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Article

Larval Competition between *Aedes* and *Culex* Mosquitoes Carries Over to Higher Arboviral Infection during their Adult Stage

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Abstract: The common house mosquito (*Culex pipiens*) is a native vector for West Nile virus (WNV). Invasive species like the tiger mosquito (*Aedes albopictus*) and Asian bush mosquito (*Aedes japonicus*) are rapidly spreading through Europe, posing a major threat as vectors for dengue, chikungunya (CHIKV), and Japanese Encephalitis virus (JEV). These mosquitoes share a similar ecological niche as larvae, but the carry-over effects of aquatic larval interactions to terrestrial adult stage remain largely unknown and their medical relevance requires further investigation. This study examines the context-dependency of larval interactions among *Aedes albopictus*, *Aedes japonicus* and *Culex pipiens*. The survival, development time, growth, and energetic storage were measured in different European populations within density-response experiments (intraspecific) and replacement experiments (interspecific) at 20°C and 26°C. Overall, *Ae. japonicus* was the weakest competitor, while competition between *Ae. albopictus* and *Cx. pipiens* varied with temperature. *Culex pipiens* was infected with WNV, *Ae. albopictus* with CHIKV and *Ae. japonicus* with JEV. While no JEV infection was observed, mosquitoes experiencing interspecific interactions during their larval stages exhibited higher infection rates and viral RNA titers for CHIKV and WNV. This increased susceptibility for viral infection after larval competition suggests a higher risk of arbovirus transmission in co-occurring populations.

Keywords: *Aedes albopictus*; *Aedes japonicus*; arbovirus; viral RNA titer; CHIKV; *Culex pipiens*; infection rate; JEV; WNV

1. Introduction

Over the past 50 years, the incidence of dengue has increased by 30-fold [1]. By 2023, more than 100 countries reported the circulation of chikungunya virus (CHIKV), putting an estimation of 1.3 billion people at risk of chikungunya fever globally [2]. A growing amount of autochthonous dengue cases have been observed in Europe, with a total of 74 cases reported between 2010 and 2021. In the past two years, this number increased considerably, with 71 cases in 2022 and 130 cases in 2023 ECDC [3]. The first autochthonous Chikungunya outbreak in Europe occurred in Italy in 2007, with approximately 330 cases identified. Since then, sporadic outbreaks of Chikungunya have been observed, which were often associated with extreme climate events [4]. In 2017, a total of 270 confirmed and 219 probable autochthonous Chikungunya cases have been observed [5]. An increase in autochthonous cases of WNV were recorded, with 1471 cases between 2000 and 2017, 1503 cases and 180 deaths in 2018 alone, and 1113 cases and 92 deaths in 2022 [6].

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Aedes mosquitoes are the primary vectors of dengue (DENV) and CHIKV virus [7]. In Europe, these invasive species include the Asian tiger mosquito *Ae. albopictus* (Skuse, 1894), the Asian bush mosquito *Ae. japonicus japonicus* (Theobald, 1901), and the Korean bush mosquito *Ae. koreicus* (Edwards, 1917) [8]. There are four subspecies of *Ae. japonicus*, however, only *Ae. japonicus japonicus* is found in Europe [9], therefore it will be referred to as *Ae. japonicus*. *Aedes albopictus* and *Ae. japonicus* are considered to be one of the fastest spreading invasive species [9]. Their spread and establishment to new regions is largely influenced by climate change and international trade [8,10]. Additionally, anthropization of the landscape is a significant factor influencing the dynamics of vector-borne pathogens [1]. One of the main consequences, apart from nuisance due to high abundances, are outbreaks of mosquito-borne diseases. It has been observed that local outbreaks typically manifest within a time frame of 5 to 15 years subsequent to the establishment of *Ae. albopictus* populations [11]. This statement is supported by the recent autochthonous outbreaks of DENV, and CHIKV in Europe [12].

Additionally, *Culex pipiens* Linnaeus, 1758, is a known vector for WNV. This species is endemic, widespread, and abundant in Europe [13]. The species consists of two bioforms: *Cx. pipiens pipiens* and *Cx. pipiens molestus* [14]. With the arrival of *Ae. albopictus* (Albania: 1979 and Italy: 1990) [10], and *Ae. japonicus* (Belgium: 2002) [8,15], it now encounters these invaders in shared larval habitats [16]. All three species occur in artificial containers [17,18].

It is hypothesized that interspecific competition among mosquito larvae may enhance their vector competence for arboviruses [19–21]. Competitive stress negatively affects mosquito fitness, which in turn has a detrimental impact on the mosquito immune system and physical barriers against viral infection. This carry-over effect of the aquatic larval interactions to the terrestrial adult stage might be important to be considered when estimating the vector competence of arboviral vectors. For example, Alto, Lounibos [19] observed that *Ae. albopictus* females were smaller, had a higher infection rate, viral RNA titer and dissemination rate of SINV following larval competition with *Ae. aegypti.* Interspecific competition led to more intense competition compared to intraspecific competition. Similarly, Alto, Lounibos [20] discovered an elevated infection and dissemination rate of DENV in *Ae. albopictus* after larval competition with *Ae. aegypti*. In addition, Bevins [21] reported that *Ae. triseriatus* females had an increased mortality, larger size, and higher infection and dissemination rate of LACV following larval competition with *Ae. albopictus*.

Aedes albopictus has often been found to be a superior competitor during the larval stage in the aquatic environment [22–29]. However, other studies suggest that a balanced coexistence between *Cx. pipiens* and *Ae. albopictus* is possible when sufficient food resources are available [17,28,30]. *Aedes japonicus* is expected to not strongly interact with *Cx. pipiens* [31,32]. It is considered a weak larval competitor when compared to *Ae. albopictus* [24,33].

Larval density and competition are found to affect egg production, body size, energy reserves, and longevity of adult females [34–37]. Generally, larger females exhibit higher levels of protein, glycogen, and lipid content upon emergence [38]. These energy reserves are important while searching for a suitable host. They show more biting persistence, higher longevity [21] and vector capacity [39]. Additionally, lipids also regulate the immune response [35,39,40]. The innate immune system of mosquitoes consists of various lines of defense mechanics. The epithelium-lined midgut serves as the initial barrier, while hemocytes play a crucial role as cellular components of innate immunity. Additionally, Toll and Imd pathways are responsible for the signaling of the production of antimicrobial peptides (AMP), contributing to the humoral defenses of mosquitoes [35]. Lipids facilitate membrane biogenesis at infection sites and in hemocytes [40]. In addition, lipid droplets could potentially serve as an energy source for microflora, and have been linked with the activation of Toll-like receptors during DENV infection [39]. In *Ae. aegypti* subjected to larval nutrient stress, a decrease in the number of hemocytes was observed, however, enhanced fat body derived immune factors, such as AMPs, were found. Furthermore, transcripts of Spaetzle, a key regulator of the Toll pathway, and certain immune-related genes were less abundant but demonstrated increased expression [35].

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Bevins [21] observed larger females of *Ae. triseriatus* after interspecific larval competition with *Ae. albopictus*. These larger females were more susceptible to develop LACV infections compared to females from intraspecific experiments. Larger females of *Ae. albopictus* have more tissue for virus replication, leading to higher viral RNA titers [19]. Controversially, a study on *Ae. triseriatus* females deriving from malnourished larvae reveal smaller females with a thinner basal lamina [41], a membrane that envelops the midgut and hinder virus movement [20]. These females were also associated with higher infection, dissemination and transmission rates of a bunyavirus. Telang, Qayum [35] observed that basal lamina thickness was not affected by the size of the female. It was noted that certain immune-related genes were less expressed in stressed larvae, but exhibited increased expression in females derived from these stressed larvae [35]. These findings suggest that nutritional stress during the larval stage may result in weaker immune responses in adults [35,41,42] , which potentially increase their vector competence. However, these smaller females will have a shorter longevity, and thus vector capacity, which might be too short to complete the extrinsic incubation period [21,36].

The objective of this study was to investigate the carry-over effect of larval interactions between *Cx. pipiens* and *Ae. albopictus* as well as *Ae. japonicus* in recently established populations from Central Europe. This carry-over effect was analyzed via intra- and interspecific larval competition on arboviral infection of *Ae. albopictus*, *Ae. japonicus*, and *Cx. pipiens* during their adult stage. The effect of synecological patterns at 20 and 26°C was assessed by collecting data on larval mortality, development time, behavior, pupal size, and the content of energy reserves. Afterwards, an infection experiment was conducted with the medically relevant CHIKV, JEV, and WNV to test whether significant ecological patterns from larval competition would carry over to an increased infection rate and viral RNA titer, potentially elevating the risk of arbovirus transmission.

2. Material & Methods

2.1. Larval Competition Study

2.1.1. Mosquito Material

Culex pipiens s.s./*Cx. torrentium* egg rafts were sampled in Frankfurt (Germany) in September 2021, June and August 2022. The egg rafts were stored for several days on humid cotton at 10°C, before they were placed in softened water at 20°C or 26°C to hatch. To analyze the biotypes, a multiplex quantitative real-time PCR (qRT-PCR) was performed as described by Rudolf, Czajka [43], with all specimens used during the infection experiments. A total of 85 *Culex* specimens were tested, of which 77 were *Cx. pipiens pipiens*, 7 *Cx. torrentium*, and 1 hybrid *Cx. pipiens pipiens* x *molestus*. The *Ae. albopictus* strain (20AAlb.DE-KABS.12) originated from egg collections in Achern (Germany) in September 2020 and was reared at 28°C with 80% relative humidity and a 16:8 L:D photoperiod. The *Cx. pipiens* biotype *molestus* strain (20CPip.BE-ITMf.6) originated from larval collections in Hove (Belgium) in 2020 and was reared as a colony with overlapping generations at 23°C with relative humidity of 80% and a 16:8 L:D photoperiod. Since the strain also contains genetically mixed forms with the bioform *pipiens* (nondiscriminating multiple generations hybrids and backcross, from here on called hybrids), a PCR was conducted on the used specimens [44]. A total of 182 *Cx. pipiens* s.l. were tested, and revealed 130 *Cx. pipiens molestus*, 46 hybrid *Cx. pipiens pipiens* x *molestus*, and 6 *Cx. pipiens pipiens*. The *Ae. japonicus japonicus* experiments were executed with field larvae reared from collected eggs. Oviposition traps were placed and collected in Havelange (Belgium) in June and July 2022 and June 2023. The oviposition sticks were stored at 10°C up to several months upon experimental use. All rearing and competition experiments took place at 20°C or 26°C with relative humidity of 80% and a 16:8 L:D photoperiod in climatic cupboards (CPS-P530 Climatic Cabinet, RUMED Germany) at the Merian insectary of the Institute of Tropical Medicine (ITM), Antwerp, Belgium.

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2.1.2. Larval Replacement-Series Experiments

Intraspecific interactions were studied at 20°C and 26°C with density response experiments for 3, 5, 15, 30 and 45 specimens in 1L cups with 600 ml soft water in triplicate. To identify the number of larvae that results in low mortality rates for interspecific experiments, the percentage of mortality was documented per larval density (Supplementary file 1 Figure S1). Interspecific interactions were investigated at 20°C and 26°C with replacement experiments for *Aedes*:*Culex* 30:0, 20:10, 15:15, 10:20 and 0:30 combinations in 1L cups with 600 ml soft water in triplicate. Within 24h after hatching, first instar larvae were placed in the experiments. Larvae were fed three times per week with sieved TetraMin (Tetra, Germany). A dose of 0.5 mg food per larvae was provided during the first four doses, from the fifth dose onwards 1 mg of food was provided per larvae to receive 6 mg per larvae in total based on Müller, Knautz [17], Müller, Knautz [45]. The time of pupation was registered for each experiment to measure development time and mortality. Three pupal growth parameters (area of cephalothorax, length and width of abdomen) were considered to correct for pupal size.

2.1.3. Video Tracking of Behavioral Variables

Fourth instar larvae from the same batch as the larval competition experiments were maintained at 20°C and used for behavioral observations using a high-quality video tracking system with digital image recognition. DanioVision hardware and Ethovision software were used to track social interactions, activity, and larval behavior such as total distance moved, velocity and body contact. Larvae were placed in a petri dish with soft water at room temperature and recording started after 30 seconds to allow acclimatization. Larvae were recorded for two minutes per experiment. Intraspecific experiments were conducted in triplicate for 1, 2, 5, 10 and 18 larvae per species. Intraspecific experiments were run in triplicate with *Aedes*:*Culex* ratios 18:0, 12:6, 9:9, 6:12 and 0:18.

2.1.4. Photometric Assays on Pupal Lipid, Glycogen, and Protein Content

The first five pupae per experiment were stored at -20°C to test for energy storage. The total content of glycogen, lipid and protein per pupae was analyzed via photometric assays according to Van Handel [46], Van Handel [47], and Bradford [48] respectively, as described by Bock, Kuch [49]. Pupal growth parameters (area of cephalothorax, length and width of abdomen) were taken. The abdominal width was considered the most robust metric (selection of parameter based on the lowest coefficient of variation, see Supplementary file 5 Table S1) and was used to correct for pupal size. The following equation was used:

$$\text{Corrected energy content} = \frac{\text{total energy content}}{\text{abdominal width}} \text{ per mosquito} \quad (1)$$

2.1.5. Data Analysis

The Relative Crowding Coefficient (RCC) is used as a measure of competition. An RCC value of 1 shows that both species are equal competitors. Values below or greater than 1 indicate out-competition [50,51]. The RCC values were calculated for the development time, pupal size, pupal energy content, protein content, and larval behavior (total distance moved, velocity, and body contact) of the three species from the means of three replicate experiments. The formula used was described by Harper (1977) and adapted by Novak, Higley [50] and Oberg, Young [51] as follows according to Müller, Knautz [17]:

$$RCC_{\text{species A}} = \frac{\left[0.5\left({SpeciesA^{20:10}}/{SpeciesB20:10}\right) + \left({SpeciesA^{15:15}}/{SpeciesB15:15}\right) + 2\left({SpeciesA^{10:20}}/{SpeciesB10:20}\right)\right]/3}{{SpeciesA^{30:00}}/{SpeciesB^{30:00}}} \quad (2)$$

There is currently no test available to determine if these RCC results are significantly different [51], therefore we conducted two-way ANOVA's, Kruskal-Wallis or Friedman tests on the raw data (see supplementary files). A two-way ANOVA was conducted to test for significant differences in larval density, species or species ratio and this interaction. Tukey's multiple comparisons test was used to compare all data. All statistical tests were conducted using Prism (version 10.1.2, GraphPad Software INC., USA). Statistical significance was defined as $P < 0.05$. Kolmogorov-Smirnov test and Shapiro-Wilk test were used to test for normality, and homoscedasticity was tested by curve fitting

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for appropriate weighting of residuals (version 10.1.2, GraphPad Software INC., USA). When assumptions for normality were violated, a Kruskal-Wallis test or Friedman test was performed instead.

2.2. Infection Study

2.2.1. Infectious Blood Feeding of Mosquitoes Which Experienced Larval Competition

Selected treatments from previously conducted larval competition experiments were repeatedly conducted at ITM in Belgium and freshly emerged adult mosquitoes were transferred to the Bernhard Nocht Institute for Tropical Medicine (BNITM) in Germany to determine the rate of infection in mosquitoes which experienced larval competition. All experiments at BNITM were performed under BSL-3 conditions. Seven to ten days after eclosion, mosquitoes were anesthetized with carbon dioxide, sorted into vials, and starved for one (*Aedes*) or two (*Culex*) night(s). Infection was performed as described by Heitmann, Jansen [53]. Blood meal, containing 50% human blood (expired banked blood), 30% of an 8% fructose solution, 10% filtrated bovine serum (FBS), and 10% virus stock; was offered with a final concentration of 10^7 plaque forming units (PFU)/ml for WNV (clade 1a, strain TOS-09, Genbank HM991273/HM641225), 10^6 PFU/ml for CHIKV (strain CNR 24/2014, supplied by the European virus Archive goes global project), and 10^6 PFU/ml for JEV (strain SA-14, GenBank accession number EU073992). All virus stocks were propagated using Vero cells (*Chlorocebus sabaeus*; CVCL 0059, obtained from ATCC, Cat# CCL-81). *Culex pipiens* was infected with West- Nile virus (WNV), *Ae. albopictus* with chikungunya virus (CHIKV) and *Ae. japonicus* with Japanese Encephalitis virus (JEV). Artificial blood meal was offered for *Cx. pipiens* via cotton stick overnight, for *Ae. albopictus* and *Ae. japonicus* in two droplets (50 µl each) per vial for 2 h. Fully engorged mosquitoes were sorted and kept at 24 +/- 5°C (mimicking fluctuating temperatures between day and night), with a relative humidity of 70%, 12:12 L:D photoperiod, and continuous fructose supply. Mosquitoes were kept for WNV and JEV for two weeks; for CHIKV for one week.

2.2.2. Quantification of Infection Rate

A total of 267 *Cx. pipiens*, 57 *Ae. albopictus*, and 47 *Ae. japonicus* were analyzed individually. Afterwards, specimens were homogenized in 500 µl Dulbecco's modified eagle medium (DMEM). RNA was extracted using the MagMAX CORE nucleic acid purification kit (Applied Biosystems, Thermo Fisher Scientific Corporation, Waltham, MA, USA). By using a RT-qPCR, viral RNA titer was determined (RealStar Chikungunya RT-PCR Kit 2.0; RealStar WNV RT-PCR 2.0, both from altona Diagnostics, Hamburg, Germany; and for JEV as described by Huber, Jansen [54], using QuantiTect Probe RT-PCR Kit, Qiagen, Hilden, Germany). From these results, the infection rate was calculated as follows:

$$\text{Infection rate (IR)} = \frac{virus{-}positive\ mosquito\ bodies}{number\ of\ fed\ mosquitoes} \quad (3)$$

To exclude natural arbovirus infections, ten randomly selected adult mosquitoes per species were tested by pan-Orthobunya-, pan-Flavivirus-, and pan- Alphavirus-PCR, confirming all specimens as negative [55–57].

2.2.3. Statistical Analysis

A principal component analysis (PCA) was conducted on the 14 variables measured during the competition and infection experiments: larval ratio, pupal size (cephalothorax area, abdominal length and width), energy (lipid, glycogen), protein content, mortality, larval development time, behavioral variables (distance moved, velocity, and duration of the body contact), infection rate, and viral RNA titer. Statistical tests were conducted using Prism (version 10.1.2, GraphPad Software INC., USA).

3. Results

Our research shows that interspecific competition had a significant impact on all three species, although out-competition was rarely observed among the 14 variables tested. The synecological

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patterns were predominantly noticeable at the often overlooked metabolic and behavioral levels. Specifically, *Cx. pipiens* showed pronounced responses at low temperatures, *Ae. albopictus* at high temperatures, and *Ae. japonicus* at both temperatures.

3.1. Competition Study

3.1.1. Mortality

The larval mortality of these three mosquito species in the interspecific competition treatments was low. The mortality of *Cx. pipiens* s.s./*Cx. torrentium* was higher if compared to the *Ae. japonicus* x *Cx. pipiens* bioform *molestus* competitive treatments (Supplementary file 2 Figure S2). Friedman tests were conducted to test for difference between the species and larval densities, however, results were not significant. Low mortality after intraspecific competition in microhabitats containing 30 larvae confirmed the use of 30 larvae to be a suitable amount for interspecific experiments with the three mosquito species (Supplementary file 1 Figure S1).

3.1.2. Development Time

A two-way ANOVA conducted to compare the development time for 50 % of the larvae to emerge revealed that the factors *Species*, *Larval ratio* and its *Interaction* were significant for the *Ae. albopictus* x *Cx. pipiens* s.s./*Cx. torrentium* competitive treatments at both test temperatures, with *Ae. albopictus* developing faster than *Cx. pipiens* s.s./*Cx. torrentium* larvae at 26°C (Supplementary file 3 Figure S3a and c). The *Ae. japonicus* x *Cx. p. molestus* competitive treatments showed significant differences for the factors *Species* and *Interaction* at 20°C, for which *Ae. japonicus* developed faster compared to *Cx. p. molestus* (Supplementary file 3 Figure S3b). At 26°C, the factors *Larval ratio* and *Interaction* were significantly different, with *Cx. p. molestus* developing much faster during interspecific competition (Supplementary file 3 Figure S3d).

3.1.3. Larval Behavior

Larval behavioral data on total distance moved, velocity, and body contact showed significant differences only for the duration of the body contact between the species competitive treatments, with body contact being avoided when more *Ae. japonicus* were present (Supplementary file 6 Figure S5).

3.1.4. Pupal Size

There were significant differences in pupal size between the species and ratios. Generally, pupae from interspecific competition were significantly larger compared to intraspecific ones. Only the *Cx. pipiens* s.s./*Cx. torrentium* showed smaller cephalothorax size in interspecific competition (Supplementary file 4 Figure S4).

3.1.5. Energy and Protein Storage

Lipid storage in the *Ae. albopictus* x *Cx. pipiens* s.s./*Cx. torrentium* competitive treatments is significantly lower at the higher temperature of 26°C. At 20°C lipid content is lowest in the intraspecific treatments if compared to interspecific treatments, at 26°C a slightly higher lipid content is found in Ae. albopictus. The *Ae. japonicus* x *Cx. p. molestus* competitive treatments showed a higher lipid content at 26°C (Supplementary file 7 Figure S6a-b,g-h). The glycogen content is always higher in *Ae. albopictus* compared to *Cx. pipiens* s.s./*Cx. torrentium* and *Ae. japonicus* (Supplementary file 7 Figure S6c-d,i-j). Protein content was highest at 20°C for both *Ae. albopictus* and *Cx. pipiens* s.s./*Cx. torrentium*, however, no difference was observed in *Ae. japonicus* (Supplementary file 7 Figure S6e-f,k-l).

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3.1.6. Relative Crowding Coefficient

The Relative Crowding Coefficient was computed as a metric of competition. A RCC value of one indicates equal competitiveness between both species, whereas a value below or above one suggests one species outcompeting the other. For both species combinations, the effects of the competition on the larval development were clearest at 26°C. *Aedes albopictus* has an advantage towards *Cx. pipiens* s.s./*Cx. torrentium* at 20°C while the opposite is true at 26°C, and *Cx. p. molestus* could out-compete *Ae. japonicus* at both temperatures (Figure 1a). The RCC for size showed a smaller difference, however, *Ae. albopictus* had a slight advantage towards *Cx. pipiens* s.s./*Cx. torrentium*. For the other species combination, *Cx. p. molestus* showed an advantage regarding larger size at 20°C and *Ae. japonicus* had an advantage at 26°C, respectively (Figure 1b).

In general, larval (out)competition between species became more apparent in the glycogen and protein content rather than development time and pupal size. For the lipid content, an advantage towards higher lipid content is seen for *Ae. albopictus* at 20°C, however, at 26°C competition is very clear with *Cx. pipiens* s.s./*Cx. torrentium* having a negative impact resulting in less the lipid storage in *Ae. albopictus*. For *Ae. japonicus*, there was a disadvantage in lipid content compared to *Cx. p. molestus* at both temperatures (Figure 1c). Larval competition clearly influences the glycogen content of all species at both tested temperature regimes. At 20°C, *Ae. albopictus* had a competitive advantage with regard to higher glycogen content over *Cx. pipiens* s.s./*Cx. torrentium*, however, at 26°C the opposite was noted with *Ae. albopictus* being negatively affected by *Cx. pipiens* s.s./*Cx. torrentium*. For the other species combination, *Cx. p. molestus* always outcompeted *Ae. japonicus* in glycogen content, resulting in a higher glycogen content in *Cx. p. molestus* in interspecific competition (Figure 1d). For protein content, *Ae. albopictus* had the competitive advantage over *Cx. p. molestus* at both temperatures, with more protein content during interspecific competition. *Aedes japonicus* had a competitive disadvantage at both temperatures compared to *Cx. p. molestus* (Figure 1e).

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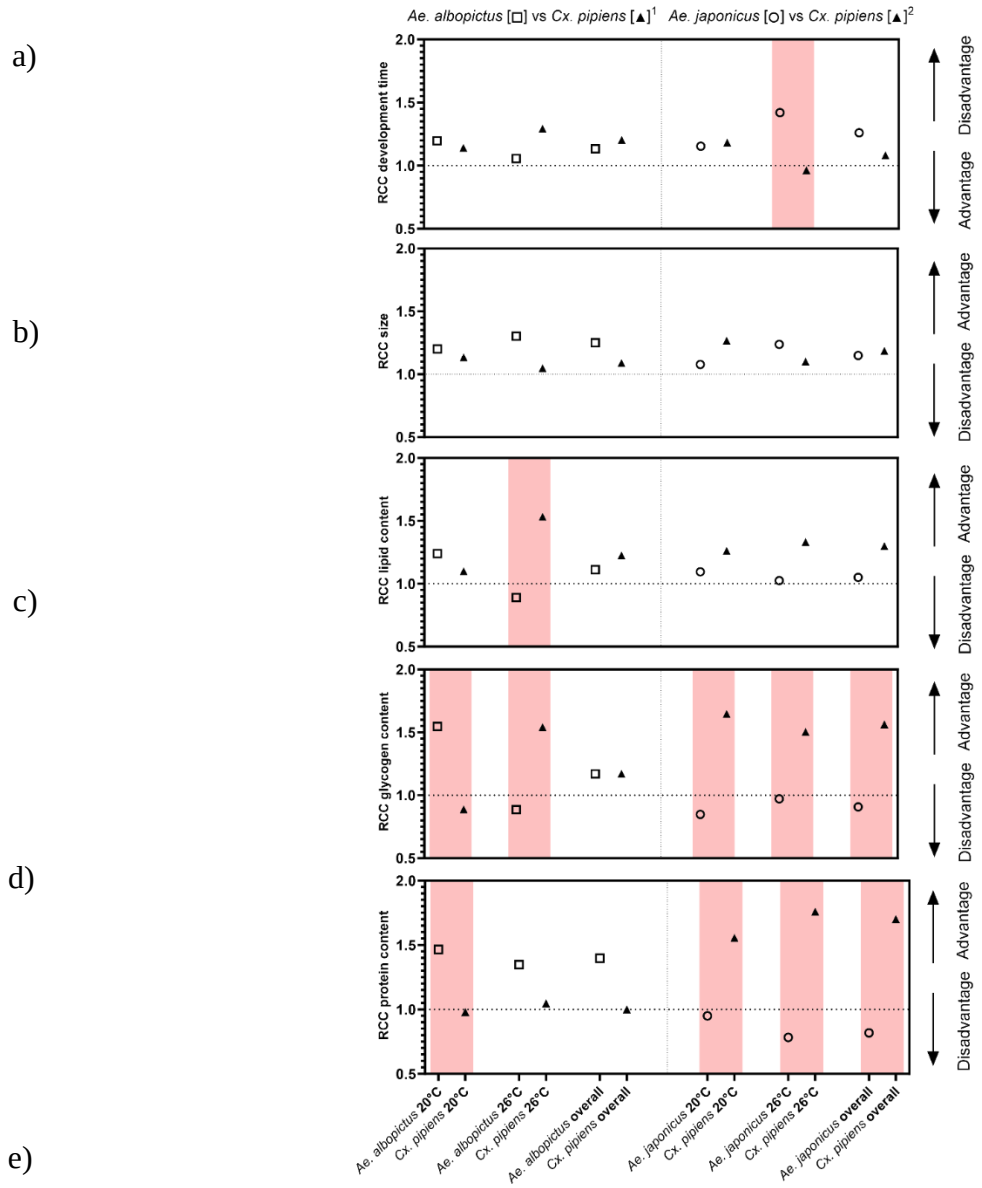

Figure 1. Relative Crowding Coefficient with advantage or disadvantage. a) development time for 50% of the pupae to emerge, b) the pupal size, c) lipid, d) glycogen, e) protein content (size corrected) of combination 1) *Ae. albopictus* vs *Cx. pipiens* s.s./*Cx. torrentium*, and 2) *Ae. japonicus* vs *Cx. p. molestus* during interspecific competition at 20°C and 26°C. In red are the differences with one of the species having a RCC below 1 indicating out-competition, according to Oberg, Young [51].

Both distance moved and velocity measured from larval behavior show more activity for *Aedes* compared to *Culex*. In contrast, *Culex* shows longer body contact compared to *Aedes* (Figure 2).

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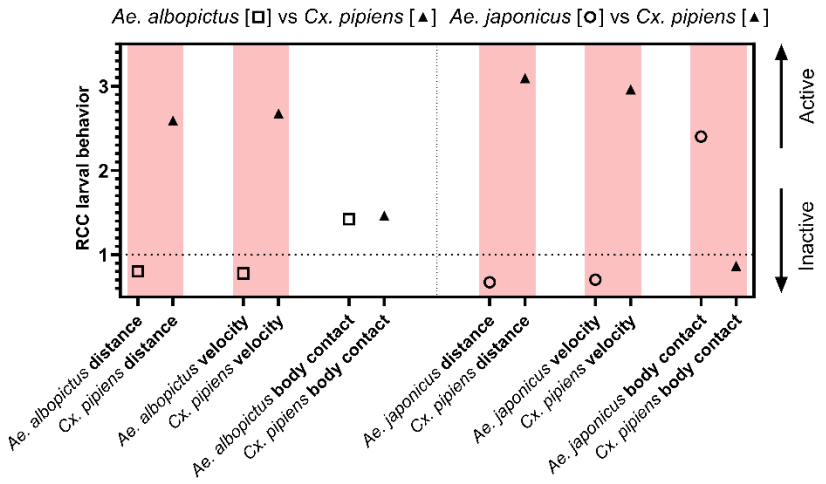

Figure 2. Relative Crowding Coefficient on activity or inactivity. Behavior variables total distance moved, velocity and duration of the body contact between intra- and interspecific larval competition ratios of *Ae. albopictus*, *Cx. pipiens* s.s./*Cx. torrentium*, *Ae. japonicus*, and *Cx. p. molestus*. In red are the differences with one of the species having a RCC below 1 indicating significant differences in behavior in a competitive environment.

3.2. Infection Study

Infection was successful for *Ae. albopictus* and *Cx. pipiens* s.s./*Cx. torrentium*, however, no infection was found for *Ae. japonicus*. A difference in infection rate and viral RNA titer was found in response to the competition treatment. Assumptions for normality were violated, therefore a Kruskal-Wallis test was performed on the viral RNA titer data and a Friedman test on infection rate.

For the *Ae. albopictus* vs *Cx. pipiens* s.s./*Cx. torrentium* combination, the former species showed an infection rate of 100 % with CHIKV. The mean number of CHIKV RNA copies per specimen of *Ae. albopictus* was 6.58 x 10^9, 2.89 x 10^{10}, and 1.97 x 10^{10} genome copies for the intraspecific combination, interspecific *Culex*:*Aedes* 10:20, and 20:10 combination respectively (Figure 3a). The difference in viral RNA copies per specimen per competitive treatment was significant (Kruskal-Wallis test, $p = 0.04$), Kruskal-Wallis multiple comparisons test showed a significant difference between *Culex*:*Aedes* 0:30 intraspecific and 10:20 interspecific ($p = 0.04$). The two other comparisons (*Culex*:*Aedes* 0:30 intraspecific vs 20:10, and 10:20 vs 20:10 interspecific) were not significantly different ($p = 0.55$ and $p = 0.69$, respectively). *Culex pipiens* s.s./*Cx. torrentium* from this combination had an infection rate of 54.8 %, 50 %, and 66.7 % for the intraspecific *Culex*:*Aedes* 30:0 combination, interspecific *Culex*:*Aedes* 20:10 and 10:20 combination respectively for WNV (Figure 3b). The mean number of WNV RNA copies per specimen of *Cx. pipiens* s.s./*Cx. torrentium* was 4.34 x 10^7, 1.43 x10^8, and 5.19 x 10^7 genome copies for the intraspecific *Culex*:*Aedes* 30:0 combination, interspecific *Culex*:*Aedes* 20:10 and 10:20 combination respectively (Figure 3c). Differences in viral RNA titer per competition combination were not significant (Kruskal-Wallis test, $p = 0.62$).

Table 1. Infection rates (IR), viral RNA copy number/body (mean log10 RNA copies/specimen), and statistical significance for *Ae. albopictus*, *Cx. pipiens* s.s./*Cx. torrentium*, and *Cx. p. molestus*.

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Virus	Mosquito Species	Species ratio	Dpi	n	IR [%]	Viral RNA Copy Number/Body [log10]	Significance
CHIKV	*Ae. albopictus*	30 *Aedes* intra	7	20	100	8.99 ± 1.58	0.04
		20 *Aedes* inter		23	100	9.36 ± 1.81	
		10 *Aedes* inter		14	100	9.41 ± 0.94	ns
WNV	*Cx. pipiens* s.s. / *Cx. torrentium*	30 *Culex* intra	14	42	54.76	5.39 ± 1.7	
		20 *Culex* inter		34	50	5.07 ± 1.59	ns
		10 *Culex* inter		9	66.67	5.69 ± 1.78	
	Cx. p. molestus	30 *Culex* intra	14	91	43.96	6.61 ± 2.1	
		20 *Culex* inter		64	54.69	6.34 ± 2.05	ns
		10 *Culex* inter		27	59.26	5.78 ± 1.8	

For the *Ae. japonicus* with *Cx. p. molestus* combination, no infection with JEV was found for the former species. *Culex p. molestus* had an infection rate of 43.9 %, 54.7 %, and 59.3 % for the intraspecific *Culex*:*Aedes* 30:0 combination, interspecific *Culex*:*Aedes* 20:10 and 10:20 combination for WNV respectively (Figure 3b). None of the infection rates were significantly different (Friedman test, p = 0.17). The mean number of WNV RNA copies per specimen was 3.90 x 10^8, 4.42 x 10^8, and 2.77 x 10^8 genome copies for the intraspecific *Culex*:*Aedes* 30:0 combination, interspecific *Culex*:*Aedes* 20:10 and 10:20 combination respectively (Figure 3d). Differences in the number of WNV RNA copies per specimen were not significant between mosquitoes which previously (non-)experienced larval competition (Kruskal-Wallis test, p = 0.43).

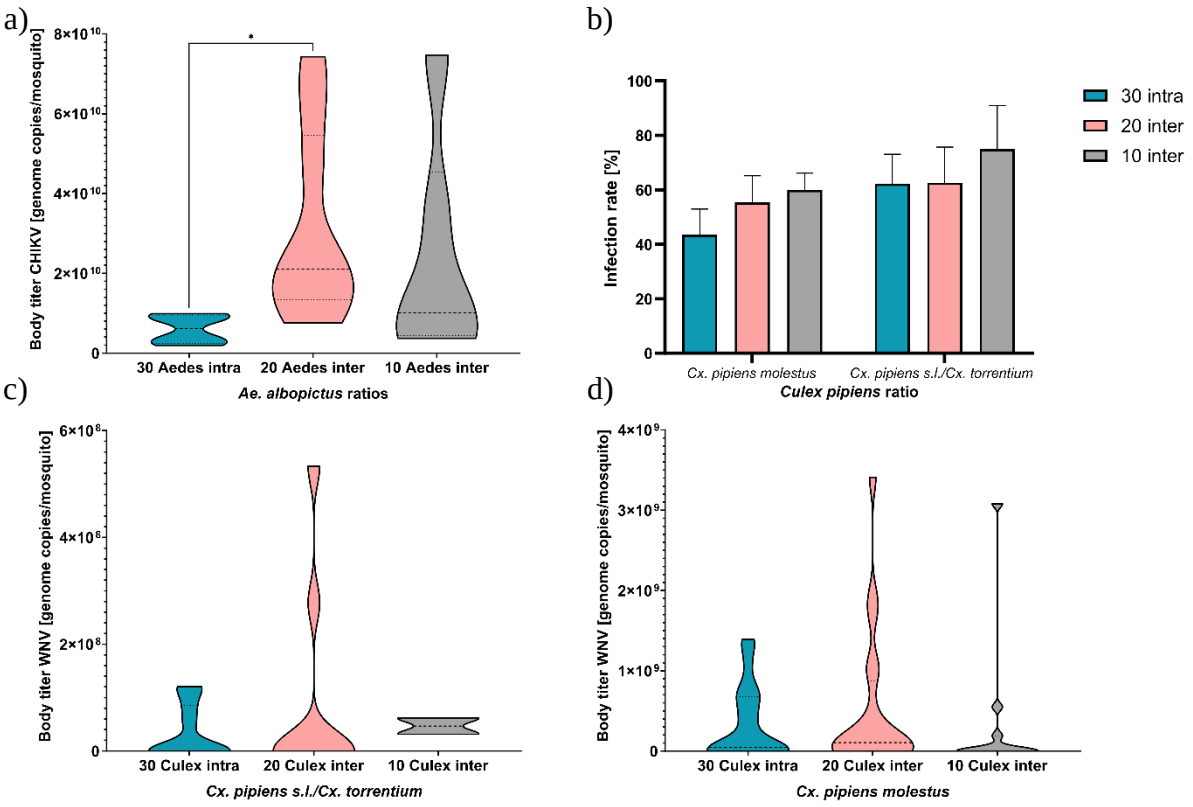

Figure 3. Mean viral RNA body titer per specimen and infection rate per species ratio. a) Mean CHIKV RNA body titer per specimen of *Ae. albopictus*, b) Infection rate of WNV of both *Cx. pipiens* strains, c) Mean WNV RNA body titer per specimen of *Cx. pipiens* s.s./*Cx. torrentium* (see Zenodo repository in data availability statement for separated results for bioforms) from *Ae. albopictus*

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combination, d) Mean WNV RNA body titer per specimen of *Cx. p. molestus* from *Ae. japonicus* combination. Infection rate was 100% and 0% for all *Ae. albopictus* and *Ae. japonicus* respectively, body titer was 0 genome copies per mosquito for *Ae. japonicus*.

For *Cx. p. pipiens*, *Cx. torrentium*, and *Cx. p. pipiens* x *molestus*, infection rate was 51.95 %, 71.43 %, and 100% respectively (Figure 4). Viral RNA titer was 8.1 x 10^7, 1.4 x 10^8, and 2.4 x 10^4 genome copies respectively. Infection rate was 50 %, 47.83 %, and 66.67 % for *Cx. p. molestus*, *Cx. p. pipiens* x *molestus*, and *Cx. p. pipiens* respectively (Figure 4), viral RNA titer was similar with 3.99, 3.85, and 3.95 x 10^8 genome copies.

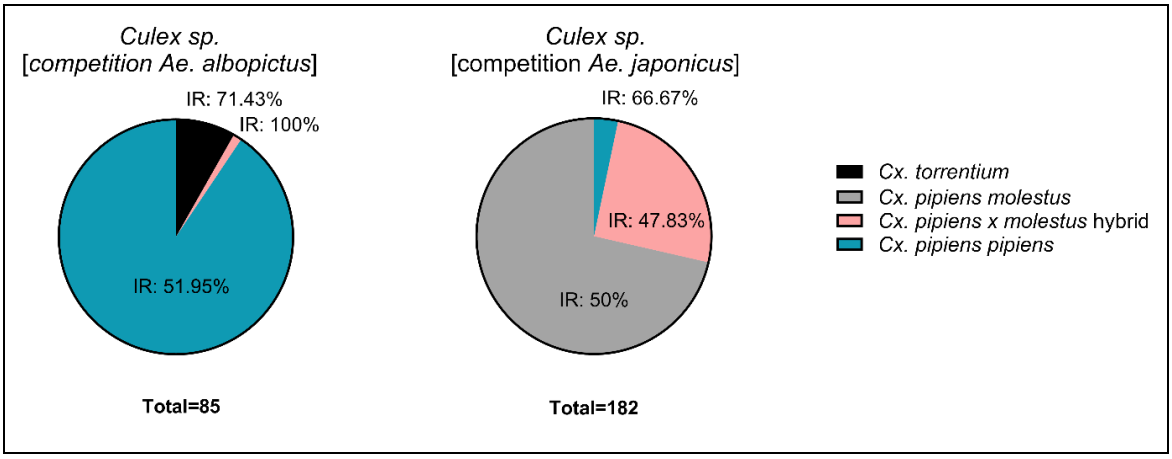

Figure 4. *Culex* species and *Cx. pipiens* biotypes used during infection study.

A Principal Component (PC) Analysis examined the 14 variables (Development, larval ratio, mortality, CL, AL, AW, L, P, G, distance, velocity, body contact, BT, IR) and reduced them to two PC axes, which explained about 54.71 ± 6.81 % of the variation in these variables (Figure 5, Table 1). Overall, pupal size (AL, AW, CL), energy reserves (L, G) and protein content are positively correlated. Total distance moved and velocity are also grouped together. For *Ae. albopictus*, the PCs explained a total of 64.68 % of the proportion of variance (Table 1). Larval ratio and development were correlated, and negatively linked with pupal size and protein content, therefore there is a link on the PC1 between interspecific larval ratio, shorter development, larger pupal size and more protein content. The PC2 showed a link between a high viral RNA titer and interspecific competition, low contents of energy storage, and less larval movement (Figure 5a, Table 1).

For the corresponding *Cx. pipiens* s.s./*Cx. torrentium* strain, the PCs explained a total of 46.74 % of the proportion of variance (Table 1). On PC1 the variables velocity, distance moved, protein content, body contact, mortality and development were correlated, meaning that lower values for these variables relate to higher viral RNA titer at interspecific competition. The velocity and distance moved was negatively correlated to cephalothorax size, larval ratio, viral RNA titer, and lipid content in PC2. This also indicates that less larval movement contributes to higher viral RNA titer, but also that larger pupae with more lipid content increase the viral RNA titer. Infection rate was negatively correlated with larval ratio, pupal size, energy reserves, protein content, mortality, and development time (Figure 5b, Table 1). For both *Ae. albopictus* and *Cx. pipiens* s.s./*Cx. torrentium* the viral RNA titer was higher in interspecific ratios, large pupae, and less movement. *Aedes albopictus* showed a higher viral RNA titer when there were less energy reserves, while *Cx. pipiens* s.s./*Cx. torrentium* had a higher viral RNA titer when more lipid was stored.

The PCs for *Ae. japonicus* together explained 56.93 % of the proportion of variance (Table 2). No infection with JEV was found, therefore viral RNA titer and infection rate were excluded. Size, energy and protein content were grouped together on PC1, and were negatively correlated to larval ratio and velocity. On PC2, body contact was negatively correlated with velocity, larval ratio, distance moved, and mortality. This means that interspecific ratios with lower mobility rates and mortality

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have a larger pupal size, more body contact, and more lipid, protein, and glycogen content (Figure 5c, Table 2).

In the corresponding *Cx. p. molestus* strain, 50.48 % of the proportion of variance is explained by both PCs (Table 2). On the PC1, the distance moved, velocity, cephalothorax area, and protein content were grouped and negatively correlated to larval ratio and development time. The PC2 indicated that viral RNA titer and velocity were negatively correlated to size and body contact. This indicates that within interspecific ratios, there was a fast development, larger pupae, more protein content, more velocity, and a higher viral RNA titer (Figure 5d).

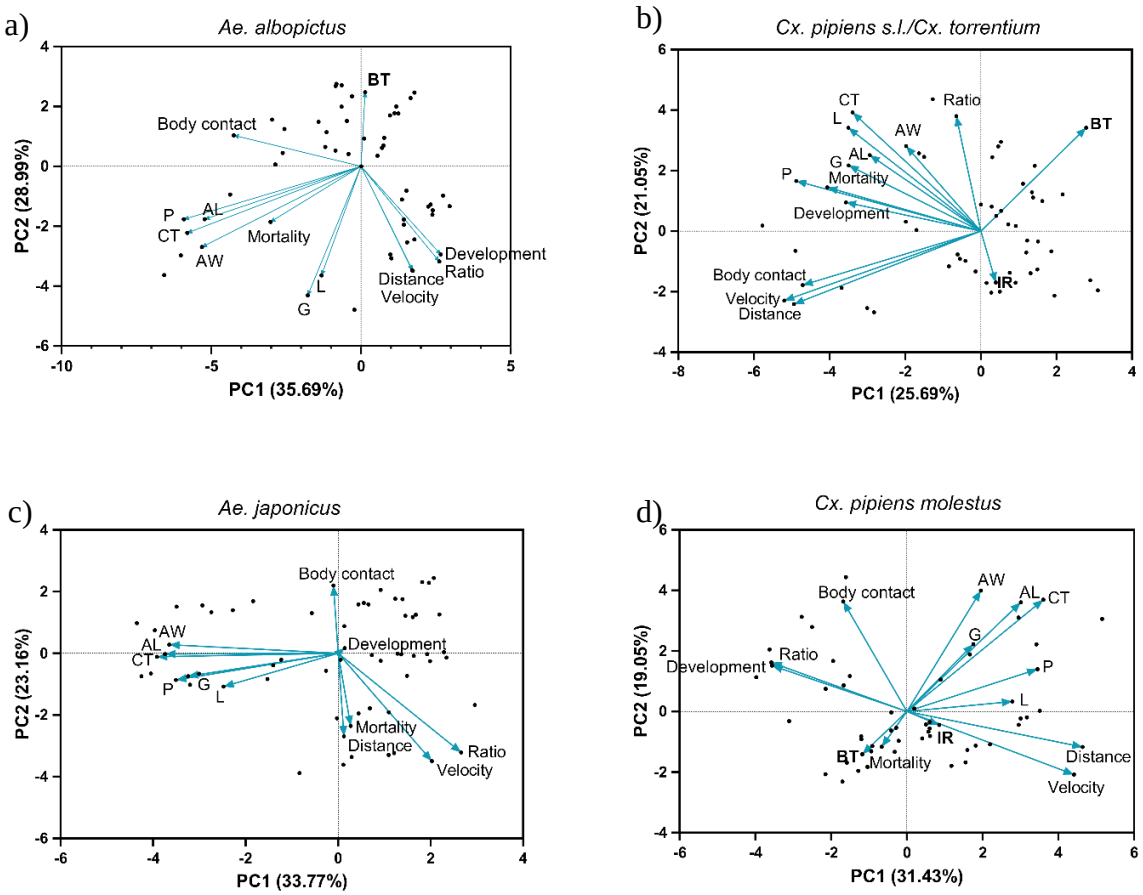

Figure 5: Principal Component Analysis (PCA) of total data set with variables. Development time, larval ratio, mortality, larval ratio, cephalothorax length (CL), abdominal length (AL), abdominal width (AW), lipid (L), protein (P), glycogen (G), mortality, total distance moved, velocity, body contact, viral RNA body titer (BT), and infection rate (IR) for different species competition treatments in a, b) *Ae. albopictus* and *Cx. pipiens* s.s./*Cx. torrentium* and c, d) *Ae. japonicus* and *Cx. p. molestus* combinations at 26°C. Infection rate is not included for *Ae. albopictus* and *Ae. japonicus*, viral RNA body titer is not included for *Ae. japonicus*.

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Table 2. Overview of the loadings per variable for each species and principal component (PC). The six highest variables (development time, larval ratio, mortality, larval ratio, cephalothorax length (CL), abdominal length (AL), abdominal width (AW), lipid (L), protein (P), glycogen (G), mortality, total distance moved, velocity, body contact, viral RNA body titer (BT), and infection rate (IR)) per species and principal component are included in this overview.

PC1

Ae. albopictus		***Cx. pipiens* s.s./*Cx. torrentium***		*Ae. japonicus*		*Cx. p. molestus*	
Development	**0.78**	**Development**	**-0.51**	L	-0.58	Distance	0.81
Ratio	0.77	Mortality	-0.58	G	-0.76	Velocity	0.77
AL	-0.71	Body contact	-0.67	P	-0.82	CT	0.63
AW	-0.73	P	-0.69	AW	-0.86	P	0.60
CT	-0.79	Distance	-0.70	AL	-0.88	Development	-0.83
P	-0.81	Velocity	-0.74	CT	-0.92	Ratio	-0.84

PC2

Ae. albopictus		***Cx. pipiens* s.s./*Cx. torrentium***		*Ae. japonicus*		*Cx. p. molestus*	
BT	**0.58**	**CT**	**0.57**	Body contact	0.61	AW	0.68
Ratio	-0.59	Ratio	0.55	L	-0.27	CT	0.63
Velocity	-0.65	BT	0.50	Mortality	-0.59	Body contact	0.62
Distance	-0.65	L	0.50	Distance	-0.68	AL	0.62
L	-0.68	Velocity	-0.63	Ratio	-0.81	BT	-0.38
G	-0.80	Distance	-0.66	Velocity	-0.88	Velocity	-0.56

4. Discussion

The present study confirmed that larval interactions between *Ae. albopictus* and *Cx. p. molestus*, and *Ae. japonicus* and *Cx. pipiens* s.s./*Cx. torrentium*, carry-over to the capability for arboviral infection in the adult stage, at least for *Ae. albopictus* and *Cx. pipiens*. Our findings indicate that all three species were significantly affected by interspecific competition, although out-competition become rarely evident at the level of 14 variables tested. Synecological pattern was in general most expressed at often neglected metabolic and behavioral levels, and specifically pronounces at low temperature in *Cx. pipiens*, at high temperature in *Ae. albopictus* and at both temperatures in *Ae. japonicus*.

4.1. Aedes Albopictus vs Culex pipiens s.s./Cx. Torrentium

Development time for *Ae. albopictus* and *Cx. pipiens* s.s./*Cx. torrentium* was similar at 20°C, however, at 26°C the thermophilic *Ae. albopictus* was faster, especially during interspecific competition, which is also found by Müller, Knautz [17]. Activity was higher in both *Aedes* species compared to *Cx. pipiens* s.s./*Cx. torrentium*. *Aedes* species are known to actively search for their food, browsing on a surface, while *Cx. pipiens* is a filter feeder hanging near the water surface [33,58,59]. This could imply that the foraging behavior of *Cx. pipiens* is well-adapted in scenarios with abundant food, in which case they conserve more energy than their competitor [17]. *Aedes albopictus* is known to be the strongest competitor in resource-limiting conditions due to their active search for food [16,23,25,27,28], however, in eutrophic conditions it might be less successful compared to *Cx. pipiens* [17,28,30]. In addition, the lipid and glycogen content of *Ae. albopictus* was positively affected by the presence of *Cx. pipiens* s.s./*Cx. torrentium* at 20°C, whereas at 26°C *Ae. albopictus* was negatively affected by its presence. During protein storage, *Ae. albopictus* was always positively affected by the presence of *Cx. pipiens* s.s./*Cx. torrentium* while the latter species remained unaffected.

4.2. Aedes Japonicus vs Culex Pipiens Bioform Molestus

At 20°C no influence of interspecific competition was found for larval development time, and at 26°C *Cx. p. molestus* developed faster in interspecific competition, outcompeting *Ae. japonicus*. This is

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in line with Andreadis and Wolfe [31] and Giunti, Becker [60], who observed that *Ae. japonicus* does not tolerate high temperatures and prefers colder habitats. *Culex p. molestus* remained unaffected by *Ae. japonicus* during lipid acquisition, moreover, it even benefitted from its presence during glycogen uptake, indicating having more energy reserves available in interspecific combinations. During protein uptake, *Aedes japonicus* was not affected by the presence of *Cx. p. molestus*, but the latter species benefitted from interspecific competition. This observation is in line with Andreadis and Wolfe [31], and Hardstone and Andreadis [32] who predicted that *Ae. japonicus* would not outcompete *Cx. pipiens*. It is considered a weak larval competitor compared to *Ae. albopictus* [24,33].

4.3. Effect of Interspecific Competition On Viral Infection

This study indicates that interspecific larval competition may enhance at least the infection rates and body titers of *Ae. albopictus* and *Cx. pipiens*. Our experiments demonstrate that interspecific competition results in larger mosquitoes, a higher arboviral infection rate, and increased arboviral RNA titer. Large mosquitoes benefit of an improved longevity, blood feeding, and vector capacity (Ratnayake et al. 2023). These are crucial factors to complete the extrinsic incubation period in order to transmit a virus [21,36]. Additionally, Alto, Lounibos [19] and Bevins [21] observed that these stressed, larger females attain higher viral titers for SINDV and were more likely to disseminate LACV and develop midgut infection. Conversely, smaller and stressed females may have a weaker immune response (Grimstad and Walker 1991; Telang et al. 2012; Paige et al. 2019), potentially enhancing their vector competence. However, their shorter longevity raises uncertainty about whether they will survive long enough to complete the extrinsic incubation period [21,36]. Interspecific larval competition might therefore have an impact of expression of important factors of the immune system, influencing vector competence.

The Relative Crowding Coefficient (RCC) was calculated to use as a measure of competition. A RCC value of 1 indicates that both species are equal competitors, while a value below or greater than 1 indicates out-competition. The calculated RCC revealed that *Ae. albopictus* was subject to competition, which was particularly evident in its reduced lipid intake. This finding suggests a potential increase in sensitivity to viral infection, since lipids are involved in the regulation of the immune response [35,39,40].

The influence of the reduced lipid intake in *Ae. albopictus* was evidenced by the negative correlation with the viral RNA titer of *Ae. albopictus* in the PCA. Additionally, infection rate and viral RNA titer were positively correlated with interspecific competition. Similarly, infection rate in both *Cx. pipiens* strains were higher during interspecific competition. Consistent with previous research by Bevins [21], females subjected to intraspecific treatments were found to be less susceptible to develop LACV infections. Those findings suggest that competitive larval interactions may not directly influence DENV replication, but may contribute to a reduction in barriers that impede viral transmission [20].

In this study, we observed that interspecific competition among larvae resulted in larger pupae with elevated infection rates and increased virus-RNA titers in the female adult stage. However, interspecifically challenged *Ae. albopictus* had less energy storage and a higher viral RNA titer, while *Cx. pipiens* had a higher viral RNA titer when more lipid was stored. This might be explained by the fact that lipids are essential for flaviviruses in order to infect cells, replicate, and spread throughout the body. They facilitate its release from infected cells into new ones. This interaction between lipids and DENV in *Ae. albopictus* and *Ae. aegypti* has been well studied by Perera, Riley [61], Chotiwan, Andre [62], and Koh, Islam [63].

4.4. Implications on Bigger Scale

This study demonstrates a combination of the factors mentioned above: after interspecific competition mosquitoes were larger, had a higher infection rate, as well as a higher virus-RNA titer. This indicates that interspecific larval competition may enhance the vector competence of *Ae. albopictus* and *Cx. pipiens*. Large mosquitoes benefit of an improved longevity, blood feeding, and vector capacity [39]. These are crucial factors to complete the extrinsic incubation period in order to

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transmit a virus [21,36]. Additionally, these larger females attain higher viral titers [19,21]. Conversely, smaller and stressed females may have a weaker immune response [35,41,64], potentially enhancing their vector competence. However, their shorter longevity raises uncertainty about whether they will survive long enough to complete the extrinsic incubation period [21,36].

4.5. Limitations of the study

No virus transmission rate has been investigated in this study due to the large number of experimental treatment and species tested. However, the relevance of carry-over effects from larval to adult stage for the vector competence of mosquitoes merits further research. Additionally, the patterns found in laboratory environments may be different than the ones in the field [21]. Additional factors shaping larval microhabitats such as temperature, food source, water quality, larval density, species composition, physical characteristics, as well as, behavioral and immune responses of resulting adult mosquitoes of the tested strains, and finally, microbiome and viral doses, need further to be considered. A very promising avenue of research is the effect of qualitative and quantitative lipid accumulation during larval stage on arboviral vector competence to better understand the causative links in semi-aquatic environments. We observed an opposite pattern between *Ae. albopictus* and *Cx. pipiens* related to their lipid storage and infection rate. Additionally, we found that interspecific combinations develop faster and had larger pupae. This paradoxical observation requires further research.

5. Conclusions

This study provides an in-depth insight into the larval competition between *Ae. albopictus*, *Ae. japonicus*, and *Cx. pipiens*, and the associated carry-over effect of synecological patterns to a higher arboviral infection during their adult stage. During interspecific competition, all species developed faster, had larger pupae and more protein storage. The competition between *Ae. albopictus* and *Cx. pipiens* varied, but indicated that *Ae. albopictus* is the better competitor in resource limited habitats while *Cx. pipiens* thrives in eutrophic situations. *Aedes japonicus* was always a weaker competitor compared to *Cx. pipiens*. *Aedes albopictus* and *Cx. pipiens* were more susceptible to arboviral infection after interspecific competition and storage of lipids was lower in *Ae. albopictus* while it was higher in *Cx. pipiens*. This could suggest that lipids are involved in both the regulation of the immune response as in virus infection in the mosquito. No infection with JEV nor natural arbovirus infections were found in *Ae. japonicus*.

Author Contributions: AV: Conceptualization, Funding acquisition, Data Curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft. SJ: Conceptualization: Resources, Supervision, Validation, Writing – review and editing. JDW: Resources: Writing – review and editing. CJ: Resources, Writing – review and editing. SV: Resources, Writing – review and editing. MH: Investigation, Writing – review and editing. UL: Investigation, Writing – review and editing. RL: Conceptualization, Funding acquisition, Validation, Writing – review and editing. JSC: Conceptualization, Funding acquisition, Writing – review and editing. AH: Conceptualization, Funding acquisition, Supervision, Validation, Writing – review and editing. RM: Conceptualization, Funding acquisition, Methodology, Visualization, Project administration, Supervision, Validation, Writing – review and editing.

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Data availability statement: Data supporting the conclusions of this article are included within the article. The datasets generated and analyzed during the current study are available in the Zenodo repository, DOI number.

Ethics approval and consent to participate: Not applicable.

Consent for publications: Not applicable.

Competing interests: The authors declare that they have no competing interests.

Abbreviations: AL: Abdominal length; ANOVA: analysis of variance; AW: Abdominal width; BNITM: Bernhard Nocht Institute for Tropical Medicine; BT: body titer; CHIKV: Chikungunya virus; CT: cephalothorax area; DENV: Dengue virus; G: glycogen; IR: infection rate; ITM : Institute of Tropical Medicine; JEV: Japanese Encephalitis virus ; L : lipid ; LACV: La Crosse encephalitis virus ; P : protein ; PCA: principal component analysis; WNV: West Nile virus.

References

1. Perrin A, Glaizot O, Christe P. Worldwide impacts of landscape anthropization on mosquito abundance and diversity: A meta-analysis. *Global Change Biol*. **2022**;*28*(23):6857-71.
2. Bartholomeeusen K, Daniel M, LaBeaud DA, Gasque P, Peeling RW, Stephenson KE, et al. Chikungunya fever. *Nat Rev Dis Primers*. **2023**;*9*(1):17.
3. Autochthonous vectorial transmission of dengue virus in mainland EU/EEA, 2010-present [Internet]. ECDC. 2024a [cited 17/06/2024].
4. Laverdeur J, Desmecht D, Hayette MP, Darcis G. Dengue and chikungunya: future threats for Northern Europe? *Front Epidemiol*. **2024**;*4*:1342723.
5. Autochthonous transmission of chikungunya virus in mainland EU/EEA, 2007–present [Internet]. ECDC. 2024b [cited 17/06/2024].
6. Historical data by year - West Nile virus seasonal surveillance [Internet]. ECDC. 2024c [cited 18/06/2024]. Available from: https://www.ecdc.europa.eu/en/west-nile-fever/surveillance-and-disease-data/historical.
7. Ryan SJ, Carlson CJ, Mordecai EA, Johnson LR. Global expansion and redistribution of Aedes-borne virus transmission risk with climate change. *PLoS Negl Trop Dis*. **2019**;*13*(3):e0007213.
8. Deblauwe I, De Wolf K, De Witte J, Schneider A, Verle I, Vanslembrouck A, et al. From a long-distance threat to the invasion front: a review of the invasive Aedes mosquito species in Belgium between 2007 and 2020. *Parasit Vectors*. **2022**;*15*(1):206.
9. Eritja R, Ruiz-Arrondo I, Delacour-Estrella S, Schaffner F, Álvarez-Chachero J, Bengoa M, et al. First detection of Aedes japonicus in Spain: an unexpected finding triggered by citizen science. *Parasites & Vectors*. **2019**;*12*(1):53.
10. Sherpa S, Blum MGB, Capblancq T, Cumer T, Rioux D, Despres L. Unravelling the invasion history of the Asian tiger mosquito in Europe. *Mol Ecol*. **2019**;*28*(9):2360-77.
11. Kraemer MUG, Reiner RC, Brady OJ, Messina JP, Gilbert M, Pigott DM, et al. Past and future spread of the arbovirus vectors Aedes aegypti and Aedes albopictus. *Nature Microbiology*. **2019**;*4*(5):854-63.
12. Worsening spread of mosquito-borne disease outbreaks in EU/EEA, according to latest ECDC figures [press release]. European Center for Disease Prevention and Control2024d.
13. Soto A, De Coninck L, Devlies AS, Van De Wiele C, Rosales Rosas AL, Wang L, et al. Belgian Culex pipiens pipiens are competent vectors for West Nile virus while Culex modestus are competent vectors for Usutu virus. *PLoS Negl Trop Dis*. **2023**;*17*(9):e0011649.
14. Haba Y, McBride L. Origin and status of Culex pipiens mosquito ecotypes. *Curr Biol*. **2022**;*32*(5):R237-R46.
15. Versteirt V, Schaffner F, Garros C, Dekoninck W, Coosemans M, Van Bortel W. Introduction and establishment of the exotic mosquito species Aedes japonicus japonicus (Diptera: Culicidae) in Belgium. *J Med Entomol*. **2009**;*46*(6):1464-7.
16. Carrieri M, Bacchi M, Bellini R, Maini S. On the competition occuring between Aedes albopictus and Culex pipiens (Diptera: Culicidae) in Italy. *Entomological Society of America*. **2003**;*32*(6):8.
17. Müller R, Knautz T, Vollroth S, Berger R, Kress A, Reuss F, et al. Larval superiority of Culex pipiens to Aedes albopictus in a replacement series experiment: prospects for coexistence in Germany. *Parasit Vectors*. **2018**;*11*(1):80.
18. Seidel B, Montarsi F, Huemer HP, Indra A, Capelli G, Allerberger F, et al. First record of the Asian bush mosquito, Aedes japonicus japonicus, in Italy: invasion from an established Austrian population. *Parasit Vectors*. **2016**;*9*(1):284.

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19. Alto BW, Lounibos LP, Higgs S, Juliano SA. Larval Competition Differentially Affects Arbovirus Infection in Aedes Mosquitoes. *Ecology*. **2005**;*86*(12):3279-88.
20. Alto BW, Lounibos LP, Mores CN, Reiskind MH. Larval competition alters susceptibility of adult Aedes mosquitoes to dengue infection. *Proc Biol Sci*. **2008**;*275*(1633):463-71.
21. Bevins SN. Invasive mosquitoes, larval competition, and indirect effects on the vector competence of native mosquito species (Diptera: Culicidae). *Biol Invasions*. **2007**;*10*:1109-17.
22. Braks MAH, Honório NA, Lounibos LP, Lourenço-De-Oliveira R, Juliano SA. Interspecific Competition Between Two Invasive Species of Container Mosquitoes, Aedes aegypti and Aedes albopictus (Diptera: Culicidae), in Brazil. *Annals of the Entomological Society of America*. **2004**;*97*(1):130-9.
23. Costanzo KS, Mormann KM, Juliano SA. Asymmetrical Competition and Patterns of Abundance of Aedes albopictus and Culex pipiens (Diptera: Culicidae). *Journal of Medical Entomology*. **2005**;*42*:20.
24. Armistead JS, Arias JR, Nishimura N, Lounibos LP. Interspecific larval competition between Aedes albopictus and Aedes japonicus (Diptera: Culicidae) in northern Virginia. *J Med Entomol*. **2008**;*45*.
25. Costanzo KS, Muturi EJ, Lampman RL, Alto BW. The effects of resource type and ratio on competition with Aedes albopictus and Culex pipiens (Diptera:Culicidae). *J Med Entomol*. **2011**;*48*(1):29-38.
26. Bonizzoni M, Gasperi G, Chen X, James AA. The invasive mosquito species Aedes albopictus: current knowledge and future perspectives. *Trends Parasitol*. **2013**;*29*(9):460-8.
27. Marini G, Guzzetta G, Baldacchino F, Arnoldi D, Montarsi F, Capelli G, et al. The effect of interspecific competition on the temporal dynamics of Aedes albopictus and Culex pipiens. *Parasit Vectors*. **2017**;*10*(1):102.
28. Leisnham PT, LaDeau SL, Saunders MEM, Villena OC. Condition-Specific Competitive Effects of the Invasive Mosquito Aedes albopictus on the Resident Culex pipiens among Different Urban Container Habitats May Explain Their Coexistence in the Field. *Insects*. **2021**;*12*(11).
29. Giatropoulos A, Papachristos D, Michaelakis A, Kapranas A, Emmanouel N. Laboratory study on larval competition between two related mosquito species: Aedes (Stegomyia) albopictus and Aedes (Stegomyia) cretinus. *Acta Trop*. **2022**;*230*:106389.
30. Rau J, Fischer S, Werner D, Kampen H. Impact of larvae of the Asian tiger mosquito Aedes albopictus on larvae of the Culex pipiens complex from Germany in laboratory co-breeding studies. *Med Vet Entomol*. **2023**;*37*(3):523-33.
31. Andreadis TG, Wolfe RJ. Evidence for reduction of native mosquitoes with increased expansion of invasive Ochlerotatus japonicus japonicus (Diptera: Culicidae) in the northeastern United States. *J Med Entomol*. **2010**;*47*(1):43-52.
32. Hardstone MC, Andreadis TG. Weak larval competition between the invasive mosquito Aedes japonicus japonicus (Diptera: Culicidae) and three resident container-inhabiting mosquitoes in the laboratory. *J Med Entomol*. **2012**;*49*(2):277-85.
33. O'Donnell DL, Armbruster P. Comparison of larval foraging behavior of Aedes albopictus and Aedes japonicus (Diptera: Culicidae). *J Med Entomol*. **2007**;*44*(6):984-9.
34. Reiskind MH, Lounibos LP. Effects of intraspecific larval competition on adult longevity in the mosquitoes Aedes aegypti and Aedes albopictus. *Med Vet Entomol*. **2009**;*23*(1):62-8.
35. Telang A, Qayum AA, Parker A, Sacchetta BR, Byrnes GR. Larval nutritional stress affects vector immune traits in adult yellow fever mosquito Aedes aegypti (Stegomyia aegypti). *Med Vet Entomol*. **2012**;*26*(3):271-81.
36. Bara J, Rapti Z, Caceres CE, Muturi EJ. Effect of Larval Competition on Extrinsic Incubation Period and Vectorial Capacity of Aedes albopictus for Dengue Virus. *PLoS One*. **2015**;*10*(5):e0126703.
37. Muturi EJ, Kim CH, Alto BW, Berenbaum MR, Schuler MA. Larval environmental stress alters Aedes aegypti competence for Sindbis virus. *Trop Med Int Health*. **2011**;*16*(8):955-64.
38. Takken W, Klowden MJ, Chambers GM. Effect of body size on host seeking and blood meal utilization in Anopheles gambiae sensu stricto (Diptera: Culicidae): the disadvantage of being small. *J Med Entomol*. **1998**;*35*(5):639-45.
39. Ratnayake OC, Chotiwan N, Saavedra-Rodriguez K, Perera R. The buzz in the field: the interaction between viruses, mosquitoes, and metabolism. *Front Cell Infect Microbiol*. **2023**;*13*:1128577.
40. Arrese EL, Soulages JL. Insect fat body: energy, metabolism, and regulation. *Annu Rev Entomol*. **2010**;*55*:207-25.
41. Grimstad PR, Walker ED. Aedes triseriatus (Diptera: Culicidae) and La Crosse virus. IV. Nutritional deprivation of larvae affects the adult barriers to infection and transmission. *J Med Entomol*. **1991**;*28*(3):378-86.
42. Hauser G, Thievent K, Koella JC. Consequences of larval competition and exposure to permethrin for the development of the rodent malaria Plasmodium berghei in the mosquito Anopheles gambiae. *Parasit Vectors*. **2020**;*13*(1):107.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 26 June 2024 doi:10.20944/preprints202406.1878.v1

43. Rudolf M, Czajka C, Borstler J, Melaun C, Jost H, von Thien H, et al. First nationwide surveillance of Culex pipiens complex and Culex torrentium mosquitoes demonstrated the presence of Culex pipiens biotype pipiens/molestus hybrids in Germany. *PLoS One*. **2013**;*8*(9):e71832.
44. Vereecken S, Vanslembrouck A, Kramer IM, Muller R. Phenotypic insecticide resistance status of the Culex pipiens complex: a European perspective. *Parasit Vectors*. **2022**;*15*(1):423.
45. Müller R, Knautz T, Volker J, Kress A, Kuch U, Oehlmann J. Appropriate larval food quality and quantity for Aedes albopictus (Diptera: Culicidae). *J Med Entomol*. **2013**;*50*(3):668-73.
46. Van Handel E. Rapid determination of glycogen and sugars in mosquitoes. *J Am Mosq Control Assoc*. **1985a**;*1*(3):299-301.
47. Van Handel E. Rapid determination of total lipids in mosquitoes. *J Am Mosq Control Assoc*. **1985b**;*1*(3):302-4.
48. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. **1976**;*72*:248-54.
49. Bock F, Kuch U, Pfenninger M, Muller R. Standardized Laboratory Feeding of Larval Aedes japonicus japonicus (Diptera: Culicidae). *J Insect Sci*. **2015**;*15*(1).
50. Novak MG, Higley LG, Christianssen CA, Rowley WA. Evaluating Larval Competition between Aedes-Albopictus and a-Triseriatus (Diptera, Culicidae) through Replacement Series Experiments. *Environ Entomol*. **1993**;*22*(2):311-8.
51. Oberg AL, Young LJ, Higley LG. A study of the statistical properties of two measures of competition. Applied statistics in agriculture1994.
52. Harper JL. Population biology of plants.: New York: Academic Press; 1977.
53. Heitmann A, Jansen S, Luhken R, Leggewie M, Schmidt-Chanasit J, Tannich E. Forced Salivation As a Method to Analyze Vector Competence of Mosquitoes. *J Vis Exp*. **2018**(138).
54. Huber K, Jansen S, Leggewie M, Badusche M, Schmidt-Chanasit J, Becker N, et al. Aedes japonicus japonicus (Diptera: Culicidae) from Germany have vector competence for Japan encephalitis virus but are refractory to infection with West Nile virus. *Parasitol Res*. **2014**;*113*(9):3195-9.
55. Lambert AJ, Lanciotti RS. Consensus amplification and novel multiplex sequencing method for S segment species identification of 47 viruses of the Orthobunyavirus, Phlebovirus, and Nairovirus genera of the family Bunyaviridae. *J Clin Microbiol*. **2009**;*47*(8):2398-404.
56. Chao DY, Davis BS, Chang GJ. Development of multiplex real-time reverse transcriptase PCR assays for detecting eight medically important flaviviruses in mosquitoes. *J Clin Microbiol*. **2007**;*45*(2):584-9.
57. Eshoo MW, Whitehouse CA, Zoll ST, Massire C, Pennella TT, Blyn LB, et al. Direct broad-range detection of alphaviruses in mosquito extracts. *Virology*. **2007**;*368*(2):286-95.
58. Yee DA, Kesavaraju B, Juliano SA. Larval feeding behavior of three co-occurring species of container mosquitoes. *J Vector Ecol*. **2004**;*29*(2):315-22.
59. Vanslembrouck A, Scheers K, Vermeersch X, Hendrickx R, Schneider A, De Witte J, et al. Exploring the efficacy of predaceous diving beetles as potential nature-based solution for combating the invasive mosquito Aedes albopictus. *Neobiota*. **Under review**.
60. Giunti G, Becker N, Benelli G. Invasive mosquito vectors in Europe: From bioecology to surveillance and management. *Acta Trop*. **2023**;*239*:106832.
61. Perera R, Riley C, Isaac G, Hopf-Jannasch AS, Moore RJ, Weitz KW, et al. Dengue virus infection perturbs lipid homeostasis in infected mosquito cells. *PLoS Pathog*. **2012**;*8*(3):e1002584.
62. Chotiwan N, Andre BG, Sanchez-Vargas I, Islam MN, Grabowski JM, Hopf-Jannasch A, et al. Dynamic remodeling of lipids coincides with dengue virus replication in the midgut of Aedes aegypti mosquitoes. *PLoS Pathog*. **2018**;*14*(2):e1006853.
63. Koh C, Islam MN, Ye YH, Chotiwan N, Graham B, Belisle JT, et al. Dengue virus dominates lipid metabolism modulations in Wolbachia-coinfected Aedes aegypti. *Commun Biol*. **2020**;*3*(1):518.
64. Paige AS, Bellamy SK, Alto BW, Dean CL, Yee DA. Linking nutrient stoichiometry to Zika virus transmission in a mosquito. *Oecologia*. **2019**;*191*(1):1-10.

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