and including timepoint 96 hpi due to the re-sampling of the same wells, from which virus had already been removed. This flaw in the method affects the comparability of later timepoints to the initial 72 hours of the experiment. Nonetheless, due to the consistently low amounts of RNA in the supernatant, between 2 and 4 orders of magnitude below the inoculated dose, it is unlikely that SINV can efficiently replicate in Hsu cells. Furthermore, the amount of viral RNA tended to decline also during the first 72 hours, even though an increase of SINV RNA is expected within the first 48 hours or sooner, based on previous studies of SINV replication in mosquito cells using comparable MOIs (29,79,91).

An interesting observation is the comparatively higher SINV RNA levels detected in resuspended cells than in the supernatant (Figure 11). Although the supernatant was not sampled before the cells were washed and resuspended, data from an earlier experiment (Figure 10) would not suggest that RNA should have increased in the supernatant simultaneously. Substantial detachment of cells inoculated with MOI 0.1, most likely due to the non-treated surface of the plate, coincided with the tendency to detect higher RNA levels in the samples as compared to MOI 0.5. Since the samples were not centrifuged prior to RNA extraction, it is reasonable to assume that some of the detected RNA could have originated from the suspended cells. These observations suggest that SINV is potentially able to attach to or enter Hsu cells, but does seem not replicate inside of them. Since the virus stocks used for these experiments replicated successfully in Vero cells (data not shown), the virions are

assumed infectious and the lack of replication is likely due to Hsu cells being non-permissive to SINV.

Hsu cells are persistently infected with Merida virus (MERDV), an insect-specific virus (ISV) belonging to the family *Rhabdoviridae* (92), which could potentially impact SINV replication. Some arboviruses replicate less efficiently in C6/36 and C7/10 *Ae. albopictus* cells (93–95) and are transmitted to a lesser extent by *Culex* mosquitoes (96,97) when the hosts are infected with ISVs belonging to the same genus, including *Alphavirus*. However, vector competence for the phlebovirus Rift Valley Fever Virus (RVFV) is unaffected by the infection of *Cx. pipiens* by Culex flavivirus (98). Nonetheless, ISVs are most often vertically transmitted (99,100), meaning the effect of superinfection exclusion could be different in cells derived from ovaries, such as Hsu cells, as opposed to embryonic cell lines, such as C6/36 and C7/10, and neither is ovarian tissue part of the barriers for arbovirus transmission. The effect of ISVs on vertical transmission of arboviruses has, to our knowledge, not been previously studied. Further investigation on the localisation of SINV in Hsu cells is needed to elucidate the barriers to its replication and potential interactions with MERDV.

Other arboviruses, such as West Nile Virus (WNV), are known to replicate in Hsu cells (90,101,102). A *Wolbachia*-infected line of these cells would therefore be useful for studying the effects of *Wolbachia* on the replication of other human-infecting arboviruses transmitted by *Culex*, e.g. WNV, Usutu virus and Japanese Encephalitis virus (103,104).

Vector competence of *Cx. pipiens molestus* for SINV

An attempt to determine the vector competence of *Cx. pipiens molestus* carrying their natural *w*Pip strain was performed in this study. However, due to inefficient blood feeding, the numbers presented in this report are uncertain. An early study on the vector competence of *Cx. pipiens pipiens* reports an infection rate of near 50% and a transmission rate of 14% after a blood meal containing similar titers of SINV (51). The low infection rate observed in the current study is presumably due to the low amounts of blood ingested by the females, of which only a few were fully engorged after the blood meal. The reason for this is likely that this colony has not yet been adapted for artificial blood-feeding. This line originates from a nuisance population, notorious for feeding on humans in a natural setting (58).

It is possible to drastically increase the feeding rate by adding sugar to the blood-meal, but this could potentially give misleading results due to differences in storage and digestion of blood and sugar inside the mosquito (105). For future experiments on vector competence,

only fully engorged females should be included in the study group to ensure similar exposure to the virus.

Conclusions

Several key initial steps have been taken to the establishment of *in vivo* and *in vitro* platforms for the investigation of *Wolbachia*'s impact on viral replication in *Culex pipiens molestus*. Colony mosquitoes have been cured of their infection and will be ready for vector competence experiments within two generations. Embryonic cells have been successfully isolated and could potentially form a new *Culex* cell line within a year. Although no stable *Wolbachia* infection has yet been achieved in the *Cx. quinquefasciatus* Hsu cell line, a functioning protocol has been established and success is possible within a few iterations. All three platforms could potentially be applied to studies of several arboviruses, in addition to SINV, in the future.

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