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No impact of biocontrol predator on development time or size of surviving *Aedes albopictus* under optimal nutritional availability

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Graphical abstract

Abstract

Background: Cyclopoid copepods have been applied successfully to limit populations of highly invasive *Aedes albopictus* mosquitoes that can vector diseases, including chikungunya, dengue, yellow fever, and Zika, to humans. However, there is concern that changes in certain vector traits, induced by exposure to copepod predation, might increase the risk of disease

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transmission. In this study, we tested whether cyclopoid copepod predation has sublethal effects on the development time or adult size of *Ae. albopictus* under the scenario of an invasion in southeast England, which lies at the northern edge of the vector's expanding global range.

Methods: Third instar *Ae. albopictus* larvae, hereafter referred to as "focal individuals", were placed in individual Petri dishes, each containing four newly-hatched *Ae. albopictus* larvae, which were counted, removed, and replaced daily. All focal individuals were provided with fish food *ad libitum*, and half were exposed to *Megacyclops viridis* copepod predators. The day of pupation was recorded for each focal individual, and the wing length of each focal adult was also measured.

Results: Exposing late instar *Ae. albopictus* to predation decreased their chances of surviving to adulthood. Mortality of the focal individuals was 12.9% in the predator treatment, and 2.9% in the controls. Three focal larvae that died in the predator treatment showed signs of melanization, indicative of wounding. Among surviving *Ae. albopictus*, no significant difference in either pupation day or wing length was observed due to copepod predation.

Conclusions: We found that *M. viridis* predation on smaller *Ae. albopictus* larvae does not significantly affect the pupation day or adult size of surrounding larger larvae that are less vulnerable to copepod attacks. This study controlled for density effects on size by maintaining a constant number of newly-hatched prey larvae surrounding each focal larva. Those working to control *Ae. albopictus* populations in the field should be made aware that increased adult body size can occur if copepod biocontrol agents are applied at lower than necessary levels. The absence of a significant sublethal impact from *M. viridis* copepod predation on surviving later-stage larvae in this analysis supports the use of *M. viridis* as a biocontrol agent.

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Keywords

predator-prey interactions, sublethal effects, vector traits

Background

Aedes albopictus is an important vector of dengue, chikungunya, yellow fever, and Zika [1]. This species is highly invasive due, in part, to its ability to lay desiccation-resistant eggs that can be transported across long distances, often by the shipment of used tires [2-6]. The northern limits of the global range of *Ae. albopictus* include North American populations in New York and Connecticut, USA [7], as well as European populations throughout Italy and France [8].

Several different methods have been proposed for controlling aedine mosquitoes in Europe, including chemical, genetic, and biological techniques [9]. Although space spraying with pyrethroid insecticide has been found to be effective against *Ae. albopictus* in Catalonia, this control strategy would require regular monitoring for insecticide resistance [10]. The Sterile Insect Technique (SIT) has previously been tested on *Ae. albopictus* mosquitoes from Italy, but it was not found to significantly reduce their population due to the reduced mating competitiveness of irradiated males [11]. Cyclopoid copepods have been used successfully as biocontrol agents against mosquito larvae in the US [12], Australia [13], Vietnam [14], and Italy [15]. Copepods are an especially convenient type of biocontrol because they are small enough to be distributed through a backpack sprayer [16]. When local predators are used to limit populations of a disease vector, there is a risk that these predation interactions could have unintended effects on the ability of surviving prey to reproduce and transmit disease.

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Copepod predators are much more effective at killing first and second instar than third and fourth instar *Ae. albopictus* [17]. Thus, after an application of copepod predators to *Ae. albopictus* larval habitats, it is likely that any immatures that were third and fourth instar larvae at the time of application would still emerge as adults. These individuals may have withstood both unsuccessful copepod attacks and exposure to the deaths of surrounding smaller conspecifics. Such indirect interactions between predator and prey species can strongly impact prey traits [18, 19].

In aquatic ecosystems, sublethal effects are governed by olfactory cues, including predator kairomones and chemical alarm cues from the prey [20]. For example, *Culex pipiens* larvae significantly reduce their movement when exposed to conspecifics that have been killed by notonectid predators [21]. In *Cx. restuans*, both freezing (hanging at the water's surface or drifting in the water column) and fleeing (moving for nearly a full minute without resting) behaviors were observed as responses to conspecific alarm cues [22]. *Cx. restuans* larvae have also learned to recognize a salamander predator's odor as a threat, after experiencing the predator's odor paired with conspecific alarm cues [23]. When *Ae. triseriatus* larvae were exposed to chemical cues of *Toxorhynchites rutilus* predation until adult emergence, females exhibited significantly shorter development times under high nutrient availability [24].

Adult body size is an important vector trait that can be affected by predation. For example, exposure to a backswimmer predator, *Anisops jaczewskii,* was found to reduce the size of *Anopheles coluzzi*, one of the main vectors of the human malaria parasite *Plasmodium falciparum* in Africa [25]. Similarly, larval exposure to chemical cues from the predatory fish *Hypseleotris galii* reduces adult size of *Ae. notoscriptus*, the main vector of canine heartworm, *Dirofilaria immitis* in Australia [26]. However, in comparison to how other

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mosquito species respond to predators, studies on *Ae. albopictus* indicate this species may be less sensitive to predator cues [27, 28].

As an invader, *Ae. albopictus* is less likely to share an evolutionary history with the predators that it encounters. A lack of a shared evolutionary history between predator and prey species has been shown to weaken non-consumptive predator effects in arthropods [29]. Previous work has shown that second instar *Ae. albopictus* reduce their movement in response to cues from the act of predation by *C. appendiculata*, which likely include dead conspecifics and predator feces, but not in response to cues from a non-feeding predator [30]. However, larger fourth instar *Ae. albopictus* are less vulnerable to predation and show no behavioral response to cues from *C. appendiculata*, even to cues from the act of predation [31].

If copepods are to be used as biocontrol agents, it is important to understand how predation may alter the adult traits of larvae that are too large to be consumed at the time of application. The body size of aedine mosquitoes can be altered by predation cues [26, 32], and changes in size can have important consequences for population dynamics and disease transmission. In *Ae. albopictus*, larger adults have greater reproductive success [33, 34]. Larger males have been shown to produce more sperm [34], and in larger females, more spermathecae have been observed to contain sperm [35]. Larger female body size consistently correlates with higher fecundity, when measured as the number of mature follicles in the ovaries [36], or as the number of eggs laid [33]. Thus, larger emerging *Ae. albopictus* could lead to increased mosquito abundance.

In addition, larger *Ae. albopictus* adults have displayed significantly longer median lifespans, when controlling for differences due to sex and diet [37]. However, smaller *Ae. albopictus*

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females are more likely to become infected with dengue and to disseminate the virus than larger females [38]. Furthermore, small females have been shown to be more likely than large females to take multiple bloodmeals within a single gonotrophic cycle [39], and the lifetime cumulative number of bloodmeals is also higher among smaller females [40]. This higher contact frequency with hosts increases the risk for disease transmission by small-sized *Ae. albopictus* vectors.

Previous studies have shown an increase in *Ae. albopictus* adult size after exposure to predation, but these studies were not designed to control for the greater per capita nutrition or decreased intraspecific competition that often occur when the population density has been significantly lowered due to successful predation [27, 41-43]. The phenomenon of increasing animal body size with decreasing population density has been documented across taxa [44]. This study is designed to test for sublethal effects of copepod predation on the development and adult size of *Ae. albopictus* that have been exposed to the act of predation during the later larval stages, while controlling for density effects. Due to the suitability of some areas in southeast England for *Ae. albopictus* populations [45], we used *Megacyclops viridis*, a likely copepod species for future biocontrol applications [46], collected from Longside Lake in Egham, UK, and *Ae. albopictus* that were originally collected in Montpellier, France. Our results show that while cyclopoid copepod predation by *M. viridis* significantly increases mortality of late-instar *Ae. albopictus*, the related predation cues do not significantly change development time or adult size of those late-instar *Ae. albopictus* that survive, assuming optimal nutritional availability.

Methods

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Local copepod collections:

One hundred thirty adult female *M. viridis* copepods were collected during the third week of September in 2019 from the edge of Longside Lake in Egham, Surrey, UK (N 51° 24.298', W 00° 32.599'). Copepods were identified as *M. viridis* (Jurine, 1820) by morphology. The copepods were kept in ten 1 L containers, each holding approximately 500 mL of spring water (Highland Spring, UK) at a 12:12 light/dark cycle, and 20 ± 1°C. *Paramecium caudatum* were provided *ad libitum* as food for the copepods, and boiled wheat seeds were added to the containers to provide a food source for the ciliates [47].

Temperate *Ae. albopictus* colony care:

A colony of *Ae. albopictus* mosquitoes (original collection Montpellier, France 2016 obtained through Infravec2) was maintained at 27 ± 1°C, 70% relative humidity, and a 12:12 light/dark cycle. Larvae were fed fish food (Cichlid Gold Hikari®, Japan), and adults were given 10% sucrose solution and horse blood administered through a membrane feeding system (Hemotek®, Blackburn, UK). *Ae. albopictus* eggs were collected from the colony on filter papers and stored in plastic bags containing damp paper towels to maintain humidity.

Experimental procedure:

On the morning of the tenth day after the last *M. viridis* were collected from the field, *Ae. albopictus* larvae were hatched over a 3-hour period at 27 ± 1°C. The hatching temperature was kept high, relative to the temperature of the experiment (20 ± 1°C), to maximize the yield of larvae over a semi-synchronous period. Stored egg papers were submerged in 3 mg/L nutrient broth solution (Sigma-Aldrich © 70122 Nutrient Broth No 1) and oxygen was displaced by vacuum suction for 30 min. Immediately following oxygen displacement, ground fish food (Cichlid Gold Hikari®, Japan) was added *ad libitum*. After three hours, 500

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larvae were counted and placed into spring water to dilute residual food from the hatching media. These focal larvae were then placed in a 1 L container with 600 mL of spring water and twelve pellets (each 50 mg) of fish food at 20 ± 1°C. The water and fish food were changed every other day.

The focal larvae were held at a constant temperature of 20°C because a previous median regression showed that summer temperatures in Southeast England rose from 14.9°C to 17.0°C between 1971 and 1997 [48]. This warming trend is very likely to continue, with the London climate projected to resemble that of present-day Barcelona by 2050 [49]. The average summer maximum temperature for the Greater London area from 1976 to 2003 was 22.3°C [50]; therefore, 20°C is within the range of realistic summer temperatures to be experienced in Southeast England during the next few decades.

On the sixth day of focal larvae development, 70 female *M. viridis* copepods were each placed in a Petri dish (diameter: 50 mm, height: 20.3 mm) holding 20 mL of spring water to begin a 24-hour starvation period. A second set of *Ae. albopictus* eggs were hatched according to the previously described method, except that the hatch was held at 27 ± 1°C for 18 hours.

On the seventh day of focal larvae development, 140 third instar focal *Ae. albopictus* larvae were each placed in a Petri dish holding 20 mL of spring water, a 50 mg pellet of fish food, and four first instar larvae from the hatch that was started on the previous day. A subset of 30 third instar focal larvae were preserved in 80% ethanol for head capsule width measurements [51]. Seventy starved *M. viridis* copepods were then introduced to 70 out of the 140 Petri dishes (Fig 1).

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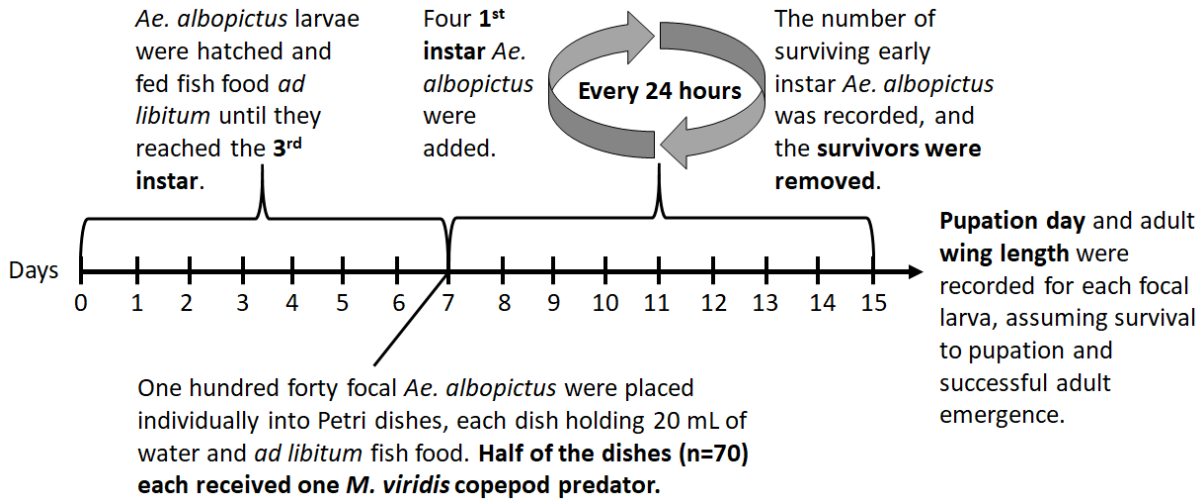

Fig 1. Experiment schedule

Each day, the number of surviving first or second instar larvae in each of the 140 Petri dishes was recorded, surviving first or second instars were removed, and four new first instars from the 18-hour hatch started on the previous day were added to each replicate (Fig 1). The status (dead or alive) of each focal larva was recorded daily, and in predator treatment replicates, the status of the copepod was also recorded. In the case of a focal larva death, the larva was preserved in 80% ethanol, and that replicate was removed from further observation. In the case of a copepod death, the copepod was preserved in 80% ethanol, and a new adult female *M. viridis* copepod from the September field collections was randomly chosen to replace it.

Pupation among focal individuals was recorded each day at 18:00 hrs. Pupae were transferred to 10 mL of spring water in a graduated cylinder with a mesh cover for emergence. Emerged adults were frozen at -20°C. Wings were removed and measured as a proxy for body size [52].

Data analysis:

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All analyses were completed in R version 3.4.2 [53]. Welch two sample t-tests for samples of unequal variance were used to compare percent survival of first or second instar larvae between copepod absent and copepod present treatments. The possibility of a difference in the proportion of focal larvae emerging as adults based on copepod presence was examined using a Pearson's chi-squared test without Yates' continuity correction. Two Kruskal-Wallis tests, one for males and one for females, were used to compare adult wing lengths between copepod absent and copepod present treatments. A Kruskal-Wallis test was also used to compare pupation day distributions between predator present and predator absent treatments. A non-parametric local regression method ("loess", "ggplot2" package) was used to present the cumulative proportion of focal larvae pupated over time, by predator presence.

Results

Head capsule width measurements (mean = 0.59 mm, sd = 0.062 mm) of a subset of the focal mosquito larvae (n = 30) confirmed that they were third instars on the first day of exposure to copepod predation [51]. One hundred fifteen copepods (mean length = 1.75 mm, sd = 0.16 mm) were used throughout the experiment across the 70 predator treatment replicates.

Out of the 70 focal larvae that were not exposed to copepod predators, 68, or 97.1%, emerged successfully as adults; one died in the larval stage, and one died in the pupal stage. Out of the 70 focal larvae that were exposed to copepod predators, 61, or 87.1%, emerged successfully as adults; five died in the larval stage, and four died in the pupal stage. Results of a Pearson's chi-squared test showed that the probability of successful adult emergence was higher in the absence of copepod predators (p-value = 0.0279). Three of the five focal larvae that died in

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the presence of a predator showed clear signs of melanization in the abdominal region, most likely due to wounding from copepod attacks (Fig 2).

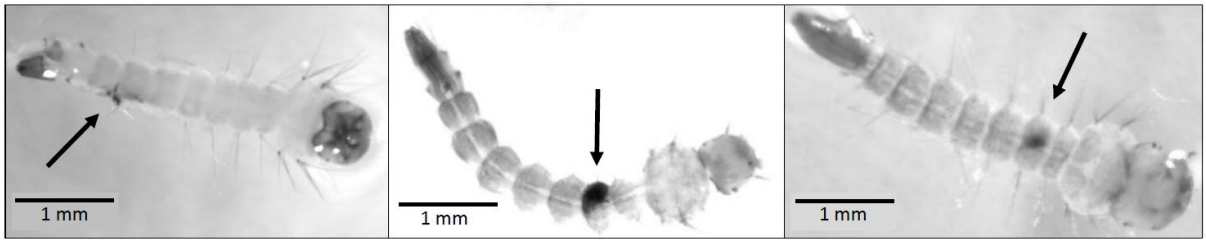

Fig 2. Evidence of melanization in focal larvae that died in the presence of a copepod predator on the eighth, ninth, and tenth days of observation, respectively

The percentage of first instar *Ae. albopictus* surviving each day was significantly lower in the presence of a copepod predator throughout the six days immediately following predator introduction (Table 1). No significant difference in first instar survival was observed on the last two days, when the number of remaining replicates was very low (Table 1).

Table 1. First instar percent survival by day and predator presence

Day of Experiment	**Predator Absent (mean ± se)**	**Number of Predator Absent Replicates**	**Predator Present (mean ± se)**	**Number of Predator Present Replicates**	**P-value[a]**
8	99.3 ± 0.5	70	6.8 ± 2.0	70	<0.0001
9	99.3 ± 0.5	70	30.1 ± 4.1	69	<0.0001
10	99.3 ± 0.5	70	47.8 ± 3.9	68	<0.0001
11	100 ± 0.0	70	42.9 ± 4.3	67	<0.0001
12	100 ± 0.0	42	55.9 ± 6.6	34	<0.0001
13	100 ± 0.0	12	58.3 ± 8.9	12	0.0007
14	100 ± 0.0	5	33.3 ± 33.3	3	0.1835
15	100	1	25 ± 14.4	3	NA

[a]P-value corresponds to a Welch two sample t-test for samples of unequal variance used to determine if there was a difference in first instar survival based on predator presence.

Ae. albopictus adult wing length data were left-skewed among both males and females. Female wing lengths (median = 2.87 mm) were significantly larger than male wing lengths (median = 2.33 mm, Kruskal-Wallis rank sum test, p-value < 0.0001). However, there were no significant differences in male wing length (Kruskal-Wallis rank sum test, p-value =

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0.6387) or in female wing length (Kruskal-Wallis rank sum test, p-value = 0.1769), due to copepod presence (Fig 3a).

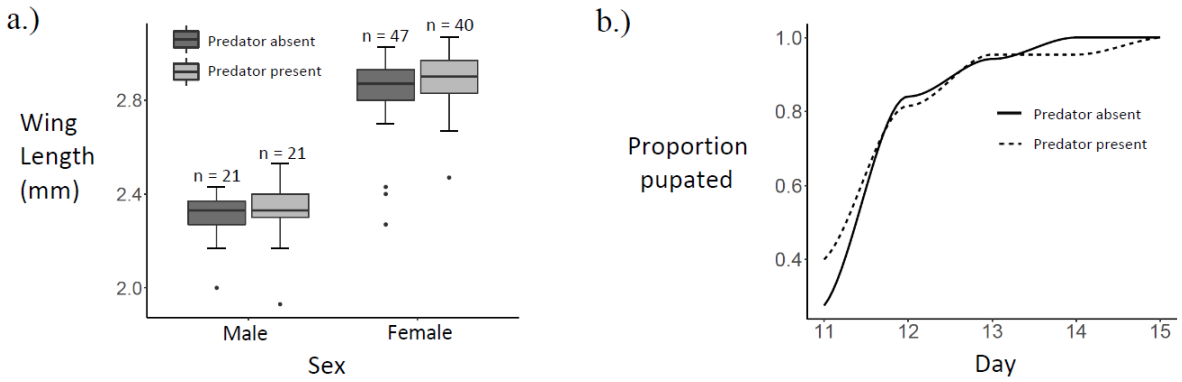

Fig 3. Size and development: a.) Boxplot of wing lengths by sex and predator presence, b.) Cumulative proportion pupated by predator presence

Pupation day data were right-skewed among both males and females. Sex did not affect the day of pupation (Kruskal-Wallis rank sum test, geometric mean ± sd: males = 11.8 ± 1.1, females = 11.9 ± 1.1; p-value = 0.2607). In addition, neither male pupation day (geometric mean ± sd: copepod present = 11.8 ± 1.1, copepod absent = 11.7 ± 1.1; Kruskal-Wallis rank sum test, p-value = 0.7819), nor female pupation day (geometric mean ± sd: copepod present = 11.8 ± 1.1, copepod absent = 12.0 ± 1.1; Kruskal-Wallis rank sum test, p-value = 0.1580), differed with copepod presence (Fig 3b).

Discussion

Although third and fourth instar *Ae. albopictus* larvae are generally less vulnerable to copepod predators than first and second instars [17], some of the third instar, fourth instar, and pupal stage deaths observed in this study were likely due to *M. viridis* copepod attacks. Three focal individuals that died in the larval stage showed signs of melanization (Fig. 2), a response triggered locally by cuticular wounding that results in the accumulation of melanin,

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a brown-black pigment, at the wound site [54-56]. Melanization is an energetically costly response that is likely to be influenced by nutritional status in mosquitoes [54, 57, 58]. Since the focal larvae in this study were provided with fish food *ad libitum*, it is unlikely that their immune responses were limited due to poor nutritional status. The location of the three melanization sites in the abdominal region (Fig. 2) is consistent with a previous study, which found that the larval abdomen was attacked more frequently by cyclopoid copepods than either the head/thorax region or the last body segment, containing the siphon [59].

While successfully emerging males were spread evenly between the predator and control treatments, less females emerged successfully from the predator treatment. These observations are consistent with those of a previous study showing that *Ae. albopictus* survivorship is skewed towards males in response to predation by *Toxorhynchites rutilus* [41]. A longer (five-week) semi-field study found that the *Ae. albopictus* sex ratio was skewed towards females after extended exposure to cyclopoid copepod predation [42]. However, lower larval densities have been shown to produce lower proportions of males in *Ae. albopictus* rearing [60]. One possible explanation for this is that the increased nutrient availability for each larva at lower densities might better support the larger body size of females. Thus, lower larval density resulting from predation, is likely to be the main cause of the female-dominated sex ratio that was previously observed [42].

In order for cyclopoid copepods to be effective biocontrols, enough adult female copepods need to be applied in order to quickly eliminate first and second instar mosquito larvae, which are the most vulnerable stages to copepod predation [17]. In some cases of incomplete control, *Ae. albopictus* adults that developed in the presence of predators emerged larger than adults that developed in control conditions because the lower larval density produced by

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predators resulted in less intraspecific competition and greater per capita nutrition [41-43]. It has also been suggested that first instar larvae benefit nutritionally from decomposing dead conspecifics [27]. First and second instar larvae are likely to benefit the most from increased nutrition because most larval growth occurs between the first and third instar stages. Under the scenario of incomplete biocontrol leading to the emergence of larger *Ae. albopictus* adults, it is important to consider that *Ae. albopictus* females do not avoid copepods during oviposition [17, 42]. Therefore, the higher fecundity that is associated with larger female size [33, 36] is likely to be strongly counteracted by copepod predation against newly-hatched *Ae. albopictus* larvae of the next generation.

Among *Ae. albopictus* adults that emerged successfully in this experiment, there was a significant difference in wing length due to sex (Fig. 3a), but there was not a significant difference in pupation day between sexes. *Ae. albopictus* males have previously been observed to be 17-20% smaller than females [61]. Accordingly, the male median wing length in this study is 18.8% smaller than the female median wing length, and wing length is known to correlate positively with mass in *Ae. albopictus* [52]. Previous work has shown that while mass clearly differs due to sex, development time in *Ae. albopictus* is less sexually dimorphic [62].

There was no significant difference in wing length or pupation day due to *M. viridis* predation cues (Fig. 3). Therefore, neither the greater reproductive success observed among larger *Ae. albopictus* [33-36], nor the higher risk for disease transmission observed among smaller female *Ae. albopictus* [38-40], is likely to result directly from *M. viridis* predation. A similar lack of predator impact on mosquito size and development time was observed when *Toxorhynchites amboinensis* was tested against newly-hatched *Ae. polynesiensis* in coconuts

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at three different larval densities [63]. In addition, a study spanning four generations of *Ae. albopictus* did not find any evidence of an evolutionary response to predator exposure in the larval stage [64].

Conclusions

We found evidence of lethal attacks on late instar larvae that resulted in a small, but significant, reduction in the probability of emergence. However, we did not find any evidence that experiencing *M. viridis* predation, both directly and on smaller conspecifics, affects size or development time of surviving *Ae. albopictus* adults at optimal nutritional availability. Previous work has shown that *M. viridis* exhibits a type II functional response curve and a relatively high predation efficiency against *Ae. albopictus* prey at temperatures representative of UK larval habitats [46]. This study builds on recent work and further supports the use of *M. viridis* as a biocontrol agent for *Ae. albopictus* in the southeastern UK, at the northern edge of the vector's expanding global range.

Declarations

Ethics approval and consent to participate:

Not applicable

Consent for publication:

Not applicable

Availability of data and materials:

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All data that were collected during this study will be made publicly accessible from the Dryad Digital Repository.

Competing interests:

The authors declare that they have no competing interests.

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Authors' contributions:

LJC and MCR conceptualized the experiment and determined the methodology, including the type of statistical analyses that were employed. MCR collected the data, performed the data analysis, produced the tables and figures, and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

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