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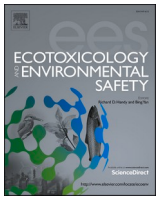

# Microplastic pollution differentially affects development of disease-vectoring *Aedes* and *Culex* mosquitoes

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ABSTRACT

Plastic in the form of microplastic particles (MPs) is now recognized as a major pollutant of unknown consequences in aquatic habitats. Mosquitoes, with aquatic eggs, larvae, and pupae, are likely to encounter microplastic, particularly those species that are abundant in close proximity to human development, including those that vector human and animal disease. We examined the effects of polyethylene MPs, the most common microplastic documented in environmental samples, on the development and survival of the mosquitoes *Aedes albopictus* and *Culex quinquefasciatus*. In laboratory egg-laying and larval development container environments similar to those used by both species in the field, a mix of 1–53 μm MPs at concentrations of 60, 600, and 6000 MP $ml^{-1}$ increased early instar larval mortality in both species relative to control treatments. A significant difference was found in the response of each species to microplastic at the lowest microplastic concentration tested, with *Cx. quinquefasciatus* survival equivalent to that in control conditions but with *Ae. albopictus* larvae mortality elevated to 37% within 48 h. These results differ from those of previous studies in which larvae were only exposed to MPs during the last aquatic instar stage and from which it was concluded that microplastic was ontogenically transferred without negatively affecting development. Increasing plastic pollutant concentrations could therefore act as selective pressures on aquatic larvae and ultimately influence outcomes of ecological interactions among mosquito vector populations.

## 1. Introduction

The use of plastic is an integral component of modern civilization. Its use has steadily increased over the past five decades, with hundreds of millions of metric tons having been produced (Geyer et al., 2017). Unsurprisingly, much of this plastic is discarded as waste, which infiltrates marine, freshwater, and terrestrial ecosystems (Horton et al., 2017). Many of these plastics persist in environments as fragments less than 5 mm (Arthur et al., 2008), called microplastics (MPs). Because these appear to be easily ingested by a wide range of organisms, MPs are increasingly recognized as a potential ecological concern. Microplastics are categorized as primary MPs or secondary MPs. Primary MPs are manufactured for commercial applications such as facial cleansers, cosmetics, and air-blasting technologies (Cole et al., 2011) and enter the environment as MPs (Moore, 2008). Secondary MPs arise from the breakdown of larger plastics already present in the environment into smaller fragments from mechanical abrasion or UV exposure (Barnes et al., 2009).

Microplastics have been found in the digestive systems or other tissues and organs of zooplankton (Cole et al., 2013), coral (Hall et al., 2015), mayflies and caddisflies (Windsor et al., 2019), mollusks (Naji et al., 2018), fish (Vendel et al., 2017), seabirds (Youngren et al., 2018), small terrestrial mammals (Thrift et al., 2022) and even humans (Leslie et al., 2022). Adverse health outcomes for MP ingestion have been documented in some species (Green, 2016; López-Rojo et al., 2020), but considering the relatively recent attention to this problem, little information exists on the long-term effects on organismal health from consuming microplastics. Furthermore, with the rise of One Health perspectives, new questions are emerging about the impact on the ecology of infectious diseases related to MP pollution—especially

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regarding vector-borne diseases (Loiseau and Sorci, 2022; Prata et al., 2021).

Mosquitoes are of special interest regarding the effects of MP pollution, in respect to both their life-history strategy and their role in vectoring pathogens that infect humans and other animals. Mosquitoes are holometabolous insects that undergo larval development in freshwater habitats and salt marshes but spend their adult lives in terrestrial habitats. This shift in habitat use presents a mechanism for MP dispersal between these distinct environments if MPs are ingested and retained throughout ontogeny. Furthermore, adult mosquitoes could potentially disseminate MPs deeper into terrestrial habitats through flight. Because mosquitoes are important components of both aquatic and terrestrial food webs, a concern is that they could move MPs across trophic levels leading to plastic bioaccumulation. Indeed, ingestion and ontogenic transference have been demonstrated in laboratory studies in *Culex* (Al-Jaibachi et al., 2018, 2019) and *Aedes* mosquitoes (Simakova et al., 2022). In those studies, 2 μm polystyrene beads persisted through pupation into adulthood and did not significantly affect mortality at any stage, however, it should be noted that larvae were exposed to MPs only beginning at the last aquatic instar stage.

In real aquatic environments where MPs are present, larvae are likely to be exposed throughout the entirety of their development. Any impacts on mosquito biology during the early larval stages could have important effects on the vector capacity of mosquitoes of medical and veterinary importance. Changes in development resulting from MP exposure could increase or decrease the number of mosquitoes acquiring and transmitting pathogens in areas with active mosquito-borne disease. In this study, we exposed *Aedes albopictus* and *Culex quinquefasciatus* to MPs at the first larval instar and left them exposed for the duration of their development in order to assess impacts on overall larval development and survival. We chose to expose larvae to MPs of polyethylene, the most abundantly produced plastic (Geyer et al., 2017), due to the frequency in which that plastic is found in water and sediment (Cera et al., 2020) and in plastic containers (Kouhi et al., 2021) that *Aedes* and *Culex* commonly utilize for oviposition.

## 2. Material and methods

### 2.1. Mosquito colonies

In June 2022 *Aedes albopictus* eggs were obtained from our lab colony (maintained in environmental chambers and fed on bovine blood) established from wild *Ae. albopictus* collected from Mānoa, Oahu, Hawai'i. An F21 generation was used for this experiment. In the same month *Culex quinquefasciatus* eggs were obtained from oviposition traps at the University of Hawai'i at Mānoa campus. The rearing of both species occurred under the following conditions: 12 L:12 D photoperiod, light period temperature of 28 ± 1 °C, dark period temperature of 22 °C, and humidity was kept constant at 60% for both the light and dark periods. A custom food preparation was made that consisted of a 1:1 ratio of liver powder (Argentine Grass Fed Beef Liver, CurEase) to dried yeast extract (Anthony's Brewer's Yeast, Anthonys Goods) mixed with pure water to a concentration of 18 mg per ml. Larval food was administered every 48 h at a concentration of 0.6 mg per larvae per larval enclosure.

### 2.2. Microplastic preparation and microplastic particle exposure experiments

Three types of microplastics were used: 1–5 μm fluorescent blue polyethylene microspheres (density 1.33 g/cc, excitation 445 nm, emission 407 nm, Cospheric LLC, Santa Barbara, California, US), 27–32 μm fluorescent red polyethylene microspheres (density 1.09 g/cc, excitation 607 nm, emission 575 nm, Cospheric LLC, Santa Barbara, California, US), and 45–53 μm fluorescent green polyethylene microspheres (density 1.00 g/cc, excitation 515 nm, emission 414 nm, Cospheric LLC, Santa Barbara, California, US). All microspheres received from Cospheric LLC were in dry form with an approximate concentration of 122,000,000 MPs/mg for the 1–5 μm fluorescent blue MPs, 74,400 MPs/mg for the 27–32 μm fluorescent red MPs, and 16,200 MPs/mg for the 45–53 μm fluorescent green MPs. Stock solutions (mg/ml) of each MP size were made in distilled water with Tween-20 (Uniqema Americas LLC) biological surfactant added to the solutions at a concentration of 0.1% to ensure full suspension of MPs in the water column. The same concentration of Tween-20 was also added to control treatments with no plastics.

Aquatic container environments for larval development were made by adding 100 ml pure water from a Millipore Milli-Q water filtration system (Massachusetts, USA) to 11.4 cm diameter 470 ml cylindrical jars fitted with funnel top containers to trap adults emerging after pupation (BioQuip Products, California, USA). These containers supplied with the food mix described above are accurate representations of oviposition sites used by *Aedes* and *Culex* mosquitoes. They are the same containers used as field traps for the establishment of the lab colonies and are the type of small human-made containers selected by mosquitoes in the field as suitable or preferred oviposition sites in urban areas (Wilke et al., 2019a; Wilke et al., 2019b). Environments were divided into four treatment groups, consisting of six replicates for each group and three replicates for each species. Treatments were at microplastic concentrations of 60 MP $ml^{-1}$, 600 MP $ml^{-1}$, 6000 MP $ml^{-1}$, and a control containing 0 MP $ml^{-1}$. These total MP concentrations were obtained by adding equal concentrations (by MP number) of the stock solution for each of the three MP sizes to each container environment in volumes to reach the nominal final treatment concentration. In terms of mass $ml^{-1}$ the treatment concentrations were 60 MP $ml^{-1}$ = 1.5 mg/L, 600 MP $ml^{-1}$ = 15 mg/L, and 6000 MP $ml^{-1}$ = 150 mg/L. These microplastic concentrations span those documented in urban and urban-adjacent environments but are still orders of magnitude lower than the highest shoreline sediment concentrations recorded in Hawaii (Carson et al., 2011; Waldschlager et al., 2020). Ten L1 instar larvae were placed in each container environment. Mortality was checked daily for all treatments through a count of surviving mosquitoes.

### 2.3. Statistical analyses

Larval mortality was assessed using a generalized linear model assuming a binomial distribution with a Firth correction using the R (4.2.1) package logistf. Development time, defined as the number of days to emergence as adults, was analyzed using a generalized linear model assuming a negative binomial error distribution using the glm function in the R package stats. Differences in proportion mortality between *Aedes* and *Culex* at individual MP concentration levels were tested by Fisher's Exact Test.

### 2.4. Analysis of microplastic uptake and ontogenic transference

To assess MP uptake, two surviving larvae were randomly selected at the fourth larval instar stage, removed from the container environment, washed with distilled water, placed in a 1.5 ml Eppendorf tube, and stored at 4°C. After storage, larvae were transferred to a microscope slide and viewed at 10X magnification under an epi-fluorescence microscope with UV illumination for body imaging and counting of ingested plastic microspheres. The same procedure was followed for pupae and emerged adults. Gut tissue was dissected from both pupae and adults and inspected under a stereoscope for the presence of the large (53 μm) and medium-sized (32 μm) MPs, and under a compound light microscope for the presence of the small-sized (5 μm) MPs.

### 2.5. Microplastic leaching exposure experiment

Following the microplastic particle exposure experiment, a second experiment was conducted to discern whether the observed effects of microplastics on larval mortality in this study were due to chemical

leaching into the external aquatic environment (separate from any effects of physical obstruction or physical irritation of digestive system or other tissues from microplastic fragments). Six container environments were created containing an MP concentration of 6000 MP $ml^{-1}$ following the protocols described above. The containers were placed in an environmental chamber and incubated under the following conditions: 12 L:12 D photoperiod, light period temperature of 28 ± 1 °C, dark period temperature of 22 °C, and 60% constant humidity. Containers were removed after 72 h of water exposure to plastic and the MPs were then filtered twice from the water using a 0.2 μm syringe filter. The filtrate was then transferred to a sterile cell imaging dish for microscopic inspection. Confirmation of MP removal was performed using light microscopy at 1000x magnification. After confirmation that MPs were removed from the water, 5 L1 instars were added to each container. Containers 1–3 contained *Ae. albopictus*. Containers 4–6 contained *Cx. quinquefasciatus*. Six additional containers containing only pure water were used as a control; half of these contained *Ae. albopictus* and half contained *Cx. quinquefasciatus*. Larvae were fed according to the protocol listed under mosquito colonies above. This microplastic leaching exposure experiment was run for 48 h. This span of time exceeds that in which all larval mortality occurred in any of the microplastic particle exposure experiments.

## 3. Results

Larval mortality was significantly affected by species and MP concentration (LR=154.31, df=4, p = <0.001; Fig. 1). The number of first instar larvae that died within 48 h after exposure was significantly higher in the 6000 MP $ml^{-1}$ (100%, n = 60/60, p = <0.001) and 600 MP $ml^{-1}$ (78.3%, n = 47/60, p = <0.001) environments versus the 60 MP $ml^{-1}$ environments (31.7%, n = 19/60, p = 0.07) and control (18.3%, n = 11/60, Table 1). An interaction effect between species and treatment was insignificant ($\chi^2$ =4.24, df=3, p = 0.23) at the level of the whole model. In all MP treatments, all mortality occurred within 48 h of exposure. For mosquitoes that survived beyond the first instar, exposure to MPs did not significantly impact development ($\chi^2$ =2.27, df=2, p = 0.32). Mean days to emergence (± standard error) for *Ae. albopictus* at 600 MP $ml^{-1}$ was 12.33 ± 0.28 (n = 4) days and 12.72 ± 0.28 days (n = 12) at 60 MP $ml^{-1}$ l and 12.40 ± 0.25 days in the control. For *Cx. quinquefasciatus*, mean days to emergence was 13.50 ± 0.25 (n = 9) days at 600 MP $ml^{-1}$ and 15.19 ± 0.48 days (n = 28) at 60 MP $ml^{-1}$ and 15.43 ± 0.47 days in the control. In our microplastic leaching experiment there was no mortality in any of the *Ae. albopictus* (n = 15) or *Cx. quinquefasciatus* (n = 15) containers.

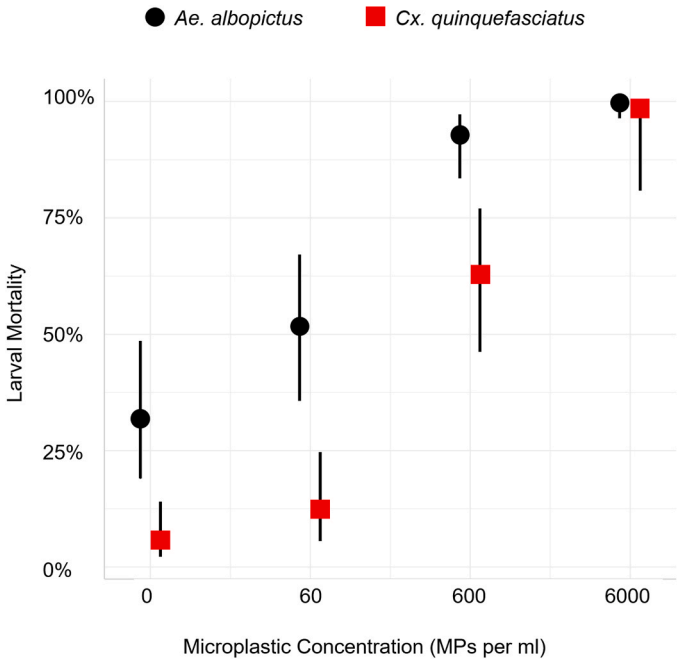


**Fig. 1.** Mean mortality of larvae by MP concentration as predicted by logistic regression. Bars represent 95% confidence intervals.

**Table 1**
Odds ratio and 95% confidence interval for each predictor in the logistic regression analysis comparing mortality by species and MP concentration.

| Full Model | Log-odds ratio | 95% CI | *p* |
|---|---|---|---|
| Intercept | -0.76 | (−1.45, 0.05) | 0.07 |
| Species *Cx. quinquefasciatus* | -2.03 | (−2.96, −1.21) | < 0.001 |
| 60 MP $ml^{-1}$ | 0.82 | (−0.07, 1.76) | 0.07 |
| 600 MP $ml^{-1}$ | 3.31 | (2.31, 4.46) | < 0.001 |
| 6000 MP $ml^{-1}$ | 7.00 | (4.82, 11.90) | < 0.001 |

A significant difference was found in the response of each species to microplastic at the lowest microplastic concentration tested with *Cx. quinquefasciatus* survival equivalent to that in control conditions but with *Ae. albopictus* larvae mortality elevated to 37% within 48 h (Fisher's Exact Test, n = 11:30, p = 0.01). Mortality was low and did not differ between species in control enclosures lacking microplastic (Fisher's Exact Test, *Aedes* 7:23, *Culex* 1:29, 0.052). At higher concentrations of microplastic most larvae of both species failed to complete larval development and converged on 100% mortality at 6000 MP $ml^{-1}$ (Fig. 1).

We were unable to observe MPs under fluorescent microscopy due to photobleaching during the course of exposure to light in our experiments, but the MPs used in this experiment are colored in the visible spectrum and could be identified using standard light microscopy. MPs of all three size classes were directly observed in the digestive track of larva sampled for microscopy from 60 and 600 MP $ml^{-1}$ environments (Fig. 2). MP uptake was common in both species during the fourth larval instar stage (see supplemental data), but no MPs of any size were

**Fig. 2.** Light microscope image showing 27–32 μm fluorescent red and 45–53 μm fluorescent green polyethylene microspheres in the digestive track of a *Culex quinquefasciatus* larva at 20X magnification.

observed in pupae or adults of either species. No MPs were observed in mosquitoes from either species from the control environments during any life stage.

## 4. Discussion

Understanding the factors that govern mosquito reproduction under real-world conditions has been recognized as an important goal in biology for over a century (Floore, 2006), with consequences in epidemiology, community composition, ecosystem dynamics, and conservation. In Hawaii alone, *Aedes* species have the potential to transmit the human diseases dengue, chikungunya, and Zika, and *Culex* mosquitoes carrying avian malaria and avian pox are a primary cause of continued extinction threat to endemic bird species (Banko et al., 2001; Atkinson and LaPointe, 2009). This study shows that microplastic pollution, one of the most widespread changes to ecosystems on a global scale (Rochman & Hoellein, 2020), can negatively affect mosquito larvae survival. Even moderate concentrations of microplastics, relative to those used in other studies (Al-Jaibachi et al., 2018, 2019; Simakova et al., 2022), led to the death of most larvae of both *Ae. albopictus* and *Cx. quinquefasciatus* in laboratory environments. The lowest concentration of microplastic tested in this study resulted in significantly elevated mortality in *Ae. albopictus* without impacting development and survival of *Cx. quinquefasciatus*, additionally indicating that microplastic pollution can have different species-specific effects within shared habitats within guilds. These results differ from those of past laboratory studies that suggested microplastics did not substantially affect mortality of these two species of mosquitoes (Al-Jaibachi et al., 2018, 2019; Simakova et al., 2022). This study is therefore the first to directly demonstrate a categorically harmful effect of the most common environmental plastic on mosquito survival through larval development. Importantly, this was observed at an MP concentration four orders of magnitude lower than that in the first study that suggested ontogenic transfer with no increase in mortality (Al-Jaibachi et al., 2018; Simakova et al., 2022) and at roughly the lowest concentration used in more recent studies that failed to demonstrate an impact on larval mortality (Al-Jaibachi et al., 2019).

Comparison of methods used in other investigations of microplastic effects on mosquitoes must be made, and the conclusions of those studies should be reassessed in light of the current findings. Although the majority of methods are similar across this and other laboratory mosquito microplastic studies, three major distinctions exist that could each serve as a basis for an explanatory hypothesis to be assessed in further research. First, the type of plastic used in this study, polyethylene, was different than the polystyrene used in Al-Jaibachi et al. (2018), (2019) and Simakova et al. (2022). Polyethylene is the most abundantly produced plastic, polystyrene is among the most abundantly produced, and together these are the most commonly documented as environmental pollutants (Geyer et al., 2017). If microplastic effects on organisms are due to chemical leaching, the different chemical compositions of these plastics could account for differences in experimental results. Second, this study investigated the effect of microplastic exposure to mosquitoes immediately after hatching, whereas the previous studies did not expose mosquitoes to microplastic until they had been reared to the final larval stage prior to emergence as adults. In demonstrating heavy early instar mortality, our study suggests that under the microplastic concentrations used in previous studies few mosquitoes would even survive to the life stages they investigated if the investigation was conducted under the realistic conditions of constant microplastic presence in the environment over the two weeks or less required for larval development. Finally, this study was conducted on mosquito lineages that had been collected from wild populations and maintained in laboratory colonies for less than a year (*Cx. quinquefasciatus*) to several years (*Ae. albopictus*), whereas the other studies were conducted on lineages that had been reproducing as laboratory colonies for several years to over a decade. The latter are spans of time shown by numerous studies to be adequate for evolution of resistance to insecticides and other anthropogenic environmental toxins (Koella et al., 2009; Paris et al., 2010). Considering the possibility of a strong selective pressure imposed by microplastic in this study, the possibility of evolved resistance to microplastic pollution in laboratory colonies, likely in constant contact with plastics, cannot be discounted and should be a factor included in the interpretation of future microplastics research. Moreover, since there was a substantially greater effect of microplastics on larval survival in *Ae. albopictus* than in *Cx. quinquefasciatus* at microplastic concentrations closest to those currently documented in field environments, both population dynamics and competitive interactions could also be altered by increasing plastic pollution.

An initial hypothesis in our study was that a high rate of larval mortality could be caused by chemical leaching from MPs. However, we demonstrated this was not the cause of mortality in our experiment rearing mosquitoes in the microplastic leaching treatments where microplastic solids were filtered from the water. No mortality was observed in these treatments. This suggests that mortality due to microplastic is caused by direct physical interaction between MPs and the developing insects. Spatial constraints within the gut could limit total ingestion rate. MPs ingested and present during gut passage might occupy volume that could otherwise be occupied by food, ultimately reducing nutrient acquisition and in some cases resulting in starvation. In early instar mosquito larvae, for example, ingested MPs even as small as 5 μm can span the internal diameter of the gut. It is also possible that ingesting MPs causes irritation or impaction in the gastrointestinal tract, leading to diminished ability of absorptive surfaces to function in nutrient uptake. Substantial physical damage to the gut might even lead directly to death from acute trauma.

Although mortality was high in the 600 MP $ml^{-1}$ environments, mosquitoes that survived at that concentration showed no developmental differences compared to their 60 MP $ml^{-1}$ and control counterparts. This could be due to a selective effect whereby it was only larvae of larger body size that overcame the exposure to MPs—either via greater surface area in the gut adequate to maintain nutrient acquisition even with some obstruction or the ability of a larger diameter gut to more rapidly evacuate MPs. Each of these mechanisms would free up space within the gut critical to increase nutrient uptake and prevent starvation. Developmental times did vary between the two species with *Ae. albopictus* emerging approximately 2.3 days faster than *Cx. quinquefasciatus*. Prior work suggests this is likely due to biological differences inherent to each species rather than due to different responses to MP exposure. The sex ratio in both species was 1:1, so differences in mean emergence times were not biased by different proportions of males, which typically emerge faster than females.

Previous studies demonstrated ontogenic transference of small polystyrene MPs (approximately 2 μm) from larvae to pupae and from pupae to adults in *Cx. pipiens* and *Ae. aegypti* (Al-Jaibachi et al., 2018, 2019; Simakova et al., 2022). We readily observed the presence of MPs in third and fourth larval instars, however, we did not observe transference of polyethylene MPs of any size in *Ae. albopictus* or *Cx. quinquefasciatus* from larvae to pupae nor from pupae to adult. One difference between polystyrene and the polyethylene investigated in this study is that polyethylene is denser than polystyrene. This density difference might affect MP fate within the mosquito, possibly allowing for easier evacuation of smaller particles from the gastrointestinal tract. It is also possible that the species used in this study have a more robust gastrointestinal clearing capacity than those species used in previous studies and that complete evacuation of the gastrointestinal tract prior to molting is the reason we did not observe ontogenic transference of any MPs. Further work would be necessary to determine if this factor explains differences between *Ae. albopictus* and *Cx. quinquefasciatus* survival.

Our results have important implications because we show for the first time that environmentally relevant concentrations (Carson et al., 2011; Bitter and Lackner, 2020) of microplastic particle size mixtures can be

toxic to larvae of two important vectors of human and animal diseases. Previous studies have largely avoided exposing first instar larvae to MPs, but this ignores the reality that larvae will likely be exposed throughout their entire developmental period if developing in MP-contaminated habitats of the types typically used by the species in this study (Wilke et al., 2019a; Wilke et al., 2019b). The impact on the vectorial capacity of mosquitoes that survive the stage-specific effects of MP exposure warrants additional investigation. Because we show that polyethylene MPs are not readily carried across mosquito life-stages polyethylene MP dispersal into aerial and terrestrial habits is unlikely to occur via *Ae. albopictus* or *Cx. quinquefasciatus* emergence from aquatic habitats as adults. However, given that microplastics are readily ingested by third and fourth instar larvae, dispersal is likely to occur through trophic interactions within aquatic systems, impacting consumers at multiple levels and presenting a novel dimension to the mosquito's role in ecological health outcomes.

## 5. Conclusion

Microplastic pollution significantly affects the mortality of common mosquitoes that can vector human and animal disease when it is present at hatching, as would be the case in aquatic environments and mosquito developmental scenarios outside of the laboratory. This is a categorically different conclusion than those from previous studies that exposed mosquito larvae to microplastics only in their final larval stage and recorded no negative effects on development or survival. Because microplastic pollution is now ubiquitous and appears to affect even ecologically similar species in different ways, future laboratory and field studies on ecology, epidemiology, and evolution of mosquitoes should be conducted with regard to this environmental factor and selective pressure.

## CRediT authorship contribution statement

**Chasen Griffin:** Methodology, Software, Formal analysis, Investigation, Data curation, Writing – original draft, Project administration, Funding acquisition. **Christine Tominiko:** Methodology, Investigation. **Matthew Medeiros:** Resources, Supervision. **Justin Walguarnery:** Conceptualization, Methodology, Writing – review & editing, Visualization, Project administration.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.115639.

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