Ex vivo midgut cultures of *Aedes aegypti* are efficiently infected by mosquito-borne alpha- and flaviviruses

Ana Lucia Rosales Rosas[a], Lanjiao Wang[a], Sara Goossens[a], Arno Cuvry[a], Li-Hsin Li[b], Nanci Santos-Ferreira[a], Kai Dallmeier[b], Leen Delang[a]#

[a]KU Leuven Department of Microbiology, Immunology and Transplantation, Rega Institute for Medical Research, Laboratory of Virology and Chemotherapy, Leuven, Belgium.

[b]KU Leuven Department of Microbiology, Immunology and Transplantation, Rega Institute for Medical Research, Molecular Vaccinology and Vaccine Discovery, Leuven, Belgium.

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#Address correspondence to Prof. Leen Delang, leen.delang@kuleuven.be.

Abstract

Aedes aegypti mosquitoes are known vectors of several arboviruses, including the chikungunya virus (CHIKV), dengue virus (DENV), and Zika virus (ZIKV). When blood-feeding on a virus-infected human, the mosquito ingests the virus into the midgut (stomach), where it must replicate and overcome the midgut barrier to disseminate to other organs (e.g., salivary glands) and ultimately be transmitted via the saliva. Tools to study mosquito-borne viruses (MBVs) include two-dimensional cell culture systems and *in vivo* mosquito infection models, which offer great advantages, yet have some limitations.

Here, we describe for the first time a long-term *ex vivo* culture of *Ae. aegypti* midguts. Cultured midguts were viable for 7 days in a 96-well plate at 28°C and displayed peristaltic movements. Several representative MBVs efficiently replicated in the midguts: ZIKV, DENV, Ross River virus (RRV) and CHIKV, as measured by qRT-PCR and plaque assay. Fluorescence microscopy revealed replication and spread of a fluorescent reporter virus (DV2/mCherry) in the midgut. Finally, we evaluated the antiviral activity of the nucleoside analogs β-D-N^4-hydroxycytidine (NHC) and 7-deaza-2'-*C*-methyladenosine (7DMA), in the *ex vivo* midgut model. At 50 µM, NHC reduced the infectious virus levels at day 3 post-infection (p.i.) for both RRV and CHIKV by 4.5- and 9.4-fold, respectively. Likewise, 7DMA at 100 µM reduced ZIKV RNA viral loads by 20-fold at day 7 p.i..

Together, our results show that *ex vivo* midguts can be efficiently infected with mosquito-borne alpha- and flaviviruses. Furthermore, the cultured midguts could potentially be used to evaluate the antiviral activity of inhibitors. *Ex vivo* midgut cultures could thus be a new model to study MBVs, offering the advantage of reduced biosafety measures compared to infecting living mosquitoes.

1. Introduction

Arboviruses are a diverse group of viruses that rely on arthropod vectors such as mosquitoes, ticks, and sandflies to infect susceptible vertebrate hosts and perpetuate transmission. Approximately 500 arboviruses have been described in the International Arbovirus Catalog by the Centers for Disease Control and Prevention (CDC), of which around 150 are known to cause disease in humans. Three viral families/orders are of clinical relevance: *Bunyavirales*, *Flaviviridae*, and *Togaviridae*, as they encompass the important arboviruses of global health concern, such as the dengue virus (DENV), chikungunya virus (CHIKV), yellow fever virus, and Zika virus (ZIKV) (1). These four mosquito-borne viruses (MBVs) are transmitted by female *Aedes* (*Ae.*) mosquitoes (e.g., *Ae. aegypti* and *Ae. albopictus*) (2). Most importantly, *Ae. aegypti* mosquitoes are considered highly competent arbovirus vectors because they prefer to feed on human blood (3).

Maintenance of MBVs in nature occurs through three key elements conveying in one geographic location: the mosquito vector, the vertebrate host(s), and adequate environmental conditions. When blood-feeding on a virus-infected human or animal, a female mosquito will ingest the virus-containing blood in the midgut (i.e., mosquito stomach), where the virus must replicate and overcome the midgut barrier to subsequently disseminate to other mosquito organs. As the mosquito becomes systemically infected, resulting in a high viral load, the virus will reach the salivary glands. The virus will be released in the saliva upon the next mosquito bite, allowing the mosquito to transmit the virus to uninfected hosts when feeding on them (4, 5). The midgut is thus the initial site for

infection and replication of any arbovirus in the mosquito. Virus replication in and spread across midgut cells are a requisite for a productive arbovirus infection in the mosquito (4). *In vitro* cell cultures are of extreme help in studying arbovirus infection and other relevant processes in mosquitoes; however, they present several limitations. Mosquito cells derived from tissues relevant to specific stages of mosquito-virus interactions, such as the salivary glands or the midgut, would be very useful to give an insight into the cells' susceptibility to a certain arbovirus prior to *in vivo* vector competence studies in mosquitoes. Yet, most of the established mosquito-derived cell lines come from larval or embryonic tissue (e.g., *C6/36* [larvae] and *Aag-2* [embryos] cells) (6). Therefore, results obtained from *in vitro* experiments are ought to be taken cautiously as cell lines are considered homogenous cultures, both genetically and phenotypically, and single cell type populations might not represent multicellular tissues, like the midgut (7, 8). Moreover, some biological differences might arise among the same cell line coming from different laboratories (7).

Another method commonly used to study mosquito-virus interactions is the mosquito infection model. Through this approach, mosquito infection occurs artificially via a blood meal (membrane filled with infectious blood), and mosquitoes are sacrificed at different time points to assess viral infection and vector competence (9). Although living infected mosquitoes provide valuable transmission data, the value of these data is directly proportional to the labor-intensive nature of the method. Transmission studies require skilled personnel for the handling of infected mosquitoes and facilities with a high biosafety level that ensure proper containment and carry the risks of prick injury with virus-infected dissection tools or the risk of infected mosquitoes escaping.

Arising as an advantageous tool, *ex vivo* organ cultures have been described in the insect research field. For instance, *ex vivo* culture of insect organs has been successfully and widely defined for ixodid ticks (8, 10). On the contrary, reports on the *ex vivo* organ cultures from mosquito tissues are scarce, mainly focused on germline and fat body tissues (11, 12). Despite the essential implication of the mosquito midgut in arbovirus replication and dissemination, no *ex vivo* mosquito midgut set-up has been properly described. To date, only a short-term *ex vivo* assay in mosquito midguts has been reported, in which the midguts were only maintained for 35 hours post dissection in tissue culture plates (13).

E*x vivo* methods can offer a unique perspective to (i) study vector-virus interactions in a relevant tissue, for example, the mosquito midgut, (ii) reveal tissue-specific responses to virus infection, or (iii) study tissue-specific metabolic processes.

Furthermore, *ex vivo* mosquito midgut cultures could provide a convenient and relevant platform to generate results that can be extrapolated to living mosquitoes. To illustrate, a new and interesting concept is the use of antiviral compounds to inhibit virus infection in the mosquito vector, thus rendering arbovirus transmission from mosquitoes to humans. This idea involves the compound being delivered to adult mosquitoes when they take a blood meal on a mammalian host undergoing antiviral treatment. As the mosquito midgut is the entry point and key replication site for MBVs, delivery of the compound to the midgut can be achieved via blood feeding. In this context, the *ex vivo* culture of mosquito midguts could serve to rationally select potential arbovirus-blocking molecules to be tested out in living mosquitoes at a later stage. Additionally, *ex vivo* midgut cultures provide a controlled environment in which many parameters can be easily adjusted, and they

constitute a safer option due to the low degree of containment required while working with BSL2/3 arboviruses, compared to working with live mosquito infection models.

On the lookout for novel tools that could be of use to study arbovirus infection within the vector, we dissected *Ae. aegypti* midguts and established their viability for a period longer than a week. We also infected the midgut tissues with several mosquito-borne alpha- and flaviviruses, confirming the production of infectious viral particles. Furthermore, the addition of previously reported antiviral drugs to the cultured midguts in an *ex vivo* antiviral assay led to a reduction in arbovirus replication in the treated midguts. In this study, we thus have developed an *ex vivo* mosquito midgut model that constitutes a valuable tool to study MBVs and complements the arsenal available to the mosquito arbovirus research field.

2. Results

Viability of *Aedes aegypti* midguts cultured *ex vivo*

We first aimed to determine the viability of dissected and *ex vivo* cultured mosquito midguts. Due to the motility observed along the gut tissue, a bioassay was performed based on the number of contractions by the hindgut (Supplementary material, Movie 1). The same area of the hindgut was analyzed for all biological replicates (Fig. 1, b). Dissected midguts presented peristalsis in the hindgut and were viable for up to 168 hours (7 days) (Fig. 1, d), suggesting that the tissue was alive and it could support arbovirus infection. At 10 days post dissection, the mean peristaltic period in the *ex vivo* cultured midguts significantly increased compared to day 0 measurements (day 0: 4.37 s vs day 10: 10.88 s) (Fig 1, d), suggesting that the midgut tissue's viability had decreased.

The responsiveness of the midgut tissue was assessed by exposing the midguts (day 0 post dissection) to either the neurotransmitter serotonin, the anticholinergic drug atropine, or midgut medium (mock exposure), after which their gut peristalsis was recorded. The peristaltic period displayed by the mock-exposed midguts was 8.68 s (mean value among 5 biological replicates, median value: 8.03 s). Midguts exposed for 1 hour to serotonin displayed a slight increase in gut motility, with a mean peristaltic period of 6.83 s (6 biological replicates, median value: 5.66 s), whereas atropine-exposed midguts exhibit a somewhat reduced peristalsis, with a mean peristaltic period of 10.05 s (6 biological replicates, median value: 7.99 s). Despite the observed trends, there were no statistically significant differences among the conditions evaluated (Supplementary Fig. S1).

Additionally, measuring the metabolic activity of the *ex vivo* cultured midguts was considered, and a few colorimetric assays were tested, as they have proved useful for other *ex vivo* organ cultures (8). When dissected midguts were incubated with either a tetrazolium salt (MTS) or a viability reagent (PrestoBlue™) for several hours, an expected change in coloration surrounding the alive organs could be visualized in each well containing individual mosquito midguts, indicating that they were metabolically active. Nonetheless, the medium color shift in the midgut-containing well was not strong enough to obtain a robust difference between the absorbances measured for the negative control (fixated midguts did not exhibit any change in medium coloration) and the alive midguts. This situation persisted even when two midguts were evaluated together in one testing well. Therefore, our observations hint at the conserved metabolic activity in the *ex vivo* cultured midguts, but no statements can be made due to the limitations exposed. Further improvements must be carried out to accurately quantify the metabolic activity of the

mosquito midguts, like employing three or more midguts in one testing well to obtain a robust readout.

Infection of dissected *Aedes aegypti* midguts with mosquito-borne alpha- and flaviviruses

Next, *ex vivo* infection was performed in the cultured midguts to test their capacity to support the replication of several arboviruses. Replication kinetics were evaluated for the Ross River virus (RRV) and CHIKV as representative members of the *Togaviridae* family. Both viruses were able to efficiently replicate in the *ex vivo* cultured midguts with an increase in viral RNA levels between 1 and 3 days post infection (p.i.), compared to viral RNA levels at 2 hours p.i. (Fig. 2, a). Infectious virus titers of RRV and CHIKV increased as well between day 1 and 3 p.i. (Fig 2, b), while no infectious virus was observed in any RRV- or CHIKV-infected midgut at 2 hours p.i. (data not shown). The peak of viral RNA and viral titer was detected at day 3 p.i. for both alphaviruses. The *ex vivo* cultured midguts appeared more permissive to RRV compared to CHIKV, as indicated by the high levels of viral RNA and infectious virus produced, reaching up to 3.0×10^9 genome copies/midgut and 3.5×10^4 PFU/midgut at day 3 p.i., respectively.

To evaluate whether *Aedes*-borne flaviviruses could replicate in the *ex vivo* midguts, the replication kinetics of DENV-2 and ZIKV were studied. As both viruses needed a longer period to replicate in the midgut tissue, viral RNA and infectious virus levels were measured starting at day 5 p.i.. Similarly to the alphaviruses, viral titers increased at day 5 p.i. and further peaked at day 7 p.i. (Fig 3). At day 7 p.i., DENV-2 reached RNA levels up to 1.5×10^8 genome copies/midgut and a viral titer of 21 PFU/midgut. On the contrary, ZIKV replication and infection presented more variability among individual midguts. The

maximum amount of viral RNA and infectious virus quantified were 1.18×10^5 genome copies/midgut and 4.8×10^2 PFU/midgut, respectively.

These replication kinetics results further confirmed the preservation of midgut tissue in an *ex vivo* culture set-up and, more specifically, the presence of midgut cells that could constitute the target for arbovirus infection.

Handling and optimization of ex vivo mosquito midguts

Initially, mosquito midguts were placed in the well of a 96-well tissue culture plate, in which they remained suspended in culture medium. However, certain procedures (washing steps) were challenging due to the small size of the midguts and the high probability of pipetting out the tissue. Therefore, other support systems were tested to immobilize the midguts and facilitate their handling to overcome this limitation.

To this end, Matrigel® (Corning®) domes were deposited in 48-well tissue culture plates (Corning® Costar®), in which the midguts were placed before the dome completely solidified. The domes provided good support for the midgut organ, as they still presented hindgut peristalsis while keeping the tissue immobilized in the center of the well. However, infection of midguts kept in Matrigel domes by incubation with ZIKV was not successful. No viral RNA was detected in these midguts after infection (data not shown), as opposed to the midguts cultured traditionally (suspended in medium, *cf. infra*). Another method tested consisted of using a foam substrate insert to line up the bottom of each well of the 96-well tissue culture plate filled with medium, as it was shown to be successful for the *ex vivo* culture of tick organs (14). Of note, the same foam substrate reported for tick organs could not be purchased due to its commercial unavailability. After dissection, mosquito midguts were laid into the sponge substrate and kept in culture. Unfortunately, the sponge

substrate used had big pores that allowed the midguts to pass through the foam to the bottom of the well and posed difficulties in the washing steps; therefore, this method was also discarded. Of note, infection by incubation with ZIKV was successful in the midguts kept on foam substrate; however, traditionally culture midguts reached higher ZIKV RNA loads as measured by qRT-PCR (Supplementary material, Fig. S2). Furthermore, no organ controls, consisting of foam substrate (no midgut) that went along all infection steps, were implemented to measure any virus inoculum that could remain embedded in the substrate. These no organ controls were shown to retain a higher amount of the virus inoculum compared to the negative controls normally used (Paraformaldehyde [PFA] fixated midguts suspended in medium).

Therefore, the culture method that provided the best outcome in terms of handling, tissue preservation and permissiveness to arbovirus infection was to keep the mosquito midguts suspended in culture medium in a black 96-well tissue culture plate with clear bottom (PerkinElmer©, USA). To reduce the risk of pipetting out the midguts during washing steps, a magnifying lamp was key to observe the midguts with the naked eye and avoid such problem. Lastly, carboxymethyl cellulose (CMC) 0.8% diluted in Leibovitz's L-15 medium was also employed and found to be suitable for long incubation periods (>3 days), as it provided a denser medium where the midguts seemed to preserve their shape better over time. However, viral RNA levels detected in the midguts incubated in CMC were significantly reduced at day 2 and 3 p.i. compared to traditionally *ex vivo* cultured midguts (Supplementary material, Fig. S3).

Infection of *ex vivo* cultured midguts with an mCherry-expressing DENV-2

To follow the progression of virus infection in the midguts by imaging, cultured mosquito midguts were infected with DENV-2 expressing the red fluorescent protein mCherry (DV2/mCherry,(15)) and imaged using the FLoid™ Cell Imaging Station (Life Technologies) at selected time points p.i.. The mCherry signal was used as a proxy for infection. DENV-2 mCherry infection was observed initially at day 3 p.i. as single or few foci in the posterior midgut region (Fig. 4, a). Over time, these focal infection points increased in number and spread to neighboring areas, primarily along the posterior region of the midgut and the hindgut (Fig. 4, b, c, d, e). No mCherry expression was observed in the negative control or mock-infected midguts (Fig. 4, f, g). Fixation of the mCherry-expressing midguts at day 7 p.i. and further imaging revealed the spread of the mCherry signal preferentially along the posterior midgut region, forming infection foci comprising multiple cells. Moreover, some infected cells were observed corresponding to the tracheal tubes that remained in the midgut tissue after dissection (Fig. 4, h).

Arbovirus replication is reduced upon treatment with antiviral drugs in midgut cultures

We next assessed whether the *ex vivo* mosquito midguts could be used to evaluate the antiviral activity of inhibitors on viral replication. To this end, the antiviral activity of β-D-N^4-hydroxycytidine was tested against RRV and CHIKV. β-D-N^4-hydroxycytidine, also known as EIDD-1931 or NHC, has been reported as a broad-spectrum inhibitor of alphaviruses *in vitro*, including CHIKV and Venezuelan equine encephalitis virus (16, 17). As a proof of concept, we first incubated the mosquito midguts with the RRV inoculum in the presence of NHC at 0 (untreated), 13, 25, or 50 µM, after which the inoculum was

removed and substituted with midgut medium containing NHC at the selected concentrations. At day 3 p.i., there was no difference in the RRV RNA levels between the untreated and treated groups (Fig. 5, a). In contrast, there was a decreasing trend in the infectious virus levels by plaque assay, following a dose-response relationship. Although there was a 4.5-fold reduction in the infectious virus levels between the untreated and the 50 µM-treated groups (0 µM: 2.6×10^4 vs 50 µM: 5.9×10^3 PFU/midgut), this difference was not significant (Fig. 5, b). Conversely, for CHIKV, NHC significantly reduced the infectious virus levels by 9.4-fold (mean values, 0 µM: 1.02×10^4 vs 50 µM: 1.08×10^3 PFU/midgut) without affecting the CHIKV RNA levels, which aligned with the known mechanism of action of NHC (Fig. 5, c-d).

As the *ex vivo* cultured midguts also supported flavivirus infection, the viral polymerase inhibitor 7-Deaza-2'-*C*-Methyladenosine (7-DMA) was selected for its described antiviral activity against ZIKV both *in vitro* and *in vivo* in a mouse model (18). While ZIKV RNA levels were reduced for all treated midguts (25, 100, and 200 µM of 7-DMA) compared to the untreated group (0 µM) at day 7.p.i., only the group treated with 7-DMA at 100 µM showed a significant difference compared to the untreated group, with a 20.5-fold reduction (mean values, 0 µM: 1.44×10^6 vs 100 µM: 7.04×10^4 genome copies/midgut) (Fig. 5, e).

3. Discussion

The study of arbovirus infection in the mosquito vector relies mainly on a combination of *in vitro* and *in vivo* approaches, which have yielded great progress in the knowledge regarding arbovirus biology, virus-vector interactions, and vector competence. While convenient and handy, *in vitro* cell culture systems have the potential shortcoming of not being as biologically relevant as using mosquito infection models, aside from the limited

selection of mosquito cell lines available. On the other hand, working with living (and infected) mosquitoes grants useful information when studying virus-vector interactions or vector competence for a specific virus, but such *in vivo* models are not always accessible and require the implementation of cumbersome safety measures ensuring the adequate containment while working with BSL2/3 pathogens (19). *Ex vivo* methods have not yet been described in-depth for the study of MBVs. Hence, in this study, we have established an *ex vivo* mosquito midgut model using dissected midguts from *Ae. aegypti* mosquitoes. When cultured *ex vivo*, the midguts displayed peristaltic contractions, mainly observed as waves along the hindgut, which is a frequent occurrence for this type of tissue (20). Although it is now outdated, intestinal peristalsis has been for a long time an indication for the assessment of bowel viability during veterinary and human intestinal surgeries, among other gross features (21, 22). Here, we used this visual feature as a proxy for viability, paired with a custom script for gut motility in zebrafish (23) to measure the peristaltic period (time between contractions) in the *ex vivo* cultured guts, which remained stable for 7 days after dissection.

The stomatogastric nervous system innervates the digestive tract muscles in insects, including the midgut, and regulates food uptake and transport (24). In *Ae. aegypti* mosquitoes, the midgut is innervated by a nerve plexus consisting of several neuron bodies and axons (25). More specifically, the mosquito gut is innervated by a small number of serotonergic neurons (26, 27). Studies have reported the stimulatory effect of serotonin on gut motility in *Ae. aegypti*'s larval anterior stomach (28, 29), as well as the localization of serotonin receptors and their expression in tracheolar cells and the hindgut of female mosquitoes (30, 31). Accordingly, incubation of the *ex vivo* cultured midguts with

serotonin resulted in an increased gut peristalsis (measured in the hindgut area), whereas atropine resulted in a slightly prolonged peristaltic period. Although not significant, such results could indicate that the peptidergic innervation and the corresponding responsive cells are present in the midgut tissue after dissection, and thus, the tissue is still viable. However, further assessment is needed with larger sample size and higher concentrations of the compounds to obtain definitive proof of their effect.

In the present study, the *ex vivo* cultured mosquito midguts could support infection with four arboviruses, despite the unusual route of infection (through incubation with virus inoculum) that differs from what occurs in nature in mosquitoes (infection via blood feeding). Furthermore, both viral RNA and infectious virus particles were detected in the mosquito midgut organs following a progressive increase over time, which confirmed the previously established viability of the midguts. In addition, viral RNA levels produced by the infected midguts at the later time points (day 3 p.i. for CHIKV or RRV, or day 7 p.i for DENV-2 and ZIKV) were distinctively higher than the viral loads of samples collected at 2 hours p.i., after removing the virus inoculum and rinsed.

Thorough optimization has been carried out to make the *ex vivo* mosquito midgut model an accessible and convenient tool for the study of arboviruses in the mosquito vector. We investigated the culture of mosquito midguts using an extracellular matrix mixture (Matrigel™) commonly used for organoid culture. Matrigel™ provides structural support for cells because of its content of laminin, collagen IV, entactin, and heparin sulfate proteoglycan perlecan, among other factors (32), which are the main components of the mosquito midgut basal lamina secreted by epithelial cells (33). The support given by the hydrogel could be readily observed when embedding and culturing the midguts in

Matrigel™. Microscopic observation of the *ex vivo* midguts cultured in medium showed some variation in the midgut organ integrity. Some midguts preserved their shape with a minimal amount of degradation over time, whereas others lost their integrity and progressively became misshapen, which has been noted before when working with mosquito tissue cultures (20). Of note, the hindgut was the region less prone to degradation. Such occurrence was improved greatly when using Matrigel™, as the midguts preserved their integrity better compared to the traditionally used culture medium. However, ZIKV infection of these midguts embedded in Matrigel™ was not successful by sole incubation with virus inoculum. Considering that the current route of infection diverges from a natural mosquito infection, the use of synthetic hydrogels could help to overcome such limitation while providing an adequate environment to culture the *ex vivo* mosquito midguts. For instance, the midguts embedded in a hydrogel are immobilized, which could allow the microinjection of virus directly into the midgut organ. Whether such approach could resemble the natural infection pattern of the midgut in mosquitoes warrants further investigation.

Infection patterns of DENV-2 in *Ae. aegypti* (Chetumal strain) mosquito midguts have been described using an indirect immunofluorescence assay (34). Individual infected epithelial cells could be detected as early as 2 days p.i.. These progressed to infection foci involving multiple cells and further spread until the whole midgut organ was covered at day 7 – 10 p.i. (34). In the *ex vivo* culture midguts, individual infected cells could be visualized at day 3 p.i., after which the mCherry signal slowly spread to neighboring cells, forming a few foci of several infected cells by day 7 p.i.. Although the infection of the midguts was performed by incubation with the inoculum, the tropism of DV2/mCherry infection

appeared to localize mainly in the posterior midgut, as it would occur when the mosquito ingests an infectious blood meal. One advantage of using a reporter virus in the *ex vivo* cultured midguts as opposed to using *in vivo* infection models is that the virus infection can be followed over time in the same midgut organ. The dark cuticle of *Aedes* mosquitoes does not allow the tracing of a reporter virus in real-time without having to first optically clear or dissect the mosquitoes to expose the organ of interest.

Although there is an apparent difference regarding the amount of virus being produced by the *ex vivo* cultured midguts, arbovirus infection is still occurring at considerable levels and thus it can greatly serve to study other aspects to arbovirus infection and facilitate the collection of preliminary data before experimenting with mosquitoes *in vivo*.

The next step in this study was to assess the antiviral activity of inhibitors on viral replication in the *ex vivo* mosquito midguts. NHC is a nucleoside analog that has been characterized as a potent antiviral against alphaviruses *in vitro*, including CHIKV and VEEV (17, 35). Various assays point to the compound acting as a pyrimidine analog that may target the viral polymerase domain of nsP4 provoking chain-termination (36). In addition, NHC also induces a high level of mutations in virus specific RNAs, leading to viral genomes unable to replicate (37). Consistent with these findings, RRV and CHIKV RNA levels were not reduced in NHC-treated midguts, but there was a marked decrease in virus infectivity for both alphaviruses when the *ex vivo* midguts were treated with 50 µM of NHC. The viral polymerase inhibitor 7DMA has shown potent anti-ZIKV activity *in vitro* and delayed disease progression in mice (18). In the *ex vivo* mosquito midguts,7DMA significantly reduced ZIKV RNA loads. Although the RNA levels presented some

variability among the ZIKV-infected midguts, the inhibitory effect of 7DMA in the *ex vivo* mosquito midguts was evident and followed a dose-response relationship.

In summary, we have established a long-term *ex vivo* mosquito midgut culture. To support this model, we have (a) assessed the viability of the *ex vivo* cultured midguts, (b) determined the replication kinetics of two alphaviruses (RRV and CHIKV) and two flaviviruses (DENV-2 and ZIKV), (c) employed a reporter virus to follow replication in the *ex vivo* mosquito midguts and, lastly, (d) evaluated the antiviral activity of two inhibitors against RRV, CHIKV, and ZIKV. Altogether, we have provided a reference for the use of *ex vivo* mosquito midguts as a tool to study arbovirus infection and related processes, while offering groundwork for developing other *ex vivo* mosquito organ cultures that can potentially provide insightful data, like salivary glands.

4. Materials and Methods

4.1. Cells

Mammalian cells

African green monkey kidney cells (Vero cells, ATCC CCL-81) and Vero E6 cells (ATCC CRL-1586) were cultured in minimum essential medium (MEM 1X) enriched with 10% Fetal Bovine Serum (FBS), 1% sodium bicarbonate, 1% L-glutamine and 1% non-essential amino acids (NEAA). Baby hamster kidney cells (BHK, ATCC CCL-10) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS, 1% sodium bicarbonate and 1% L-glutamine. Mammalian cell cultures were incubated at 37°C, with 5% CO_2.

Mosquito cells

Ae. albopictus larval cells (C6/36, obtained from ATCC, CRL-1660) were maintained in Leibovitz's L-15 medium containing 10% FBS, 1% NEAA and 1% HEPES buffer. *Ae. aegypti* larval cells (Aag2-AF5; a kind gift from Prof. Maringer, University of Surrey, Guildford, UK (38)) were maintained in Schneider's Drosophila medium supplemented with 10% FBS, 2 mM L-Glu and 1% Penicillin-Streptomycin (PenStrep). Mosquito-derived cell lines were incubated at 28°C, without CO_2.

For cell culture assays containing virus or virus-infected material, the concentration of FBS in the medium was reduced to 2%. All cell culture media and supplements were obtained from Gibco™, Thermo Fisher Scientific (Aalst, Belgium).

4.2. Viruses

Flaviviruses

Dengue virus serotype 2 (DENV-2/TH/1974, isolated in 1974 from human serum collected in Bangkok, Thailand, GenBank MK268692.1) was kindly provided by Prof. A. Failloux (Institut Pasteur, Paris, France) (39); ZIKV (SL1602, Suriname strain, GenBank KY348640.1) was acquired via the EVAg consortium (https://www.european-virus-archive.com). The DENV-2 reporter virus expressing the red fluorescent protein mCherry (DV2/mCherry, New Guinea C strain, NGC, GenBank AF038403.1) was kindly provided by Li-Hsin Li (Rega Institute for Medical Research, KU Leuven, Belgium) (15).

Alphaviruses

Ross River virus (RRV) was received from the National Collection of Pathogenic Viruses (UK; catalog number 0005281v); and CHIKV (Indian Ocean strain 899, GenBank FJ959103.1) was generously provided by Prof. Drosten (University of Bonn, Bonn, Germany) (40).

Virus stocks were prepared by passaging the isolates on Vero (for CHIKV) or C6/36 cells (for ZIKV, DENV-2, and RRV). Viral titers of the stocks were determined via plaque assay or end-point titration on Vero or BHK cells.

4.3. Compounds

7-Deaza-2'-*C*-Methyladenosine (7-DMA) was purchased from Carbosynth (Berkshire, UK) and dissolved in DMSO. β-D-N^4-hydroxycytidine (EIDD-1931 or NHC) was purchased from MedChemExpress (Monmouth Junction, NJ, USA) and dissolved in DMSO.

4.4. *Aedes aegypti* rearing

Aedes aegypti Paea (Papeete, Tahiti, collected in 1994) were obtained via the Infavec2 consortium. For each rearing, eggs were hatched in dechlorinated tap water. After hatching, groups of ±400 larvae were transferred into trays containing 3 L of dechlorinated tap water and fed every day with a yeast tablet (Gayelord Hauser, Saint-Genis-Laval, France) until the pupae stage. Pupae were placed in small plastic containers inside cardboard cups for its emergence. Adult mosquitoes were supplied with cotton balls soaked in a 10% sucrose solution supplemented with 100 U/mL of Penicillin-Streptomycin. Cardboard cups containing adults were maintained at 28 ± 1 °C with a light/dark cycle of 16/8 h and 80% relative humidity.

4.5. Mosquito midgut dissection and *ex vivo* culture

Unfed, antibiotic-treated female mosquitoes (3 – 7 days old) were cold-anaesthetized and surface sterilized by soaking in 70% ethanol for 20 s followed by soaking in Dulbecco's phosphate-buffered saline (PBS) for 20 s. Next, mosquitoes were dissected in PBS on a petri dish using the stereomicroscope (VisiScope®, VWR). In brief, the midgut was

exposed by carefully pulling the second to last segment of the mosquito abdomen. The gut of the mosquito was excised in its entirety (foregut, anterior and posterior midgut, and hindgut) to keep the tissue of interest (midgut) from degradation, and tracheal tubes were removed as much as possible without damaging the tissue. Following dissection, mosquito guts were washed twice in midgut medium, consisting of: Leibovitz's L-15 medium supplemented with 2% FBS, 100 U/mL of Penicillin-Streptomycin, 50 µg/mL of kanamycin, and 0.25 µg/mL of amphotericin B (Sigma Aldrich, USA), and finally placed in a black 96-well tissue culture plate with clear bottom (PerkinElmer©, USA) containing midgut medium. One mosquito gut was placed in each testing well. Plates were maintained at 28°C, without CO_2.

4.6. Videography of gut peristalsis and analysis

Ex vivo cultured mosquito midguts were recorded with the Leica DMi8 microscope to quantify the peristalsis observed *ex vivo*. A drop of carboxymethyl cellulose (CMC) 0.8% diluted in Leibovitz's L-15 medium was deposited on a microscope glass slide and one mosquito midgut was soaked in the drop. Forceps were used to gently arrange the hindgut in a position suitable for analysis (horizontally positioned on the slide). Each hindgut was recorded for a total of 1 minute 7 s (1 frame/0.3 s) and further discarded. This procedure was repeated at several time points during incubation starting from day 0 (right after dissection) to assess the contractibility, and hence viability, of the *ex vivo* cultured midguts over time.

For the analysis of the gut peristalsis, frames generated for each midgut were processed in IgorPro (Wavemetrics, USA) using a custom script described for the measurement of zebrafish gut motility (23). This script quantifies the changes in pixel intensity in a

designated area of analysis as it sequentially goes through all frames generated during the video recording. As output, the software indicates individual peristaltic periodicity (seconds in between contractions) per point of evaluation and overall averages of peristaltic periodicity for each midgut analyzed. These outputs were annotated manually to check for artifacts that could be generated by debris or tracheal tubes.

Serotonin hydrochloride (Sigma Aldrich, USA) and atropine sulfate salt monohydrate (Sigma Aldrich, USA) were employed in this bioassay to test the responsiveness of the mosquito midgut tissue. Midguts were incubated with either serotonin (20 µM), atropine (20 µM), or midgut medium for 1 hour, after which they were video recorded for further analysis.

4.7. Infection and antiviral assays in *ex vivo* mosquito midguts

Mosquito guts were dissected as described in 4.5 and placed into a 96-well tissue culture plate filled with midgut medium. After removing the medium, a total of 1×10^4 PFU/mL of virus was added to each well. Of note, an inoculum of 1×10^5 PFU/mL was used for DENV-2. Negative controls consisted of dissected guts fixated in 4% paraformaldehyde for 30 minutes. Midguts were incubated for two hours at 28°C, without CO_2. The virus inoculum was carefully removed, and midguts were washed two times with midgut medium. Fresh medium was finally added to all wells for incubation at 28°C, without CO_2. To test the activity of antiviral drugs in the *ex vivo* mosquito midguts, compound dilutions were prepared in midgut medium and added to the wells containing midguts to be treated, after which they were infected with $1 \times 10^{4\text{-}5}$ PFU/mL of virus. Following two hours of incubation at 28°C, without CO_2, the midguts were washed twice before adding midgut

medium alone (for virus control midguts) or containing compound (for treated midguts). Midguts were returned to the incubator at 28°C, without CO_2.

Incubation time for alphavirus-infected midguts was typically 3 days, while flavivirus-infected midguts were maintained for 7 days. Midguts were collected at several time points after infection for further analysis.

4.8. Determination of viral RNA levels and detection of infectious virus replication

Collected midguts were homogenized individually in 250 µL of PBS using bead disruption (2.8 mm beads, Precellys). The midgut homogenate was filtered using 0.8 µm MINI column filters (Sartorius, Germany) to remove debris, bacteria, or fungi. The filtered homogenate was used for further viral RNA isolation and qRT-PCR to determine viral RNA levels, and plaque assay to assess infectious virus replication.

Viral RNA isolation was performed with the NucleoSpin RNA Virus kit (Macherey-Nagel) following manufacturer's protocol. The sequences of primers and probes used for each virus are compiled in Table 1. One-Step, quantitative RT-PCR was performed for CHIKV and ZIKV in a total volume of 25 µL, consisting of 13.94 µL of RNase free water (Promega), 6.25 µL of master mix (Eurogentec), 0.375 µL of each forward and reverse primer (to a final concentration for each primer: 150 nM [CHIKV]; 900 nM [ZIKV]), 1 µL of probe (to a final concentration of 400 nM [CHIKV]; 200 nM [ZIKV]), 0.0625 µL of reverse transcriptase (Eurogentec), and 3 µL of RNA sample. For RRV, the reaction mixture was prepared in a total volume of 20 µL, containing 5.2 µL of RNase free water, 10 µL of SYBR Green master mix (BioRad), 1 µL of each forward and reverse primer (to a final concentration of 125 nM for each primer), 0.3 µL of reverse transcriptase (BioRad), and 4 µL of RNA sample. For DENV-2, the reaction mixture was prepared to a final

volume of 20 µL, consisting of 3 µL of RNase free water, 10 µL of SYBR Green master mix (BioRad), 0.3 µL of each forward and reverse primer (to a final concentration of 900 nM for each primer), 0.4 µL of reverse transcriptase (BioRad) and 6 µL of RNA sample. The qRT-PCR assays were performed with the QuanStudio™ 5 Real-Time PCR System (ThermoFisher Scientific) with the following cycling conditions for CHIKV and ZIKV: 30 min at 48°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. Cycling conditions for RRV were as follows: 10 min at 50°C, 3 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 30 s at 60°C. For DENV-2, the following cycling conditions were used: 30 min at 48°C, 3 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 62°C.

For absolute quantification, standard curves were generated each run using 10-fold serial dilutions of: cDNA of CHIKV-nsP1, viral RNA isolated from RRV and DENV-2 Bangkok virus stocks or synthesized gBlocks™ gene fragments (Integrated DNA Technologies) for ZIKV.

Plaque assays were performed to quantify infectious virus particles from infected midgut samples. Mammalian cells (Vero cells were employed for RRV and CHIKV; BHK cells were used for ZIKV and DENV-2) were seeded at a density of $2.5x10^5$ cells per well in 24-well tissue culture plates. One-day confluent monolayers of cells were inoculated with serial 10-fold dilutions of midgut samples, dilutions were prepared in 2% assay medium, and the inoculum was allowed to infect the cells for 2 hours at 37°C, with 5% CO_2. A negative control consisting of 2% assay medium used for preparing the dilutions was included in all plaque assays. The inoculum was removed and replaced with CMC 0.8% overlay diluted in RPMI medium (supplemented with 1% HEPES, 1% sodium bicarbonate,

521 1% L-glutamine, 1% PenStrep and 2% FBS). Infected cells were incubated for 3
522 (*Alphaviruses*) or 7 (*Flaviviruses*) days. After incubation, 4% PFA was added to each well
523 on top of the overlay medium and allowed for fixation for 1 hour, after which the overlay
524 mixture was discarded, and the wells carefully washed with water. The culture plates were
525 allowed to air dry, and then the wells were stained with 1% crystal violet solution (Sigma
526 Aldrich, USA). Plaques were counted and titer of each sample was calculated.

527 **4.9. Infection of *ex vivo* cultured midguts with DENV-2 expressing mCherry and**
528 **imaging**

529 Mosquito guts were dissected and infected with a DENV-2 expressing mCherry (15) as
530 described in 4.5 and 4.7, respectively. Of note, an inoculum of 1×10^5 PFU/mL was used
531 to maximize DENV-2 chance of infection. Negative controls and mock-infected midguts
532 were included. Midguts were monitored every day during the incubation period and
533 checked under the FLoid™ Cell Imaging Station (Life Technologies) for any signal that
534 indicated mCherry expression. At day 7 p.i., the mosquito midguts were fixated with PFA
535 for one hour at room temperature. Fixated midguts were transferred to a µ-slide 18 well
536 (Ibidi GmbH, Germany) plate and rinsed with PBS five times, for 5 minutes each time.
537 Midguts were then saturated with PBS-T (0.1% Triton X-100 [Sigma Aldrich, USA] and
538 1% bovine serum albumin [BSA, Sigma Aldrich, USA] in PBS 1X) for 2 hours at room
539 temperature. This was followed by phalloidin (diluted in 1% BSA in PBS 1X solution, final
540 concentration 100 nm) incubation for 10 minutes. DAPI (diluted in PBS 1X, final
541 concentration 100 nm) was added on top of the phalloidin solution and allowed to incubate
542 for 10 minutes. Finally, phalloidin/DAPI solution was removed, and midguts were rinsed
543 with PBS. Midgut samples were mounted in microscope slides using Glycergel® mounting

medium (Agilent, USA) and allowed to dry before imaging with a Leica DMi8 microscope (Leica Microsystems).

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Figures and tables

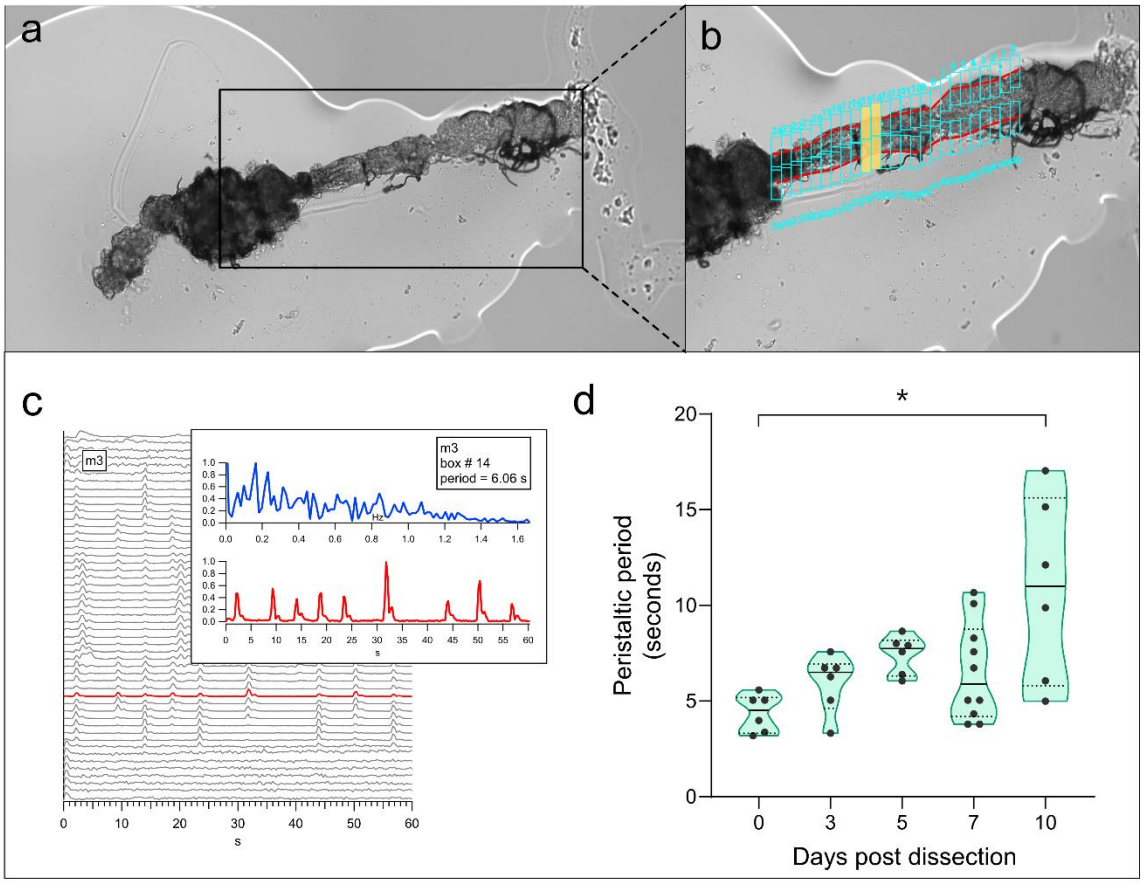

Figure 1. ***Ex vivo* cultured mosquito guts presented peristalsis in the hindgut, which is reduced upon 10 days post dissection**. **a**, Representative image of a mosquito gut placed in a CMC drop for videography. **b**, Close-up of the hindgut which comprises the area of analysis (AOA) manually set and outlined by the red lines. Cyan boxes denoted the regions to be analyzed along the AOA. The number and size of the regions (cyan boxes) were adjusted to be 25 boxes on each side of the hindgut. **c**, Representative kymograph showing the output for one mosquito gut analysis. Each line in the kymograph corresponded to an individual region, which was further investigated in a plot. In the figure, the red highlighted line in the kymograph correlates to the presented plot displaying a representative analysis readout for this region. **d**, Peristaltic period in function of time post dissection in days. Each point represents one individual mosquito midgut. The black line represents the median value. The graph shows data from three independent experiments. Statistical significance was assessed with a Kruskal-Wallis test. Significantly different values are indicated by asterisks: *, $P < 0.05$.

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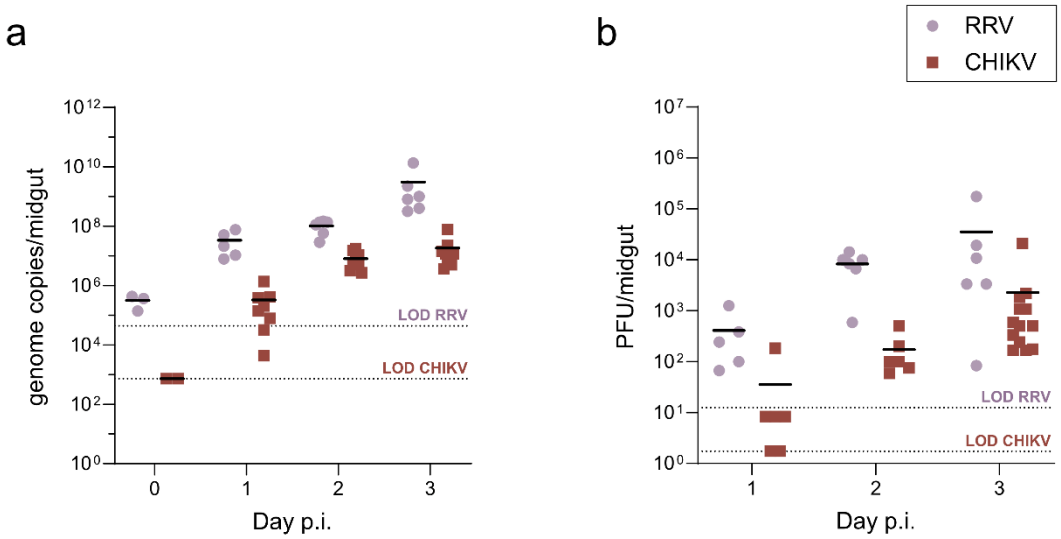

Figure 2. ***Ex vivo* cultured midguts supported Ross River (RRV) and chikungunya virus (CHIKV) infection. a**, Viral RNA levels in the mosquito midguts were quantified at 2 hours (day 0), 1, 2 and 3 days p.i. by means of qRT-PCR. **b**, Infectious virus loads in the mosquito midguts were quantified by means of plaque assay. Each dot represents an individual midgut organ. The black line represents the mean value. Data correspond to at least two independent replication kinetics assays. LOD: Limit of detection of the corresponding assay.

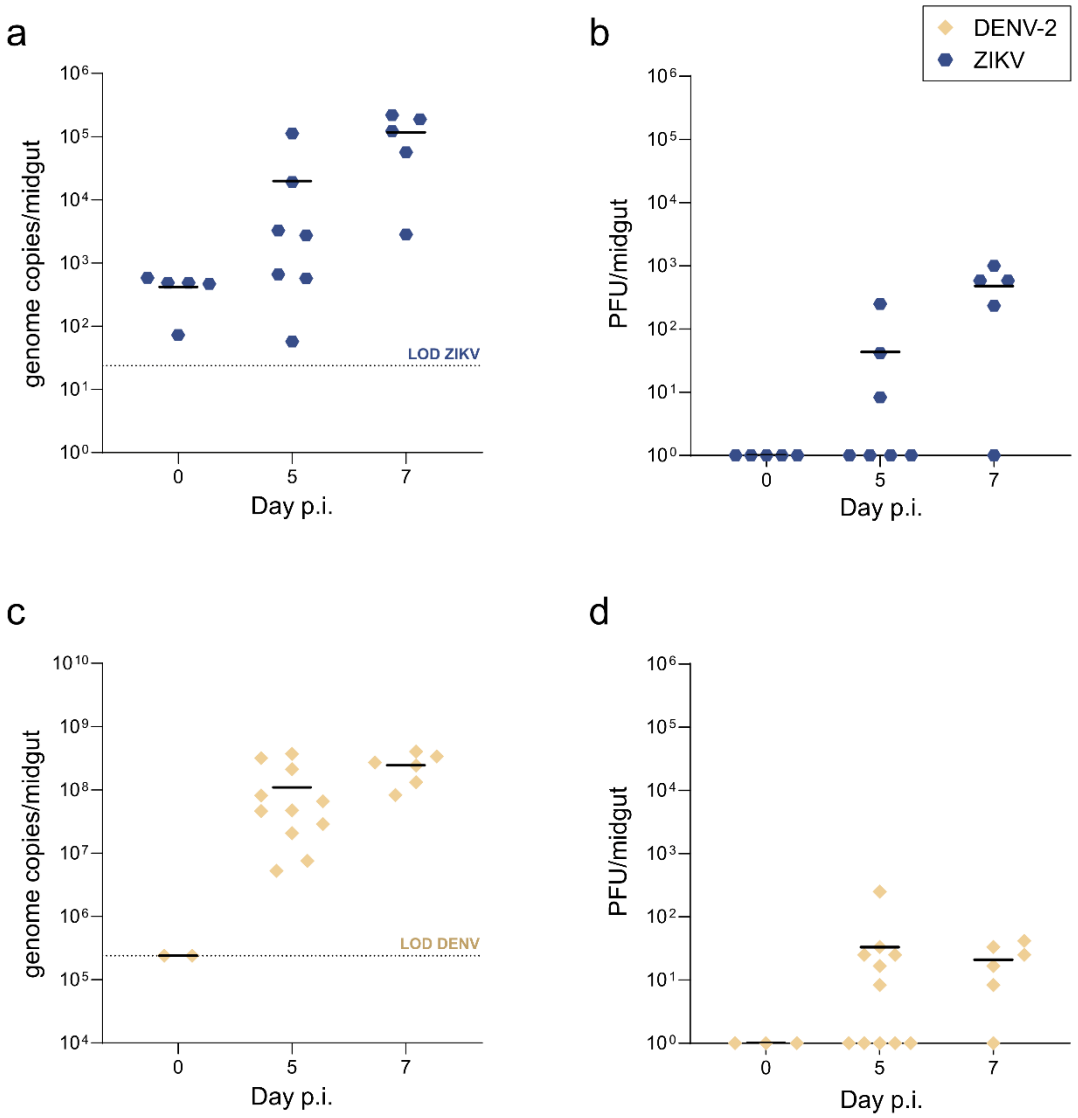

Figure 3. ***Ex vivo* cultured midguts supported dengue virus serotype 2 (DENV-2) and Zika virus (ZIKV) infection. a**, **c**, Viral RNA levels in the mosquito midguts were quantified at 2 hours, 5 and 7 days p.i. by means of qRT-PCR. **b**, **d**, Infectious virus loads in the mosquito midguts were quantified by means of plaque assay. Each dot represents an individual midgut organ. The black line represents the mean value. Data correspond to two independent replication kinetics assays. LOD: Limit of detection of the corresponding assay.

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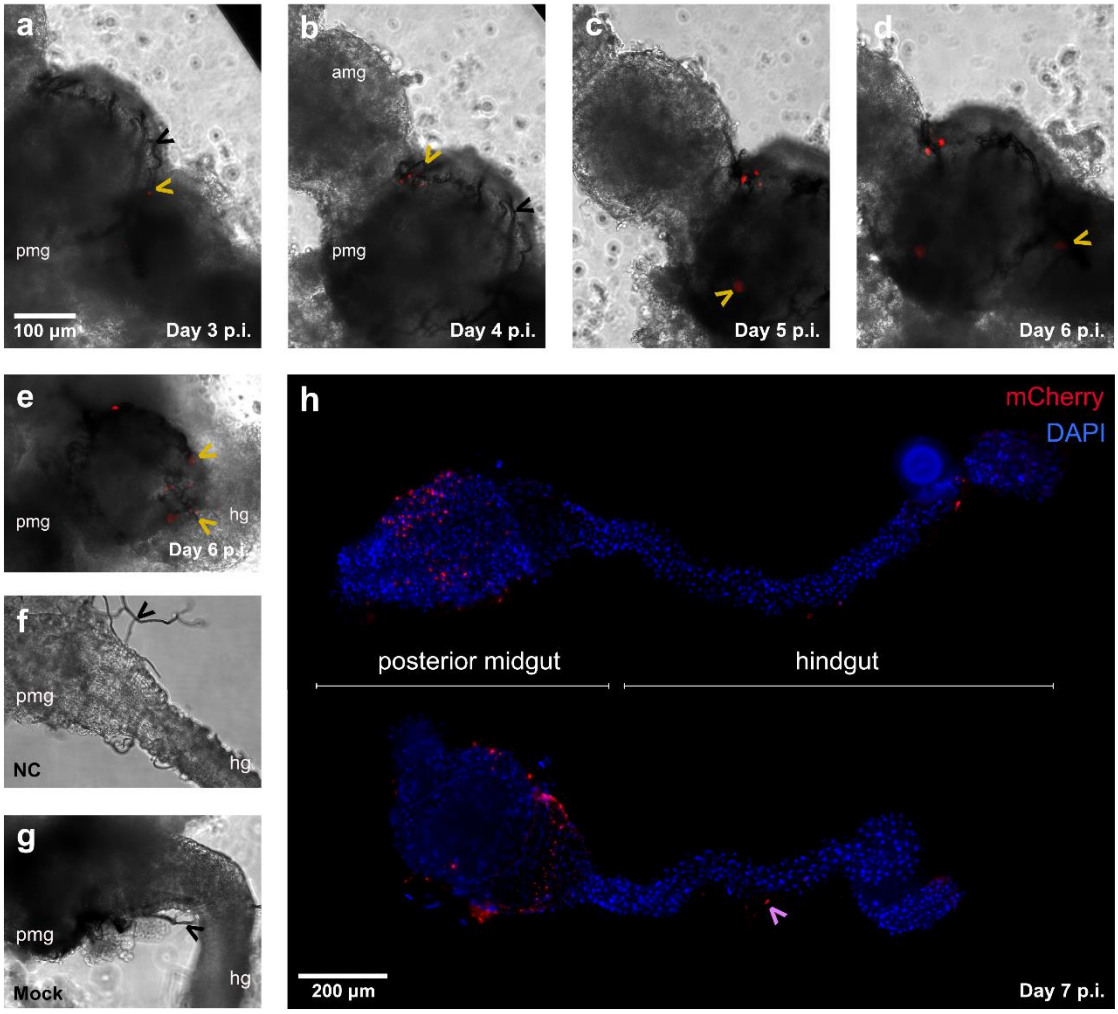

Figure 4. **Replication of DENV-2 expressing mCherry in the *ex vivo* cultured midguts. a**, **b**, **c**, **d**, Magnification, 20X. Overlay of bright field and red filter imaging. Panels display the DENV-2 infection and spread in the midguts as seen by the mCherry red signal on days 3 to 6 p.i. All four images correspond to the same organ imaged over the selected time points. The scale bar, represented by the white line, can be applied to panels a-g. **e**, mCherry expression in another biological replicate at day 6 p.i., showing several infection foci in the posterior midgut region. **f**, **g**, NC: negative control. No mCherry expression was detected in fixated or mock-infected midguts. **h**, mCherry signal in DENV-2 infected midgut, magnification, 10X. amg: anterior midgut, pmg: posterior midgut, hg: hindgut. Black arrows indicate the presence of tracheae. Yellow arrows indicate mCherry signal. Magenta arrow indicates mCherry signal present in tracheal tubes that remained in the hindgut.

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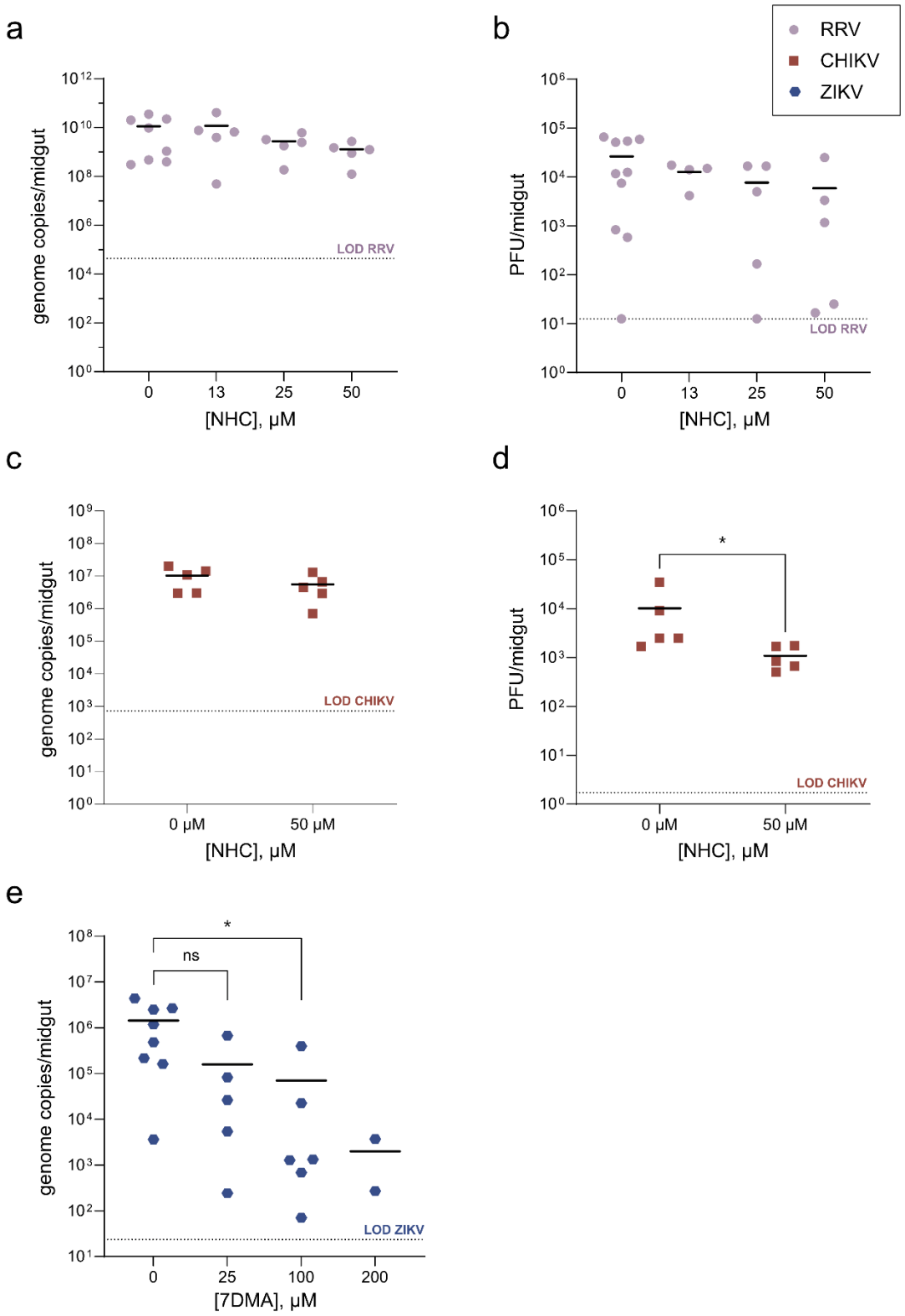
a
genome copies/midgut
LOD RRV
[NHC], μM
b
RRV
CHIKV
ZIKV
PFU/midgut
LOD RRV
[NHC], μM
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genome copies/midgut
LOD CHIKV
0 μM
50 μM
[NHC], μM
d
PFU/midgut
*
LOD CHIKV
0 μM
50 μM
[NHC], μM
e
genome copies/midgut
*
ns
LOD ZIKV
[7DMA], μM

Figure 5. **Antiviral activity of NHC and 7DMA in the *ex vivo* cultured mosquito midguts. a**, RRV RNA loads in the mosquito midguts were quantified at day 3 p.i. by qRT-PCR. **b**, RRV infectious virus loads quantified at day 3 p.i. by plaque assay. **c**, CHIKV RNA loads in the mosquito midguts were quantified at day 3 p.i. by qRT-PCR. **d**, CHIKV infectious virus loads were quantified at day 3 p.i. by plaque assay. Statistical significance was assessed with a Mann-Whitney test. Significantly different values are indicated by asterisks: *, $P<0.05$. **e**, ZIKV RNA levels in the mosquito midguts were quantified at day 7 p.i. by qRT-PCR. Statistical significance was assessed with a Kruskal-Wallis test. Significantly different values are indicated by asterisks: *, $P<0.05$. ns: not significant. Each dot represents an individual midgut. The black line represents the mean value. LOD: Limit of detection of the corresponding assay.

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712 **Table 1. Sequences of primers and probes for qRT-PCR used in this study**

Virus [target protein]	Forward primer (5' → 3')	Reverse primer (5' → 3')	Probe (5' → 3')	Reference
Ross River virus [E2]	TACAAGCACGACCCATTGCCG	GATAGTCCTGCCGCCTGCTGT	N/A	(41)
Chikungunya virus [nsP1]	CCGACTCAACCATCCTGGAT	GGCAGACGCAGTGGTACTTCCT	FAM-TCCGACATCATCCTCCTTGCTGGC-TAMRA	(42)
Zika virus [E]	CCGCTGCCCAACACAAG	CCACTAACGTTCTTTTGCAGACAT	FAM-AGCCTACCT-ZEN-TGACAAGCAATCAGACACTCAA-IABkFQ	(43)
Dengue virus serotype 2 [NS3]	CAGATCGGAGCTGGAGTTTAC	TTTTGACGTCCGCCCATGAA	N/A	*In-house*

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