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Effects of Biochar on *Aedes Aegypti* Oviposition Site Choice, Microbiome and Metabolism

By

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DISSERTATION

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Acknowledgments

To everyone who has been there throughout this journey, or for parts of it, whether you are still here or no longer on this mortal coil—you know who you are. You have my sincerest gratitude! And a special shoutout to all the fluffy and scaly critters I've had the privilege of petting along the way!

Abstract

Wildfire is an important ecological disturbance in California that occurs in forested, urban and suburban areas. These fires generate biochar and other partially burned plant materials capable of entering urban and suburban aquatic environments. However, little is known about how biochar may interact with mosquitoes that utilize these aquatic habitats for reproduction. This thesis investigated the effects of wood derived biochar on *Aedes aegypti* across multiple stages of the mosquito life cycle, from oviposition site selection to larval development and adult physiology, while also examining changes in the microbial communities of larval rearing water.

The first chapter reviews the current understanding of *Aedes aegypti* oviposition behavior and the sensory and environmental cues that influence female selection of oviposition sites. This review provides a framework for understanding how changes in aquatic habitat characteristics may alter oviposition behavior and highlights the importance of chemical, olfactory, visual, and microbial cues in site selection.

The second chapter investigates the effects of wildfire and lab generated wood biochar on *Aedes aegypti* oviposition under laboratory and field conditions. Initial observations suggested that, contrary to the original hypothesis that biochar would repel ovipositing females,

biochar associated water could increase egg deposition. Subsequent experiments examined the effects of biochar concentration, preparation, and the presence of other potential oviposition attractants. These experiments demonstrated that biochar can influence oviposition-site selection and provide evidence that wildfire-derived materials may alter the attractiveness of aquatic habitats to gravid females.

The third chapter examines the effects of biochar exposure beyond oviposition behavior. *Aedes aegypti* larval development was assessed following exposure to biochar associated water, while metabolomic and lipidomic analyses were used to investigate changes in adult *Aedes aegypti* female physiology. The microbial communities of larval rearing water were also characterized to determine whether biochar was associated with changes in the aquatic microbial environment. Together, these analyses indicate that biochar exposure is associated with changes in *Aedes aegypti* larval development, adult female metabolism, and the aquatic microbial community composition of *Aedes aegypti* breeding sites.

Collectively, this work demonstrates that biochar has the potential to influence *Aedes aegypti* at multiple stages of its life cycle. These findings provide a foundation for further investigation into how biochar, aquatic habitat characteristics, and microbial communities interact to influence mosquito ecology and behavior.

Chapter 1

Maternal Instincts: A Review of the Role of Chemosensory Cues and Larval Habitat Conditions on Oviposition Site Choice by Gravid *Aedes aegypti* (Diptera: Culicidae)

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Abstract :

Aedes aegypti (*Ae. aegypti*) is a global vector of arboviruses, including those causing dengue, chikungunya, Zika, and yellow fever. Effective management of *Ae. aegypti* populations remain challenging due to their anthropophilic feeding behavior, their exploitation of urban environments, their complex oviposition behavior, and their egg physiology. The characteristics of larval habitats can influence various aspects of larval development, thereby altering adult physiology and behavior. These include differences in blood-feeding behavior, reproductive capacity, and lifespan. Gravid females select oviposition sites based on a diverse array of visual, textural, chemical and biological cues that attract/repel females from sites and/or stimulate/inhibit oviposition, including volatile organic compounds from microorganisms, detritus, conspecific/heterospecific larvae and adults, predator activity, and environmental factors. This review focuses on the chemosensory aspects of oviposition site selection. Understanding the effects of concentration-dependent chemical cues on oviposition behavior in gravid female *Ae.*

aegypti offers critical insights into the ecology of this species. Integrating this knowledge into vector control strategies, particularly attract-and-kill ovitraps incorporating optimized attractants and larvicides, could enhance surveillance efficacy and reduce *Ae. aegypti* populations. As climates become more favorable and urbanization expands, *Ae. aegypti*'s geographic range is likely to expand, making it essential to leverage detailed behavioral and ecological insights to improve surveillance, vector management, and to mitigate arboviral disease risks globally.

Key words: *Aedes aegypti*, oviposition, behavior, breeding site, geosmin, conspecific densities, heterospecific densities

1. Introduction

Ae. aegypti (L.) is a successful invasive vector due to its adaptations to urban environments and its eggs' ability to survive desiccation (OECD 2018). This urban-suburban mosquito is commonly known as the yellow fever mosquito. *Ae. aegypti* is a primary vector of numerous medically important arboviruses, including yellow fever, dengue, chikungunya, and Zika (OECD 2018, Carvajal-Lago et al. 2021, Egid et al. 2021, Mosquera et al. 2023). These diseases are a growing global concern with an expanding distribution and increasing incidence among human populations (Wang et al. 2019, Egid et al. 2021). Vector control-based strategies are among the most effective means of preventing vector-borne diseases (Carvajal-Lago et al. 2021).

Historically, *Ae. aegypti* was a forest-dwelling mosquito that bred in tree holes across sub-Saharan Africa. Approximately five centuries ago, global trade and human movement facilitated its spread beyond its native range, enabling it to invade new regions across the globe (Powell and Tabachnick 2013, Powell et al. 2018). Today, *Ae. aegypti* has two distinct subpopulations: *Ae. aegypti formosus*, the ancestral form still found in African forests, and *Ae. aegypti aegypti*, which has adapted to urban environments (OECD 2018). Female *Ae. aegypti*

formosus prefer non-human blood meals for egg production and lay their eggs in coconut husks or tree holes (OECD 2018, Pruszyński et al. 2020).

For this review, references to *Ae. aegypti* will specifically pertain to *Ae. aegypti aegypti*, the urban-adapted form. This urban mosquito species is distributed between 40°N and 40°S latitude.

Although females strongly prefer human hosts, they will feed on other vertebrates (Gulia-Nuss et al. 2015, Pruszyński et al. 2020, Egid et al. 2021). Gravid (egg-carrying) females use chemosensory (e.g. olfactory) cues (e.g. chemicals produced by microbes) as well as visual, gustatory, and tactile cues to select an oviposition site (Afify and Galizia 2015). They preferentially deposit eggs in artificial containers found in urban environments, including discarded trash, tires, flower pots and other water-holding items, which make up an estimated 95% of oviposition sites (McGregor and Connelly 2021, Walker et al. 2021, OECD 2018, Egid et al. 2021, Wilke et al. 2019). In field and lab experiments, black ovitraps have more eggs deposited than blue, green and transparent ovitraps (Khan A. et al. 2022). Human infrastructure and behavior often provide conditions that support *Ae. aegypti* breeding including sewage treatment plants, storm drains, and irrigated lawns (Wilke et al. 2019). Improved public outreach and communication about this would enable the public to be aware of the disease risks and lessen the risks of misinformation (Thomas et al. 2024).

Ae. aegypti eggs, typically ~1 mm in length and ovoid in shape, are white when freshly laid and turn black after a few hours (OECD 2018). The eggs are resistant to desiccation and capable of surviving for months until rehydration triggers hatching. Depending on resource availability and temperature, larvae can develop into adults in as little as a week or as long as 90 days (Walker et al. 2021). Their diet consists primarily of detritus, microbes, and small invertebrates, from

which they derive essential nutrients required for growth, including sugars, salts, lipids, amino acids, and vitamins (Singh and Brown 1957).

Research has made strides in examining factors influencing oviposition by mosquitoes.

Oviposition site selection is a critical decision gravid females make to give their offspring the best chance at survival, as the immobile larvae cannot move to new aquatic environments if resources become scarce or conditions deteriorate. Site selection can be influenced by olfactory cues emitted from aquatic habitats. These cues that draw gravid females to potential oviposition sites are called oviposition attractants (Khan et al. 2023, Affi and Galizia 2015). While an oviposition repellent is an olfactory cue that repels gravid females from an oviposition site. An oviposition stimulant is a chemosensory (not necessarily olfaction) cue that elicits oviposition behavior once at a site. The opposite of a stimulant is a deterrent, a chemosensory cue that deters oviposition. Attractants and repellents work from long distances from the oviposition site while stimulants and deterrents work closer to the oviposition site.

After choosing an oviposition site, to create a more supportive larval environment, *Ae. aegypti* can shape the microbial community as oviposition causes a decrease in microbial alpha diversity (Mosquera et al. 2023). After ovipositing, gravid *Ae. aegypti* may also defecate, introducing beneficial microbes to the breeding site (Mosquera et al. 2021). For instance, *Ae. aegypti* can inoculate the bacteria *Elizabethkingia* into the oviposition site (Mosquera et al. 2023). The presence of these microbes (e.g. *Elizabethkingia*) can support larval health and development (Mosquera et al. 2023).

Temperature also plays a key role in development and behavior. The viable range for *Ae. aegypti* is approximately from 14°C to 30°C (OECD 2018). At 25°C, development from egg to adult may take slightly over a week (OECD 2018). Lower temperatures slow development and can extend the larval period significantly (e.g. months), while higher temperatures enable larval

growth, development, and movement. In adults, higher temperatures compared to lower ones reduce their lifespans (OECD 2018). The progression of climate change is resulting in new geographic regions, such as parts of the United States, that are becoming more hospitable to *Ae. aegypti* (Reinhold et al. 2018, CDC 2020, Walker et al. 2021, Zhang et al. 2025, CDC 2024, Heath et al. 2025, Abbasi 2025). The ability of this species to overwinter successfully in new locations has allowed the mosquito to expand its range, as seen in California, where *Ae. aegypti* arrived in the summer of 2013, likely following multiple undetected introductions and has continued to spread through the state (Gloria-Soria et al. 2014, Pless et al. 2017, VectorSurv Maps).

Aquatic insects are classic bioindicators used to monitor the quality of underwater environments (Fadel et al. 2023). Because they depend on water for much of their development, changes in their presence, density, and behavior can reflect subtle environmental changes, such as variations in nutrient availability, pollution, or habitat disturbance. This principle not only applies to ecologically important species but also underscores the broader relevance of understanding *Ae. aegypti* oviposition behavior in relation to environmental health. For instance, *Ae. aegypti* larval density can be correlated to differences in water conductivity, pH, ammonium, and phosphate levels (Avramov et al. 2024).

Oviposition site selection by *Ae. aegypti* is influenced by a complex array of chemical signals, including bacterial volatiles, conspecific byproducts, and physical attributes such as container size, color and water quality (Boullis et al. 2021, Prasad et al. 2023, Khan et al. 2023, Khan A. et al. 2022). The primary focus of this review will be on chemical stimuli of mosquito oviposition behavior. Experiments have demonstrated that gravid females use these cues to evaluate potential egg-deposition sites, balancing the risks of predation, competition, and habitat suitability (Mosquera et al. 2021, Albeny-Simões et al. 2014). This review aims to synthesize

current knowledge on *Ae. aegypti* oviposition behavior as well as the chemical and biological factors that influence egg-laying decisions.

Due to their strong preference for urban environments and human-derived habitats for reproduction, *Ae. aegypti* exploit a wide array of breeding sites created by human activities, making their populations difficult to control and monitor (Prasad et al. 2023, Wilke et al. 2019). Traps can help reduce *Ae. aegypti* populations, however source reduction is needed prior to trap deployment (Perich et al. 2003, Khan A. et al. 2022, Eiras et al. 2021). Understanding these processes and gaining a deeper understanding of site selection will aid the development of cheaper, novel and lethal oviposition traps, and targeted interventions to control and monitor *Ae. aegypti* populations.

2. Oviposition Behavior in *Ae. aegypti*

Ae. aegypti females exhibit complex oviposition behavior driven by ecological pressures and reproductive biology. A key strategy is skip oviposition, where a female distributes her eggs across multiple sites within a single gonotrophic cycle (Craig et al. 2008, Powell et al. 2018, Walker et al. 2021). This behavior minimizes the risk of larval overcrowding and competition for limited resources such as food and space, thereby improving the survival chances of her offspring (Craig et al. 2008, Walker et al. 2021). One study using oviposition traps in *Ae. aegypti* endemic areas found that around 77% of ovitraps contained fewer than 30 eggs, with each gravid female typically laying 11–30 eggs per site (Chadee 2009).

Another noteworthy behavior is superoviposition, in which females lay eggs at sites that already contain conspecific eggs or larvae (Ganesan et al. 2006, Craig et al. 2008). Field and laboratory studies have shown that superoviposition tends to occur when egg densities at a site are low, typically fewer than 25–30 eggs (Craig et al. 2008, Chadee 2009). While skip oviposition generally favors unoccupied sites, low densities of existing eggs or larvae may actually

encourage additional oviposition (Chadee 2009, Khan et al. 2023). The presence of conspecific eggs or larvae at low densities can signal a resource-rich environment, this can stimulate egg-laying, whereas sites that are heavily contaminated or exhibit signs of high larval densities act as oviposition deterrents due to resource competition (Khan et al. 2023, Shragai et al. 2019, Chadee 2009, Gonzalez et al. 2016, Boullis et al. 2021, Mosquera et al. 2021). This behavior is seen in *Aedes albopictus* (*Ae. albopictus*) as well.

While laboratory experiments have shown that when only a single oviposition site is available, even one with a high egg density, females are unlikely to retain their eggs and will lay them there (Craig et al. 2008). However, when multiple sites are available, skip oviposition dominates (Craig et al. 2008). Older eggs may also be less effective at deterring further oviposition, possibly because egg-associated chemical markers fade over time (Chadee 2009).

The presence of late-stage larvae (3rd- and 4th-instar) also stimulate oviposition in both *Ae. aegypti* and *Ae. albopictus*, likely because their development indicates a productive and resource-rich site (Khan et al. 2023, Boullis et al. 2021, Mosquera et al. 2021). In an oviposition choice assay, females were offered a control site containing larval food in water alongside experimental sites containing either eggs frozen at -20 °C for 30 min, 2nd- or 4th-instar larvae, or pupae and pupal exuviae. Females preferentially oviposited in sites containing 4th-instar larvae (Khan et al. 2023).

Ae. albopictus and *Ae. aegypti* larvae can be found coexisting in the same habitats, such as artificial containers (Gonzalez et al. 2016). The larvae of both species are very competitive during the larval stage (Gonzalez et al. 2016). When *Ae. albopictus* has the choice of breeding sites with no larvae, breeding sites of conspecific (same species, i.e. *Ae. albopictus*) or heterospecific (different species, i.e. *Ae. aegypti*) larvae, they chose the breeding sites with conspecific/heterospecific larvae (Shragai et al. 2019, Tuñón et al. 2025). This preference is

influenced by both the species and density of larvae present. Conspecific larvae are generally attractive for oviposition, but the response is density-dependent: moderate densities (e.g., 100 larvae per 3.7 L) promote oviposition, whereas very high densities (e.g., more than 130 larvae per 0.37 L) act as a deterrent (Shragai et al. 2019). In sites containing between 10 and 100 heterospecific larvae, no oviposition effect was observed for either *Ae. aegypti* or *Ae. albopictus* (Gonzalez et al. 2016). . In an experiment when conspecific and heterospecific larvae were removed, *Ae. aegypti* did not avoid heterospecific breeding sites for oviposition—this may indicate the visual cue of larvae enables *Ae. aegypti* to detect other species (Tuñón et al. 2025). These dynamics reflect the delicate balance that gravid females strike between resource availability and competitive pressures .

Not only do gravid *Ae. aegypti* use conspecific immatures in breeding sites as cues for oviposition; they also use other conspecific female mosquitoes as cues. Aggregations of female *Ae. aegypti* at breeding sites can occur and females use chemosensory dependent communal cues to regulate their population densities to either aggregate or disperse (Costa-da-Silva et al. 2024). Aggregation occurs independently of oviposition activity, water interaction and skip oviposition. Lower densities of aggregation may indicate a suitable breeding site. There may be as of yet unidentified intraspecific adult VOCs that enable adult female mosquitoes to locate each other. Understanding these nuanced behaviors is essential for developing effective vector control strategies. By leveraging the tendency of gravid *Ae. aegypti* to respond to specific environmental signals and their avoidance of overcrowded sites; targeted interventions such as oviposition traps, habitat management and lures that aggregate female mosquitoes could be designed to reduce mosquito population growth and disease transmission.

3. Chemical Cues and Predators in Oviposition Site Selection

It is crucial for gravid female *Ae. aegypti* to choose sites that provide the best chance for survival and optimal development for their offspring. Factors such as the presence of nutrients,

reduced competition from conspecifics as well as heterospecifics, and an absence of predators optimizes the chances for proper development and survival of offspring (Mosquera et al. 2021, Khan et al. 2022, 2023). Site selection is in part influenced by a complex interplay of chemical cues in the environment, which signal the availability of resources, the risk of competition or predation, and the overall suitability of the habitat for offspring survival. Physicochemical properties such as conductivity, total dissolved solids, and water hardness are important factors for oviposition (Motta-Yanac et al. 2025). Polarized light can also enable mosquitoes to detect aspects of the breeding site such as pH but they use other senses, such as touch, to detect suitable habitats. Prior to oviposition, gravid females will touch the water of a site with their legs and mouthparts. Experiments removing different sensory organs of the mosquito found that chemoreceptors on the tarsi and potentially tibia are required for perception of compounds that act as oviposition stimulants or deterrents (Hudson 1956).

For instance, high salt concentrations are lethal to *Ae. aegypti* larvae, so gravid females will avoid ovipositing in sites with high levels of sodium (Hudson 1956, Matthews et al. 2019). Experiments showed that they can detect the difference between 0 and 0.136 M of NaCl in water with fewer eggs laid in solutions at or above 0.085 M NaCl (Hudson 1956). Additional experiments demonstrated that high concentrations of KCl, MgCl₂ and Na₂SO₄ have similar effects on oviposition (Hudson 1956). Certain sensory neurons express ppk301, a member of the DEG/ENaC (degenerin/epithelial sodium channel) family, and play a key role in initiating oviposition (Matthews et al. 2019). These neurons exhibit two distinct responses: a ppk301-dependent response to water that drives gravid females to oviposit in sites with low salt concentrations, and a ppk301-independent response to salt that inhibits oviposition under high-salt conditions (Matthews et al. 2019).

CO₂ also appears to function as an oviposition cue. It is a well known cue for host seeking behavior. However, the function of CO₂ changes in gravid female mosquitoes with CO₂ levels

enabling female *Ae. aegypti* to either aggregate or disperse at breeding sites (Costa-da-Silva et al. 2024). High CO₂ levels can act as a communal cue to signal dispersion thereby preventing high densities of female mosquitoes aggregating around a single breeding site, thus preventing competition among offspring. This dispersal cue may also result from the presence of high densities of conspecific immatures or hosts or predators. CO₂ levels do not differ between *Ae. albopictus* and *Ae. aegypti* larvae and does not appear to act as a specific cue of conspecific or heterospecific larvae in a site (Tuñón et al. 2025).

The presence of mosquito larvae is not the only biological factor considered during breeding site selection. Predator presence also influences oviposition site selection. For example, *Toxorhynchites* (*Tx.*) larvae, known predators of mosquito larvae, can be found in containers where *Ae. aegypti* lay their eggs. All instars of their larvae feed on *Ae. aegypti* larvae and they can be cannibalistic as well (Collins and Blackwell 2000). Prior to pupation *Tx.* 4th-instar larvae will kill other organisms in the breeding site, leaving the corpses of their prey uneaten. This is thought to be a defensive behavior to eliminate organisms that could threaten the survival of the vulnerable pupal stage (Collins and Blackwell 2000). One might expect these predators to deter oviposition; however, this behavior makes aquatic habitats more attractive for oviposition. The predatory activity results in reduced competition in aquatic habitats by consumption of early instar *Ae. aegypti*. Studies show that gravid *Ae. aegypti* females are not repelled by the presence of *Tx.* larvae in potential oviposition sites (Albeny-Simões et al. 2014). Predator-driven trophic cascades in small containers can indirectly increase microbial abundance and resource availability (Albeny-Simões et al. 2014). Their predatory activities reduce the density of competing larvae and can stimulate microbial growth through various mechanisms, such as partially eaten prey and fecal matter serving as substrates for microbial growth. Such effects are more pronounced in small aquatic habitats when only a few predators present. This creates

conditions that favor egg-laying by gravid females, despite the predation risk (Albeny-Simões et al. 2014).

There are multiple reasons why *Ae. aegypti* have not evolved a strong preference for predator-free oviposition sites. Gravid females move more slowly, and the longer they spend searching for ideal sites increases the probability of predation by organisms such as spiders or dragonflies, prior to egg deposition (Albeny-Simões et al. 2014). Furthermore, their skip oviposition behavior increases the likelihood of offspring survival even in the presence of aquatic predation.

Overall, the ability of *Ae. aegypti* females to detect and respond to this complex array of chemical cues underscores the importance of these signals in shaping oviposition behavior. Understanding these factors can inform the development of targeted surveillance and control strategies, such as attractant-based oviposition traps and habitat management interventions.

4. Volatile Organic Compounds and Other Oviposition Cues

Gravid *Ae. aegypti* females rely heavily on airborne and water-borne volatile organic compounds (VOCs) to evaluate potential oviposition sites. VOCs serve as evidence of microbial activity, of conspecific and heterospecific eggs and larvae, and of decaying organic matter, within aquatic habitats (Mosquera et al. 2021, Khan et al. 2023, Collins and Blackwell 2000). Gravid females use VOCs to assess site quality, balancing the benefits of nutrient-rich environments against the risks of overcrowding, cannibalism, or predation.

VOC cues from conspecific and heterospecific immature stages enable gravid *Ae. aegypti* to perceive the characteristics of a site. These include the presence (or absence) of site-specific resources, overcrowding from larval conspecifics/heterospecifics, sensation of the developmental stages of con/heterospecifics, and the water quality of the oviposition site. An

analysis of cuticular hydrocarbons from eggs and 1st-3rd instar larvae demonstrated that those life stages exhibit similar chemical profiles after hexane extraction, followed by GC/MS analysis (Wang et al. 2019). Pupae do not appear to provide chemical signals that would influence females' oviposition decisions (Khan et al. 2023). A subsequent experiment examined VOCs from each of those groups and treated the oviposition sites with multiple concentrations of a VOC blend in hexane as well as hexane alone. The study found that at high concentrations, fewer eggs were deposited, whereas at lower concentrations, oviposition was stimulated (Khan et al. 2023). As late-stage larvae transition into the non-feeding pupal stage, competition for resources decreases, however some resources may be reduced or depleted as a result of their prior development (Khan et al. 2023).

Infusion of potential oviposition sites with plants such as Bermuda grass can result in the release of p-cresol as a VOC (Afify and Galizia 2014). Laboratory assays have shown that low concentrations of p-cresol stimulate egg-laying behavior in *Ae. aegypti*, while higher concentrations (10^{-8} – 10^3 ppm) deter it (Afify and Galizia 2014). Similar effects have been observed in other mosquito species, such as gravid *Culex (Cx.) quinquefasciatus* and *Cx. tarsalis*, which are attracted to p-cresol at 10^{-4} ppm. A concentration of 10^{-1} ppm stimulates egg-laying in *Cx. quinquefasciatus*. In two species of *Tx.* mosquitoes (*Tx. brevipalpis* and *Tx. amboinensis*), p-cresol at concentrations of 1, 10, and 50 ppm stimulates oviposition, while for *Tx. splendens*, 10 ppm is effective. In *Ae. albopictus*, p-cresol stimulates oviposition at 10^{-5} ppm, while 8.3 ppm acts as a repellent.

In contrast, m-cresol is largely inactive for *Ae. aegypti*, except at the highest dose tested, 10^3 ppm, which acts as a deterrent. For other mosquito species, including *Ae. triseriatus*, *Tx. moctezuma*, and *Tx. amboinensis*, m-cresol at 3 ppm stimulates egg-deposition. When both p- and m-cresols are present together in a 1:1 mix, they act as an oviposition deterrent. When presented in separate cups within the same cage at 10^2 ppm, both cresols resulted in

significantly fewer eggs than in water controls. If p-cresol and m-cresol are near each other in neighboring breeding sites, they have a repelling effect on gravid *Ae. aegypti* mosquitoes. In concentration-choice tests, both cresols were deterrents only at the highest concentration.

Organic detritus forms the base of the aquatic food web. Detritus such as dead larvae and decaying plant matter supports microbial growth, making these sites more attractive to gravid females. Organic infusions of fermenting leaves and other plant matter are also significant cues for oviposition. Different leaf infusions can have attractive effects. For instance, hay infusions that contain 3-methylindole are attractive to *Ae. aegypti* (Ganesan et al. 2006). In laboratory settings, sites with fermenting leaf infusions, such as those from white oak and bamboo, also attract more gravid mosquitoes to deposit eggs than sites containing filtered water alone (Ponnusamy et al. 2008). Microbial fermentation of this matter produces kairomones, chemical signals highly attractive to gravid *Ae. aegypti*, which act as oviposition stimulants (Ponnusamy et al. 2008). When these infusions are sterilized, they lose much of their attractiveness for egg-laying, as microbial activity is removed; however, they can still lure gravid females to the site (Ponnusamy et al. 2008).

Bacteria are important as they play critical roles in larval development by aiding in energy storage and the biosynthesis of vitamin B9 as well as acting as a source of raw nutrients (Romoli et al. 2021). Aquatic microbial communities produce a variety of oviposition cues. *Pseudomonas aeruginosa* produces decanoic acid, which also serves as a cue for egg deposition (Benzon and Apperson 1988). The bacterial species *Acinetobacter calcoaceticus*, has also been implicated in the selection of oviposition sites. In laboratory studies, water containing larvae and *Acinetobacter calcoaceticus* attracted egg-laying females, whereas water containing only larvae acted as a repellent (Benzon and Apperson 1988). Bacteria produce a mix of carboxylic acids, ranging from nonanoic acid to octadecanoic acid, along with several carboxylic acid methyl esters that can affect oviposition choice (Ponnusamy et al. 2008).

Compounds such as nonanoic acid, tetradecanoic acid, and methyl tetradecanoate promote egg-laying at low concentrations but deter it at higher doses (Ponnusamy et al. 2008). A 10 ng blend containing 16% nonanoic acid, 83% tetradecanoic acid, and 1% methyl tetradecanoate was highly stimulatory for egg deposition; these ratios were based on profiles from lyophilized bacteria (Ponnusamy et al. 2008). Hexadecanoic acid methyl ester, on the other hand, acts as a repellent, and high bacterial densities in infusions can similarly deter oviposition. These bacterial cues thus provide gravid mosquitoes with nuanced information about habitat quality.

Bacteria can produce VOCs that stimulate oviposition (Ponnusamy et al. 2008). For instance, *Klebsiella sp.* and *Pseudomonas aeruginosa* produce VOCs that elicit contact stimulation or strong antennal responses in gravid *Ae. aegypti* and stimulated oviposition (Benzon and Apperson 1988, Mosquera et al. 2023). These VOCs often act in a dose-dependent manner, with low concentrations promoting oviposition, and higher concentrations acting as repellents (Seenivasagan et al. 2009, Boullis et al. 2021). Experimental work with a binary blend of 2-ethyl-1-hexanol and 2,4-di-tert-butylphenol produced by *Klebsiella sp.* is detected by gravid females' antennae and elicits a dose-dependent oviposition preference (Mosquera et al. 2023). This binary blend successfully recapitulated the egg-laying responses of gravid mosquitoes. When these cues were tested in an *orco* mutant strain of *Ae. aegypti*, which lacks a functional odorant receptor coreceptor, the mosquitoes failed to respond both behaviorally and physiologically. This finding highlights the critical role of the odorant receptor pathway in mediating chemical communication during oviposition-site selection. This may be because gravid females introduce these bacteria to aquatic sites during egg-laying, making the presence of the bacteria and their volatiles a reliable indicator of viable breeding sites to other conspecific females.

Carboxylic acid compounds also serve as cues for oviposition choice and are produced by *Ae. aegypti* larvae, with the highest production in the fourth instar (Wang et al. 2019). Dodecanoic

acid (C₁₂) and tetradecanoic acid (C₁₄) isomers are found in egg cuticles and in all larval and pupae stages. Other egg- and larva-associated compounds include (Z)-9-hexadecenoic acid (C₁₆) and octadecanoic acid (C₁₈). The egg cuticle also contains fatty acids of carbon chain-length C₁₂ to C₁ as well as methyl esters (methyl dodecanoate, methyl tetradecanoate, methyl (Z)-9-hexadecenoate, methyl hexadecanoate, hexadecanoic acid, methyl (Z)-9-octadecenoate, methyl octadecanoate, (Z)-9-octadecenoic acid) along with a lactone, 6-hexanolactone (Ganesan et al. 2006). Dodecanoic acid and (Z)-9-hexadecenoic acid show positive oviposition responses at 10–100 ppm, with (Z)-9-hexadecenoic acid peaking in attractiveness at 1–10 ppm and remaining constant up to 100 ppm (Ganesan et al. 2006). Tetradecanoic acid is most attractive at 1 and 10 ppm but loses effectiveness at higher concentrations, while octadecanoic acid induces a positive response at one ppm and a negative response at higher concentrations (Ganesan et al. 2006).

The straight-chain hydrocarbon n-heneicosane (C₂₁), found on late-stage *Ae. aegypti* larvae cuticles and *Ae. albopictus* larvae, acts as an attractant at 10⁻⁶–10⁻⁵ g odor plumes or 10 ppm solutions, but repels at concentrations such as 10⁻³ g, which likely indicates an overcrowded site (Seenivasagan et al. 2009, Gonzalez et al. 2016).

Other compounds, such as isopropyl esters, dodecyl lactone, octadecenoic acid, and medium-chain fatty acids, increase in fourth instar larvae (Wang et al. 2019). Isovaleric acid, myristoleic acid, myristic acid, and pentadecanoic acid are found in the fourth instar. Pupae were found to be associated with myristoleic acid, myristic acid, and pentadecanoic acid. Isovaleric acid is also present in larval food infusions and human sweat, indicating bacterial activity and elicits antennal responses from *Ae. aegypti* (Boullis et al. 2021). At 10 ppm, pentadecanoic acid acts as a positive oviposition stimulation cue. At concentrations of 10–100 ppm, myristoleic acid and isovaleric acid deter oviposition. A blend of isovaleric acid,

tetradecanoic acid, myristoleic acid, and pentadecanoic acid at 1 ppm acts as an attractant, while higher concentrations act as a deterrent (Boullis et al. 2021).

C₉ acids induce significant negative oviposition responses in *Ae. aegypti* at lower concentrations. When the ester concentration increases from 1 to 100 ppm, the ovipositional deterrent effect increases in all cases except for an ester of a saturated C₁₈ acid. In addition, methyl octadecanoate acts as a repellent at 10 ppm (Ganesan et al. 2006). The esters contained in eggs may act as oviposition deterrents to prevent conspecific overcrowding, a phenomenon observed in lepidopteran and other dipteran species as well. Higher concentrations appear to be associated with higher egg densities, resulting in a repellent effect.

Across all these chemical classes, a consistent pattern emerges: low concentrations indicate a productive, lightly used habitat, while high concentrations signal overcrowding, decay, or competition. This concentration-dependent switch in behavioral responses highlights how sensitive *Ae. aegypti* females are to nuanced chemical cues. Understanding these cues could guide the development of attract-and-kill ovitraps and other lure-based surveillance tools that exploit the mosquito's finely tuned chemosensory system.

5. Geosmin and Cyanobacteria

Geosmin is a microbial metabolite responsible for the characteristic earthy or musty odor found in surface water, soil, and certain foods (Wnorowski 1992, Liato and Aider 2017). For humans, geosmin is typically perceived as an unpleasant odor and is resistant to removal by common water purification methods, such as chlorination or oxidation (Wnorowski 1992). Its synthesis involves the methylation of L-methionine and folic acid (Wnorowski 1992). Cyanobacteria, such as *Fischerella muscicola*, produce the highest geosmin concentrations during their lag phase, with yields decreasing as the bacterial culture ages (Wnorowski 1992). Geosmin can be produced under both aerobic and anaerobic conditions (Wnorowski 1992).

The odors present in water bodies depend on the type and bloom of cyanobacterial species, with intensity and chemical profile varying accordingly (Lee et al. 2017). Different types of cyanobacteria produce either toxins or geosmin, serving as primary producers in both aquatic and terrestrial habitats (Churro et al. 2020). Cyanobacteria, such as *Oscillatoria simplicissima* and *Anabaena scheremetievi*, are notable producers of geosmin in water (Lee et al. 2017). The gene coding for the enzyme responsible for geosmin production is found across cyanobacteria, actinobacteria, and delta- and Gammaproteobacteria, and it exhibits high sequence similarity among soil and freshwater strains (Churro et al. 2020).

Cyanobacteria can be found throughout the water column with some species localized to the surface of the water, some migrate to different depths in response to environmental stimuli, and others are found primarily in the sediment (Carratalà et al. 2023). Cyanobacteria regulate their depth through a combination of buoyant gas containing vesicles and use carbohydrates as ballast to sink; with water column depth preferences varying depending on species (Carratalà et al. 2023). These bacteria are accessible to larvae and may provide nutritional benefits (Carratalà et al. 2023, Vázquez-Martínez et al. 2002). Cyanobacteria are a key food source for some mosquito larvae, including *Anopheles albimanus Wiedemann* (Vázquez-Martínez et al. 2002). In southern Mexico, cyanobacteria were prevalent in larval habitats, particularly in permanent freshwater bodies (Vázquez-Martínez et al. 2002). *Ae. aegypti* larvae have been documented feeding on cyanobacteria (García-Sánchez et al. 2017). It is hypothesized that *Ae. aegypti* larvae associate the presence of geosmin with cyanobacteria, which signals the presence of a primary food source (Vázquez-Martínez et al. 2002).

Environmental factors can modulate geosmin production; higher nitrogen-to-phosphorus ratios limit geosmin synthesis by cyanobacteria (Lee et al. 2017). Ozone treatment can reduce geosmin odors, replacing them with a fruity odor instead (Lee et al. 2017). *Gravid Ae. aegypti* correlates the presence of geosmin with the presence of microbes, a larval food source, in the

water, and thus choose to lay their eggs in that site (Melo et al. 2020). In vector control applications, geosmin has been successfully used as bait in oviposition traps to capture gravid *Ae. aegypti* females (Melo et al. 2020). Beetroot peel can serve as a natural substitute for geosmin in such traps (Melo et al. 2020).

6. Impact of Larval Nutrition on Adult Behavior

There can be many factors, including genetic and environmental factors, as well as factors related to pathogen populations, that determine mosquitoes' capacity to serve as effective disease vectors (Carvajal-Lago et al. 2021). A key predictor of an adult mosquito's ability to serve as a disease vector is the nutrition of its larvae.

The quality of nutrition available to *Ae. aegypti* larvae can significantly shape various aspects of adult behavior and physiology. Well-nourished larvae typically develop into larger, more robust adults with higher fecundity, greater energy reserves, longer lifespans, and increased success in blood feeding (Klowden et al. 1988, Carvajal-Lago et al. 2021). Better energy reserves retained from their larval stage enable the adult to obtain its initial meal and increase survivability (Carvajal-Lago et al. 2021). These adults are better equipped to fight parasites and avoid infection. In contrast, larvae that experience poor nutrition due to lack of food or the necessity of rapid development in an ephemeral water source, grow into smaller adults, which may require two blood meals to produce a single batch of eggs (Klowden et al. 1988). Smaller adults are also less inclined to immediately seek hosts for blood meals post-eclosion and/or have reduced ability to do so. As such they may prioritize nectar feeding to provide additional energy for flight and developmental processes (Klowden et al. 1988, Carvajal-Lago et al. 2021). These mosquitoes may have increased susceptibility to arbovirus infection as adults because, as larvae, limited nutritional resources were allocated to development and survival rather than to immunity (Carvajal-Lago et al. 2021). For instance, with the Sindbis virus, adults that had poor

nutrition as larvae had a higher infection rate than those with proper nutrition (Carvajal-Lago et al. 2021). Also, *Ae. aegypti* adults derived from larvae that had low-insect detritus related to a high nitrogen percentage presented the highest rates of infection with the Zika virus (Carvajal-Lago et al. 2021). However, the data from lab-based experiments on Sindbis virus and Zika virus may not entirely reflect real-world conditions due to the complexity of ecological interactions and physiological states. These factors could incorporate additional variables that could complicate our understanding of the true relationship between larval nutrition and adult vectorial capacity. A longer lifespan can also allow an infected mosquito to infect more hosts in its quest for blood meals for egg production. There are different effects regarding poor larval nutrition and the ability to vector different diseases as adults (Carvajal-Lago et al. 2021).

Host-seeking behavior in female *Ae. aegypti* is further modulated by access to sugar after blood feeding. When provided with sucrose after a blood meal, females reduce their host-seeking activity as they develop their eggs. However, if sucrose access is restricted, they maintain active host-seeking behavior (Klowden et al. 1988). Competition among larvae during development can also influence adult reproductive ability, suggesting that increased competition may lead to less successful reproduction in emerging adults (Klowden et al. 1988).

In laboratory studies, larvae provided with ample nutrition develop synchronously and produce large, healthy adults (Klowden and Lea 1978). Larvae fed poor diets emerge as smaller adults at staggered times (Klowden and Lea 1978). Field-collected adults are typically smaller than laboratory-reared ones, often requiring two blood meals to complete one reproductive cycle (Chadee 2012).

Blood meal size in adult females is a critical factor in determining reproductive success and the cessation of host-seeking behavior. Depending on the size of the blood meal and the adult mosquito, the amount of blood may be sufficient to produce a batch of eggs and may cause

enough abdominal distension to signal the female to cease host-seeking activities (Klowden and Lea 1978).

A well-fed larva that develops into a robust female will require between 2.6 and 3.5 μL of blood to stop seeking hosts. If the blood meal exceeds 4 μL , host-seeking behavior ceases entirely. Smaller-sized females require less blood (1.6–2.5 μL) to suppress host-seeking behavior, underscoring the relationship between blood meal volume and adult mosquito size (Klowden and Lea 1978). Additionally, the density of mosquitoes feeding on a host can affect blood meal size, as high densities may make hosts more defensive, leading to smaller blood meals and the need to feed again (Klowden and Lea 1978).

7. Knowledge Gaps and Application of New Technologies to Old Questions

Considerable research progress in this field has identified an array of chemosensory cues that mediate *Ae. aegypti* oviposition site selection. However, many knowledge gaps remain to be explored to deepen and add nuance to our current understanding. These gaps are in part due to the experimental difficulties of controlling complex multivariate systems while maintaining fidelity with field conditions. One of these challenges comes from the use of long colonized strains of *Ae. aegypti*, such as the Rockefeller strain which has been in culture for decades and lacks the myriad genetic adaptations and environmental cues that guide the behavior of wild mosquitoes. These experiments are often performed in small cages that cannot completely reflect environmental contexts. This leaves open whether identified attractants, repellents, stimulants, and deterrents retain their behavioral activity for wild-type populations navigating complex three-dimensional environments with competing odor plumes and multiple candidate sites. Cue validation using recently colonized populations under semi-field or full-field conditions is important for translation of laboratory findings into vector surveillance and control tools.

Another challenge faced by researchers is the development of ways to integrate the effects of crosstalk across multiple sensory modalities including vision, olfaction, gustation, and mechanosensory stimuli. The question of how different stimuli are weighted, integrated, and acted on during decisions on whether a site would be a good habitat for offspring is important to understand, but also technically difficult. However, this knowledge would be extremely informative in understanding how different stimuli should be integrated into ovitraps to optimize their function as well as improving our understanding of the ecological dynamics site choice in urban and sub-urban environments.

Additional investigation would benefit the development of a deeper understanding of the role inorganic water chemistry plays in site preference. The role of sodium concentration on site selection by *Ae. aegypti* is fairly well understood. However, there is relatively little information available on the influence of other ions and minerals such as ammonium, phosphate, calcium, and magnesium. More information on the role these ions play in site selection in terms of dose response, effects on volatile organic compounds, and dynamics of microbial communities at different concentrations could provide important understandings of the role of water chemistry on chemosensory stimuli.

Finally, while microbial communities are known to be important for the production of chemosensory cues and as a source of nutrients for developing larvae, the field lacks information on the metabolic functions provided by the various microbial taxa found in oviposition sites and how these functions interact with environmental substrates to produce chemosensory cues. Identification of key microbial constituents or specific metabolic functions required for production of chemosensory products would also provide key information for production of standardized and deployable microbial lures for oviposition trap optimization.

These are all difficult subject areas to approach. However, the development of new high throughput technologies such as metabolomic analyses, metagenomic sequencing, new spectral analyses (such as Raman and infrared spectroscopy), and advanced statistical analyses and modeling of complex multivariate datasets allow for detailed investigations of these complex systems. Leveraging these new technologies to expand on and deepen existing understandings could provide novel insights into oviposition biology and significant improvements on existing gravid trap technologies.

8. Conclusion: Application in Vector Surveillance and Control

A detailed understanding of *Ae. aegypti* oviposition behavior, including its regulation by VOCs, microbial metabolites, conspecific and heterospecific cues, and environmental context, holds considerable promise for enhancing vector surveillance and control strategies. Ovitrap have proven highly effective for monitoring *Ae. aegypti* populations because they exploit the natural tendency of gravid females to lay eggs in man-made containers (CDC 2020, McGregor and Connelly 2021, Walker et al. 2021, Otero 2025). The traps are useful for surveillance and population control, are typically monitored weekly, and are designed to prevent the emergence of adult mosquitoes. They are cost-effective, easy to move, and are not detrimental to the location where they are placed. Additionally, eggs can be reared to identify the mosquito species present in the area.

However, ovitraps do have some drawbacks that limit their efficacy relative to traps that utilize host cues which target blood meal seeking females. Gravid autocidal traps have some requirements which can complicate the logistics of their implementation. Multiple traps per household are required to compete with natural oviposition sites and source reduction prior to deployment is needed to optimize efficacy. Traps also require regular maintenance to remain operational. These factors require community cooperation and high logistical capacity which can

be difficult to maintain in under-resourced areas (Acevedo V. et al. 2016, Barrera R. et al. 2014). Beyond the logistical issues, optimization of traps with the right oviposition attractants and stimulants is essential to their function.

In spite of these caveats, gravid autocidal traps remain a promising and environmentally friendly method of vector control which has the potential to be significantly improved via attractant optimization, implementation of public education and outreach efforts, and modification of suburban and urban infrastructure development practices to minimize the presence of human derived breeding sites. Incorporating attractants mentioned in this review, such as p-cresol, 1 ppm of caproic acid, bacterial VOCs, or microbial kairomones, can further enhance ovitrap efficacy and specificity, while adding larvicides, such as granular temephos, an organophosphorus larvicide, creates lethal lures that reduce mosquito populations at the source (Ong and Jaal 2015, Martínez-Mercado et al. 2022).

As *Ae. aegypti*'s range continues to expand due to urbanization and climate change, deploying surveillance and control tools that align with the species' oviposition ecology will become increasingly important. Continued integration of physiological and behavioral data from laboratory and field studies will support the refinement of mosquito monitoring and control. Ultimately, translating oviposition behavior into field-applicable technologies will strengthen proactive vector management and mitigate the risk of arbovirus transmission in both endemic and newly affected regions.

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Chapter 2

Title: Examining Wood Biochar Effects on *Aedes aegypti* Oviposition Behavior in Laboratory and Field Settings

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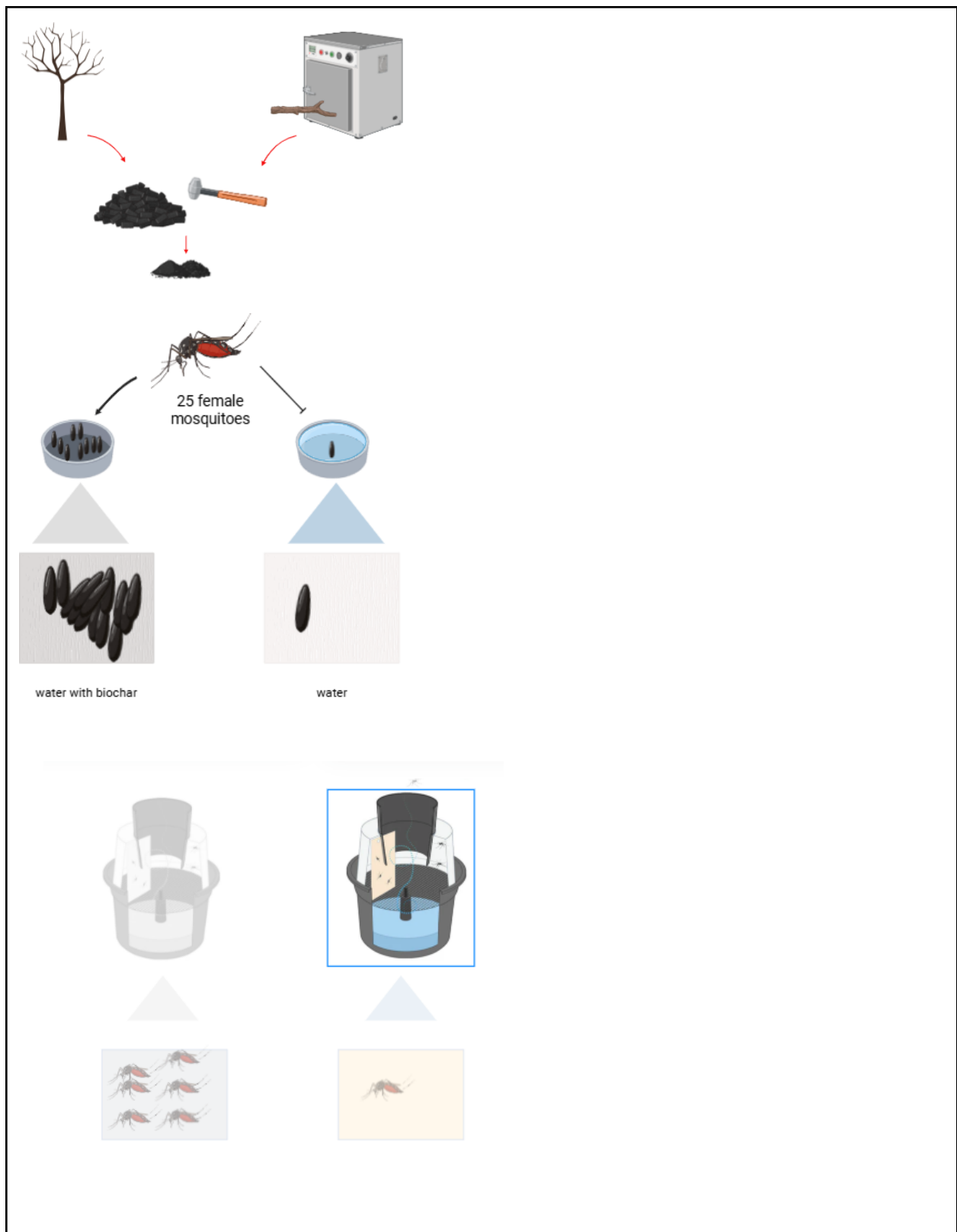
Abstract:

Ae. aegypti is a major vector of arboviruses such as dengue, Zika, chikungunya, and yellow fever, and poses a major global public health threat. Climate change-induced temperature shifts and anthropogenically modified environments compound these threats by creating conditions that facilitate the expansion of *Ae. aegypti*'s range. Climate change has also intensified wildfire activity, which can deposit biochar into standing water sources within urban and suburban

landscapes, potentially creating novel breeding habitat as *Ae. aegypti*'s range continues to expand into fire-prone areas. This paper explores the effects of infusing wood biochar (forest-fire-derived and lab-generated) on *Ae. aegypti* oviposition behavior and biology. It focuses on the impact of biochar on oviposition behavior and larval development. Biochar is defined as the product of any organic matter, such as wood, being heated in the absence of oxygen (pyrolysis). Experiments evaluating the oviposition preferences of gravid *Ae. aegypti*, in response to oviposition sites containing various substrates, including hay, wood biochar, unburned wood, and larval water, demonstrates a pronounced preference for wood biochar. The results showed that more than 60% of eggs were deposited in biochar-containing oviposition sites. Furthermore, oviposition sites treated with Zeolites, silicon-based compounds with adsorptive properties similar to biochar, also show similar increases in attractivity. Additionally, oviposition choice assays show that gravid females prefer biochar-infused water, indicating that biochar affects the chemosensory profile of potential breeding sites. To determine whether this effect could improve the function of oviposition traps, biochar supplementation of Biogents Gravid Autocidal Ovitrap in a suburban neighborhood shows significant increases in catches of *Ae. aegypti* in the biochar-supplemented versus non-supplemented traps. These findings suggest that biochar could be a low-cost, accessible, and effective means of improving the efficacy of gravid trapping for *Ae. aegypti* as well as providing new insights into the behavioral and biological underpinnings of oviposition site selection in this important vector species. While the exact mechanisms underlying biochar's behavioral and developmental effects remain unclear, ongoing investigations seek to elucidate this phenomenon.

Keywords: *Aedes aegypti*, biochar, wildfire, ecological impact, oviposition behavior

Graphical abstract:



Introduction:

The yellow fever mosquito, *Ae. aegypti*, is an efficient and prolific vector of multiple arboviruses, such as yellow fever, dengue, Zika, and chikungunya (Lopes et al. 2014; M. U. G. Kraemer et al. 2015; M. U. Kraemer et al. 2015; Souza-Neto et al. 2019). Its effectiveness as a vector has driven the re-emergence of dengue fever in many areas, including the US, where a record of 3798 cases reported in 2024—this was a 359% increase over the previous 14 years annual average (Sandra J. Kiplagat et al. 2026). An estimated ~400 million dengue infections occur annually, with as many as 5.6 billion people at risk of dengue and other overlapping arboviral infections (Bhatt et al. 2013; Lim et al. 2025).

One factor contributing to *Ae. aegypti*'s success as a disease vector is its ability to thrive in urban and suburban environments. Gravid (egg-laying) females prefer to lay eggs in artificial water-holding sites, such as discarded containers, plant pots, tires, and other man-made standing-water holding structures, which account for the large majority of egg deposition sites (David et al. 2023; McGregor and Connelly 2021). They engage in “skip oviposition,” distributing eggs derived from a single gonotrophic cycle across multiple sites (Colton et al. 2003).

Documented oviposition attractants include water from previously inhabited larval breeding sites which contain remnants of larval sheddings and fish food as well as fermented hay/alfalfa-infused water. The hay/alfalfa infusion is commonly used as an attractant in oviposition traps (Wong et al. 2011; Allan and Kline 1998). However, the exact factors/mechanisms by which *Ae. aegypti* make site selection choices are not fully understood.

Furthermore, the difficulty of locating and eliminating the numerous artificial containers used as breeding sites by *Ae. aegypti* presents an important challenge for vector control. Current control programs commonly rely on source reduction, larvicides, and adulticides, but these approaches can be difficult to implement consistently across urban environments and may be limited by

insecticide resistance (Achee et al. 2015; Moyes et al. 2017). These limitations have increased interest in complementary control strategies that target gravid females during the process of selecting oviposition sites. Oviposition traps are widely used for surveillance, but increasing their attractiveness through the use of effective chemical or organic attractants may improve their ability to detect and target gravid females (Fay and Eliason 1966; Allan and Kline 1998). More effective attractants could therefore enhance trap-based approaches by drawing females toward artificial oviposition sites and away from naturally occurring containers, potentially providing a reduced insecticide approach to *Ae. aegypti* management. Understanding the environmental and chemical cues that influence oviposition site selection is thus important for both improving surveillance and developing novel control strategies.

Among these challenges, with the spread of urbanization, *Ae. aegypti* populations have thrived and also expanded into new geographic regions. For instance, *Ae. aegypti* have spread into California, where vector control personnel in the Central Valley first detected established, persistent populations during the summer of 2013 (Gloria-Soria et al. 2014). Since then, *Ae. aegypti* has continued to spread across the state, with its range expanding each year (Metzger et al. 2017; “VectorSurv Maps,” n.d.).

In parallel with the *Ae. aegypti* invasion, wildfires in California have become larger and more intense, moving beyond forested areas and causing catastrophic damage in suburban and urban areas (Moritz et al. 2014). Wildfires play a key role in shaping and regulating forest ecosystems, but fire suppression by western forestry policies has resulted in the accumulation of fuel loads, driving larger, hotter, and more frequent fires. Depending on severity, it can take years for a burned area to recover its biodiversity (Ruchin et al. 2021). Projections suggest that climate change will lengthen the fire season in Southern California (C. Dong et al. 2022).

Wildfires occurring in urban and suburban environments alter the physical and hydrological conditions of burned areas and surrounding locations that can result in breeding site production. Because fire reduces soil infiltration, rainfall over burn scars can generate substantial surface runoff, causing water to pool in low-lying areas and other surface features (Cotton 2021; US Department of Commerce, n.d.). These effects can persist for months, as burn scars alter local surface temperature, moisture, and precipitation patterns well after the fire itself has been contained (Blackford et al. 2026). Firefighting activities may also contribute to the accumulation of standing water, while ash, vegetation, and other fire-related debris can enter and collect in these water sources. These newly created or altered water holding sites can provide potential oviposition habitats for *Ae. aegypti*.

Urban wildfire affected areas may contain numerous potential *Ae. aegypti* breeding sites, including swimming pools, spas, containers, and other water-retaining structures. Following the 2025 Eaton and Palisades fires in California, 1,475 pools were identified with broken equipment, stagnant water, and wildfire debris (Seidman 2025). Within the Eaton Fire burn zone, approximately 1,700 parcels containing pools or spas required monitoring or pesticide treatment by the San Gabriel Valley Vector Control District to address potential mosquito breeding sites (Seidman 2025). These observations demonstrate that wildfire affected neighborhoods can contain abundant standing water habitats capable of supporting mosquito development, creating a potential pathway through which wildfire-derived materials such as biochar, may interact with *Ae. aegypti* breeding-site ecology.

The ecological effects of wildfire byproducts, such as biochar, have not been extensively investigated in relation to mosquito ecology, and there is little to no research on how fire-modified landscapes influence mosquito ecology, behavior, and physiology. Biochar itself, however, is well studied in the context of restorative agriculture, carbon sequestration, and soil remediation (Spokas et al. 2012). Biochar is produced via pyrolysis, which involves heating

organic material (e.g., wood) in the absence of oxygen. Pyrolysis conditions such as temperature, pressure, oxygen levels, and the type of biomaterial, can affect the composition and mechanical properties of the resulting biochar, resulting in variations in biochar characteristics, such as surface area, pore size, stability, nutrient and mineral content, and the abundance of surface functional groups (Tomczyk et al. 2020). In particular, temperature has significant effects on biochar composition with lower temperatures resulting in less exposed surface area and more nutrients due to incomplete pyrolysis and offgassing; while higher temperatures are associated with a larger surface area and fewer remaining nutrients (He et al. 2021). The intricate structure that remains after pyrolysis results from the xylem and phloem channels that transport water and nutrients throughout the plant. During pyrolysis, the internal components of the tissue are gasified and vaporized away, leaving behind the carbon skeleton of these intricate structures. The remaining material has a tremendous surface-area-to-volume ratio. The resulting surface area is coated with charged molecular functional groups that have high cationic and anionic exchange capacities and can adsorb large amounts of molecules from the surrounding environment, acting as a filter.

These bio/physical properties could have significant effects on water chemistry/quality in potential *Aedes aegypti* breeding sites. However, the effect of biochar on aquatic invertebrates such as mosquitoes and the associated aquatic larval ecosystems is understudied. Most prior studies focus on effects of biochar in soil as a bioremediation agent. In that context it facilitates sequestration and conversion of environmental contaminants, including heavy metals and persistent organic pollutants, into less bioavailable and soluble forms, and its stability in soil (a lifespan of over a century) supports long term carbon sequestration (Guo et al. 2020; Cara et al. 2022). As a non-corrosive alkali, it improves soil quality by reducing aluminum and manganese toxicity, lowering soil acidity, and increases bioavailability of key micronutrients (Yu et al. 2019). Additionally, biochar contains bioavailable carbon and nutrients— including calcium, nitrogen,

potassium, phosphorus, magnesium, and sulfur—that can supplement animal feed and support soil microbial activity (Schmidt et al. 2019). In urban home gardens, biochar replenishes soil nutrients and enhances soil health. Following wildfires, nitrogen and phosphorus levels in affected ecosystems can increase by 5 to 60-fold, further influencing soil composition and nutrient cycling (Spencer et al. 2003).

In aquatic environments, biochar generated at different temperatures exhibits varying properties that influence its ability to adsorb various pollutants, including endocrine-disrupting chemicals (which mimic hormones found in living organisms) and pesticides (M. Dong et al. 2023). Ash and nutrient-rich runoff entering nearby water bodies can alter local nutrient dynamics and water quality (Woods and Balfour 2008). These effects are not uniformly beneficial: under oxygen-limited conditions, biochar rich in free radicals can elevate reactive oxygen species in aquatic systems, inducing oxidative stress in aquatic organisms (Zhang et al. 2019).

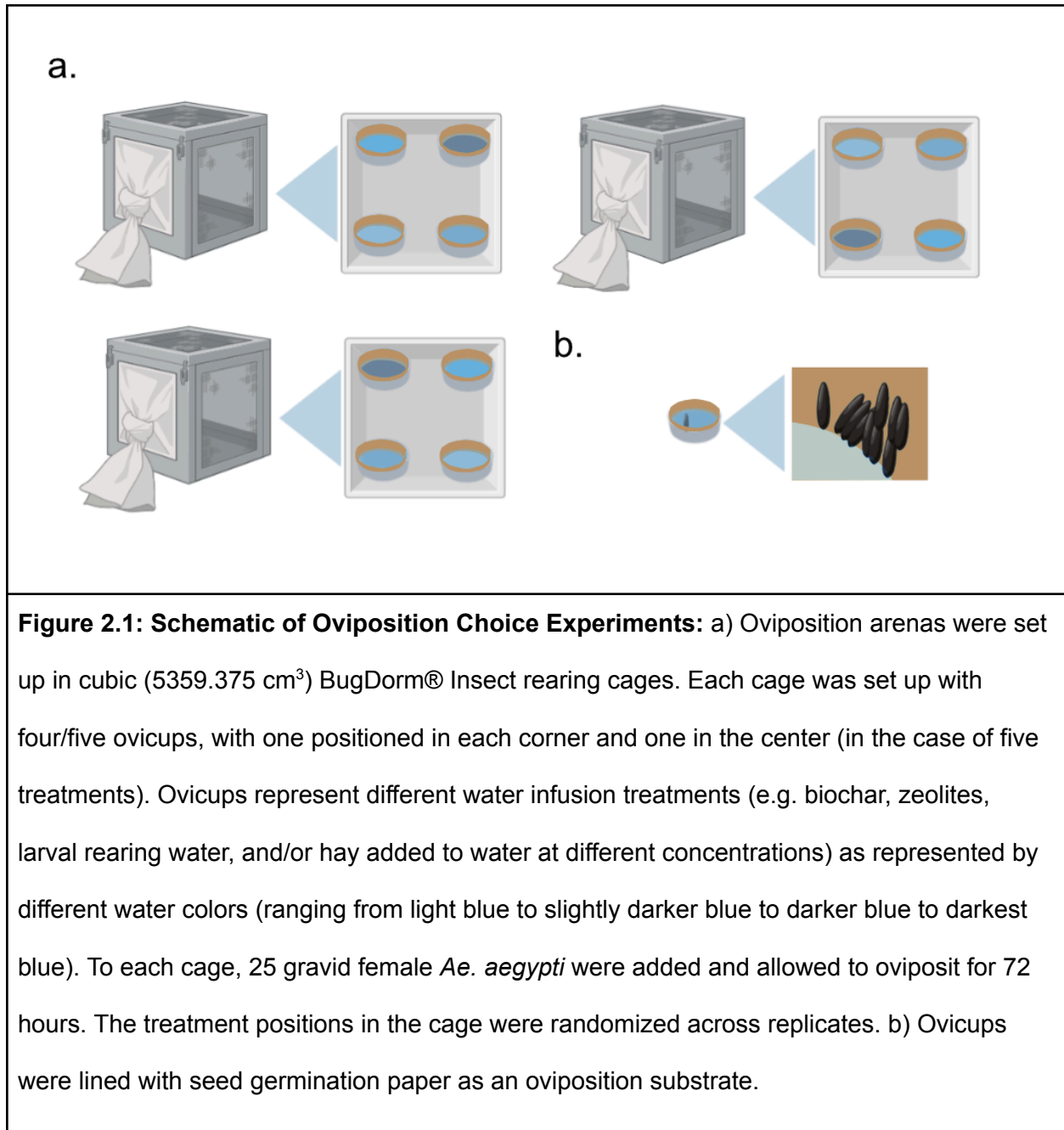
Biochar's high surface area and adsorptive capacity also give it the potential to act as a volatile sink, sequestering organic compounds from the surrounding environment (Ahmad et al. 2014). This property may be especially relevant to *Ae. aegypti* ecology, since volatile chemical cues influence oviposition site selection (Mwingira et al. 2020). Compounds associated with high densities of conspecific larvae can repel gravid females, while others (e.g. larval compounds released at low density, beneficial microbes) can attract them and stimulate oviposition (Melo et al. 2020; Wong et al. 2011).

Given biochar's high surface area-to-volume ratio, adsorptive capacity, nutrient/micronutrient supplementation, and microbial habitat provisioning, its physical and chemical properties are well positioned to influence the composition and properties of environmental cues and resources that mediate *Ae. aegypti* breeding-site selection and oviposition behavior. Our hypothesis is that wood-based biochar will have a positive effect on *Ae. aegypti* breeding site

selection due chemosensory cues derived from its effects on water chemistry, nutritional availability and microbial diversity. Here we examine the potential for biochar to influence oviposition behavior through a combination of lab and field assays to better understand the effects of biochar infusion on the *Aedes aegypti* oviposition behavior and as a potential accessible and cost-effective supplement in gravid autocidal traps to improve monitoring and control activities by vector control districts.

Results

To measure biochar's effects on oviposition behavior in *Ae. aegypti*, a choice assay was developed to quantify site choice by gravid females. Assays were performed in cubic cages containing 4-5 oviposition sites, with each site consisting of a plastic condiment cup with 30 mL of dechlorinated tap water, seed germination paper as the oviposition substrate. Depending on the experiment, Infusion treatments included biochar (percentage dry weight/volume) in different concentrations, derived from different plant tissues, or generated under different temperatures. In addition, Non-biochar treatments included zeolite infusions, and previously described *Aedes aegypti* oviposition attractants including previously inhabited breeding site water, and hay infused water (Figure 2.1). The biochar used in most of these experiments was produced from Manzanita wood (*Arctostaphylos densiflora*) pyrolyzed in a muffle furnace. This wood type was chosen as initial experiments employed biochar recovered from a recent local wildfire in Frenchtown California in a stand of predominantly Manzanita trees. Manzanita are native to the Western United States and are frequently used as ornamental plants due to their hardiness and drought tolerance.

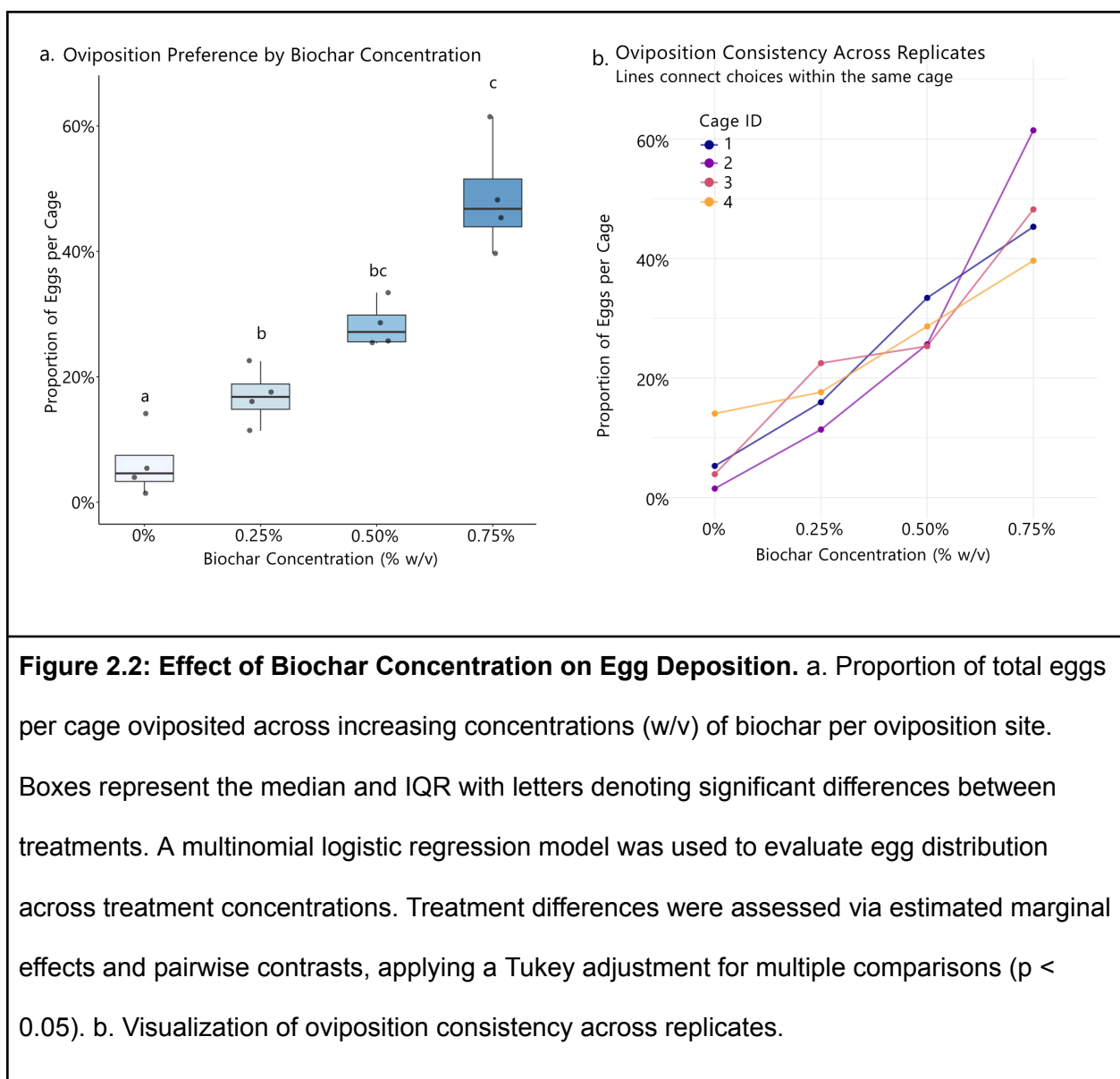


Effect of Biochar Concentration on Oviposition: Biochar Concentration Increases Egg Deposition.

Gravid females were provided access to four oviposition sites containing a range of concentrations (0%, 0.25%, 0.5%, and 0.75% w/v) of water infused with wildfire-derived biochar. After 72 hours, egg papers were removed from the cages, and egg counts per treatment were quantified by microscopic examination.

Our initial hypothesis was that the presence of biochar could have a positive effect on oviposition due to its physical properties, including its adsorptive capacity, large surface area, porosity, pH buffering capacity, sequestration or precipitation of heavy metals, adsorption and facilitated degradation of organic compounds, and alteration of the availability of nutrients, along with other dissolved materials.

The resulting data indicates that biochar produces a concentration-dependent attractive effect on gravid females (Figure 2.2). Ovicups containing the wildfire-derived Manzanita biochar showed consistently higher egg proportions than water containing no biochar, with the proportion of eggs showing a strong positive correlation with biochar concentration.



Effect of Biochar Pretreatment and Filtration of Breeding Site Water on Oviposition

Behavior

The physical properties of biochar, suggest that the biochar itself may not be the attractant, but that the modification of water chemistry induced by its infusion could mediate the attraction

To test this hypothesis, gravid mosquitoes were exposed to water treated with increasing concentrations of biochar for 24 hours. However, prior to use in the assay, the biochar was removed from the water via filtration, and oviposition treatment sites were filled with the filtered biochar-treated water. The results indicate that biochar's attractivity effects on water do not persist after removal, as egg distribution was uniform across the four treatments, with no significant differences observed between treatments (Figure 2.3). This suggests that the physical presence of biochar in the water is required for oviposition attractivity.

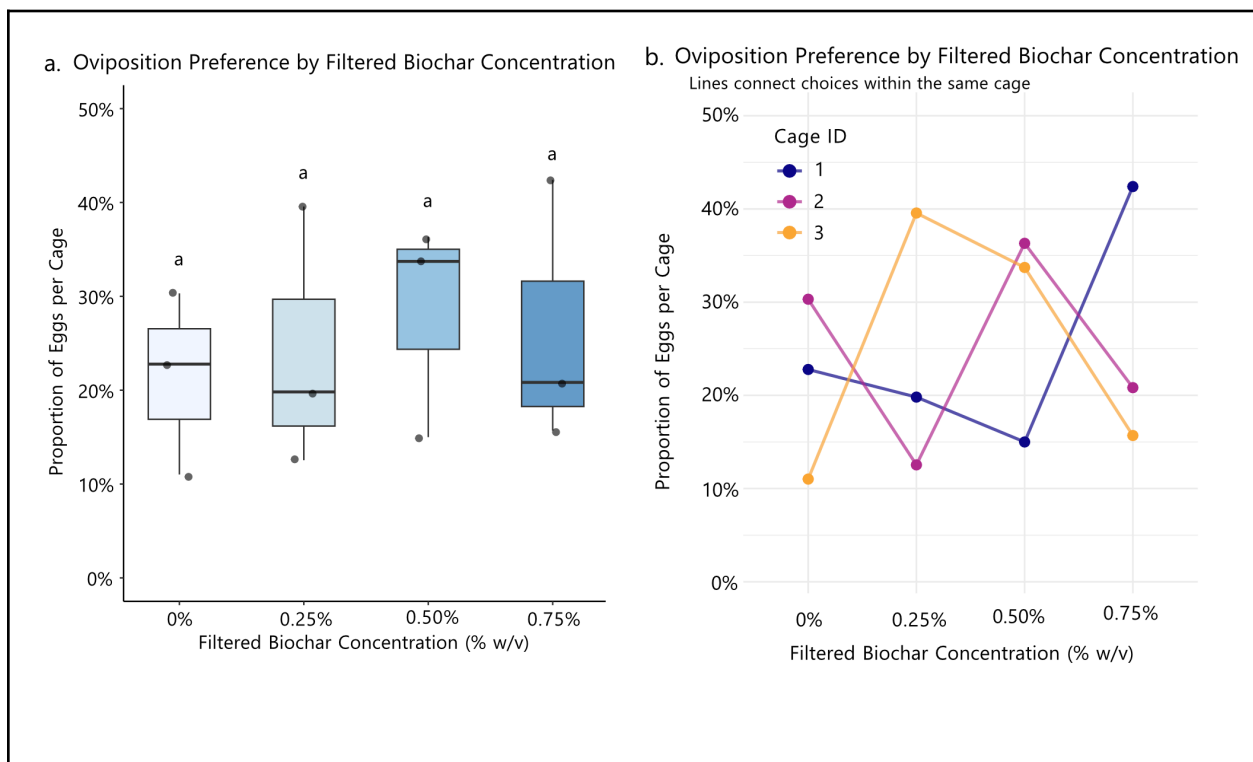


Figure 2.3: Filtered Biochar Infused Water Effects Egg Deposition: a. Proportion of total eggs per cage oviposited across increasing concentrations (w/v) of filtered biochar infused water per oviposition site. A multinomial logistic regression model was used to evaluate egg distribution across treatment concentrations. Treatment differences were assessed via estimated marginal effects and pairwise contrasts, applying a Tukey adjustment for multiple comparisons ($p < 0.05$). b. Visualization of oviposition consistency across replicates.

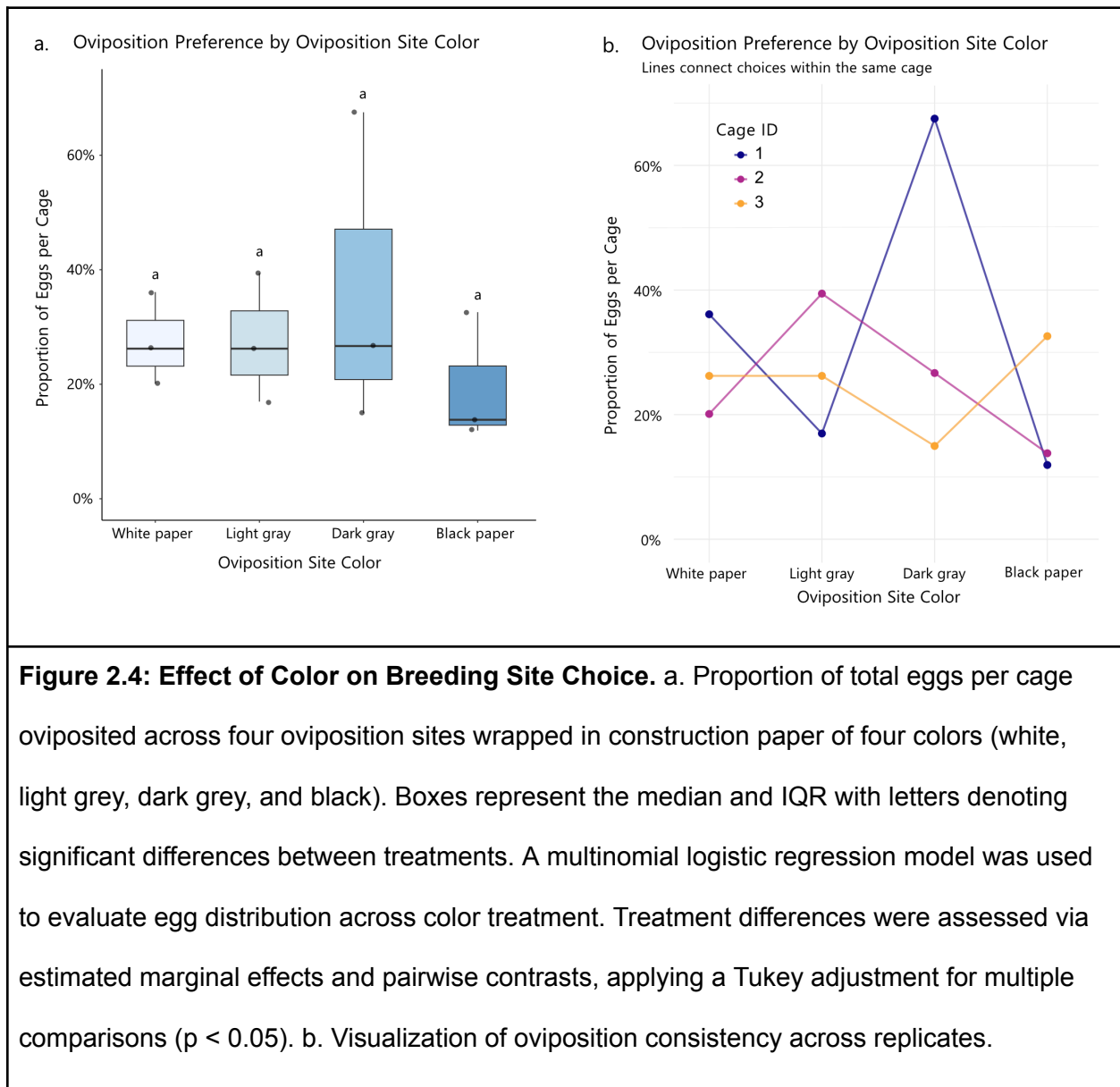
Effect of Oviposition Site Color on Egg Deposition Behavior

The introduction of biochar into oviposition sites alters water color, shifting it from clear to grayish-black, with the degree of darkness depending on the biochar concentration. Darker breeding sites are preferred by *Ae. aegypti* suggesting that the color change resulting from the addition of biochar could underlie the attractive effect of the treatment. To determine whether the site's attractiveness is due to coloration by the addition of biochar, the transparent oviposition containers were modified by covering their sides and bottoms with construction paper in four greyscale shades: white, light grey, dark grey, and black. The water and oviposition paper used in the ovicups remained unaltered. The results of this assay suggest that the change in color is not responsible for the effect as the color treatments did not have a significant effect on the site selection by gravid females (Figure 2.4). However, while this partially accounts for the container color, this experiment does not fully account for the potential of color to affect oviposition behavior as direct coloration of the water itself or the oviposition paper substrate was not performed as it would be difficult to discern if a dissolvable coloring agent would be attractive due to visual stimuli or via an alternative chemosensory mechanism.

Effect of Plant Tissue Type on Biochar Attractivity

To determine whether biochar derived from different tissues of the tree differed in their effects, leaves, bark, and wood were sampled from manzanita cuttings derived from the Frenchtown burn site. The different tissue types were pyrolyzed to biochar at 550°C for 30 minutes in a muffle furnace. The biochars were tested via the competitive choice assay against water alone and the original forest fire-generated biochar originally collected from the site after the fire (Figure 2.5). The results show that egg deposition was low in water alone and water containing leaf and bark-derived biochar. The wood-based biochar had higher egg counts than biochar made from other parts of the tree and showed a higher but not significantly different response to

the char originally collected from the site after the wildfire. Notably, the proportion of eggs deposited in the treatment with lab-produced wood biochar exceeded that observed in wildfire-derived biochar. However, this difference did not reach statistical significance.



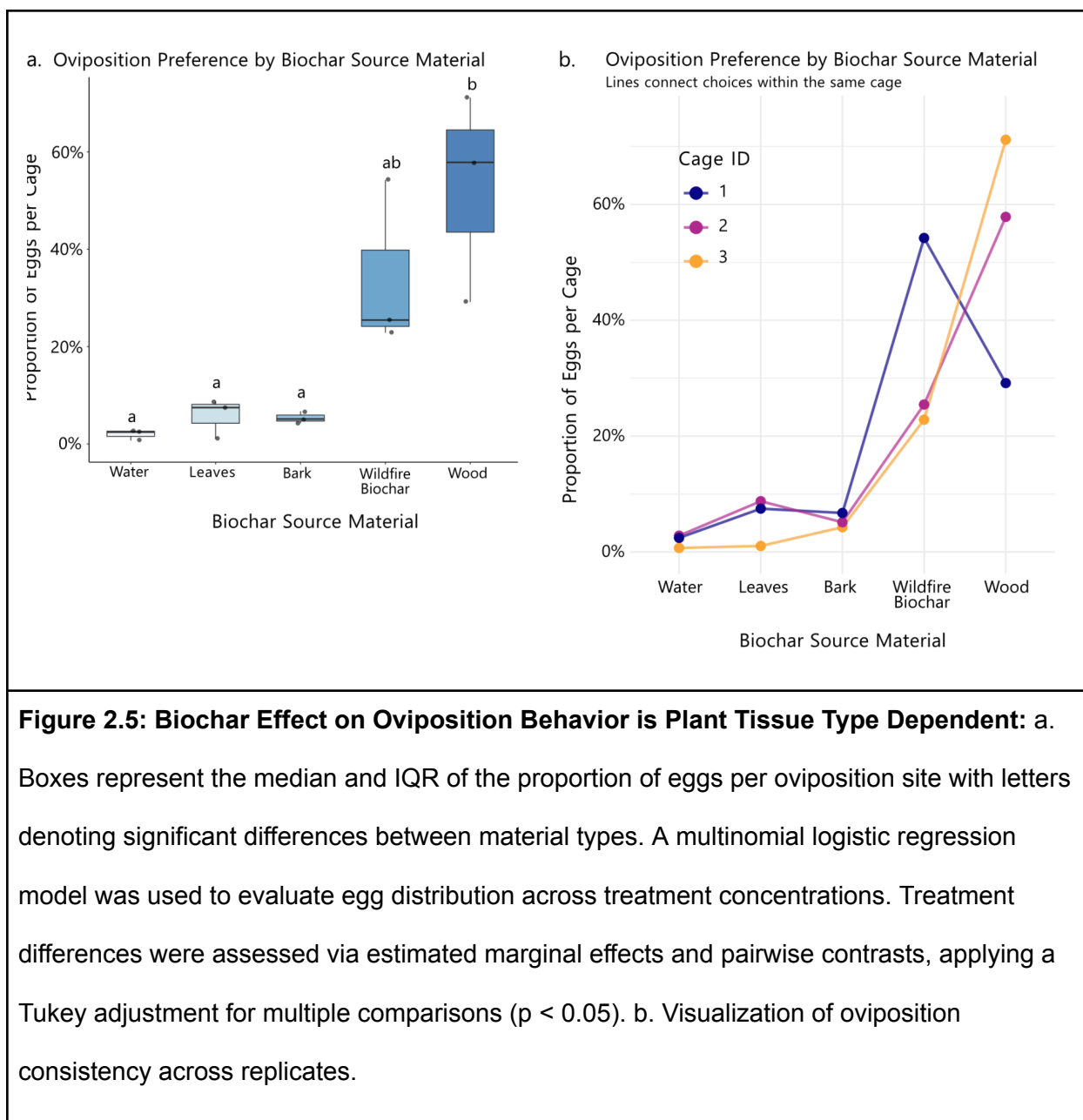


Figure 2.5: Biochar Effect on Oviposition Behavior is Plant Tissue Type Dependent: a.

Boxes represent the median and IQR of the proportion of eggs per oviposition site with letters denoting significant differences between material types. A multinomial logistic regression model was used to evaluate egg distribution across treatment concentrations. Treatment differences were assessed via estimated marginal effects and pairwise contrasts, applying a Tukey adjustment for multiple comparisons ($p < 0.05$). b. Visualization of oviposition consistency across replicates.

Effect of Biochar Pyrolysis Temperature on Oviposition Behavior

The physical properties and composition of biochar, including the available surface area and nutrient content, can vary depending on the temperature at which it is produced. To determine the effect of pyrolysis temperature on Manzanita wood-based biochar, wood was pyrolyzed in a

muffle furnace at three temperatures (350°C, 550°C, 750°C) to determine if production temperature affects attractivity. These temperatures are representative of those occurring during natural wildfires ranging from low to high intensity. Higher intensity fires are usually associated with the accumulation of dry fuels, low humidity, and high winds.

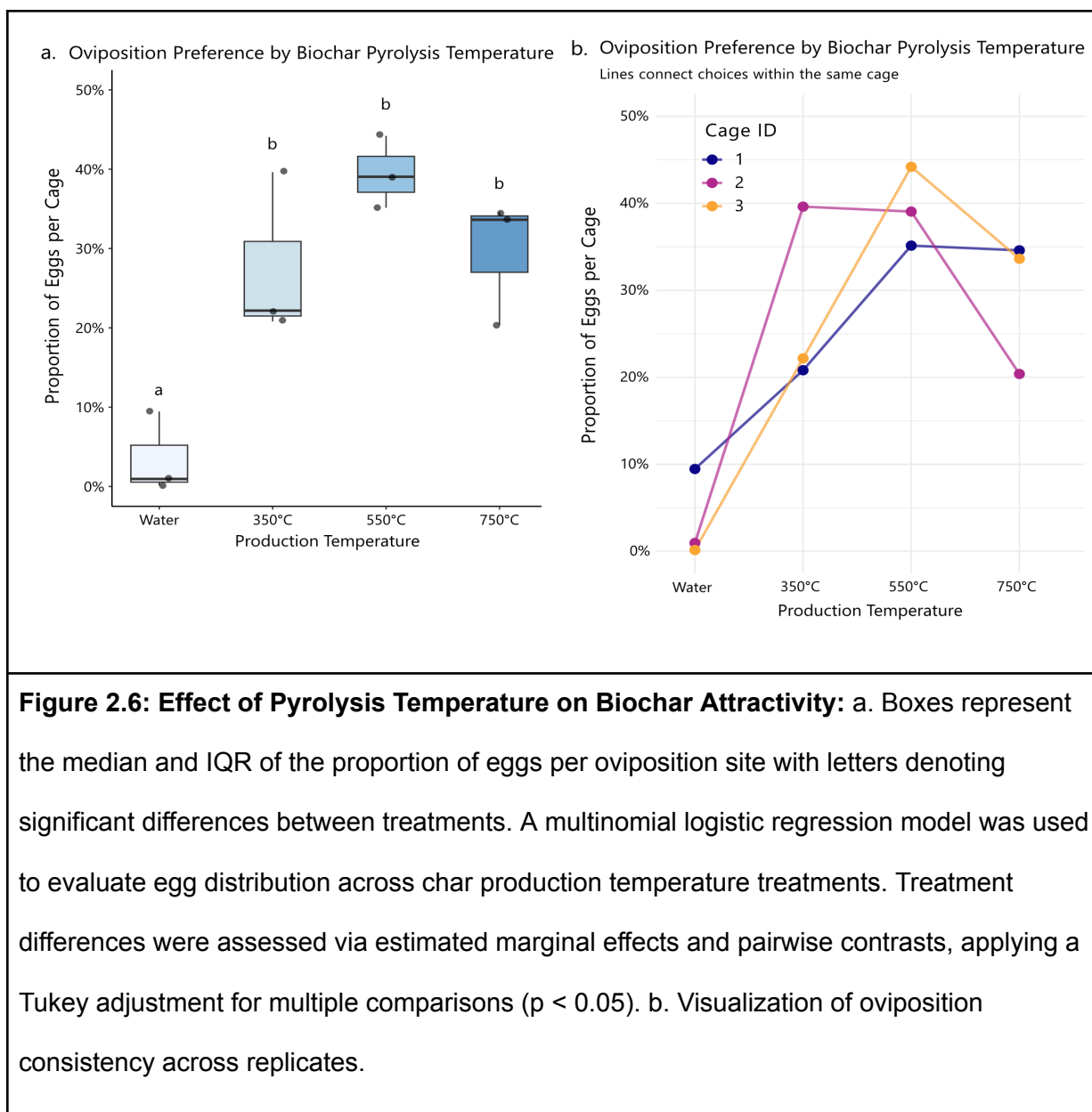
Relative to water alone, the biochar derived from all three temperature regimes showed significantly higher egg deposition proportions of eggs than water alone (Figure 2.6). The 550°C treatment trended toward higher egg counts than the 350°C and 750°C treatments, though this difference did not reach statistical significance.

Biochar Infused Water Outcompetes Other Oviposition Attractants

While biochar increases oviposition site attractivity relative to water alone, the question remains as to its attractivity relative to other documented oviposition attractants. Hay-infused water and water in which larvae had previously developed are known attractants for gravid *Ae. aegypti*.

An oviposition experiment was conducted incorporating these treatments relative to an infusion of 0.75% Manzanita biochar to determine the relative competitiveness of the different attractants. Uncharred Manzanita wood was also included as a treatment to determine if the wood itself could function as an attractant in the absence of pyrolysis. (Figure 2.7a+b).

The results show a clear and significant preference for the manzanita biochar infusion relative to the other treatments. The hay-infused water showed the second-highest proportion of eggs and was also significantly higher than either the larval water or the uncharred Manzanita wood. The uncharred Manzanita was the third most attractive, with the larval breeding water being the least attractive. Combined, the hay infusion and biochar-containing sites were the dominant attractants in the assay (Figure 2.7a+b).



Effects of Biochar in Combination with Other Oviposition Attractants

To determine the effect of biochar in combination with other documented attractants, experiments combining its infusion with larval breeding water or hay-infused water were performed. The results from these assays suggest that combining biochar with attractants appears to have an additive effect as these treatments showed higher median egg proportions. However, these differences did not achieve a statistical difference from biochar alone. The larval water and biochar treatment showed a significantly increased proportion of eggs relative to the rest of the other treatments with the exception of biochar alone (Figure 2.7c+d). The experiment combining biochar with hay infusion showed that the combined treatment had the highest median egg proportion. However, high levels of variability between replicates resulted in none of the observed proportional differences between treatments being statistically significant (Figure 2.7e+f). The observed trends from these experiments suggest that the combination of biochar with other oviposition attractants, however, additional experimentation is needed to determine if these trends reflect a real functional difference.

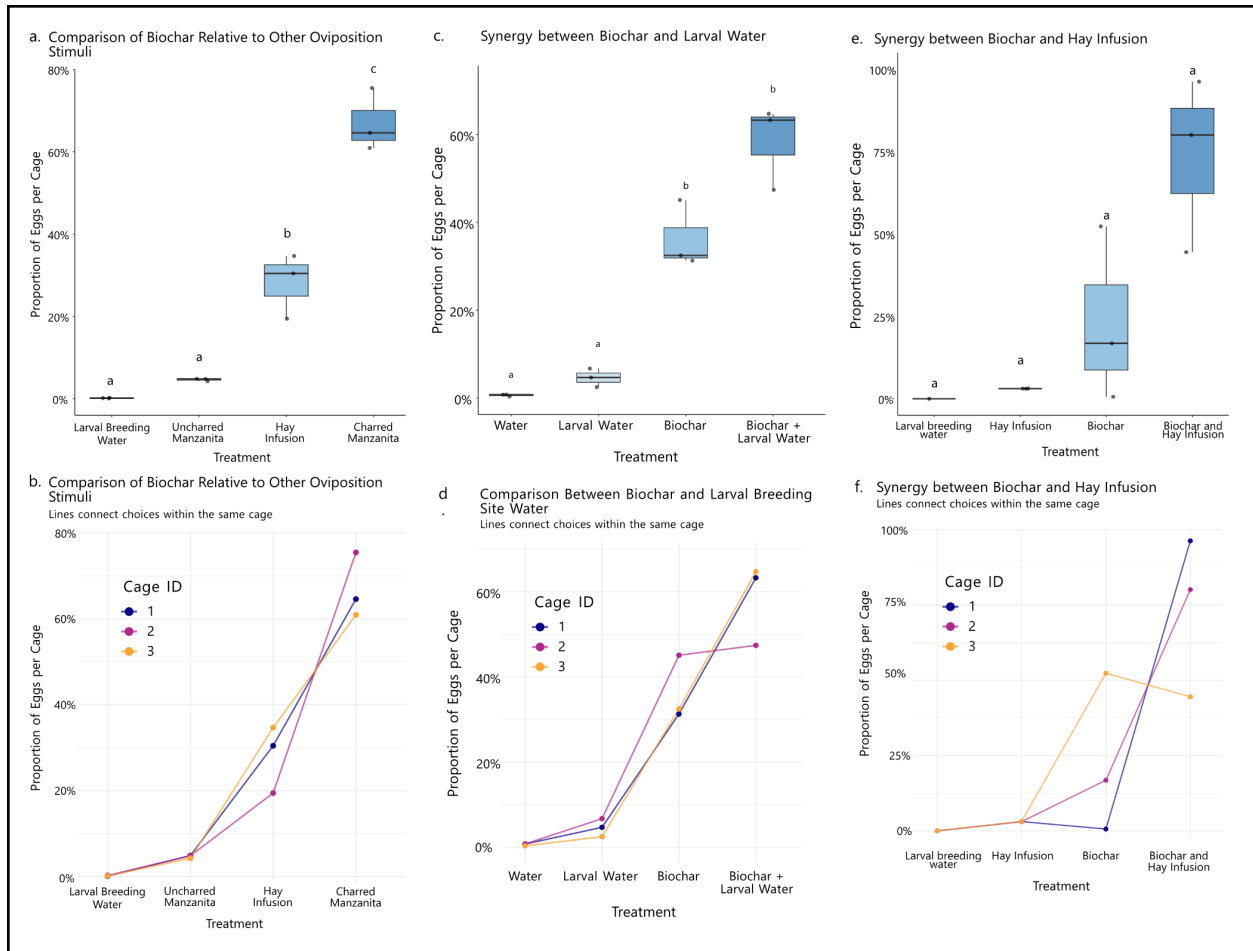


Figure 2.7: Effect of Manzanita Biochar Infusion on Oviposition Site Selection in

Competition and Combination with Other Oviposition Attractants: Boxes represent the median and IQR, with letters denoting significant differences between treatments. Top panels: multinomial logistic regression model was used to evaluate egg distribution across attractant treatments. Treatment differences were assessed via estimated marginal effects and pairwise contrasts, applying a Tukey adjustment for multiple comparisons ($p < 0.05$). Bottom panels: Visualization of oviposition consistency across replicates.. a+b. Choice assay comparing biochar against uncharred wood, larval breeding water, and hay-infused water. c+d. Choice assay comparing biochar-infused larval water versus individual attractants. e+f. Choice assay comparing biochar-infused hay infusion versus individual attractants.

Effect of Non-Biochar Derived Adsorbent Compounds on Oviposition Site Selection Behavior

The adsorptive and cation-exchange capacities of biochar arise from its intricate structure and surface functional groups. To test if biochar's adsorbance was important for its behavioral effects, a substitute material with similar adsorptive capacity was tested to determine if this property was mediating this effect.

Zeolites are materials with adsorptive and cation-exchange capacities similar to biochar. However, they are composed of aluminosilicate crystals rather than carbon, eliminating potential nutritional benefits. To test the relative attractiveness of zeolite-treated oviposition sites, a two-treatment comparison between water and 0.5% (w/v) zeolite-infused water was performed.

The results show a significant preference for the oviposition sites containing the zeolite infusion (Figure 2.8). This finding suggests that the similarities in the physical properties between zeolites and biochar are, at least in part, conferring the attractiveness to infused oviposition sites.

Field Testing Biochar Efficacy as an Attractant in Biogent Gravid *Aedes* Traps (BG-GAT)

To test if biochar supplementation would improve gravid autocidal trap performance, BG-GAT traps by Biogents (Cary, NC) were utilized in a pairwise competitive assay across 50 houses in a gated neighborhood in Madera, California. The wood used to generate the biochar was commercially available Oak wood, a hard wood like Manzanita. The results of the study show a significant increase in overall female *Ae. aegypti* caught in the GATs containing the 0.75% biochar infusion (2391) relative to those containing alfalfa water alone (1656) (IRR = 1.55, 95% CI 1.34–1.79; likelihood-ratio $\chi^2_1 = 32.7$, $p = 1.1 \times 10^{-8}$), representing an approximate 55% increase in efficacy. (Figure 2.9a).

The majority of mosquitoes caught in the traps were female *Ae. aegypti*. However, a small number of males were caught with a total of 38 in the alfalfa treatment and 66 in the biochar-supplemented traps, which may be reflective of the greater number of females associated with the supplemented traps.

The biochar-infused traps show consistently higher average trap counts over time than the alfalfa infusion alone (Figure 2.9b). Analysis of the treatment $\times$ date interaction proved non-significant ($\chi^2_4 = 3.39$, $p = 0.49$), demonstrating that the biochar enhancement remained stable throughout the study duration; specifically, per-date contrasts were statistically significant for four of the five intervals. The greatest impact was observed early in the season (15 Aug, IRR = 1.93, $p < 0.001$), with the difference between trap types narrowing by the final assessment (17 Oct, IRR = 1.28, $p = 0.099$). Examination of the proportion of total *Ae. aegypti* catch per house between the two treatments showed that in 34 out of the 50 houses (68%) the biochar-treated traps outperformed the alfalfa water alone (Figure 2.9c). The variance in observed catch between houses is likely due to multiple variables affecting the microhabitat around the traps, such as shade level, proximity to plants and hosts, and other unknowns that may vary between houses. However, the overall result indicates that biochar supplementation significantly improved GAT performance in a suburban neighborhood.

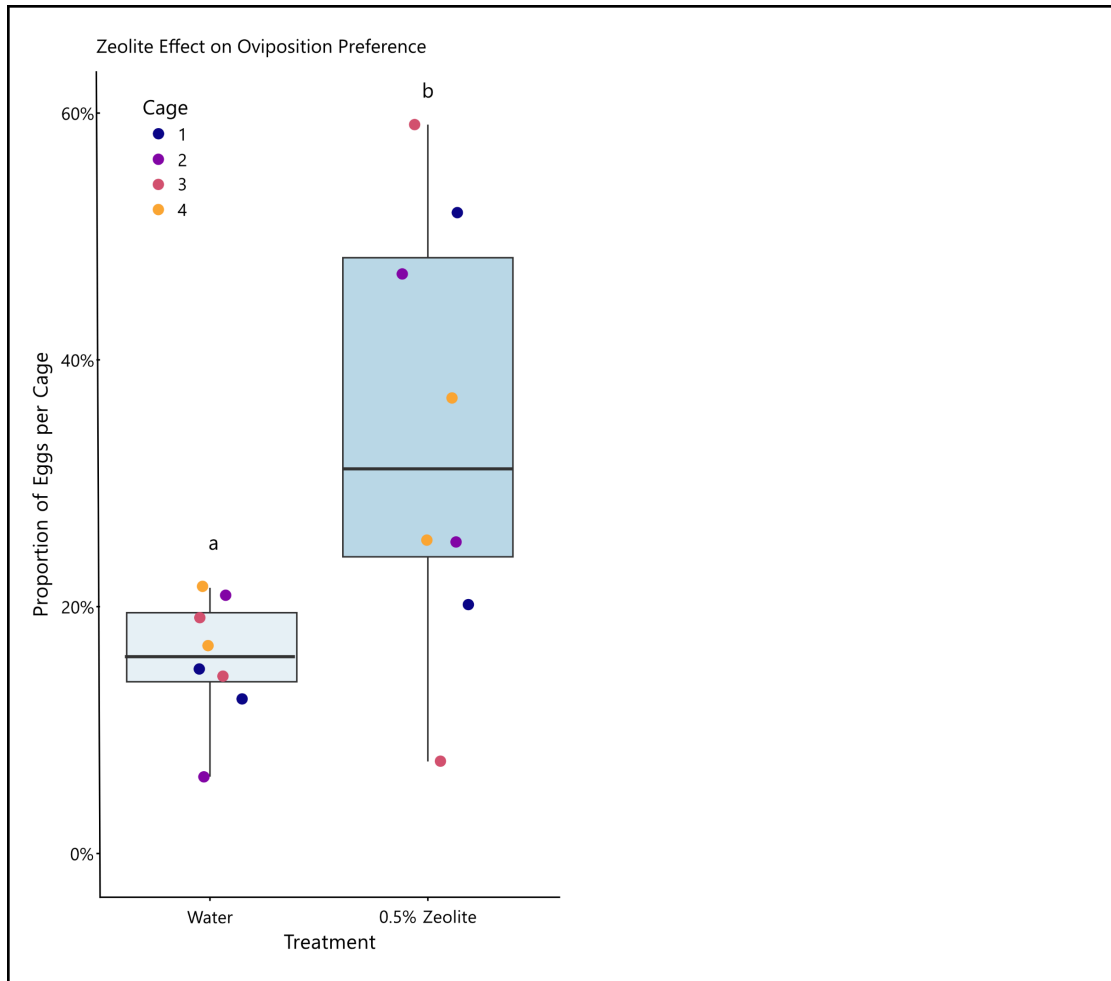


Figure 2.8: Effect of Zeolite infusion on mosquito oviposition site choice:

Boxes represent the median and IQR, with letters denoting significant differences between treatments. Cages included two cups per treatment with positional randomization across replicates. Datapoints represent the proportion of egg counts per cup per cage for each treatment. A multinomial (conditional logit) choice model was used to evaluate egg distribution between treatments. Difference between treatments was assessed via estimated marginal effects ($p < 0.05$).

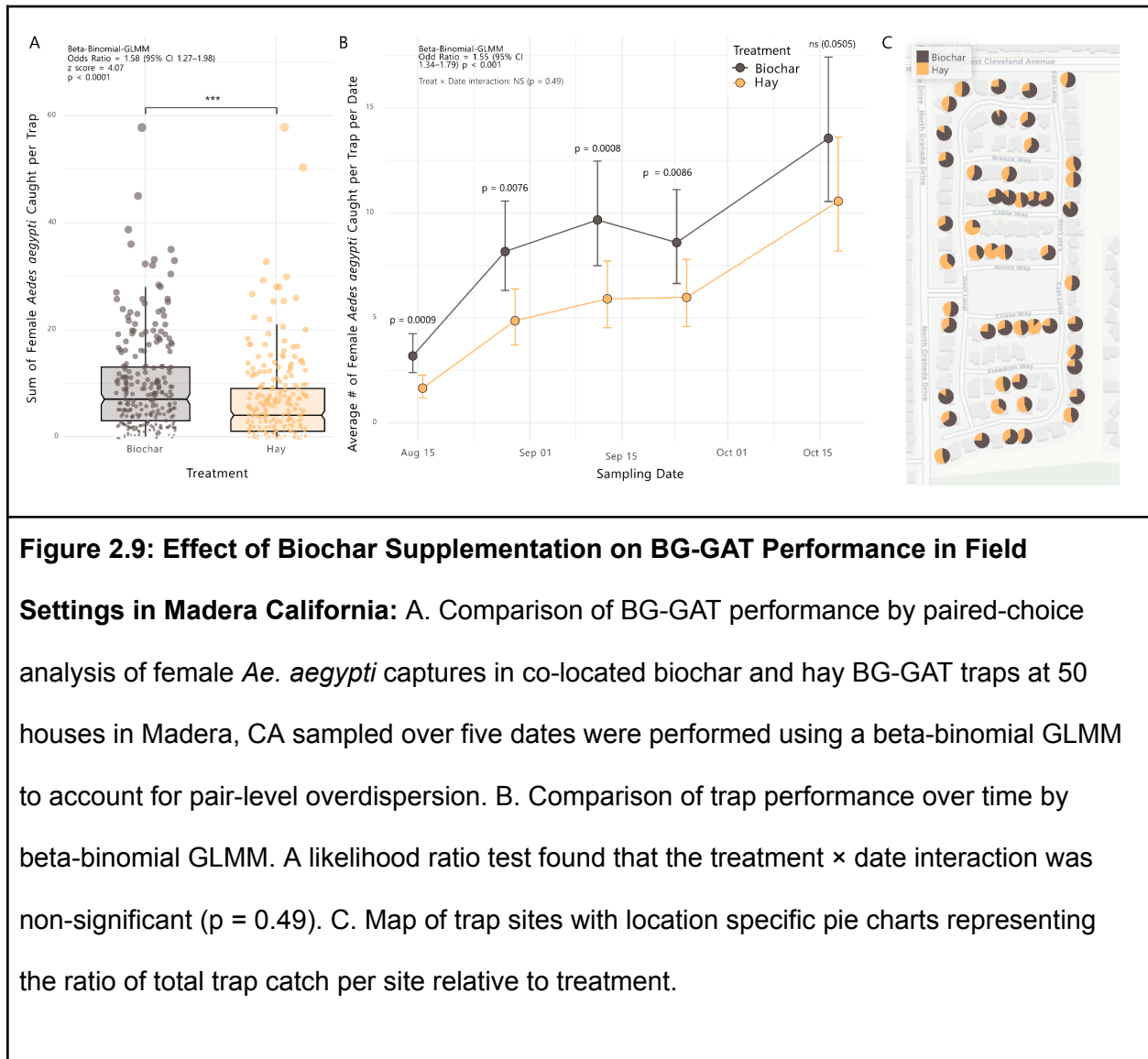


Figure 2.9: Effect of Biochar Supplementation on BG-GAT Performance in Field

Settings in Madera California: A. Comparison of BG-GAT performance by paired-choice analysis of female *Ae. aegypti* captures in co-located biochar and hay BG-GAT traps at 50 houses in Madera, CA sampled over five dates were performed using a beta-binomial GLMM to account for pair-level overdispersion. B. Comparison of trap performance over time by beta-binomial GLMM. A likelihood ratio test found that the treatment × date interaction was non-significant (p = 0.49). C. Map of trap sites with location specific pie charts representing the ratio of total trap catch per site relative to treatment.

Discussion

The presence of wood-based biochar has a significant effect on oviposition behavior by *Ae. aegypti* through mechanisms extending beyond simple visual cues. Oviposition behavior increased with biochar concentration and was observed across several hard-wood-derived

biochars with the phenomenon persisting across a wide range of pyrolysis temperatures. The physical presence of biochar appears essential for maintaining its attractive effects as removal of biochar from oviposition water by filtration eliminates attractive properties observed in unfiltered biochar treatments. The results presented here raise intriguing questions spanning different aspects of *Ae. aegypti*'s biology sensory mechanics, how this phenomenon relates to their evolutionary history, the ecological significance of this phenomenon, whether biochar can improve existing vector control technologies, and the ecological significance in the context of climate change and increasing wildfire frequency and intensity.

Mechanistic Understanding of the Effect of Biochar on *Ae. aegypti* Oviposition Behavior

Experiments using biochar from different tree species indicate that the observed effect is not specific to wood type and is likely a general effect of physical properties of wood-derived biochar. The requirement of the physical presence of biochar suggests it is altering water chemistry/parameters in a way that triggers oviposition behavior. The similarity of the oviposition response to the zeolite infusion relative to biochar implies that the common physical characteristics of these materials, including high surface area-to-volume ratio, high adsorptive capacity, and high CEC, drive the mechanism underlying the perception of a high-quality oviposition site by gravid *Ae. aegypti*. This information gives rise to the hypothesis that biochar alters oviposition sites to be read as high-quality by gravid *Ae. aegypti*.

Biochar has significant beneficial effects on plant health when integrated into soil. This is accomplished through the combination of multiple functions including the adsorption of toxic compounds, provision of micronutrients, and enrichment of microbial diversity and abundance (Yu et al. 2019; Osman et al. 2022; Wang et al. 2019). Biochar could function in a similar manner in the aqueous environment of an oviposition site. The CEC of biochar can provide a steady release of essential micronutrients such as magnesium, calcium, and potassium in

exchange for alternative cations including pollutants or toxic compounds, such as ammonium (a biological waste product), microplastics, and/or oxidized heavy metals (Ahmad et al. 2014). Biochar bound toxic compounds can be processed by enzymatic activity in biochar-associated bacterial biofilms and converted into less toxic or non-toxic chemical forms, some of which such as nitrates can be used by plants and algae as nutrients (Li et al. 2026). These properties also provide buffering capacity, which also stabilizes the pH of the surrounding water. These changes have the potential to maintain stable conditions for immature mosquitos.

In addition, the observation that biochar in combination with known attractants (hay infused water or larval rearing water) is more attractive than biochar alone suggests that the biochar derived changes are enhancing the attractive aspects of these formulations. A potential explanation for this is that biochar could be adsorbing attractive volatiles and releasing them via diffusion in a controlled concentration dependent manner. This would differ from the attractant alone which diffuses in an unregulated volatilization resulting in higher concentrations and potential depletion from the breeding site over time. In addition, many oviposition cues utilized by mosquitoes can be both attractive or repellant depending on the concentration of the compound (Zaid Khan et al. 2023). The high adsorptive capacity of biochar and zeolites could function to consolidate attractants and diffuse them in a controlled manner.

Gravid mosquitoes have intricate sensory systems tuned to perceive subtle differences in complex mixtures of volatile biochemicals, chemicals, humidity, and temperature to find oviposition sites in the environment (Z. Khan et al. 2022). The sensitive chemo-ionotropic and gustatory receptors expressed in their antennae, palps, feet, and proboscis have been honed over the evolutionary history of mosquitoes to enhance their reproductive and survival capabilities (Montell 2025). Prior work measuring the ionic and osmotic detection capabilities of *Ae. aegypti* found that ovipositing females preferred oviposition sites with less than 0.085 M NaCl and that this was mediated by receptors in the tarsi (Hudson 1956). Recent work shows

that female *Ae. aegypti* use the ppk301 ion channel to detect water and a separate undetermined mechanism to detect sodium concentrations in water (Matthews et al. 2019). The findings of these results suggest that *Ae. aegypti* have the ability to perceive subtle changes in water chemistry to choose an optimal breeding site for their offspring. The specific cues remain undetermined and require further investigation with different ionic balances to determine the optimal parameters for oviposition and to determine if adsorption and sequestration of compounds by biochar could be masking or mediating detection of sensory stimuli such as geosmin, skatole, and other microbial byproducts (Baak-Baak et al. 2013; Melo et al. 2020).

These insights provide a path forward to measure and determine the exact effects of biochar on micronutrient availability, pH balance, water conductivity, ion exchange, and the sequestration of toxic compounds. It will also facilitate the development of an in-depth understanding of the more fundamental aspects of how water chemistry relates to the preferences of ovipositing *Ae. aegypti*. This could also provide novel insights into larval biology and nutrition, water-quality sensation, and optimization of trapping techniques for this prolific invasive vector species.

The Ecological and Evolutionary Significance of Biochar Attractivity

Beyond the mechanistic aspects of biochar's attractivity to *Ae. aegypti* in oviposition sites, the questions arise as to how this response would be selected for during the evolution of the species and under what natural environmental conditions would oviposition sites with similar qualities be found. Oviposition site selection is the most consequential decision made by female mosquitoes, as the quality of the selected habitat directly affects offspring survival and species reproductive success. Natural selection would favor females capable of identifying environmental cues that reliably indicate high-quality larval habitats as surviving offspring lead to continuation of species and integration of favourable cues of breeding site choice into behavioral programs. The results of this study suggest that biochar may alter water chemistry

and aquatic-habitat characteristics in ways that gravid *Ae. aegypti* perceived as favorable for offspring development and survival.

This interpretation is consistent with the evolutionary history of *Ae. aegypti*. Although modern populations of *Ae. aegypti* are known as anthropophilic container breeding species, an ancestral form, *Ae. aegypti formosus* is still found in forests and vegetative ecotones where treeholes containing water can be used as breeding sites (Lounibos 1981; Powell and Tabachnick 2013). These habitats vary considerably in nutrient availability, microbial composition, water chemistry, and the accumulation of organic detritus, factors that similarly influence the quality of modern anthropogenic container habitats used by *Ae. aegypti* for reproduction (Juma et al. 2021). Gravid *Ae. aegypti* capable of assessing environmental differences and selecting sites that maximize offspring performance would have a selective advantage, as increased offspring survival and reproductive success would favor the persistence of these behaviors over evolutionary time. Consequently, the sophisticated sensory systems used by mosquitoes to evaluate breeding sites likely evolved as mechanisms for assessing multiple features related to overall breeding site quality rather than for detecting any single feature.

The results presented support the hypothesis that biochar is not necessarily the direct target of gravid *Ae. aegypti* attraction, but rather that biochar creates conditions associated with favorable breeding sites. The observation that zeolite-infused water elicited a similar oviposition response suggests that mosquitoes may be responding to physicochemical characteristics shared by highly adsorptive materials rather than to unique compounds derived from burned wood. Likewise, the loss of attraction following removal of biochar by filtration indicates that the continued presence of the material is necessary to maintain these habitat characteristics. Together, these findings suggest that gravid females may be responding to changes in water quality generated by biochar rather than to biochar itself. The observation that the field based

biochar supplementation effect seems to fade over time indicates that the traps may require refreshment with fresh biochar over time to maintain optimal function.

The exact roles of different sensory stimuli in oviposition site selection are not well defined and likely involve a combination of stimuli. Female oviposition behavior can be influenced by olfactory, visual, and gustatory cues (Mwingira et al. 2020). In the present study, when *Ae. aegypti* females were given the choice of water infused with biochar (baywood, redwood, or manzanita biochar) or plain water, a clear preference was also observed for the arm with the biochar-infused sites, the manzanita wood biochar did not result in a significant difference in choice. The lack of response to the manzanita biochar may be due to the fact that the biochar used in the experiment was collected at a wildfire site a year prior and was potentially losing its potency while the other two wood types were generated just prior to the performance of these experiments. However, taken together, these results indicate that the presence of biochar appears to alter the volatile profile of oviposition sites, increasing their attractiveness. Whether this is due to the presence of new attractive olfactory stimuli from biochar, changes in relative humidity across the site due to differences in water chemistry, or the absence of aversive stimuli due to adsorption remains to be addressed.

So why would mosquitoes in the wild be exposed to biochar-associated conditions in breeding sites, and how could this relate to *Ae. aegypti* natural history? One possible ecological context for this response is the long history of wildfire disturbance across many regions of sub-Saharan Africa. Fire has been a recurring component of African ecosystems for millions of years, and partially burned wood (biochar) capable of retaining water (Bremond et al. 2024; Adhikari et al. 2023). Our hypothesis is as biochar could retain water, it may have occasionally served as mosquito breeding habitat. There are sedimentary charcoal records of burned biomass to indicate what sorts of biomass burned and from what biomes (Bremond et al. 2024).

Biochar-associated breeding sites could offer advantages to developing larvae through

improved nutrient retention, enhanced microbial colonization, stabilization of water chemistry, or the adsorption of harmful compounds. If these conditions resulted in improved offspring survival or performance, natural selection could favor females capable of detecting the associated environmental cues. Importantly, this does not require mosquitoes to have evolved a specific attraction to biochar itself. Rather, biochar may represent one of several environmental features capable of generating habitat conditions that mosquitoes have evolved to recognize as indicators of breeding-site quality.

The adaptive value of detecting factors associated with water-quality likely would allow gravid *Ae. aegypti* to assess habitat suitability prior to oviposition. For instance, the *Ir8a* gene pathway in *Ae. aegypti* allows them to detect standing water (Raji et al. 2019). Previous studies have demonstrated a combination of olfactory, gustatory, and ion-sensitive sensory pathways to evaluate potential breeding sites. The phenomenon observed in this study is consistent with the broader hypothesis that ovipositing females integrate multiple environmental signals to identify habitats that maximize offspring fitness. Determining the cues involved remains an important area for future investigation, as understanding how mosquitoes assess water quality could provide new insights into mosquito ecology, larval habitat selection, and the evolution of oviposition behavior.

Implications for Application and Optimization of Existing Vector Control Strategies

Beyond providing insights into oviposition site selection and mosquito ecology, gravid *Ae. aegypti* choosing to oviposit in biochar-infused water has the potential applications for vector surveillance and control. Oviposition site selection in *Ae. aegypti* is influenced by a combination of visual, physical, chemical, and olfactory characteristics of potential breeding sites, including container characteristics such as size (Mwingira et al. 2020; Harrington et al. 2008). Modifying the characteristics of oviposition sites, particularly through the addition of organic infusions, can

further increase their attractiveness and improve traps (Reiter et al. 1991). In this context biochar could be a low-cost additive to increase the efficacy of oviposition traps targeting this species. This is supported by the results of this study demonstrating biochar-infused water is highly attractive to gravid *Ae. aegypti* under laboratory conditions and that this attraction persists under field conditions, suggesting that biochar may represent a practical additive for improving existing oviposition-based trapping technologies.

The utility of biochar as a trapping additive is supported by its performance relative to established oviposition attractants. In the present study, in laboratory choice assays, biochar-infused water consistently outperformed commonly used attractants, including hay-infused water and larval hatch/pupation water (Reiter et al. 1991; Boullis et al. 2021). This indicates that biochar can produce a strong oviposition response relative to attractants that have previously been used to enhance ovitrap performance. Furthermore, combining biochar with these attractants often enhanced oviposition beyond that observed for either biochar or the attractants alone. This pattern suggests that biochar may complement existing oviposition cues rather than simply replacing them, although additional experiments specifically designed to test interactions among attractants would be necessary to establish whether these effects are truly synergistic. Importantly, these laboratory observations translated to field conditions. BG-GAT traps supplemented with biochar-infused alfalfa water captured significantly more female *Ae. aegypti* than traps containing alfalfa infusion alone, and this effect persisted throughout the majority of the field trial. Although the effect seemed to decrease in intensity after ~four weeks. The agreement between laboratory and field results indicates that the attractiveness of biochar-infused water remains effective even in the presence of the numerous competing environmental cues encountered by mosquitoes under natural conditions.

In addition to improving trap performance, biochar may provide several practical advantages that facilitate implementation in surveillance programs. During the field trial,

biochar-supplemented traps appeared to produce substantially less odor than traps containing alfalfa infusion alone. Fermented infusions are effective attractants but can generate strong odors that may reduce homeowner acceptance when traps are deployed in residential areas. The adsorptive properties of biochar may reduce the concentration of odor-producing compounds while maintaining or enhancing attractiveness to gravid mosquitoes. Although this observation was not formally quantified in the present study, reduced odor could represent an important operational benefit by increasing public acceptance and facilitating the deployment of oviposition traps in urban and suburban environments where community participation is often critical to surveillance success. It is also not hazardous waste so it is easy to dispose of such as using it for plant nutrients and water.

Biochar may also offer economic and logistical advantages compared to many existing attractant formulations. Suitable feedstocks for biochar production are widely available through forestry operations, landscape maintenance, agricultural waste streams, and routine tree trimming activities. Production can be accomplished using relatively simple pyrolysis methods, allowing biochar to be generated locally, at low cost, in low resource areas. Furthermore, because biochar's oviposition effects can be enhanced when combined with existing attractants rather than replacing them, implementation would not require substantial modifications to current surveillance strategies. Instead, biochar could be incorporated as a supplemental component of established oviposition trap formulations. The simplicity, low cost, and apparent effectiveness of this approach make it particularly attractive for large-scale surveillance programs and integrated mosquito management efforts where maximizing trap sensitivity is essential.

Broader Significance and Future Research Directions

The findings of this study have implications that extend beyond mosquito behavior and vector surveillance. Wildfires are increasing in frequency, duration, and intensity in many regions of the world as a consequence of climate change and changing land-use practices. As a result, biochar and other fire-derived materials are becoming increasingly common components of both natural and human-modified environments. Despite the widespread ecological impacts of wildfire, relatively little attention has been given to how fire derived materials could influence mosquito ecology and behavior. The results presented here suggest that biochar could be useful to help trap gravid female *Ae. aegypti*.

The study also identifies several important avenues for future research. While the results strongly suggest that biochar alters habitat characteristics that are perceived by gravid females as indicators of breeding-site quality, the specific mechanisms responsible remain unresolved. Future investigations should examine how biochar influences water chemistry, nutrient availability, conductivity, pH, microbial community composition, and volatile organic compound (VOC) production. For instance, it is known via lab experiments that VOCs from bacteria such as *Klebsiella sp.* and *Pseudomonas aeruginosa* in sites can attract *Ae. aegypti* to oviposit (Mosquera et al. 2023; Benzon and Apperson 1988). Future studies should investigate whether biochar interacts with these microbial attractants by enhancing their production, altering their persistence, or modifying their release from breeding sites. Evaluating combinations of biochar and known oviposition-associated bacteria or VOCs may reveal synergistic effects that further improve oviposition attraction and trap performance. Identifying the specific environmental cues associated with attraction would provide valuable insight into how mosquitoes assess habitat quality and could facilitate the development of more effective surveillance and control technologies.

Another question is whether the behavioral responses observed in *Ae. aegypti* are shared by other mosquito species. Mosquito taxa differ considerably in their preferred breeding habitats,

suggesting that biochar may have species-specific effects on oviposition behavior. Comparative studies across medically and ecologically important mosquito species could reveal broader principles governing mosquito habitat selection while also identifying opportunities for species-targeted surveillance and control strategies.

In conclusion, this study identifies a significant and previously unrecognized factor in mosquito reproduction by demonstrating biochar's potential as an oviposition attractant for *Ae. aegypti*. It also generates new insights and research pathways into the biotic and abiotic factors that this important vector species prioritizes when selecting breeding sites for their offspring. The research not only expands our understanding of mosquito behavior but also prompts further inquiries into the specific biochar characteristics that influence attraction and larval development. It also highlights the potential effects biochar has on water chemistry and quality. Beyond acting as a mosquito attractant, biochar could function as a low-cost water remediation agent with broader implications for invertebrate health in natural aquatic habitats. For instance, biochar-based treatments of water sources could reduce the sub-lethal exposures of mosquitoes to environmental toxins, including pesticides, agrochemicals, and urban/suburban pollutants, by its absorptive capabilities (X. Dong et al. 2024). As a result, mosquitoes developing in these environments would be under lower selective pressure for development/enhancement of resistance mechanisms. These findings hold promise for development of sustainable and effective mosquito control measures, as well as potential ecological management strategies that boost environmental health and lower insecticide resistance.

Materials and Methods:

Oviposition Behavior Experiments:

Mosquito Rearing Protocols: Mosquitoes utilized for these experiments were the Rockefeller

Table 2.1: Larval Diet	
Day	Fluval Fish Pellet Diet
1	372 mg (2 pinches)
2	46.5 mg (1 drop)
3	93 mg (2 drops)
4	186 mg (4 drops)
5	186 mg (4 drops)
6	372 mg (8 drops)
7	279 mg (6 drops)
8	186 mg (4 drops)

strain of *Ae. aegypti*. Egg papers were hatched under vacuum, and first instar larvae transferred into trays for rearing. Larvae were reared in groups of 200 per tray in 1 L of dechlorinated tap water. Larvae were reared at 27°C and 80% relative humidity on a 14:10 h (light:dark) cycle. Larvae were fed a diet of Fluval fish pellets accordingly (Table 2.1).

Upon pupation, pupae were removed from larval trays and placed in BugDorm #6 cages (27.5 x 29.5 x 29.5 cm; BugDorm Company, Taichung, Taiwan) and provided 10% sucrose ad libitum until the 5th day post-eclosion.

Experimental Setup: Adult female mosquitoes were blood-fed on the arm of a human volunteer (GMA).

At three days post-blood meal, bloodfed females were aspirated from the feeding cage and added to the experimental cages. Each treatment was repeated at least three times with each cage containing 25 females.

Each cage contained four 3.25 oz plastic condiment cups. Each cup was lined with germination paper and 30 mL of dechlorinated tap water or infusion treatments. Cups were placed in each corner of the experimental cages. The corner positions of each oviposition treatment were systematically rotated in each replicate to account for potential position effects.

Solids-based Infusion treatments were added in mass/volume ratios (g/ml*100). Gravid females were allowed 2 days to oviposit. Following oviposition, cups were removed from the cages, and

oviposition papers were allowed to dry at room temperature. Egg deposition was quantified by egg counts determined by egg counts performed under a dissecting microscope.

Hay-Infusion Treatments: The hay-infused water was generated from dried grass from Hutchison Intramural Field on the UC Davis Campus in Davis California. Collected hay was added to dechlorinated tap water and incubated in a sealed container for two weeks to ferment.

Collection of Wildfire Derived Biochar Samples: The wildfire based biochar was collected from a wildfire burn scar located in Frenchtown California (39.383922°N, 121.24684°W). The fire covered 45 acres and occurred on 7/20/2021 and was contained on 7/22/2021 (<https://www.fire.ca.gov/incidents/2021/7/20/frenchtown-fire/>). The biochar was collected on 3/31/2022.

Lab-Based Biochar Generation: A hood based Thermo Scientific Box Furnace (Gravity, 0.5 cu ft Capacity, 1,200°C Max Temp, 2,323 W Watt) was used to generate biochar under controlled conditions in the lab. A thermocouple was attached to the furnace to ensure accurate temperature readings. Wood was charred in a lidded graphite crucible or wrapped in aluminium foil packets. Prior to addition of wood, the furnace was brought up to temperature and the crucible was added to the oven using tongs and fire resistant PPE. The wood was charred for 30 minutes. Charring temperature was experiment dependent, but ranged from 350-750°C with the majority of the char generated at 550°C. After 30 minutes the crucible/aluminium foil packets was removed and the biochar was either quenched on dry ice or allowed to cool to room temperature in the hood. Manzanita wood was acquired from branch cuttings of trees at the burn site from which initial wildfire char was obtained from while the Oak wood was purchased from Home Depot.

Cage Based Assay Statistical Analyses: Statistical processing was executed utilizing the R programming language (R Core Team, 2024). To quantify how biochar and other treatments

impact *Ae. aegypti* egg deposition, we employed an intercept-only multinomial logistic regression with 0% as the reference category (R package - nnet). Pairwise comparisons across treatments on the response scale were performed by estimated marginal means using the *emmeans* package (Lenth and Piaskowski 2026) and pairwise contrasts were adjusted for multiple comparisons with the Tukey method. Raw data and R scripts used for analysis and figure generation are included in the compressed supplemental file archive.

Field Evaluation of Biochar Supplementation of BG-GAT: Untreated oak wood purchased from Home Depot was cut into small rectangular pieces and pyrolyzed in a muffle furnace at 550 °C for 30 minutes. After pyrolysis, the material was rapidly cooled on dry ice to halt combustion. The resulting biochar was stored in sealed ziplock bags and later crushed manually with a mallet to produce smaller, irregular fragments (from ~5 cm to powder in size) suitable for use in the traps. The processed biochar was then weighed using a laboratory scale and portioned into individual ziplock bags (22.5 g each) for consistent and convenient addition to the biochar treatment traps. Each trap in the biochar treatments resulted in a 0.5% biochar infusion in a volume of 3L of water.

Field Site: The field trial was conducted in Madera, California, within a gated residential neighborhood consisting of approximately 100 houses. Fifty houses within this neighborhood were selected for trap placement. The study area corresponds to the neighborhood that includes the 2 West Loop, Madera, CA 93637 address. The neighborhood is characterized by suburban housing with landscaped yards and outdoor water sources, providing suitable conditions for *Ae. aegypti* breeding and activity.

Trap Setup: A total of 100 BG-GAT oviposition traps were deployed for the field trial in collaboration with personnel from the Madera County Mosquito and Vector Control District. Each of the 50 participating houses received two traps. One containing hay infusion (control) and one

containing hay + biochar infusion (treatment). Traps were placed outdoors in the front yard of each house, approximately 10 ft or more apart to minimize potential interference between treatments. The trial ran from August 1 to October 17, with traps inspected approximately every two weeks. During each visit, sticky cards were replaced, and infusion water was replenished if the volume had decreased substantially. The hay infusion was prepared following the standard recipe used by the Madera County Vector Control District. For the biochar treatment, 22.5 g of biochar was added to each trap, corresponding to 0.75% w/v, assuming a total trap volume of 3 L.

Statistical Analyses: R programming language and RStudio were used to perform statistical analyses. The analysis of female *Ae. aegypti* capture rates across different trap types was conducted using a betabinomial GLMM model on paired counts to absorb pair-level overdispersion across the 50 residences in the study area (utilizing the “betabinomial” parameterization via the ``glmmTMB`` package, version 1.1.8, in R version 4.3.3) (Brooks et al. 2017). The model estimates relative preference conditional on arrival at one of the two traps in the context of other potential oviposition sites in the surrounding environment. To evaluate the influence of biochar supplementation, catch counts were modeled as a function of treatment, with the standard hay infusion serving as the reference. The magnitude of the treatment effect was quantified as a rate ratio (IRR), supported by a 95% Wald confidence interval and assessed using a likelihood-ratio test. To investigate potential seasonal shifts in treatment efficacy, a secondary model was developed to include a treatment × date interaction term. This interaction was evaluated via likelihood-ratio testing, with specific biochar-versus-hay contrasts for each date derived from estimated marginal means using the ``emmeans`` package.

Data visualizations were generated in R and exported as PDFs. Aesthetic modifications to graphs for publication were made in Adobe Illustrator and Affinity Designer. Experimental schematics were generated in Biorender (<https://www.biorender.com/>). Raw data and R scripts

used for analysis and figure generation are included in the compressed supplemental file archive.

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There are no conflicts of interest.

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Chapter 3

Title: Biochar effects on the microbiome of breeding sites of *Aedes aegypti* and the subsequent metabolomes of the adult female *Aedes aegypti*

Abstract

Biochar can alter the physical and chemical characteristics of aquatic habitats and influence organisms that rely on these environments for development. However, the effects of biochar on *Aedes aegypti* development and the associated microbial and metabolic changes remain poorly understood. Here, we investigated the effects of wood-derived biochar across multiple stages of the *Aedes aegypti* life cycle, examining larval development, the microbial communities of larval rearing water, and adult female metabolic profiles. Larvae reared in water containing biochar developed significantly faster than larvae reared without biochar. Biochar exposure was also associated with substantial restructuring of bacterial communities in larval rearing water. Community composition differed significantly between larvae-only and larvae+biochar water and between biochar-only and larvae+biochar water, while alpha diversity increased in larvae+biochar water relative to larvae-only water. Functional profiling further identified biochar-associated differences in microbial metabolic potential, including increased representation of genes involved in denitrification, sulfur oxidation, and aromatic/furanic compound transformation, alongside reduced representation of nitrogen fixation and sugar uptake functions. Developmental exposure to biochar was also associated with distinct adult female biogenic amine and lipid profiles. Together, these findings indicate that biochar can alter the microbial environment experienced by developing *Aedes aegypti* and is associated with changes in larval development and adult physiology that persist beyond the aquatic exposure period.

Key words: microbiome of water, *Aedes aegypti*, metabolomics, biochar

Introduction

Aedes aegypti (*Ae. aegypti*) is an important vector of arboviruses such as dengue, Zika, chikungunya, and yellow fever; *Ae. aegypti* has an ability to thrive in man-made environments (Kraemer et al., 2019; Lindsay et al., 2017). Gravid *Ae. aegypti* commonly deposit eggs in sites that contain water such as plant pots or tires, in urban and suburban environments (David et al., 2023; Wilke et al., 2019). The selection of an oviposition site represents an important component of mosquito reproduction as no two sites are alike (e.g.: water microbiome, organic detritus, container material). Female *Ae. aegypti* use a combination of environmental cues to distinguish among potential breeding sites. They use visual, chemical and olfactory cues associated with the water and its surrounding environment to examine factors such as potential larval overcrowding and food abundance (David et al., 2023; Khan et al., 2023; Wong et al., 2011). Understanding the cues used during oviposition site selection may therefore provide opportunities to improve mosquito surveillance and control strategies.

In our previous study, we examined the effects of biochar on *Ae. aegypti* oviposition site selection (Rodrigues et al. 2026). Biochar is produced from organic matter burned in the absence of oxygen (pyrolysis). For the case of the previous paper and this paper's experiments, the organic matter pyrolysed was hard wood. The previous work demonstrated in both laboratory and field settings that oviposition sites containing biochar-infused water are strongly preferred over dechlorinated tap water by ovipositing *Ae. aegypti*. In laboratory conditions, gravid *Ae. aegypti* preferentially deposited eggs in oviposition sites containing wood-derived biochar vs those without. Furthermore, supplementation of oviposition traps with biochar increased female *Ae. aegypti* captures under field conditions, demonstrating that the behavioral

response persists in the complexity of a natural environment. These findings indicate that biochar-associated aquatic habitats may be perceived by gravid *Ae. aegypti* as suitable environments for offspring development. However, the biological characteristics that contribute to this response remain unresolved. Here we examine the effect of biochar on larval development, nutrient acquisition and breeding site microbial community composition.

Biochar has been extensively investigated in agricultural and environmental applications, including soil amendment, carbon sequestration, remediation, and wastewater treatment (Díaz et al., 2024; S. Li & Tasnady, 2023; Spokas et al., 2012). Biochar can interact with nutrients, contaminants—including endocrine-disrupting chemicals and microplastics—and microorganisms in soil and water. These interactions may promote pollutant adsorption or immobilization and alter nutrient availability and microbial communities (Dong et al., 2023; Osman et al., 2022; Wu et al., 2022). Their effects depend on the biochar feedstock, pyrolysis conditions, contaminant properties, and environment (He et al., 2022). However, the ecological effects of biochar on the *Ae. aegypti* life cycle is currently not known.

Larval *Ae. aegypti* obtain nutrients by feeding on detritus, microbes, and invertebrates smaller than themselves, which provide nutrients including sugars, salts, lipids, amino acids, and vitamins for growth (Singh & Brown, 1957). Microbial communities are an important component of this nutritional environment and can actively influence mosquito development. Axenic *Ae. aegypti* larvae fail to develop normally, whereas colonization with bacteria can restore development, demonstrating that microbes are required for normal larval development (Coon et al., 2014). More recent work indicates that the microbiota also influences energy production, nitrogen metabolism, and sterol and fatty-acid metabolism during larval development (Romoli et al., 2026). Thus, the differences in microbial composition of larval habitats can potentially affect the nutritional and metabolic environment experienced by developing larvae (Dickson et al., 2017; Louie & Coffey, 2021).

Biochar may provide a mechanism through which this microbial environment is altered. In aquatic environments, biochar can alter microbial community structure by changing the availability and quality of dissolved organic matter and providing surfaces for microbial colonization (Pommier et al., 2014; Wu et al., 2022). Consequently, biochar addition could alter the abundance, composition, or activity of microorganisms available to mosquito larvae. However, whether biochar directly alters the microbial community of *Ae. aegypti* larval habitats, and whether such changes contribute to differences in larval development, remains unclear.

Changes occurring during larval development may also extend beyond the aquatic environment and influence adult mosquito physiology. Larval nutrition (access to proteins, lipids, carbohydrates, and micronutrients) as well as environmental conditions can affect development rate as well as adult phenotypes. Adult size is dependent on larval nutrition, while reproductive traits can be influenced by both larval and adult nutrition (van Schoor et al., 2020; Yan et al., 2021). These developmental effects suggest that differences in larval environments may produce physiological consequences that persist following adult emergence.

Here we examine the biological consequences of biochar exposure during *Ae. aegypti* larval development, the effects of biochar on larval habitat microbial communities, and adult female metabolic profiles. First, we characterize the effect of biochar on larval developmental timing and follow that with metagenomic analysis of microbial communities of larval rearing water under conditions with and without biochar and in the presence or absence of mosquito larvae. We then use untargeted metabolomics to characterize the biochemical composition of adult *Ae. aegypti* that had developed in biochar-infused versus biochar-free water. By examining both the larval/pupal aquatic environment and the female *Ae. aegypti* developing within it, this paper builds upon our previous demonstration of biochar associated oviposition behavior and provides insight into the potential ecological and physiological drivers behind the attractivity of biochar in *Ae. aegypti* breeding habitats.

Results

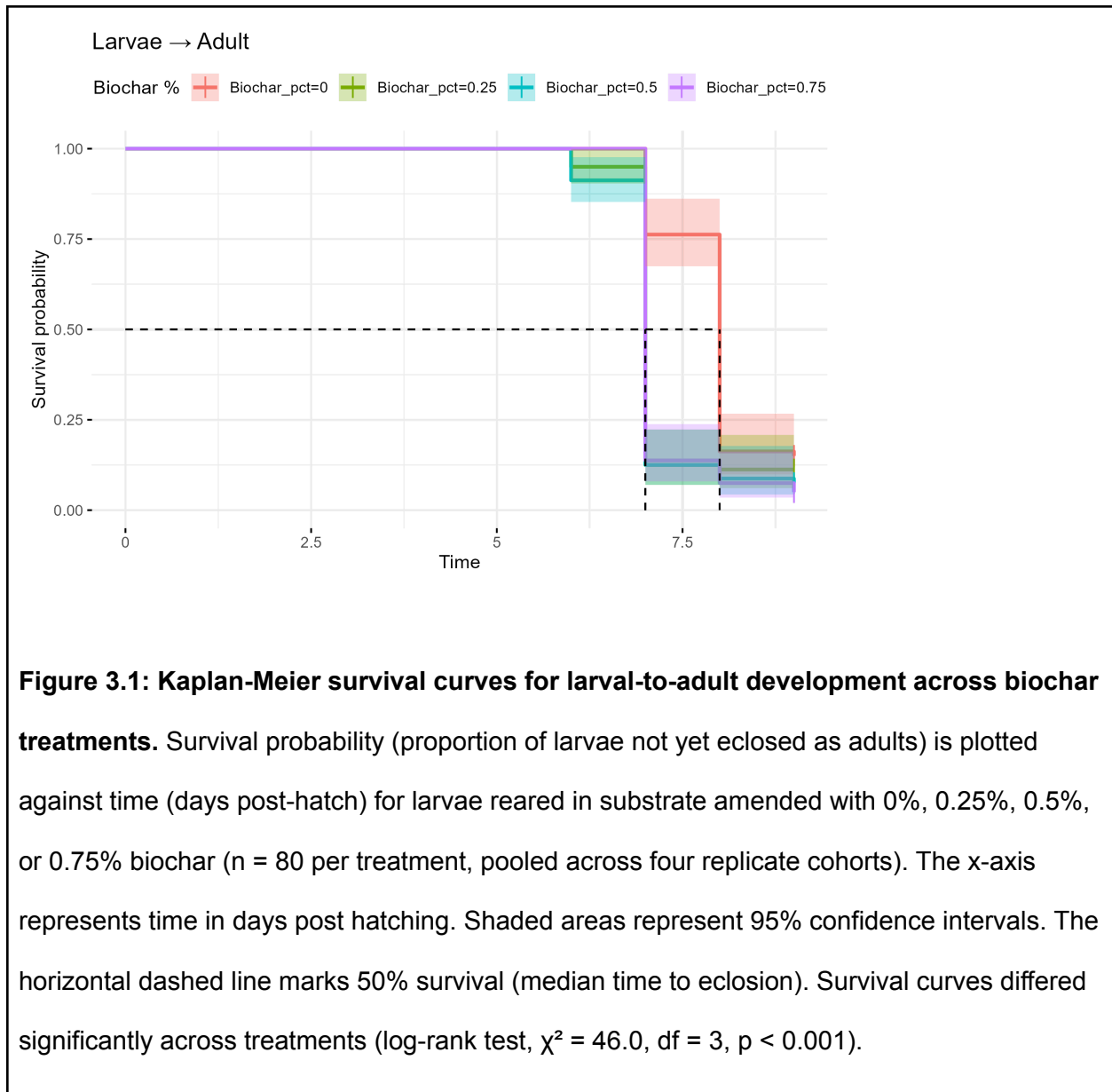
Effects of Biochar Infusion on Larval Development

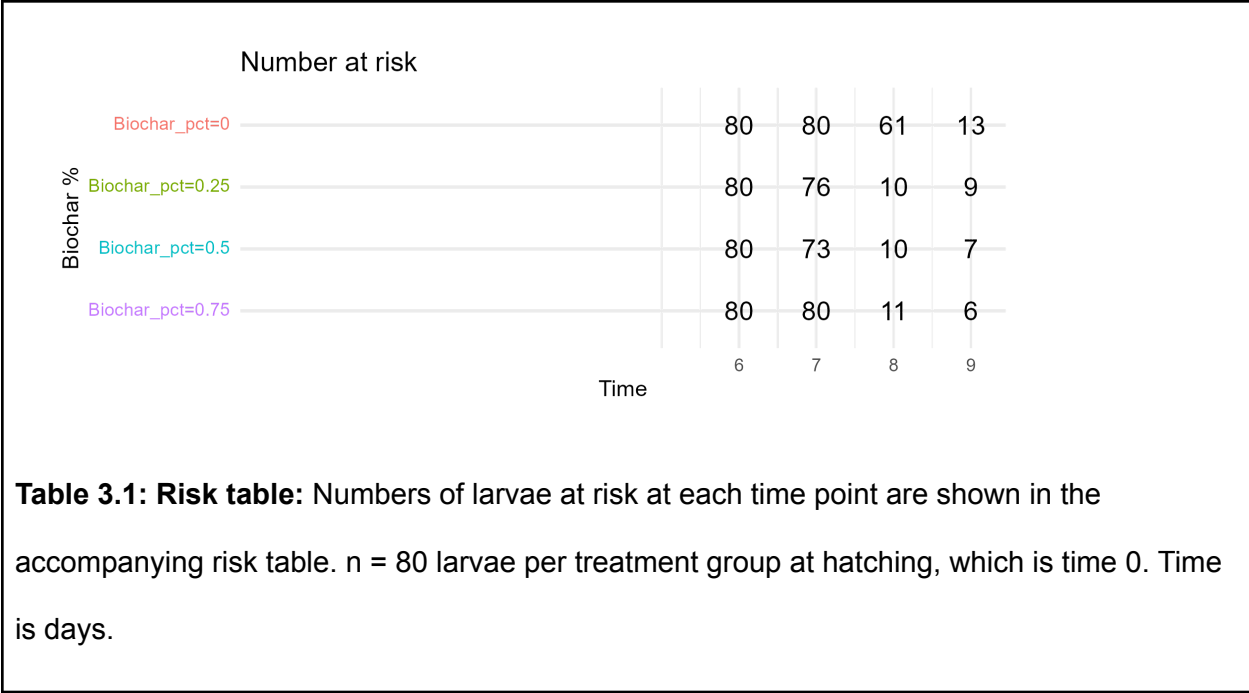
Ae. aegypti were reared in three concentrations of biochar infused water (0.25, 0.5, and 0.75% biochar/water w/v), with water alone as a control, to examine biochar's effects on larval development time. Larval development in the biochar treatment was significantly faster than the controls reared in water alone (Fig. 3.1, Table 3.1). The median time to eclosion was 8 days in the 0% biochar control, compared to 7 days in all three biochar-amended groups (0.25%, 0.5%, and 0.75%). Across the four treatments, 68/80 (85%), 71/80 (89%), 74/80 (93%), and 76/80 (95%) *Ae. aegypti* larvae eclosed by day 9 in the 0%, 0.25%, 0.5%, and 0.75% groups, respectively.

An overall log-rank test confirmed a significant difference in eclosion timing across treatments ($\chi^2 = 46.0$, $df = 3$, $p < 0.001$). A Cox proportional-hazards model, using the 0% control as the reference group, showed that *Ae. aegypti* larvae in all biochar-amended groups had a significantly higher hazard of eclosing (i.e., eclosed faster) than controls: 0.25% biochar (HR = 2.30, 95% CI 1.64–3.23, $p < 0.001$), 0.5% biochar (HR = 2.53, 95% CI 1.81–3.53, $p < 0.001$), and 0.75% biochar (HR = 2.42, 95% CI 1.74–3.38, $p < 0.001$).

Pairwise log-rank comparisons (Benjamini-Hochberg corrected) showed that each biochar-amended group differed significantly from the 0% control (0% vs. 0.25%: $p < 0.001$; 0% vs. 0.5%: $p < 0.001$; 0% vs. 0.75%: $p < 0.001$), but biochar-amended groups did not differ significantly from one another (0.25% vs. 0.5%: $p = 0.31$; 0.25% vs. 0.75%: $p = 0.76$; 0.5% vs. 0.75%: $p = 0.46$). Together, these results indicate that larval development in biochar amended

water shortens the larvae-to-adult development period relative to unamended controls, but that this effect does not appear to be concentration-dependent within the 0.25–0.75% biochar range tested.





Effects of Biochar on the Microbiome of *Ae. aegypti* Larval Aquatic Habitat

Larval Breeding Site Microbial Constitution, Diversity, Consistency, and Functional Enrichment in the Presence and Absence of Biochar

Investigation of the effect of biochar on the microbiome of the larval breeding habitat was performed by infusion of larval rearing trays with 0.75% biochar. The biochar used in most of these experiments was produced from Manzanita wood (*Arctostaphylos densiflora*) pyrolyzed in a muffle furnace. This wood type was chosen to maintain consistency with prior work as most previous experiments used Manzanita biochar (Rodrigues et al. 2026). The trays were also seeded with a representative microbiome derived from water collected from artificial breeding sites exposed to outdoor environmental conditions in Davis, California, to represent the microbial composition of an anthropogenically derived site.

Alpha and Beta Diversity of Ae. aegypti Larval Habitat Microbiomes: Biochar Restructures Water Microbiome Composition, Variability, and Diversity in the Presence of Larvae

Alpha diversity patterns (the relative species richness and homogeneity of a site) differed between comparisons focused on the differential presence of larvae or biochar. Chao1 (species richness), Shannon diversity (richness and evenness), and Simpson diversity (relative dominance/homogeneity of species) did not differ significantly between control (biochar, no larvae) and larvae+biochar water (Chao1: $p = 0.056$; Shannon: $p = 0.147$; Simpson: $p = 0.291$; Fig. 3.2A–C; Table 3.2). In contrast, all three alpha-diversity measures were significantly higher in larvae+biochar water than in larvae-only water (Chao1: $p = 2.15 \times 10^{-4}$; Shannon: $p = 8.86 \times 10^{-4}$; Simpson: $p = 0.00796$; Fig. 3.2D–F; Table 3.2). Thus, although biochar-containing communities differed in composition from both comparison groups, alpha diversity increased specifically when biochar was present in water containing larvae.

Introduction of biochar resulted in changes in bacterial community diversity and abundance in *Ae. aegypti* larval habitats based on the Alpha diversity analysis. Beta diversity (the consistency of taxonomic diversity and relative abundance between samples) was analyzed by PERMANOVA pairwise analysis (Fig. 3.3; Table 3.3). This analysis revealed significant differences in community composition between control water (biochar, no larvae) and larvae+biochar water ($R^2 = 0.478$, $p = 0.002$). In this comparison the larvae and biochar water show a significant reduction in variance between experimental replicates with the biochar alone samples showing much larger variation between replicates (Fig. 3.3A).

A similar result was observed in the comparison between larvae-only and larvae+biochar water ($R^2 = 0.306$, $p = 0.002$;). The water containing both larvae and biochar shows reduced variation relative to the water containing larvae without biochar (Fig. 3.3B). The combined effect of the

presence of both larvae and biochar reduced beta diversity significantly relative to either biochar or larvae alone.

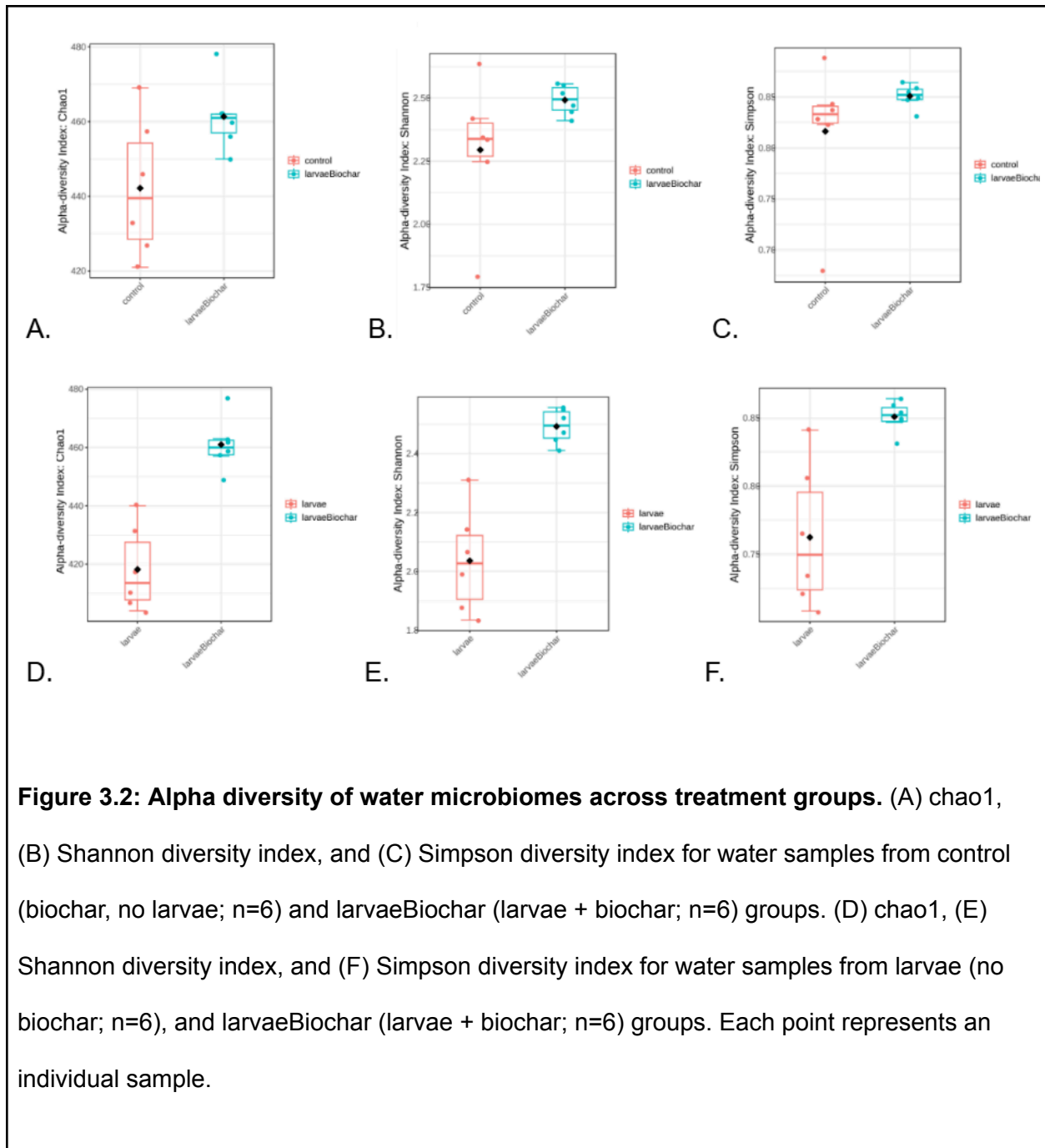
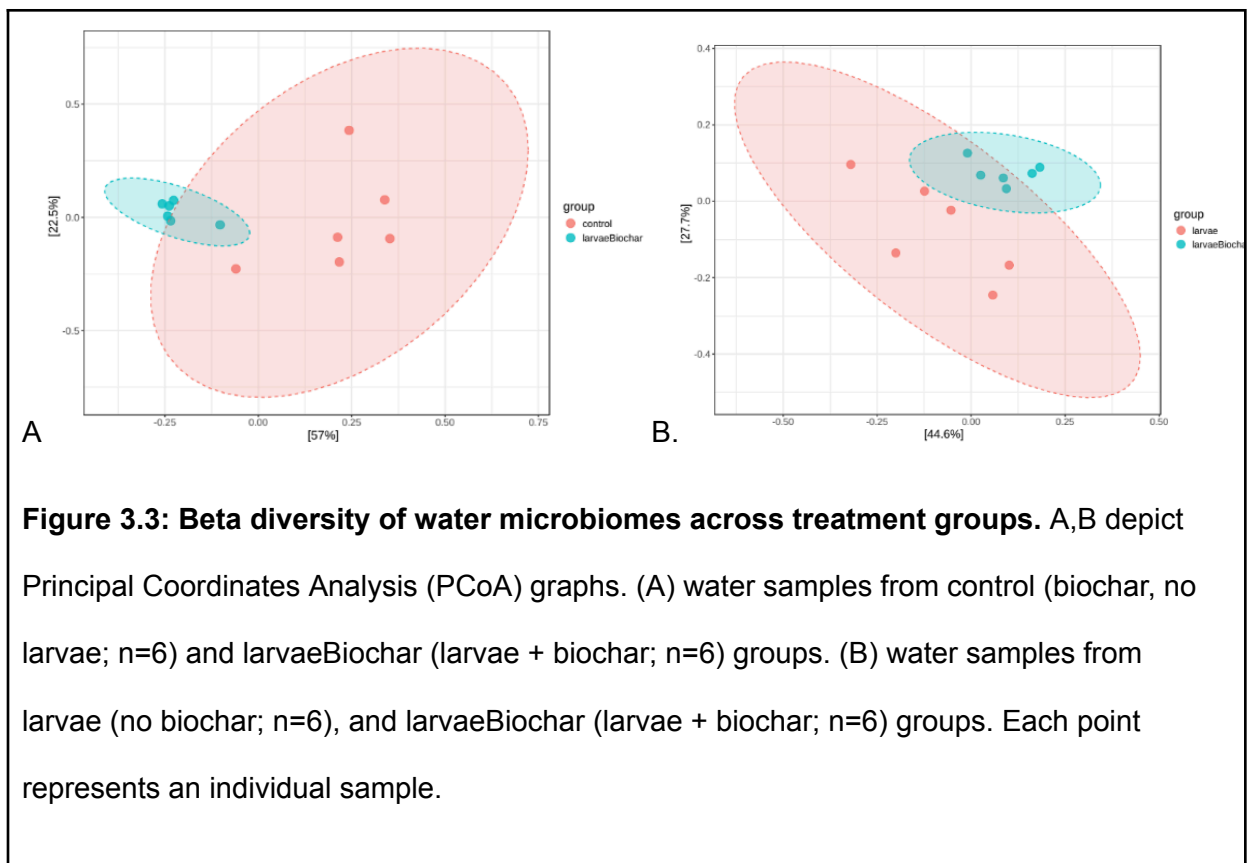


Figure 3.2: Alpha diversity of water microbiomes across treatment groups. (A) chao1, (B) Shannon diversity index, and (C) Simpson diversity index for water samples from control (biochar, no larvae; n=6) and larvaeBiochar (larvae + biochar; n=6) groups. (D) chao1, (E) Shannon diversity index, and (F) Simpson diversity index for water samples from larvae (no biochar; n=6), and larvaeBiochar (larvae + biochar; n=6) groups. Each point represents an individual sample.

Indices	Control vs. LarvaeBiochar	Larvae vs. LarvaeBiochar
Chao1 richness	p = 0.056 (ns)	p = 2.15×10^{-4} (sig)
Shannon	p = 0.147 (ns)	p = 8.86×10^{-4} (sig)
Simpson	p = 0.291 (ns)	p = 0.00796 (sig)

Table 2.2: Post-hoc pairwise comparison. (ns) not significant. (sig) significant.



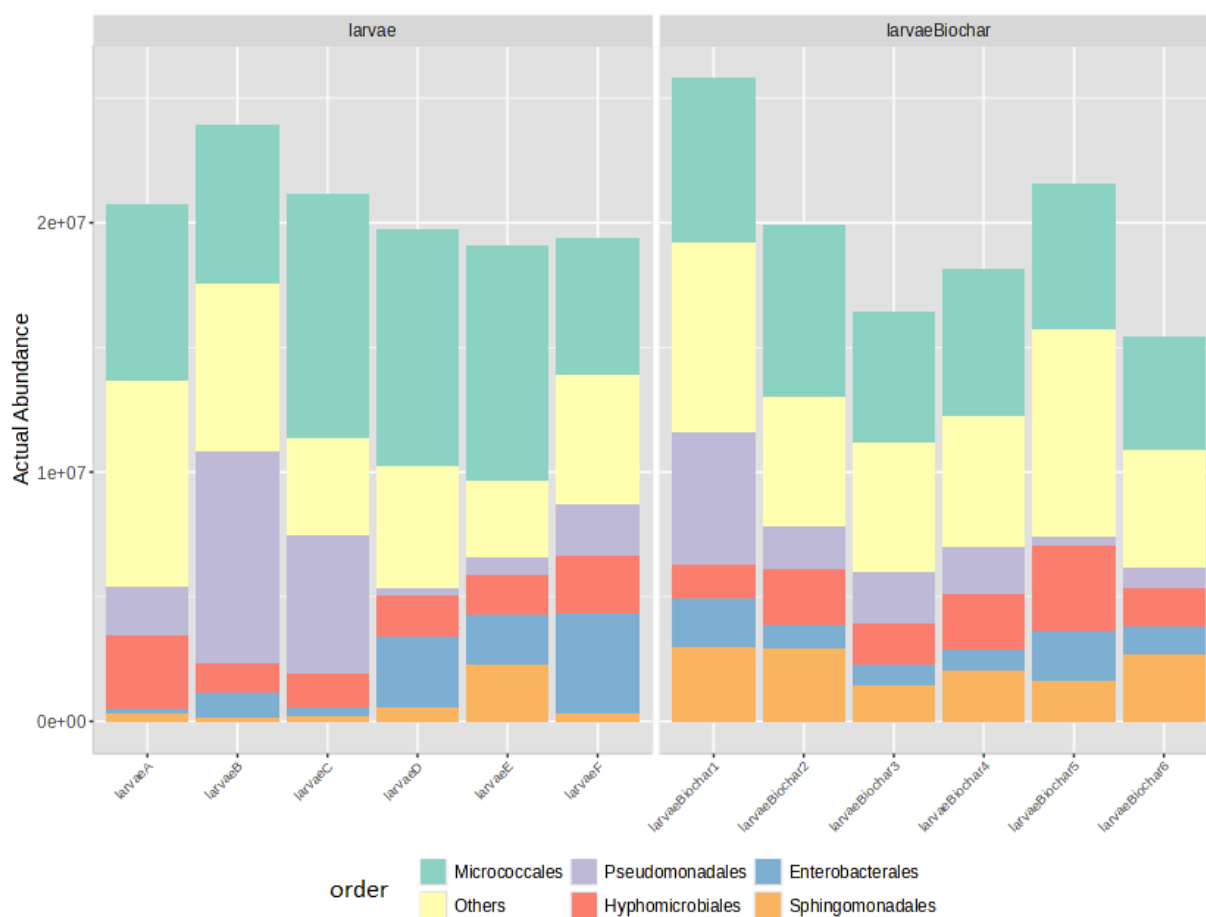
Comparison	F	R ²	p	FDR
Control vs. LarvaeBiochar	9.17	0.478	0.002	0.002
Larvae vs. LarvaeBiochar	4.42	0.306	0.002	0.002

Table 2.3: Pairwise PERMANOVA.

Taxonomic Composition Illustrates Differences Among Treatment Groups: Taxa Abundance Appears More Consistent in the larvaeBiochar Group

Relative abundance profiles showed differences in the composition of bacterial orders represented among treatment groups (Fig. 3.4). The order Micrococcales represented a substantial and relatively consistent proportion of the bacterial community across larvae-only and larvae+biochar samples and appeared more consistently abundant in larvae+biochar water. Sphingomonadales also showed greater and more consistent representation in larvae+biochar samples than in larvae-only samples, although this difference was not significant after FDR correction (Fig 2.4A). In the control versus larvae+biochar comparison, Micrococcales was more abundant in larvae+biochar water, whereas Caulobacteriales was more prominent in control water. Several other orders, including Pseudomonadales, Enterobacteriales, and Hyphomicrobiales, showed substantial variation among biological replicates (Fig 2.4B).

A.



B.

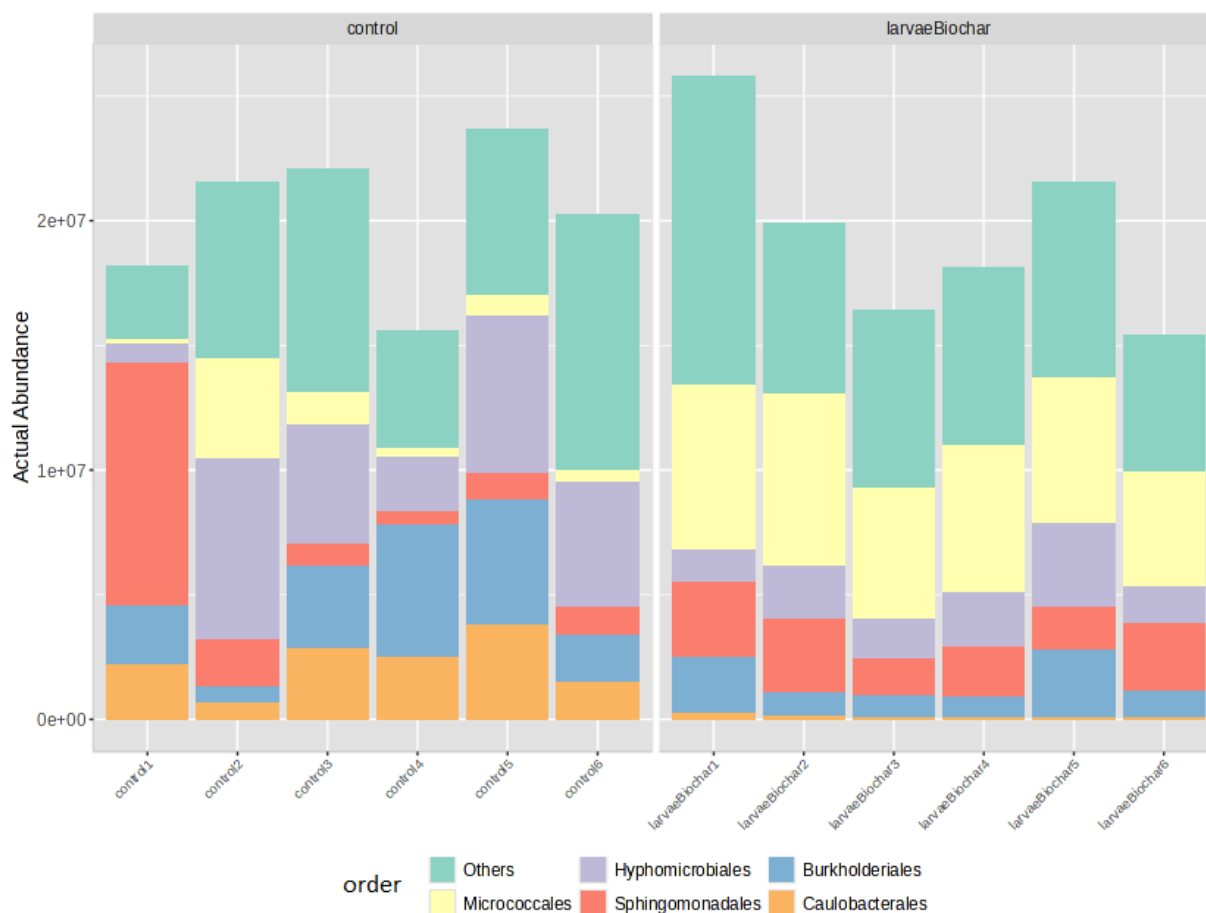


Figure 3.4: Relative taxonomic composition of water microbiomes at the order level. (A) Larvae vs. larvae+biochar groups. (B) Control (biochar/no larvae) vs. larvae+biochar groups. Bars represent sequencing read proportions of the five most abundant bacterial orders per group, with remaining taxa grouped as "Others." Each bar represents an individual replicate (n=6 per group).

Biochar Amendment Significantly Shifts Bacterial Order-Level Composition

Linear discriminant analysis Effect Size (LEfSe) analysis identifies taxa that differ significantly in relative abundance between treatment groups and estimates their effect size, highlighting taxa that most strongly distinguish the groups. LEfSe identified several bacterial orders that were differentially abundant between treatment groups (Fig. 3.5, Table 3.4). In the Larvae vs. Larvae+Biochar comparison, Burkholderiales (linear discriminant analysis (LDA) = -4.48), Rhodospirillales (-3.54), Cytophagales (-3.82), Chitinophagales (-3.87), and Flavobacteriales (-4.04) were significantly enriched in the Larvae+Biochar group after FDR correction (FDR < 0.05) (Fig. 3.5A). In the Control vs. Larvae+Biochar comparison, Caulobacteriales (4.73) and Bacillales (3.64) were significantly enriched in the Control group, while Micrococcales (-5.09), Mycobacteriales (-3.86), and Chitinophagales (-3.80) were significantly enriched in the Larvae+Biochar group (Fig. 3.5B). Notably, Chitinophagales was significantly enriched in Larvae+Biochar relative to both Larvae and Control, suggesting a consistent biochar-associated shift for this order.

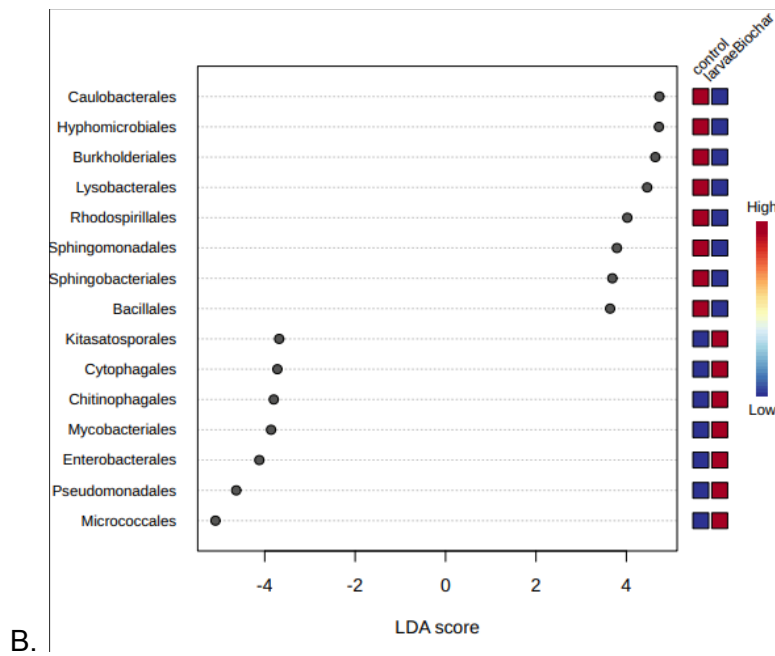
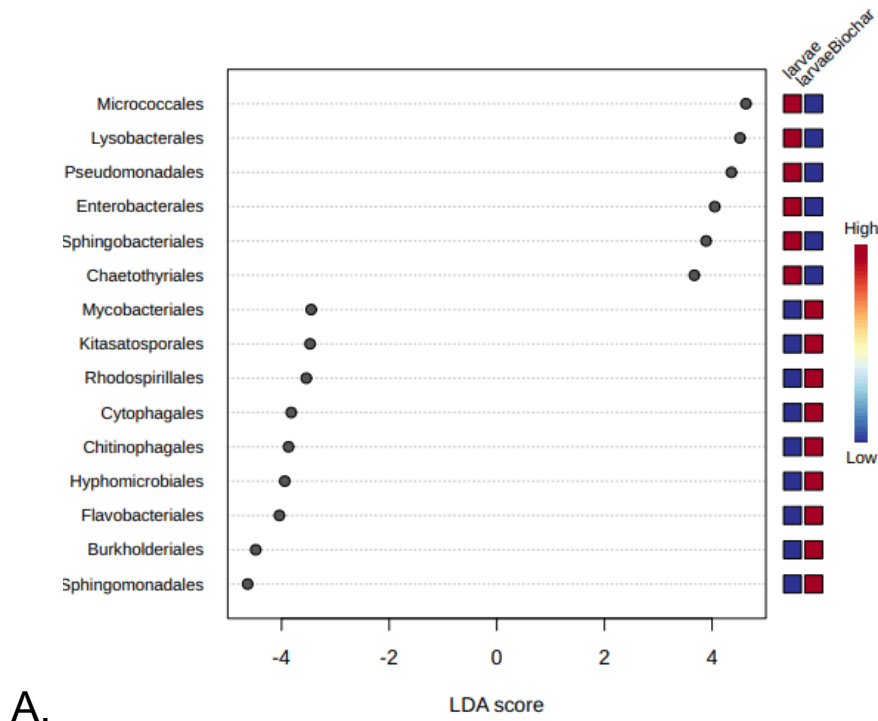


Figure 3.5: Linear discriminant analysis (LEfSe) of bacterial taxa differentially abundant between groups. (A) Larvae vs. larvae+biochar. (B) Control vs. larvae+biochar. Bars show taxa with significant differential abundance ranked by LDA score; bar color indicates the group in which each taxon was enriched.

Order	Larvae	Larvae+Biochar (vs Larvae)	Control	Larvae+Biochar (vs Control)
Micrococcales	4.63 ns	–	–	-5.09 *
Lysobacterales	4.52 ns	–	4.46 ns	–
Pseudomonadales	4.36 ns	–	–	-4.63 ns
Enterobacterales	4.05 ns	–	–	-4.12 ns
Sphingobacteriales	3.89 ns	–	3.69 ns	–
Chaetothyriales	3.67 ns	–	–	–
Caulobacterales	–	–	4.73 *	–
Hyphomicrobiales	–	-3.94 ns	4.72 ns	–
Burkholderiales	–	-4.48 *	4.64 ns	–
Rhodospirillales	–	-3.54 *	4.02 ns	–
Sphingomonadales	–	-4.63 ns	3.79 ns	–
Bacillales	–	–	3.64 *	–
Mycobacteriales	–	-3.45 ns	–	-3.86 *
Kitasatosporales	–	-3.47 ns	–	-3.68 ns
Cytophagales	–	-3.82 *	–	-3.72 ns
Chitinophagales	–	-3.87 *	–	-3.80 *
Flavobacteriales	–	-4.04 *	–	–

* $FDR < 0.05$ (significant) ns = not significant ($FDR \geq 0.05$) – = not a significant/plotted result in that comparison

Table 2.4: LDA scores for bacterial orders are differentially abundant between groups, derived from two pairwise LEfSe comparisons: Larvae vs. Larvae+Biochar, and Control vs. Larvae+Biochar. Positive scores indicate enrichment in the first-named group of each comparison; negative scores indicate enrichment in Larvae+Biochar.

Biochemical functional composition differed among treatments

Analysis of the functional composition of the microbial communities by analysis of protein coding gene representation differed significantly among the three treatments. PERMANOVA identified significant differences in community composition using both Bray–Curtis distances ($R^2 = 0.625$, $F = 12.52$, $p = 1 \times 10^{-4}$) and Aitchison distances ($R^2 = 0.446$, $F = 6.04$, $p = 1 \times 10^{-4}$). Pairwise comparisons were also significant for both contrasts, with differences between larvae + biochar and biochar-only treatments ($R^2 = 0.360$, $p = 0.0031$) and between larvae + biochar and larvae-only treatments ($R^2 = 0.366$, $p = 0.0018$).

PCoA based on Aitchison distances separated the three treatment groups (Fig. 3.6A). PCo1 explained 39.1% of the variation and primarily separated the larva-free biochar treatment from the two treatments containing larvae. PCo2 explained an additional 14.5% of the variation and separated the larvae-only and larvae + biochar treatments. Thus, the major axes of functional variation corresponded to larval presence and, among larval treatments, biochar exposure.

Community functional dispersion decreased with combined treatment

Community dispersion in protein coding gene functional representation also differed significantly among treatments. PERMDISP detected significant differences in dispersion using both Bray–Curtis distances ($F = 9.74$, $p = 0.0018$) and Aitchison distances ($F = 42.15$, $p = 1 \times 10^{-4}$). Based on Aitchison distances, mean distance to the group centroid was greatest in biochar-only water (95.5), intermediate in larvae-only water (59.3), and lowest in larvae + biochar water (40.7; Fig. 3.6B). Tukey's HSD indicated that dispersion was significantly lower in larvae + biochar water than in biochar-only water ($p = 5 \times 10^{-7}$) and larvae-only water ($p = 0.020$). For Bray–Curtis distances, dispersion was also significantly lower in larvae + biochar water than in biochar-only water ($p = 0.0014$), whereas the difference between larvae + biochar and larvae-only water was not significant ($p = 0.109$).

Differential abundance of targeted functional genes

Analysis of a targeted functional gene panel identified treatment-associated changes in several functional categories of enzyme coding genes predicted to be of functional importance *a priori* in the context of these environments. These included 27 genes representative of KEGG Orthology (KO) functions including nitrogen cycling, sulfur cycling, aromatic degradation, chitin and glycan degradation, biofilm formation, and ion transport (Fig. 3.6C). In the biochar contrast, nirK (nitrite reductase: denitrification) and nosZ (nitrous oxide reductase: denitrification) were significantly more abundant in larvae + biochar water than in larvae-only water ($q = 0.011$ and 0.014 , respectively), whereas narG (respiratory nitrate reductase: nitrate reduction) was significantly reduced ($q = 0.012$). The sulfur oxidation gene soxB was also more abundant with biochar ($q = 0.012$). In contrast, the nitrogen fixation genes nifH, nifD, and nifK (nitrogenase subunits) were reduced in the presence of biochar (all $q = 0.019$), as were the ammonium transporter amtB (ammonium transport, $q = 0.045$) and the cation transport genes kdpA ($q = 0.020$), trkA ($q = 0.021$), and nhaA ($q = 0.045$).

The larval contrast showed a different pattern of functional responses. The nitrogen fixation genes nifH, nifD, and nifK were more abundant in larvae + biochar water than in biochar-only water ($q = 0.018$ – 0.027), together with nrfA (nitrite reductase, $q = 0.045$). In contrast, napA (periplasmic nitrate reductase, $q = 0.004$), nirB (nitrite reductase, $q = 0.027$), ureC (urease: urea decomposition, $q = 0.013$), and sat (sulfate adenylyltransferase: sulphur metabolism, $q = 0.027$) were significantly reduced in the presence of larvae.

The Presence of Biochar Alters The Functional Composition of the Breeding Site

Microbiome

Functional analysis showed enrichment of genes involved in aromatic and furanic compound degradation in larvae + biochar water. Of the significant features associated with these

functions, 12 of 25 increased with biochar, exceeding the background increase rate of 11.2% (binomial $p = 4.8 \times 10^{-6}$), with monooxygenase and hydroxylase annotations also enriched among increasing features. More abundant genes included ring-hydroxylating oxygenases (hapE, hipH, nagG, resorC, npcB, rif19, tfdB, and mhpA), furanic degradation genes (hmfA and hmfF), and the alkylaromatic degradation gene cmtC (Fig. 3.7). In contrast, central aromatic ring-cleavage genes catA, pcaG, and pcaH did not differ significantly among treatments ($q = 0.069, 0.899, \text{ and } 0.849$, respectively). F420-dependent metabolism genes (fgd1 and fno) and spermidine/putrescine ABC transport genes (potA, potB, potC, and potD) were more abundant in larvae+ biochar group, whereas PTS-associated (phosphotransferase system) sugar uptake genes, including scrA, treB, gatC, and scrK, decreased. All 24 significant PTS features decreased with biochar, although the overall directional enrichment was not significant (binomial $p = 0.058$).

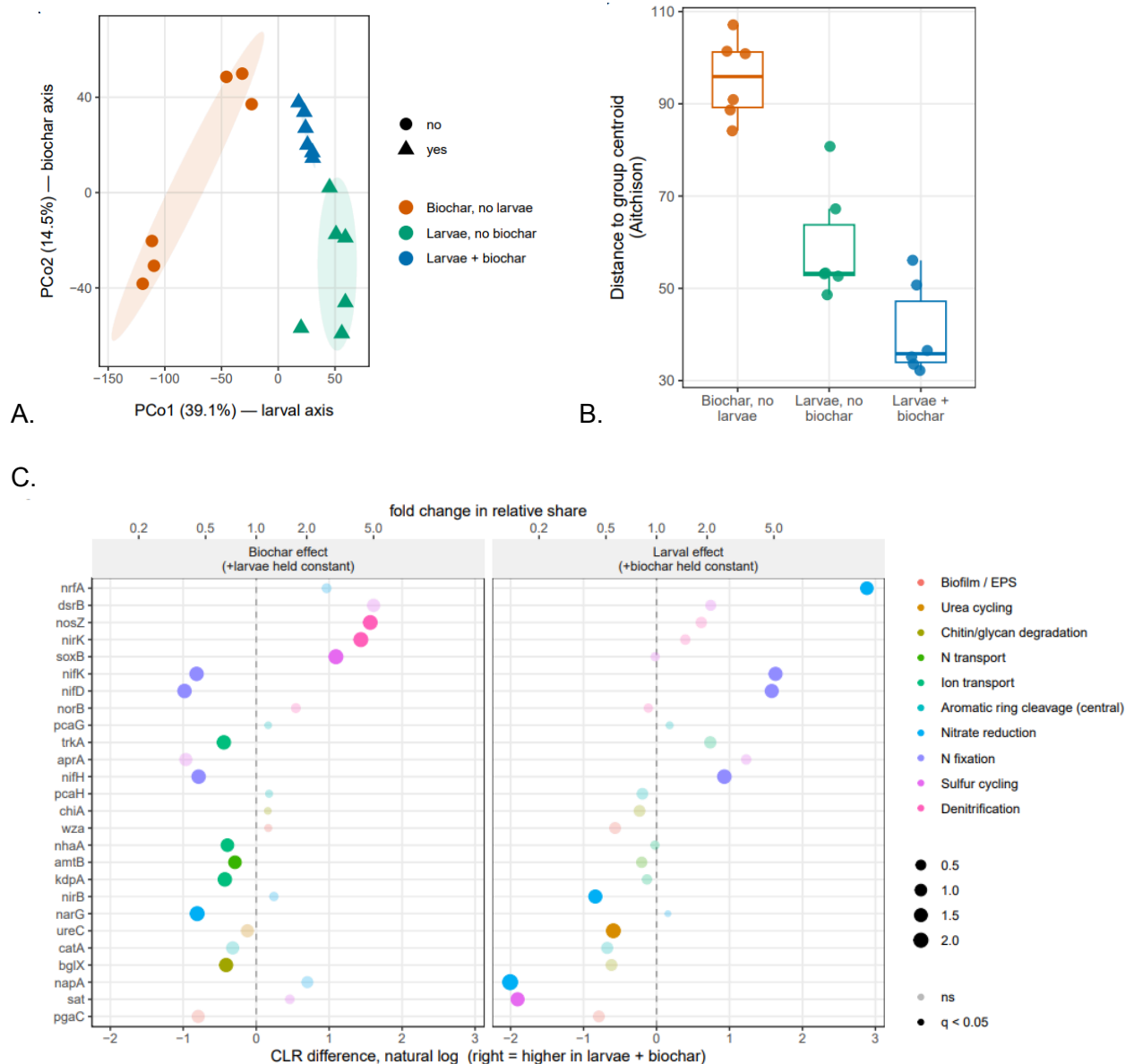
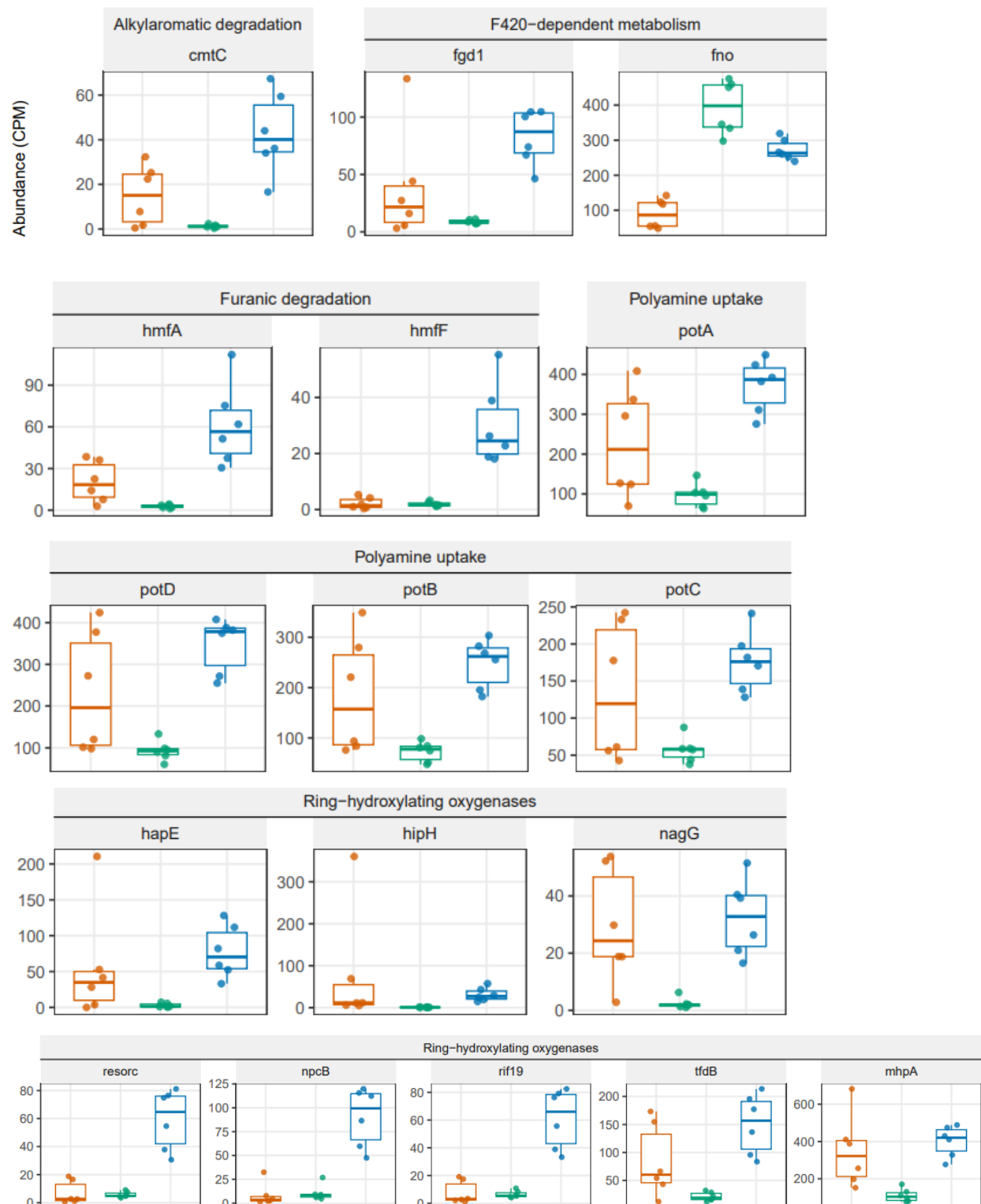


Figure 3.6: Functional composition and community dispersion of larval habitat microbial communities differ among treatments. (A) PCoA based on Aitchison distances calculated from centered-log-ratio (CLR)-transformed KEGG Orthology (KO) abundances for 18 samples. PCo1 explained 39.1% of the variation and primarily separated the larval and larva-free treatments, while PCo2 explained 14.5% of the variation and separated the two larval-containing treatments according to biochar treatment. Points represent individual samples

from the biochar-only, larvae-only, and larvae + biochar treatments. (B) Distance to group centroid for each treatment. Points represent individual samples and boxplots show the distribution of distances within each treatment. (C) Differential abundance of KOs from an a priori functional panel representing ten functional themes: biofilm/extracellular polymeric substance (EPS) production, urea cycling, chitin/glycan degradation, nitrogen transport, ion transport, central aromatic ring cleavage, nitrate reduction, nitrogen fixation, sulfur cycling, and denitrification (color for each depicted in the legend). Results are shown for the biochar effect (larvae + biochar versus larvae-only) and larval effect (larvae + biochar versus biochar-only). The x-axis represents the CLR difference (natural log), the dot sizes represent fold change. Significance was determined following Benjamini–Hochberg correction, with $q < 0.05$ considered significant.



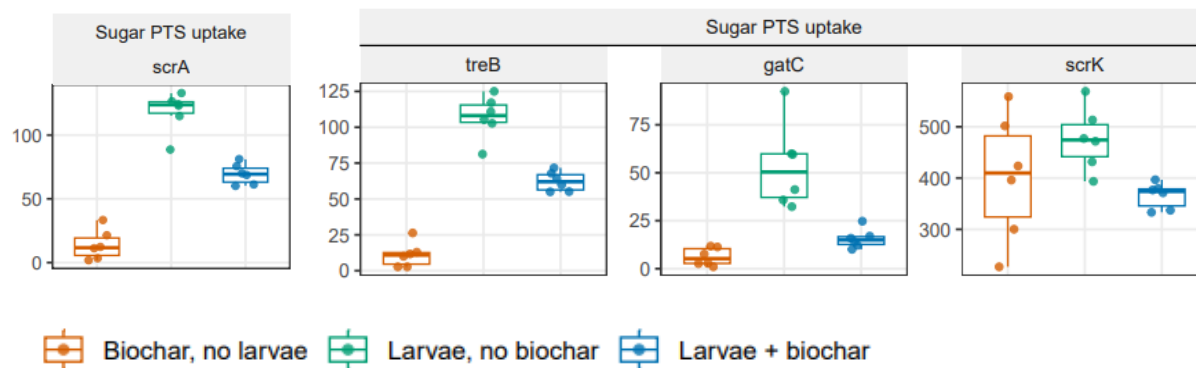


Figure 3.7: Abundance of functional genes associated with aromatic compound degradation, F420-dependent metabolism, polyamine uptake, and sugar uptake differs among treatments. Abundance of selected KEGG Orthologs (KOs), reported as counts per million (CPM), in biochar-only, larvae-only, and larvae + biochar water. Genes are grouped by functional theme, including alkylaromatic degradation (cmtC), F420-dependent metabolism (fgd1, fno), furanic degradation (hmfA, hmfF), polyamine uptake (potA, potB, potC, potD), ring-hydroxylating oxygenases (hapE, hipH, nagG, resorc, npcB, rif19, tfdB, mhpA), and sugar phosphotransferase system (PTS) uptake (scrA, treB, gatC, scrK). Boxplots show the distribution of samples within each treatment, with individual points representing samples. Ring-hydroxylating oxygenases activate aromatic compounds for subsequent ring cleavage, whereas the central ring-cleavage genes catA, pcaG, and pcaH did not differ significantly among treatments.

Adult Female *Ae. aegypti* Biochemistry: Examining Biochar Effects on Metabolism

Metabolomic Profiling of Adult Female *Ae. aegypti*: Larval Biochar Exposure Alters Biogenic Amine and Lipid Profiles

To determine whether exposure to biochar during larval development was associated with changes in adult female metabolism and constitution, whole-body metabolite profiles were compared between biochar-exposed and non-exposed *Ae. aegypti*. Each biological replicate consisted of a pool of five adult females, with six pooled samples analyzed per treatment group (n = 6 per group). Samples were analyzed for biogenic amines and lipids.

Biogenic amine metabolites: Biogenic amine profiles showed differential compositions between biochar-exposed and non-exposed females. PCA revealed clear separation between groups along PC1 (43.8% of variance explained; $p = 0.002$, Fig. 3.8A). This separation was confirmed by OPLS-DA (Fig. 3.8B). The top VIP-ranked features from the OPLS-DA model closely matched the most statistically significant univariate hits (e.g., features 2.05_228.06, 2.05_570.18, 2.21_356.15), and notably, the most strongly significant features overall were consistently elevated in the non-biochar exposed group (Fig. 3.9A). Together, these analyses indicate that larval exposure to biochar was associated with differences in the adult female biogenic amine profile.

Integrated pathway enrichment analysis, combining mummichog and gene set enrichment analysis (GSEA) p-values, identified three pathways: sphingolipid, fatty acid elongation and fatty acid degradation (Fig. 3.9B). These pathways showed convergent significance across both independent enrichment methods, distinguishing them from pathways supported by only one approach.

Lipidomics: Lipidomic profiles also differed between biochar-exposed and non-exposed females. PCA showed partial separation between groups along PC1 (30.6% of variance explained), with some overlap between groups (Fig. 3.10A). OPLS-DA showed separation between groups (Fig. 3.10B). The top VIP-ranked and most statistically significant features were largely consistent between multivariate and univariate approaches, with the majority of top-ranked discriminating features elevated in the non-biochar exposed group (Fig. 3.11A). Of the 2,899 detected lipid features, 167 met the combined fold-change and statistical significance criteria (Fig. 3.11). Among the significantly altered annotated lipid features, triglycerides accounted for 101 features (84%). Of these triglyceride features, 98 (97%) were reduced in biochar-exposed females (Fig. 3.11). The top VIP-ranked features included several triglyceride species, including TG 56:8, TG 53:6, and TG 47:2, which were consistently reduced in biochar-exposed females (Fig. 3.11A). These results demonstrate that lipidomic differences between treatment groups were particularly pronounced among triglyceride species. This is reinforced by the enriched lipid metabolic functions identified in the biogenic amine analysis.

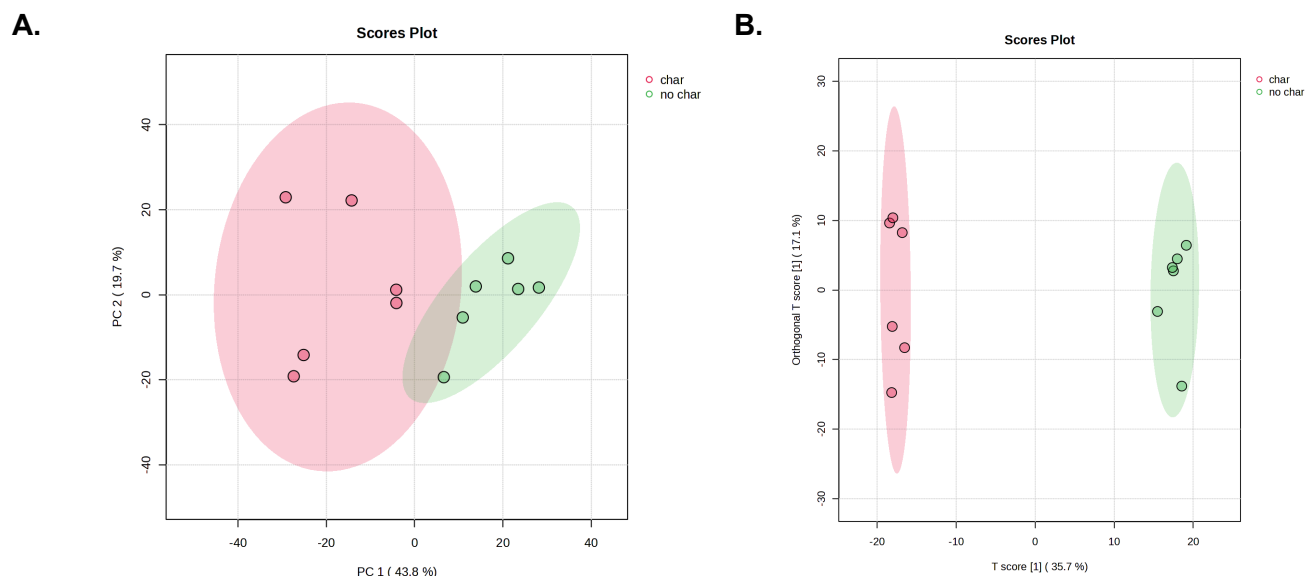
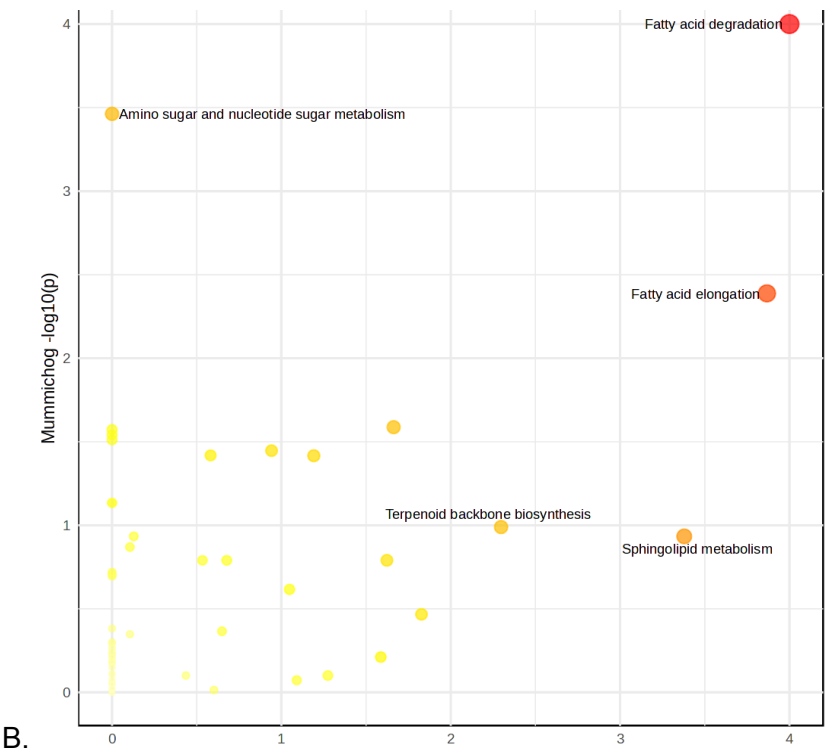
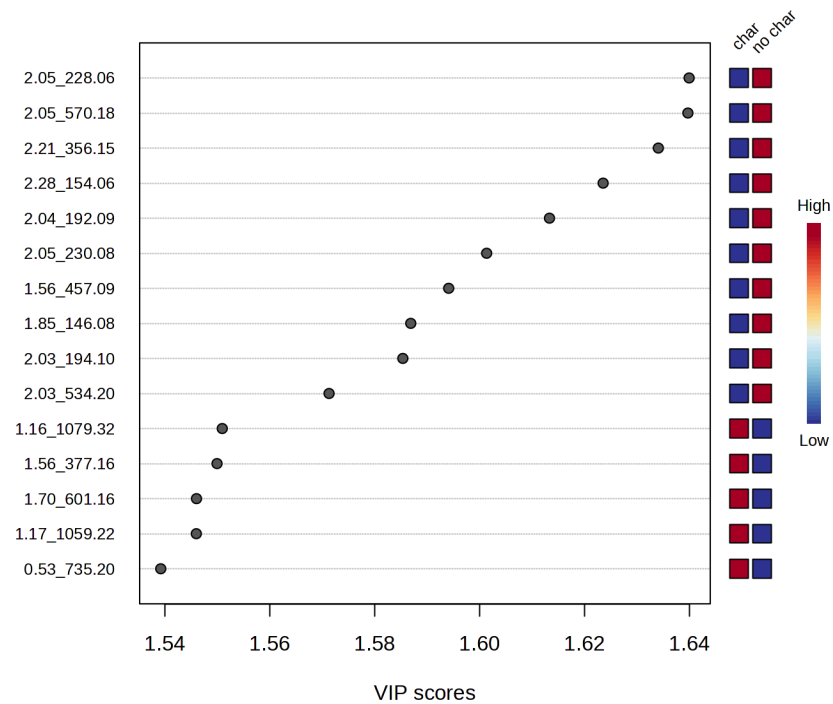


Figure 3.8. Multivariate analysis of biogenic amine profiles in adult female *Ae. aegypti*. (A)

PCA scores plot showing separation between biochar-exposed and non-exposed groups along PC1 (43.8% of variance) and PC2 (19.7% of variance); density plots show the distribution of samples along each component, with group separation along PC1 confirmed by statistical testing ($p = 0.002$). (B) OPLS-DA scores plot showing complete separation between groups along the predictive component (T score, 35.7% of variance).

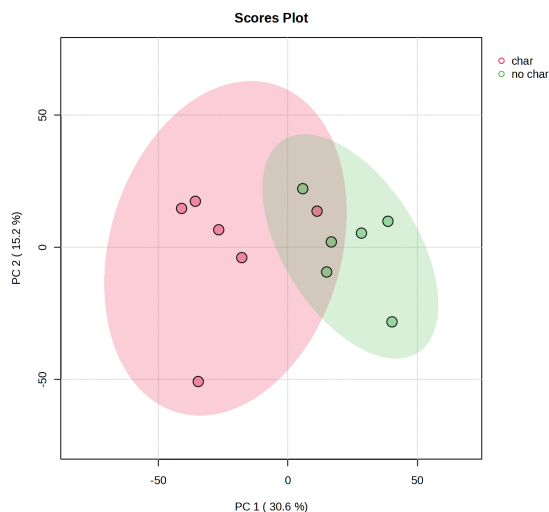
A.



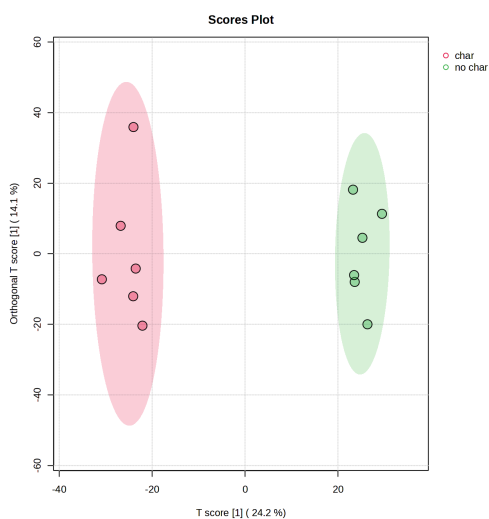
B.

Figure 3.9. Discriminating biogenic amine features between biochar-exposed and non-exposed female *Ae. aegypti*. (A) Variable Importance in Projection (VIP) scores from the OPLS-DA model, showing the top 15 features contributing most to group separation; color indicates relative abundance (red = high, blue = low) in each group. The table depicts an annotation of one known compound. (B) Integrated pathway enrichment analysis of metabolic shifts associated with biochar rearing. Bubble plot of KEGG pathways enriched among metabolite features altered between biochar-reared and control adult *Ae. aegypti*, based on two complementary approaches applied to the same untargeted LC-MS dataset: mummichog pathway analysis (y-axis, $-\log_{10} p$) and gene set enrichment analysis (GSEA; x-axis, $-\log_{10} p$). Each bubble represents one pathway; bubble size scales with the number of significantly altered features annotated to that pathway, and color intensity reflects the combined significance across both methods (Fisher's combined p-value), with red denoting the strongest, most concordant enrichment. Pathways in the upper right (large, red bubbles) are supported by both independent enrichment methods.

Feature ID (RT_m/z)	Compound Annotation
3.79_806.72	TG 47:2
3.78_882.75	TG 53:6
3.94_884.77	TG 53:5
3.54_764.71	TG O-45:2 (alt: TG O-17:1_12:0_16:1)
3.61_752.68	TG 43:1



A.



B.

Figure 3.10. Multivariate analysis of lipidomic profiles in adult female *Ae. aegypti*.

(A) PCA scores plot showing partial separation between biochar-exposed and non-exposed groups along PC1 (30.6% of variance) and PC2 (15.2% of variance). (B) OPLS-DA scores plot showing complete separation between groups along the predictive component (T score, 24.2% of variance).

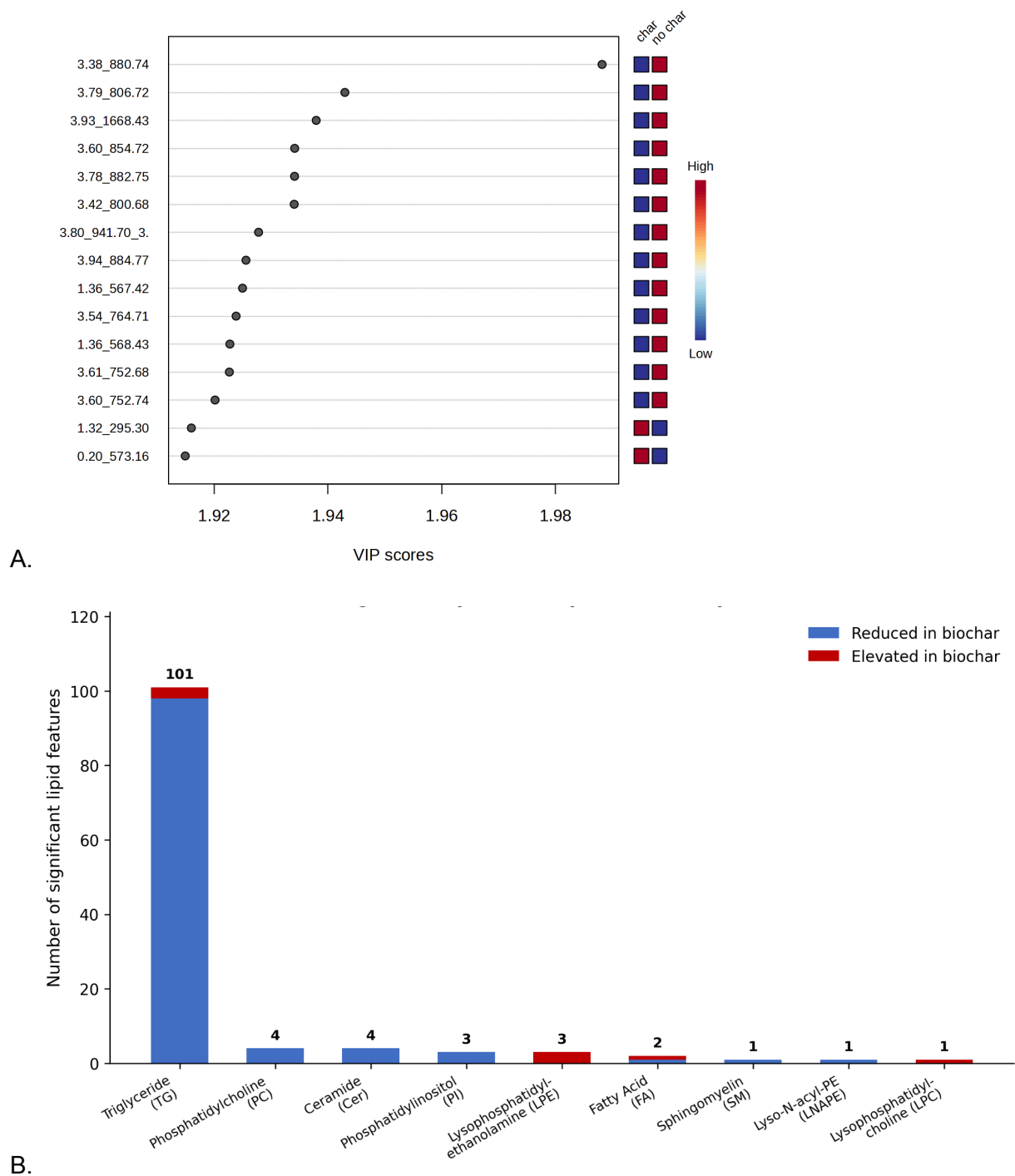


Figure 3.11. Discriminating lipid features between biochar-exposed and non-exposed female *Ae. aegypti*. (A) VIP scores from the OPLS-DA model, showing the top 15 features contributing most to group separation; color indicates relative abundance (red = high, blue = low) in each group. The table depicts annotations of known compounds. Top-ranked features

were predominantly identified as triglyceride (TG) species, consistently reduced in biochar-exposed *Ae. aegypti*. (B) Number of significantly altered lipid features ($p < 0.05$) by lipid class, colored by direction of change in biochar-exposed *Ae. aegypti* relative to non-exposed *Ae. aegypti*. Triglycerides (TG) accounted for the large majority of significant features (101 of 120, 84%), of which 98 (97%) were reduced in biochar-exposed *Ae. aegypti*.

Discussion

Our previous work demonstrated that gravid *Ae. aegypti* preferentially select biochar associated oviposition sites in both laboratory and field settings (Rodrigues et al. 2026). The present study extends these findings by examining potential biological consequences of biochar exposure during the aquatic stage of the mosquito life cycle. Biochar was associated with changes in bacterial community composition in larval rearing water, with effects observed in both the presence and absence of larvae, while differences in alpha diversity (taxa diversity in individual samples) were observed specifically when larvae were present. Larval presence was also associated with reduced beta-diversity, indicating greater consistency among replicate bacterial communities, with the greatest reduction observed in larvae+biochar water. Functional differences between larvae-only and larvae+biochar communities further indicated increased denitrification, sulfur oxidation, and aromatic compound metabolism, alongside reduced nitrogen fixation and carbohydrate uptake. Developmental exposure to biochar was also associated with distinct adult female biogenic amine and lipid profiles, particularly a pronounced reduction in triglyceride features. Together, these findings indicate that biochar can shape the constitution and function of the microbial environment experienced by developing *Ae. aegypti* and is associated with changes in larval development and adult physiology that extend beyond the aquatic exposure period.

Biochar Exposure Effects on Larval Development

The accelerated development observed in biochar-amended water indicates that biochar exposure influenced the rate at which *Ae. aegypti* progressed from larvae to adults. All three biochar concentrations significantly increased the rate of eclosion relative to the unamended control, with larvae exposed to 0.25%, 0.5%, and 0.75% biochar having approximately 2.3-, 2.5-, and 2.4-fold higher hazards of eclosion, respectively. However, the absence of significant differences among the biochar concentrations suggests that the effect was not concentration-dependent within the range tested. The mechanism underlying this accelerated development is unclear, but may involve changes in the larval environment that affect resource availability or utilization. In particular, biochar-associated changes in dissolved organic compounds, nutrients, or microbial resources could influence the resources available to developing larvae. The restructuring of bacterial communities observed in biochar-amended water provides evidence that biochar altered the larval environment, although the present study cannot determine whether these microbial changes directly contributed to accelerated development. Because developmental conditions can influence energy allocation and adult physiology, differences in larval development may also contribute to the metabolic differences observed in adult females following biochar exposure.

Biochar and the Larval Breeding Sites Microbiome

Biochar exposure was associated with substantial restructuring of the bacterial communities present in larval rearing water. Pairwise PERMANOVA demonstrated significant differences in community composition between control water and larvae+biochar water ($R^2 = 0.478$, $p = 0.002$), as well as between larvae-only and larvae+biochar water ($R^2 = 0.306$, $p = 0.002$). The

latter comparison indicates that biochar was associated with a distinct bacterial community when larvae were present, while the former shows that the community in larvae+biochar water also differed from that of biochar-only water. These results indicate that bacterial community composition varied among the experimental conditions and that biochar was associated with differences in community composition in both larval and larva-free water.

Although biochar was associated with differences in bacterial community composition across treatment groups, its effects on alpha diversity was context dependent. Chao1 richness, Shannon diversity, and Simpson diversity did not differ significantly between control (biochar, no larvae) and larvae+biochar groups, whereas all three measures were significantly higher in larvae+biochar water than in larvae-only groups. Thus, the presence of biochar did not simply increase bacterial diversity across all conditions. This pattern is consistent with evidence from other systems showing that biochar can alter microbial communities without consistently increasing alpha diversity. Meta-analyses have found that the effects of biochar on microbial diversity in soil vary with factors such as biochar feedstock and pyrolysis conditions, application rate, and environmental context (Deshoux et al., 2023; X. Li et al., 2020). A recent meta-analysis similarly found that biochar significantly altered bacterial beta diversity while having limited effects on bacterial alpha diversity, suggesting that biochar may restructure community composition without necessarily increasing within-community diversity (J. Li et al., 2026). The higher alpha diversity observed in larvae+biochar water in the present study may therefore reflect an interaction between biochar and the biological conditions created by developing larvae rather than a direct effect of biochar alone.

One possible explanation is that the porous structure of biochar provided additional surfaces or microhabitats that facilitated microbial colonization and provided refuge from grazing by larvae or predation by other microbes (Mukherjee et al., 2022; Warnock et al., 2007). However, bacterial colonization of biochar and grazing rates were not measured in this study, so this

mechanism remains speculative. The beta diversity results further support the interpretation that biochar influenced the structure of the bacterial communities rather than simply increasing overall diversity. Both larvae and biochar contributed to differences in community composition among treatments, indicating that biochar may interact with biological factors to shape the bacterial community. Together, these findings suggest that biochar can influence microbial community composition and, under some conditions, increase bacterial diversity, but that these effects are dependent on the environmental and biological context.

Bacterial Groups Of Significance in Community Composition

Differences in community composition were further supported by the taxonomic profiles and LEfSe analysis. Several bacterial orders were differentially abundant in larvae+biochar water relative to larvae-only water, including Burkholderiales, Rhodospirillales, Cytophagales, Chitinophagales, and Flavobacteriales. Similar shifts in bacterial community composition have been reported following biochar amendment in other systems. For example, biochar-amended tomato rhizospheres showed increased relative abundances of Burkholderiales, Flavobacteriales, Cytophagales, and Bacillales, while biochar-amended growth media also showed increases in Caulobacterales, Flavobacteriales, Cytophagales, and Bacillales (Jaiswal et al., 2017, 2018). These findings support the broader observation that biochar can selectively restructure bacterial communities, although the specific taxa responding can vary among environments and biochar treatments. In the comparison between control and larvae+biochar water, Micrococcales, Mycobacteriales, and Chitinophagales were enriched in the larvae+biochar group, whereas Caulobacterales (contain motile bacteria potentially susceptible to grazing) and Bacillales were enriched in control water. The direction of these responses differed from some previous studies, highlighting the context-dependent nature of biochar effects on microbial communities.

Notably, Chitinophagales was significantly enriched in larvae+biochar water relative to both larvae-only and control water, suggesting that this order may be particularly responsive to the presence of biochar under the conditions examined here. Members of Chitinophagales include bacteria capable of degrading complex carbohydrates, including chitin, and metagenomic analyses have identified the potential for Chitinophagales to participate in chitin-related carbohydrate metabolism (Ellegaard-Jensen et al., 2025). However, the ecological functions of these taxonomic shifts cannot be determined from relative abundance data alone, i.e. interpreted as evidence that chitin degradation increased as functional activity was not measured. The observed changes may reflect differences in the physicochemical environment, resource availability, microbial interactions, or other effects of biochar, but these potential mechanisms were not directly measured in this study. Although Sphingomonadales appeared visually enriched in larvae+biochar samples, this difference did not remain significant after FDR correction.

Potential Mechanisms of how Biochar Affects Bacterial Communities in *Ae. aegypti* breeding site

The mechanisms by which biochar altered bacterial community composition remain unresolved. We know that biochar has a porous structure and a high surface area that can provide surfaces for microbial attachment and alter the local chemical environment (e.g. pH, dissolved oxygen content, sequestering heavy metals and pollutants and making them less bioavailable). In aquatic systems, biochar can also interact with dissolved organic compounds and nutrients, potentially changing the resources available to microorganisms and influencing microbial community structure (Odedishemi Ajibade et al., 2021; Wu et al., 2022). These properties provide plausible explanations for the community level differences observed here. However, because water chemistry, nutrient concentrations, biochar surface colonization, and microbial

enzymatic activity were not measured, the present study cannot determine whether the observed microbial shifts resulted from direct microbial association with biochar, changes in resource availability, chemical interactions, or a combination of these processes. Future experiments incorporating measurements of water chemistry and microbial function would be necessary to identify the mechanisms underlying the observed community restructuring.

Consistent with these compositional shifts, the functional gene content of the bacterial communities also differed among treatments. PCoA of KEGG Ortholog abundances separated the larva-free biochar treatment from the two larval treatments along PCo1, with additional separation of larvae-only from larvae+biochar along PCo2, and community dispersion was lowest in larvae+biochar water, suggesting that the combination of larvae and biochar constrained functional variability among replicates in this treatment than in the biochar-only or larvae-only treatments.

Among the functional categories examined, biochar exposure was most strongly associated with enrichment of genes involved in aromatic and furanic compound transformation, including ring-hydroxylating oxygenases, F420-dependent metabolism genes, and alkylaromatic degradation genes. The enrichment of these functions suggests that the microbial community in larvae+biochar water had greater representation of metabolic potential related to aromatic compound transformation. However, the absence of significant differences in the central ring-cleavage genes *catA*, *pcaG*, and *pcaH* suggests that the observed shift was not uniform across all stages of aromatic compound degradation. Increased sulfur oxidation and polyamine transport genes, together with decreased nitrogen fixation and sugar PTS uptake genes, further indicate that biochar was associated with changes in the functional potential of the microbial community. Biochar often contains polycyclic aromatic hydrocarbons (PAHs) and a variety of other aromatic compounds (Weidemann et al., 2018). The shift in functional gene enrichment is

suggestive that biochar associated compounds such as PAHs could be shaping the metabolic composition of the bacterial population. However, because neither dissolved aromatic compounds nor biochar chemical composition was measured, it cannot be determined whether these changes resulted from compounds released by the biochar or from other changes in the aquatic environment. Direct measurements of biochar-derived and dissolved organic compounds would be needed to determine whether aromatic substrates contribute to the observed functional shift.

Biochar Effects on Adult Female *Ae. aegypti* Metabolome

The microbiome level changes were accompanied by selective differences in the adult female metabolome following developmental exposure to biochar. Because adult females were reared from larvae exposed to either biochar-containing or biochar-free water, these analyses allowed us to determine whether metabolic differences associated with the larval environment remained detectable after adult emergence. Both biogenic amine and lipid profiles showed significant separation supported by permutation testing. This pattern suggests that developmental exposure to biochar was associated with selective rather than global changes in adult metabolism.

Biochar Effects on Biogenic Amines

Biogenic amine profiles showed a clear association with developmental biochar exposure. Integrated pathway enrichment analysis (mummichog + GSEA) identified three pathways with convergent support: sphingolipid, fatty acid elongation and fatty acid degradation. The enrichment of fatty acid pathways within the biogenic amine dataset echoes the triglyceride reductions observed in the lipidomic data, suggesting lipid-related nutrient storage and metabolism may be broadly affected by developmental biochar exposure. Many of the discriminating features from the biogenic amines panel remain unannotated, which limits the

interpretation of the specific physiological pathways represented by these changes. As biogenic amines participate in diverse physiological processes, targeted identification of unidentified altered compounds will be necessary to determine their biological significance.

Biochar Effects on Lipids

The lipidomic results revealed an especially pronounced and consistent pattern of metabolic differences associated with developmental biochar exposure. Triglycerides are an important form of energy storage in the mosquito fat body, and lipid reserves accumulated during larval development can influence adult starvation resistance, flight capacity, and fecundity (van Schoor et al., 2020; Yan et al., 2021).

The predominance of triglyceride features among the discriminating lipids therefore suggests that developmental exposure to biochar was associated with differences in adult lipid storage or lipid metabolism. One possibility is that exposure to biochar altered larval nutritional acquisition or energy allocation during development, potentially through changes in the microbial environment, as larval microbiota can influence nutrient availability and developmental outcomes. However, whether the observed triglyceride differences reflect altered nutrient availability, feeding conditions, or metabolic allocation cannot be resolved from the present data. Previous work showed that larvae reared in crowded environments take longer to complete larval development and have higher levels of stored triglycerides (Silva et al., 2021). Those mosquitoes also took longer to utilize stored lipids as adults and showed increased resistance to starvation. This is hypothesized to be a response to larval stress which could indicate that the presence of biochar might reduce environmental stress possibly through the adsorption of waste byproducts. The enrichment of denitrification genes suggests a greater capacity for metabolism of nitrogenous waste products such as ammonia and nitrates. However, this study did not directly measure energy reserves, feeding, fecundity, survival, or other physiological traits.

Consequently, the functional significance of the reduced triglyceride abundance remains to be determined and warrants direct investigation of larval nutritional conditions and adult physiological phenotypes.

Biochar and the Microbiome and the Metabolome

One possible explanation for the adult metabolic differences is that biochar-associated changes in the larval microbial environment altered the nutritional or chemical conditions experienced during development. Larval *Ae. aegypti* acquire nutrients from their aquatic environment, and microbial communities can contribute to the availability and transformation of nutrients in larval habitats. Thus, changes in microbial community composition could potentially influence the resources available to developing mosquitoes and contribute to metabolic differences that remain detectable after adult emergence. The present study, however, does not directly establish a causal relationship between the microbiome and adult metabolome. Linking the two datasets mechanistically will require experiments that simultaneously characterize microbial function, larval nutritional conditions, and mosquito metabolic phenotypes.

One possible link between the microbiome and metabolome findings is the shift in microbial functional potential observed in larvae+biochar water. Bacterial genes associated with PTS-mediated sugar uptake decreased, whereas genes associated with aromatic and furanic compound transformation increased, suggesting a change in the metabolic potential of the biochar-associated community. One possible consequence of this shift is altered availability or transformation of nutrients accessible to developing larvae, which could contribute to differences in larval developmental rate and adult lipid metabolism. This interpretation is consistent with the pronounced reduction in triglyceride features observed in biochar-reared adult females, but the proposed mechanism remains untested. Water carbohydrate availability, larval feeding and nutrient assimilation, and larval lipid accumulation were not measured directly, and the

microbiome and metabolome were analyzed using separate cohorts rather than paired samples. Therefore, the present data support a potential microbiome–metabolome association but do not establish a causal pathway between the microbial functional shifts and reduced adult triglycerides. Future experiments should directly measure water chemistry parameters, nutrient composition, larval nutrient acquisition, and lipid accumulation while pairing microbiome and metabolome measurements within the same biological replicates.

Biochars Ecological Implications

Together with our previous findings that gravid *Ae. aegypti* preferentially oviposit in biochar associated oviposition sites, the present results suggest that biochar can influence larval development and nutrition via multiple components of the mosquito–microbe–environment interaction. Biochar associated water groups differed in bacterial community composition from the biochar free water group, and exposure during larval development was associated with shorter developmental time and distinct adult biogenic amine and lipid profiles. These findings raise the possibility that the effects of biochar extend beyond oviposition-site selection to subsequent stages of the mosquito life cycle. However, the adaptive or detrimental significance of the observed metabolic differences remains unknown. The reduction in triglyceride features could reflect altered lipid metabolism without necessarily indicating a negative physiological consequence, while changes in biogenic amine profiles may or may not translate into behavioral effects. Direct measurements of adult fitness, energy reserves, behavior, and vector-related traits will be necessary to determine the biological significance of these metabolic changes.

Several limitations should be considered when interpreting these findings. First, the study used a single biochar feedstock and production condition (Manzanita wood pyrolyzed at 550°C), and the effects observed here may not apply to biochars produced from other feedstocks or under different pyrolysis conditions. Second, the sample sizes were modest, with six biological

replicates per treatment for both microbiome and metabolomic analyses, and each metabolomic replicate consisted of a pool of five adult females. Pooling reduced the ability to assess individual level metabolic variation. Third, although the microbiome analysis identified clear differences in bacterial community composition, the study did not measure water chemistry, nutrient availability, microbial functional activity, or direct microbial colonization of biochar, which limits the ability to identify the mechanism responsible for the observed community shifts. Similarly, many of the metabolomic features, particularly within the biogenic amine dataset, remain unannotated, limiting interpretation at the individual-compound level. Future studies should therefore combine microbiome profiling with measurements of water chemistry and nutrient availability, targeted identification of discriminating metabolites, and direct measurements of adult physiological and behavioral phenotypes. Such approaches would help determine whether the microbial and metabolic changes observed here represent functional consequences of biochar exposure and whether they influence mosquito fitness or other traits relevant to disease transmission.

Methods

Larval Development Experiment: Larval development was assessed by rearing *Aedes aegypti* larvae in water containing different concentrations of biochar. Larval trays were prepared with 200 larvae per 1,000 mL of plain water. Approximately 700 larvae were transferred from the trays into medium-sized Petri dishes for the experiment. Each Petri dish contained 20 larvae in 100 mL of treatment water containing 0%, 0.25%, 0.5%, or 0.75% biochar (w/v).

Larvae were maintained in the Petri dishes and provided food throughout the experiment. Food was added in increasing amounts as larvae developed, with approximately ¼ drop (11.6 mg), ¼ drop, ½ drop (23.3 mg), 1 drop, 1 drop (46.5 mg), 2 drops (93 mg), and 1 drop provided over

successive feeding periods. Larval development was monitored daily, and the time to eclosion was recorded for each larva.

Statistical Analysis: Larval development was analyzed as time-to-event data, with eclosion to the next developmental stage recorded over time. Kaplan–Meier survival analysis was used to compare developmental timing among the four biochar treatments (0%, 0.25%, 0.5%, and 0.75%). Cox proportional hazards models were used to estimate the effect of biochar concentration on the rate of development, with Petri dish group included as a clustering factor. Hazard ratios were used to quantify differences in the rate of eclosion between biochar treatments relative to the 0% control. Pairwise differences among biochar treatments were assessed using log-rank tests with Benjamini–Hochberg adjustment for multiple comparisons. Analyses were conducted in R.

Glycerol Microbe Seed Stock: To generate a microbial seed stock representative of an outdoor mosquito breeding environment, 1,000 mL of tap water was added to each of three plastic mosquito-rearing trays. The trays were placed outdoors near Briggs Hall on the University of California, Davis campus, in a shaded area near vegetation to provide environmental conditions similar to those of natural mosquito breeding sites in urbanized areas (Fig. 3.12). After one week, the water and accumulated organic debris from the trays were collected and transferred to a carboy. Four days later, glycerol stocks were prepared by combining 8 mL of the microbial water with 2 mL of glycerol in sterile tubes under sterile conditions in a biosafety hood. The resulting 10-mL glycerol stocks were frozen and subsequently used to inoculate water for the microbiome and metabolomics experiments.



Figure 3.12: The plastic mosquito-rearing trays outside of Briggs Hall.

Lab Based Biochar Generation: A hood based Thermo Scientific Box Furnace (Gravity, 0.5 cu ft Capacity, 1,200°C Max Temp, 2,323 W Watt) was used to generate biochar under controlled conditions in the lab. A thermocouple was attached to the furnace to ensure accurate temperature readings. Manzanita wood was acquired from branch cuttings of trees at the burn site, Frenchtown California (39.383922°N, 121.24684°W), from which initial wildfire biochar was obtained. This biochar was used in previous experiments (Rodrigues et al. 2026). Wood was charred in a graphite crucible with a lid. Prior to addition of wood, the furnace was brought up to temperature and the crucible was added to the oven using tongs and fire-resistant PPE. The wood was charred for 30 minutes. The pyrolysis temperature was 550°C. After 30 minutes the crucible was removed and the biochar was allowed to cool to room temperature in the hood. Then the biochar was manually crushed. It was measured and placed into foil packets. These packets were placed in the furnace to sterilize 150°C for 10 mins.

Mosquito Rearing Protocol for the Metabolome Experiment: Glassware used for liquid preparation and larval hatching, as well as plastic containers used for larval counting, were bleached and allowed to dry under a clean bench. Glassware was also autoclaved. Prior to use, all materials were exposed to UV light under the clean bench for approximately 10 min. Dechlorinated tap water was filtered to remove microbial contaminants.

For larval hatching, 1,000 mL of filtered water was combined with one tube of microbial seed stock. The mixture was shaken, and larvae were allowed to hatch for 2 h. The hatching flask was connected to a vacuum source using a hose. Separately, 3 L of filtered water was combined with three tubes of microbial stock under the clean bench (mixture 1).

Twelve sterilized plastic containers with lids were used for larval rearing. Containers were opened under the clean bench, and each container was filled with 250 mL of mixture 1. Six containers received 1.875 g of biochar, resulting in a final biochar concentration of 0.75% (w/v), while the remaining six containers did not receive biochar. Sterilized larval food (0.13 g) was added to each container every 2 days under the clean bench.

Containers were monitored for pupation, and pupae were transferred to labeled tubes fitted with mesh to allow adult emergence. Following eclosion, adults were provided with sterile 10% sugar water (autoclaved) supplied on UV-sterilized cotton for 3 days. For the subsequent 2 days, autoclaved water was provided on UV-sterilized cotton. Adults were frozen on day 5 post-eclosion. Five female adults from each rearing container were pooled to constitute one sample.

Mosquito Rearing Protocol for the Rearing Water Microbiome Experiment: The mosquito rearing protocol followed what is described for the metabolome experiment, with modifications to accommodate collection of the rearing water microbiome. Eighteen containers were used, consisting of three experimental groups: six containers containing larvae and biochar, six containers containing larvae without biochar, and six containers containing biochar without larvae (control). All containers received sterilized larval food, including those without larvae, to account for potential effects of food addition on the microbial community.

Following removal of the pupae, the rearing water from each container was collected and stored in a 4°C room for a month until processing. Water samples were filtered using 0.2-µm

Whatman® filters to collect microbial biomass. The filters were subsequently cut into smaller pieces and processed using the Zymo Research HostZERO™ Microbial DNA Kit according to the manufacturer's instructions. A total of 18 microbial DNA samples to reflect the 18 groups were generated and submitted to Azenta for Illumina Paired End sequencing.

Data Management: Microbiome and metabolome datasets, including sample metadata and processed data, were organized and maintained in Google Drive. Descriptive statistics and preliminary data calculations were also performed using Google Sheets.

Metagenomic Analysis: Microbiome analysis was performed using Galaxy (The Galaxy Community, 2026) for paired-end read assembly and taxonomic classification at the order and genus levels. Output data from Galaxy were then imported into MicrobiomeAnalyst 3.0 (Lu et al., 2023) for data normalization and statistical analysis.

Data were uploaded to MicrobiomeAnalyst as an OTU/ASV table with an accompanying metadata file and taxonomy table (no phylogenetic tree included), with taxonomy labels set to "Not Specific/Other." During data filtering, a minimum count of 4 and a prevalence threshold of 10% (in sample) were applied, with 0% removed via the low variance filter; this step removed 0 low-abundance and 0 low-variance features, leaving 525 features for downstream analysis. Data were normalized using total sum scaling (TSS).

Alpha diversity (Chao1 richness, Shannon, and Simpson indices) and beta diversity were calculated to assess within- and between-group community differences, respectively. Pairwise PERMANOVA was used to test for differences in community composition between groups, with p-values corrected for multiple comparisons using the Benjamini-Hochberg false discovery rate (FDR) method. Taxonomic composition and relative abundance were visualized at the order level across treatment groups. Linear discriminant analysis effect size (LEfSe) was used to

identify bacterial taxa differentially abundant between groups, with significance determined by FDR-corrected p-values ($\text{FDR} < 0.05$).

Metagenomic Functional Enrichment Analysis:

Read processing and host depletion:

Raw paired-end reads were assessed with FASTQC and aggregated with MultiQC. Reads were quality-trimmed with fastp (Chen et al., 2018) using adapted auto detection for paired-end libraries, a 4-bp sliding window from the 5' end trimmed at mean quality below 20, a minimum retained length of 50 bp, overlap-based base correction, poly-G trimming, and duplicate removal. As mosquito larvae were present in two of the three treatment, host DNA load was confounded with treatment by design. Trimmed reads were therefore mapped against the *Aedes aegypti* AaegL5 reference genome (GCF_002204515.2) with Bowtie2 (Langmead & Salzberg, 2012) in `-very-sensitive-local` mode, and only read pairs failing to align were retained. Host-aligned fractions were recorded per sample and compared across treatments to confirm the post-depletion sequencing depth was not systemically associated with treatment.

Assembly and gene catalog construction:

Host-depleted reads from all 18 samples were co-assembled with MEGAHIT (D. Li et al., 2015) using a k-mer list of 21, 29, 39, 59, 79, 99, 119, and 141, a minimum k-mer count of 2 and a minimum contig length of 500 bp. Co-assembly was used in preference to per-sample assembly so that all samples would be quantified against a single shared gene catalog. Assembly quality was evaluated with QUAST in metagenome mode (Mikheenko et al., 2016) with reference genome download disabled, and read recruitment to the assembly was assessed separately by mapping each sample's cleaned reads back to the contigs.

Contigs were screened for eukaryotic sequences with Kraken2 (Wood et al., 2019) against the standard database at a confidence threshold of 0.1. Contigs receiving a positive assignment within Eukaryota were removed. Unclassified contigs were retained, as environmental freshwater bacteria are underrepresented in reference databases and a substantial unclassified fraction is expected. Protein coding genes were predicted from the filtered assembly with Prodigal (Hyatt et al., 2010) in metagenomic (-p meta) mode, retaining genes running off contig ends.

Functional annotation:

Predicted proteins were annotated with eggNOG-mapper v2 (Cantalapiedra et al., 2021) against the eggNOG 5.0.2 database using DIAMOND in *more-sensitive* mode with automatic taxonomic scope adjustment, a seed orthog e-value threshold of 0.001, and a minimum bit score of 60. The higher sensitivity setting was selected because the taxa in these habitats are phylogenetically distant from the well sampled clades that dominate eggNOG

Quantification and feature table construction:

Cleaned reads from each sample were mapped to the nucleotide gene catalog with Salmon (Patro et al., 2017) in selective-alignment mode using a k-mer size of 31, with sequence and GC bias correction enabled. Salmon apportions multi-mapping reads probabilistically, which is necessary due to the homology among genes in a metagenomic catalog. Per-gene read counts were joined to eggNOG annotations by gene identifier. Four feature tables were constructed by aggregating gene-level abundance to KEGG Orthology (KO), KEGG Module, KEGG Transporter Classification (TC), and COG functional category. Genes carrying multiple annotations within a category were assigned fractionally, with abundance divided by the number of assignment, so that per-sample totals were conserved. Genes without an assignment in a given category were excluded from that table.

Statistical Analysis:

All analyses were performed in R v4.4.1. Features were retained if present in at least 50% of samples within at least one treatment group. The filter was applied within groups so that treatment-specific features were not discarded. The experimental design is a 2 x 2 factorial with one cell absent: no unamended (food-only, no biochar, no larvae) arm was included. Two contrasts were estimable:

- Larval effect, biochar held constant: larvae+biochar vs. biochar only
- Biochar effect, larvae held constant: larvae+biochar vs larvae only

Compositional structure was addressed by centered log-ratio (CLR) transformation.

Community-level differences were tested by PERMANOVA (*adonis2*, *vegan* v2.7-3, 9,999 permutations) on both Bray-Curtis distances of relative abundances and Aitchison distances (Euclidean distance on CLR values) (Dixon, 2003). Because PERMANOVA is sensitive to heterogeneity of multivariate dispersion, homogeneity was tested in parallel with PERMDISP (*betadisper* with *permutest*, 9,999 permutations), followed by Tukey's HSD on distances to group centroids.

Differential abundance was assessed with two methods and the intersection reported as the primary result. ALDEx2 v1.38.0 (Fernandes et al., 2014) was applied to each contrast separately using 128 Monte Carlo Dirichlet instances, with features considered significant at Benjamini-Hochberg-corrected Welch's $p < 0.05$ and absolute effect size > 1 . MaAsLin2 v 1.20.0 (Mallick et al., 2021) was applied to the same contrasts with total-sum-scaling normalization, log transformation, a linear model, a minimum prevalence of 0.25, and Benjamini-Hochberg correction at $q < 0.05$.

A panel of 27 KOs spanning nitrogen cycling, sulfur cycling, aromatic degradation, chitin and glycan degradation, biofilm formation, and ion transport was specified a priori and tested as its

own family, with Benjamini–Hochberg correction applied across the panel only. Enrichment of functional themes among differentially abundant features was assessed by comparing the proportion of significant features in a given theme that increased against the background proportion across all significant features, using a binomial test.

Metabolomic Profiling (Kelly et al., 2024): For metabolomic profiling, 6 samples of 5 adult female *Ae. aegypti* were submitted for each of the two groups. Frozen samples were submitted to the University of California, Davis West Coast Metabolomics Center for analysis using mass spectrometry (MS)-based assays, designed to measure lipids, and biogenic amines. Lipidomic analysis was performed by liquid chromatography (LC) (Agilent 6530 Q-TOF LC/MS UPLC) coupled to a quadrupole time-of flight (QTOF) charged surface hybrid column (CSH) mass spectrometer (Waters MS Technologies, Manchester, UK). and with MS-Dial 3.98, after filtering for a minimum peak intensity of 1000. Biogenic amines were measured using a hydrophilic interaction liquid chromatography quadrupole time-of-flight mass spectrometry with tandem mass spectrometry (HILIC QTOF MS/MS) Agilent 6530 Q-TOF LC/MS UPLC, fitted with a Waters Acquity UPLC BEH Amide VanGuard pre-column, Waters Acquity UPLC BEH Amide Column (Waters, Pleasanton, CA, USA). Data was analyzed using Metaboanalyst 6.0. Samples were normalized by the sum of internal standards, log transformed and mean centered prior to performing principal component analysis.

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