

## RESEARCH ARTICLE

# The Goldilocks principle: Finding the balance between water volume and nutrients for ovipositing *Culex* mosquitoes (Diptera: Culicidae)

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## Abstract

Females of container-breeding mosquito species use visual and chemical cues to determine suitable habitats to oviposit their eggs. Female *Culex* mosquitoes oviposit single egg rafts containing hundreds of eggs on the surface of water in container habitats. In this project, the effects of water volume and nutrient concentration were studied using three semi-controlled field assays to determine the role these parameters play on female *Culex* mosquito oviposition preference. The results of this study suggest female *Culex* prefer to oviposit in larger volumes of water and higher concentrations of nutrients separately, but chose intermediate conditions when presented with a combination of these two variables, which follows the Goldilocks principle. This choice may provide their offspring with optimal conditions for development by reducing intraspecific competition, thereby maximizing the biological fitness of the ovipositing *Culex* females.

## Introduction

The Goldilocks principle is a phenomenon in which organisms select the intermediate value when presented with choices along a gradient [1, 2]. This principle is named after the tale, *Goldilocks and the Three Bears*. In this fairytale, Goldilocks comes across a house in the woods and tries out each of the three family member's chairs, beds, and porridge. Goldilocks repeatedly chooses the option that falls not at either extreme of the range, but directly in the middle ("just right"). The Goldilocks Principle has been observed widely across the animal kingdom, including in insects [1–4]. For example, Enjin et al. (2016) demonstrated that *Drosophila melanogaster* prefer moderate humidity when presented with a range of humidity levels suggesting that the humidity preference for this fly species is in the intermediate range between too wet and too dry [3]. While the Goldilocks Principle has been observed in a range of animals, including insects, it has not been documented in mosquitoes.

Females of container-breeding mosquito species oviposit in a wide range of container habitats from naturally occurring tree holes to man-made flowerpots and garbage cans [5–8]. The

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resulting mosquito larvae developing within these container habitats compete for space and nutritional resources. The density of larvae and intensity of competition for space and nutritional resources within the container can affect adult life history traits including survival to adulthood, adult body size, and longevity [9–13]. Thus, it is important for the ovipositing females to select the optimum conditions within the container habitat to ensure survival to adulthood for their resulting offspring.

Oviposition choice is influenced by several environmental factors such as the volume and depth of water in the container, the nutrient concentration of that water, and the presence or absence of other mosquito larvae [5, 14, 15]. The concentration of nutrients within a container habitat strongly affects oviposition choice. High nutrient concentrations provide ample resources for larval growth and development while minimizing intraspecific and interspecific competition [16–19]. However, excess nutrients can lead to high levels of microbial activity, creating biofilms that cover the surface of the water and prevent larval respiration [20]. Too little nutrients can hinder larval development, increase intraspecific and interspecific competition for nutrients, and lead to starvation [17, 18]. The volume and depth of water within the container can also influence oviposition choice [21, 22]. Too much water can lead to water spillover, and therefore potential spillover of larvae, out of the container during rain events [23]. Too little water can increase competition for space and food resources or lead to evaporation [24, 25]. The volume of water can also impact the concentration of nutrients within the habitat. Larger volumes of water can disperse nutrients decreasing the nutrient concentration whereas smaller volumes of water can create higher concentrations of nutrients even if the total nutrient availability is equal in both volumes of water [18]. Female mosquitoes must consider the interaction between nutrient availability and water volume when deciding which environment provides optimal resources for larval development.

*Culex* mosquitoes are container-breeding mosquitoes that oviposit a single egg raft on the surface of the water each gonotrophic cycle [26]. Egg rafts generally contain between 100 and 300 eggs [27]. Since *Culex* females lay all their eggs in one container, selecting the best habitat for all of her offspring to develop is key to increasing her fitness. Female *Culex* can sense numerous visual and olfactory cues of the container and water within the container, including water volume and nutrient concentration [28, 29]. Previous research suggests that female *Culex* prefer to oviposit in larger containers with greater volumes of water [6, 30], but some studies show that this preference is negated if nutrient levels are too low [19]. Since *Culex* females take both water volume and nutrients into consideration when deciding where to lay their eggs, females could be selecting the habitat that is “just right” for both water volume and nutrient concentration thereby following the Goldilocks Principle.

In this study, we examine how the interaction of water volume and nutrient concentration in identical container habitats influence female *Culex* oviposition choice. We hypothesized that females would examine both water volume and nutrient concentration and select the optimum combination of the two following the Goldilocks principle. Understanding how this interaction impacts female *Culex* oviposition choice provides deeper insight into the ecology of these mosquitoes.

## Materials and methods

### Ethics statement

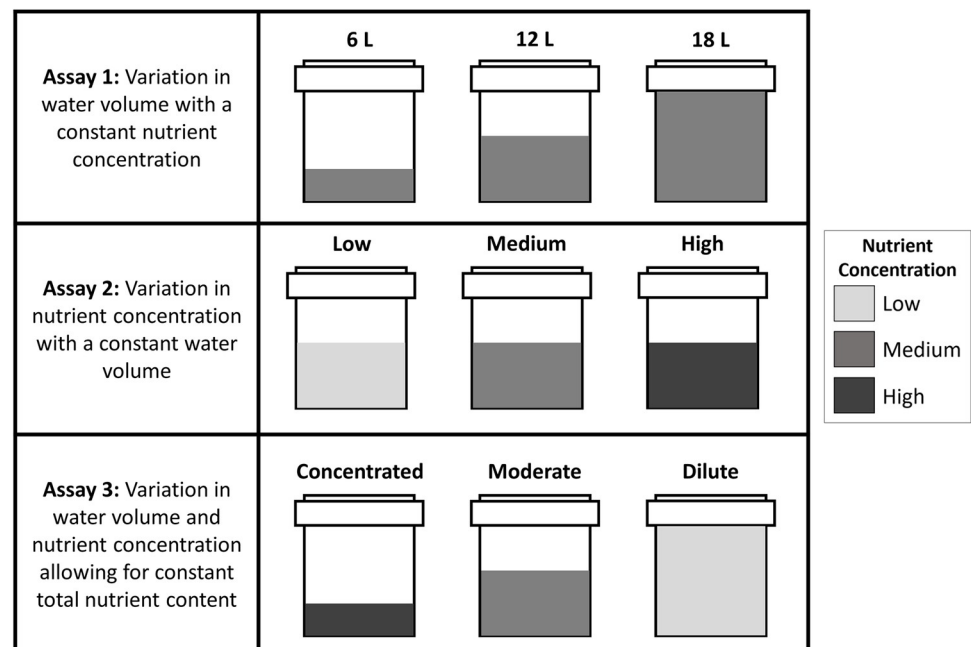
Field collection and handling of mosquito eggs were conducted under a permit granted by the Northern Kentucky University: Research and Education Field Station (REFS) and the Campbell County Conservation District. All handling of mosquito eggs was conducted in accordance with institutional ethical standards.

## Study design

Three semi-controlled field assays were conducted at the Northern Kentucky University Research and Education Field Station (REFS), in Melbourne, Kentucky. At each of the fifteen field sites (each assay was conducted independently at five different sites), three container habitats (19-liter, white, cylindrical buckets) were evenly spaced in a triangle. To induce oviposition by *Culex* females, containers were filled with grass infusion to a predetermined water volume (6 liters (15 cm deep), 12 liters (25 cm deep), 18 liters (35 cm deep), depending on the assay). Three nutrient levels of Bermuda grass infusion were made using dried grass clippings wrapped in cheesecloth which were steeped in 185 liters of water in a 50-gallon rain barrel. Low nutrient infusion was made using 10 grams of grass clippings. Medium nutrient infusion was made using 20 grams of grass clippings. High nutrient infusion was made using 30 grams of grass clippings. Pilot studies were conducted to ensure adequate nutrition was available in each infusion level to support larval development. If evaporation had occurred, infusion was added daily to each bucket to maintain a constant volume. Once per week and after heavy rains, old infusion was removed and new grass infusion was added to each container. The three assay designs are as follows (Fig 1):

### Assay 1: Variation in water volume with a constant nutrient concentration

The first assay varied the water volume while keeping the nutrient concentration constant. At each of the five sites, one container held 6 liters of medium-nutrient grass infusion, one held 12 liters of medium-nutrient infusion, and one held 18 liters of medium-nutrient infusion.



**Fig 1. Visual representation of the three assays examining *Culex* female oviposition preferences for water volume and nutrient availability.** Assay 1 examined variation in water volume (6L, 12L, 18L) using medium nutrient infusion in all containers. Assay 2 examined variation in total available nutrients (low, medium, high) across a constant water volume. Assay 3 examined variation in water volume and nutrient concentration (concentrated, moderate, dilute).

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### **Assay 2: Variation in nutrient concentration with a constant water volume**

The second assay varied the nutrient concentration while keeping the water volume constant. At each of the five sites, all three containers had the same water volume (12 liters) but varying nutrient concentrations (low, medium, and high).

### **Assay 3: Variation in water volume and nutrient concentration allowing for constant total nutrient content**

The third assay varied the water volume and nutrient concentration. This allowed for the total nutrient content available in the container habitat to remain constant across the treatments. At each of the five sites, one container held 6 liters of high-nutrient infusion, one held 12 liters of medium-nutrient infusion, and one held 18 liters of low-nutrient infusion (concentrated, moderate, dilute).

Every day for 12 weeks, the total number of egg rafts in each container was recorded, and a maximum of four egg rafts per container was collected for up to a total of 180 eggs rafts collected daily. All remaining egg rafts were removed from the container habitat daily. The collected egg rafts were allowed to hatch, and the emerged larvae were enumerated to species using taxonomic keys [31, 32].

### **Statistical analysis**

Generalized linear mixed-effects models (GLMERs) were used to analyze the fixed main effect of each assay (water volume in Assay 1, nutrient concentration in Assay 2, or total nutrient content in Assay 3) and the random effects of site by date on the total number of collected egg rafts. Overdispersion was assessed for each model [33]. No overdispersion was detected for Assay 1 (variation in water volume with a constant nutrient concentration) or for Assay 3 (variation in water volume and nutrient concentration allowing for constant total nutrient content). Since no overdispersion was found for Assay 1 or Assay 3, GLMERs using a Poisson regression were used. Overdispersion was detected for Assay 2 (variation in nutrient concentration with a constant water volume), so a quasiPoisson was used. Least square mean separation tests were used to detect significant pairwise differences between the three levels of each treatment for each assay. All analyses were carried out using an alpha level of 0.05 in R (version 3.5.1, R Core Team 2018, [34]) using RStudio (RStudio, Inc. Boston, MA 2016).

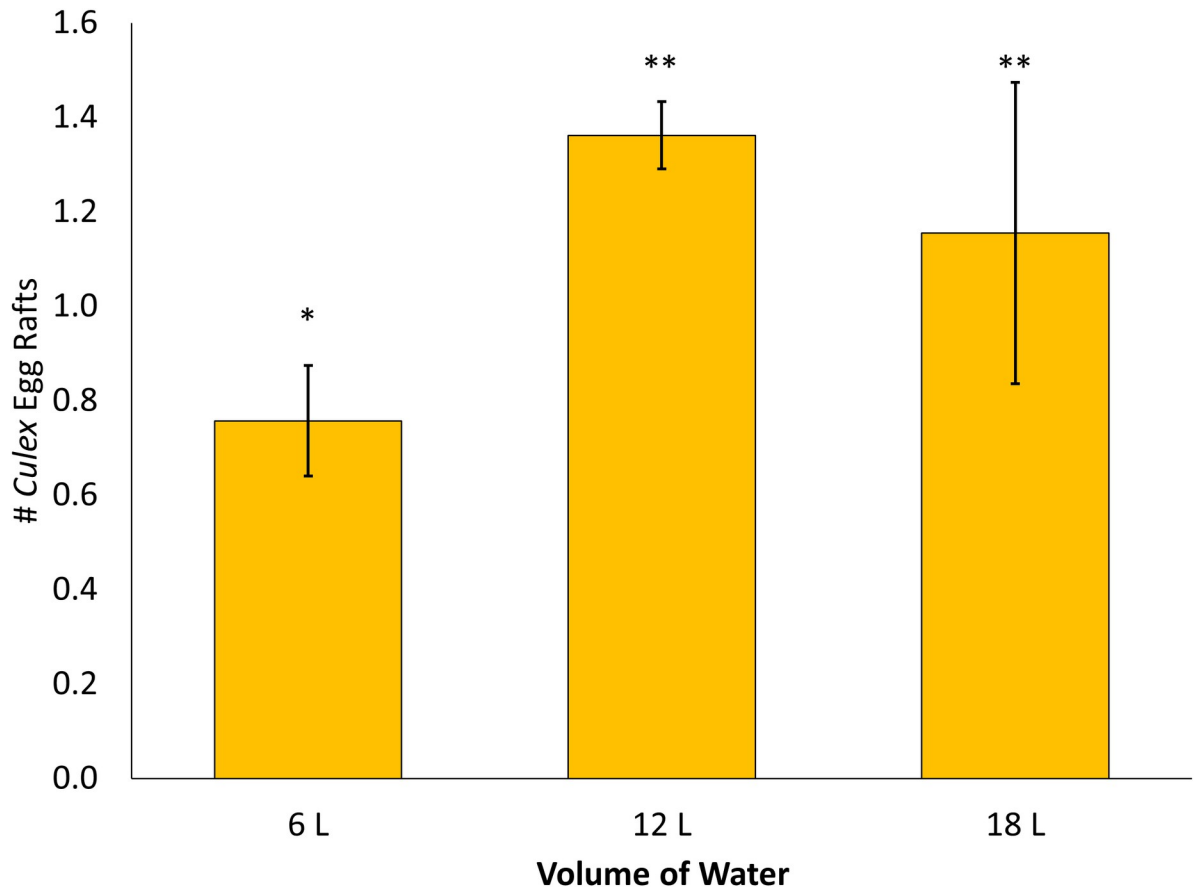
## **Results**

### **Assay 1: Variation in water volume with a constant nutrient concentration**

The number of egg rafts laid was significantly influenced by the main effect of water volume (Table 2). Significantly fewer egg rafts were laid in the 6-liter treatment compared to the 12-liter and 18-liter volumes, and no significant difference was detected between the 12-liter and 18-liter volumes (Fig 2, Table 1).

### **Assay 2: Variation in nutrient concentration with a constant water volume**

The number of egg rafts laid was significantly influenced by the main effect of nutrient concentration (Table 2). Significantly fewer egg rafts were laid in the low treatment compared to the medium and high treatments, and no significant difference was detected for the number of egg rafts laid between the medium and high treatments (Fig 3, Table 2).



**Fig 2. Number of egg rafts laid comparing three treatments with a constant nutrient content across multiple volumes of infusion.** The three treatments in Assay 1 include 6 liters (6 liters of medium infusion), 12 liters (12 liters of medium infusion), and 18 liters (18 liters of medium infusion). Asterisks denote significant differences at  $P < 0.05$ .

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**Table 1. Poisson regression model constructed for number of egg rafts laid in Assay 1 comparing three treatments with a varied total nutrient content, fixed nutrient concentration across multiple volumes of infusion (6 liters: 6 liters of medium infusion, 12 liters: 12 liters of medium infusion, 18 liters: 18 liters of medium infusion).**

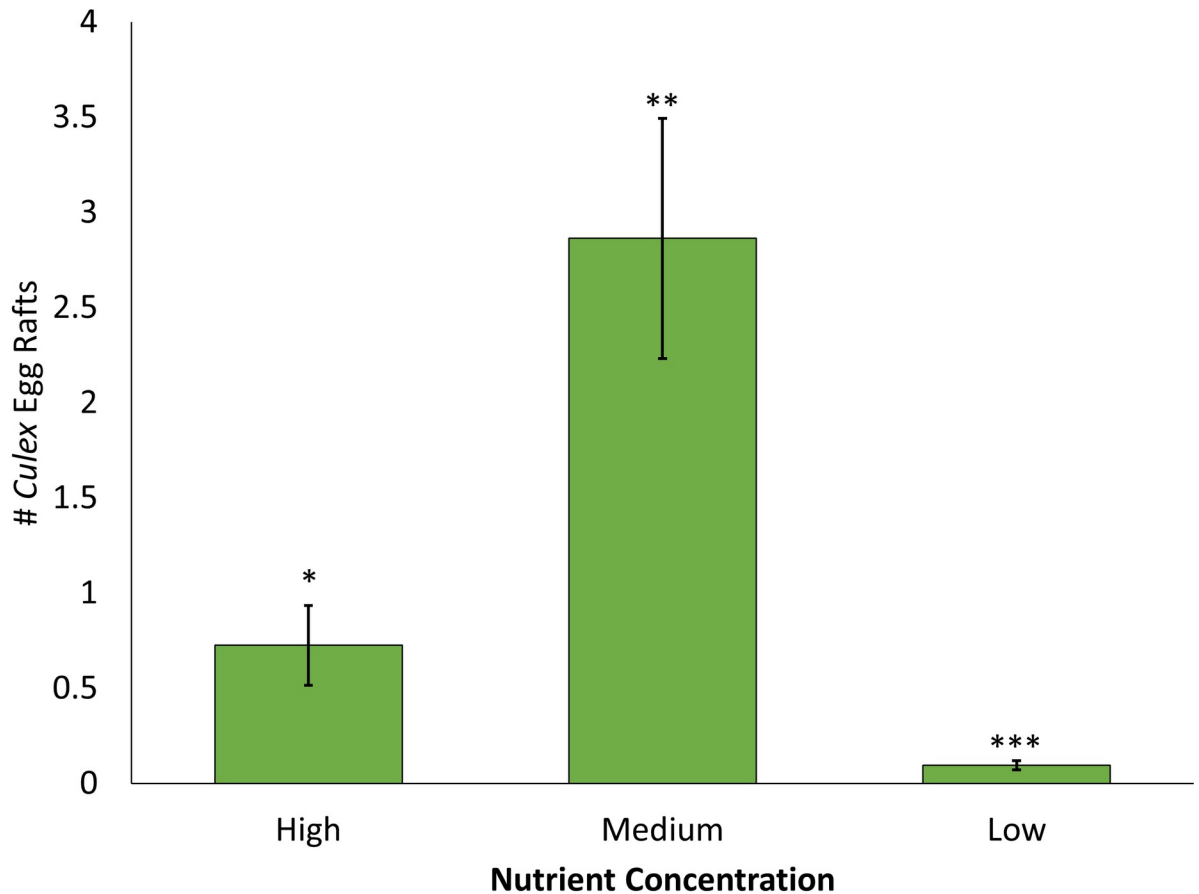
	Estimate	Standard error	P-value
<b>Intercept</b>	-1.854	0.263	<0.001
<b>6 liters</b>	-0.449	0.073	<0.001
<b>12 liters</b>	0.167	0.062	<0.001

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**Table 2. QuasiPoisson regression model constructed for number of egg rafts laid in Assay 2 comparing three treatments with a variation in nutrient concentration with a fixed water volume (Low: 12 liters of low infusion, Medium: 12 liters of medium infusion, High: 12 liters of high infusion).**

	Estimate	Standard error	P-value
<b>Intercept</b>	-6.547	0.489	<0.001
<b>High</b>	2.031	0.168	<0.001
<b>Medium</b>	3.404	0.161	<0.001

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**Fig 3. Number of egg rafts laid comparing three treatments with a variation in nutrient concentration with a fixed water volume.** The three treatments in Assay 2 include Low (12 liters of low infusion), Medium (12 liters of medium infusion), and High (12 liters of high infusion). Asterisks denote significant differences at  $P < 0.05$ .

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### Assay 3: Variation in water volume and nutrient concentration allowing for constant total nutrient content

The number of egg rafts laid was significantly influenced by the main effect of total nutrient content (Table 3). The greatest number of egg rafts were laid in the moderate treatment, the second-most amount of egg rafts were laid in the concentrated treatment, and the lowest number of eggs rafts were laid in the dilute treatment (Fig 4, Table 3).

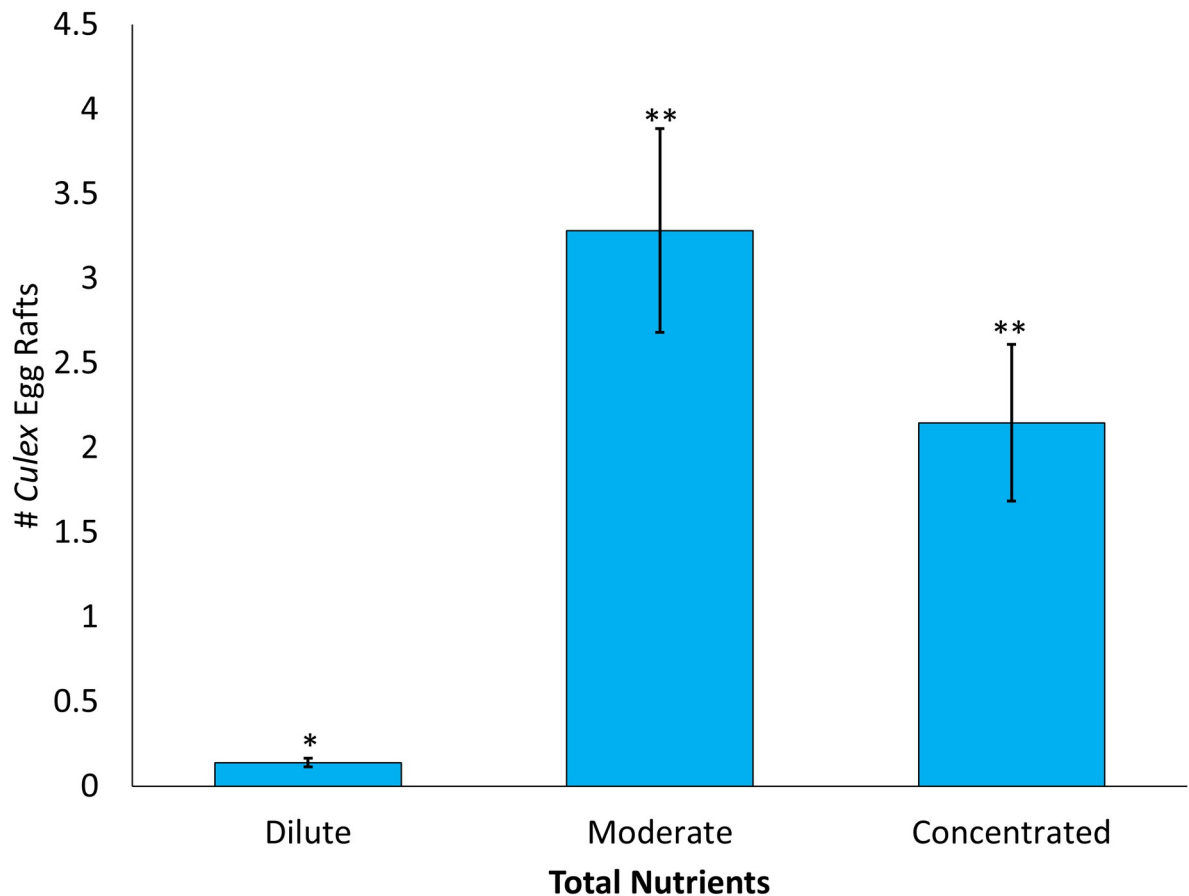
### Discussion

The effects of nutrient concentration and water volume on oviposition choice have been studied separately in various experiments but studying the interaction between these two

**Table 3. Poisson regression model constructed for number of egg rafts laid in Assay 3 comparing three treatments with variation in water volume and nutrient concentration allowing for constant total nutrient content (concentrated: 6 liters of high infusion, moderate: 12 liters of medium infusion, dilute: 18 liters of low infusion).**

	Estimate	Standard error	P-value
Intercept	-2.581	0.257	<0.001
Dilute	-2.496	0.260	<0.001
Moderate	0.227	0.183	0.215

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**Fig 4. Number of egg rafts laid comparing three treatments with variation in water volume and nutrient concentration allowing for constant total nutrient content.** The three treatments in Assay 3 include Concentrated (6 liters of high infusion), Moderate (12 liters of medium infusion), and Dilute (18 liters of low infusion). Asterisks denote significant differences at  $P < 0.05$ .

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environmental parameters can be challenging. In this study, *Culex* females show that both water volume and nutrient concentration are being taken into account when deciding on the location where they oviposit their eggs. The results from this study suggest that female *Culex* are following the Goldilocks principle and looking for the container habitat that is the “just right” combination of water volume and nutrient concentration. Our results suggest that females showed a preference for a moderate volume of water and a medium nutrient concentration compared to high nutrients in a small volume of water or to a diluted concentration of nutrients in a large volume of water. This may be an evolutionary strategy to ensure sufficient nutrients for their offspring while preventing either the over dispersion of those nutrients in large container habitats or overcrowding and increased intraspecific competition in small volumes of water.

Previous research on the effects of water volume on oviposition choice suggests that females prefer to lay their egg rafts in larger volumes of water compared to smaller volumes. Parker et al. (2020) showed that *Culex* females prefer larger containers and thus larger volumes of water, and in the absence of the large containers, there was a significant decrease in the number of egg rafts laid by *Culex* females suggesting that the females choose to look for other, more suitable habitats [6]. However, the main limitation of this study was that there was no control for nutrients since the infusion used was the same across all container sizes. Thus, larger

containers harbored more nutrients compared to smaller containers holding smaller volumes of water. In Assay 1 of this study, we found similar results where female *Culex* preferred to oviposit in containers with larger volumes of water, but in Assay 1, water volume was also conflated with nutrient availability with larger volumes of water having a greater amount of nutrients. Females may have been selecting containers with higher nutrient concentrations instead of larger water volumes. Additional assays in this study allowed for the examination of the interaction between these two parameters to better elucidate the driving factors in oviposition choice for *Culex* females.

The amount of nutrients and type of nutrients in the container habitat can also impact *Culex* oviposition preferences. Previous research shows that female *Culex* mosquitoes prefer to oviposit in containers harboring certain species of plant and animal detritus [35, 36] suggesting that females are using the chemical cues being emitted from the container habitat to determine habitat suitability for their offspring. In this project, Bermuda grass infusion was used in all treatments because it is a common plant that can enter into container habitats and grass infusion has been shown to be attractive to *Culex* mosquitoes [36, 37]. Using the same grass infusion across all containers and trials ensured that chemical cues from different plant species were not eliciting the observed oviposition choices. Oviposition rates have also been shown to be significantly higher in containers with higher nutrient concentrations of the same food resources [19, 38] suggesting that female *Culex* are inspecting both the strength and type of chemical cues being emitted from a container habitat. In Assay 2 of this project, water volume was held constant while nutrient concentration was varied. Our results suggest that females preferentially oviposit in containers with medium and high nutrient concentrations compared to containers with low nutrient concentration when water volume is held constant. This shows that *Culex* females are taking nutrient concentration into consideration when deciding where to oviposit.

In addition to chemical cues, visual cues, such as water color and depth, also play an important role in oviposition choice for female mosquitoes. Water color may be an indication of the concentration of nutrients in the container habitat or of the water volume. *Culex* females have been shown to preferentially oviposit in containers with darker colored water suggesting that females may be using this visual cue as an initial indicator of nutrient concentration [39, 40]. Color of the container habitat has been shown to impact oviposition choice among female mosquitoes with females preferring darker-colored containers [41]. In this project, all the containers were white, which may have impacted the results of the study as *Culex* females may be using the visual cue of the water height in relationship to the exposed height of the white container as a way to determine suitable habitats for their offspring [42]. Two major limitations of this study was that the height of the water to the top of the container and water depth was not fixed but varied with water volume with smaller volumes of water being lower in the container habitat and having a smaller depth of water and larger volumes of water coming closer to the top of the container and having a greater depth of water. A future experiment could include controlling for water surface height to eliminate variation based on water depth. This would indicate whether the height of the water in relationship to the height of the container plays a role in oviposition choice. A different result may have been observed if each container habitat had the same distance from the surface of the water to the top of the container but with different water volumes. Since water depth was also confounded with water volume, another future experiment may examine how changes in water volume while keeping water depth constant (changes in pool size) can impact oviposition choice.

Since no parental care is provided for the developing *Culex* larvae, the ability of adult females to select the highest quality habitat for their offspring is essential for achieving high levels of biological fitness. The results of this study show that *Culex* females are indeed looking



at the interaction between nutrients and water volume when deciding where to oviposit. Higher nutrient content allows for faster development and greater survivorship to adulthood [17, 43]. Females may also be selecting container habitats that buffer potential competition between their offspring and other larvae developing in the container habitat. In some cases, superior competitors such as *Aedes albopictus* lose their competitive advantage at higher nutrient levels or due to certain nutrients being available within the container habitat [44, 45] and larger volumes of water in a habitat might allow for higher nutrient concentrations since larger volumes of water typically occur in larger container habitats. Thus, female *Culex* may be selecting larger volumes of water while also having sufficient nutrients to allow for a reduction in the competitive advantage of superior larval competitor leading to the observation of the Goldilocks principle for ovipositing female *Culex* mosquitoes. Elucidating the role of oviposition choice in the distribution of mosquito species can aid in our understanding of overall mosquitoes species distribution.

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**Visualization:** Noah C. Weidig.

**Writing – original draft:** Noah C. Weidig, Amber L. Miller, Allison T. Parker.

**Writing – review & editing:** Noah C. Weidig, Amber L. Miller, Allison T. Parker.

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