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Do container size and predator presence affect Culex (Diptera: Culicidae) oviposition preferences?

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A B S T R A C T

Organisms with complex life cycles typically do not exhibit parental care. Hence, the ability of adult females to choose quality oviposition sites is critical for offspring success. Gravid females of many insect taxa have the capability to detect environmental conditions in water-holding containers (e.g., resource level, presence of competitors or predators) and to choose the sites that are most suitable for offspring growth and development. Mosquitoes may also detect physical container characteristics related to water permanence such as surface area, volume, or container size, and some species such as those in the genus Culex have been shown to prefer larger containers. However, predators may also preferentially colonize larger containers; thus, ovipositing females may face decisions based on cues of site quality that balance the costs and benefits for offspring. We used a field experiment to evaluate the oviposition preferences of two Culex species in response to experimental container size and predator abundances within the containers. We found that both species avoided ovipositing in the largest containers, which have high abundances of Chaoborus sp. and dragonfly larvae (predators). However, the container size most commonly chosen for oviposition (15-L buckets) also had high mean abundance per liter of dragonfly larvae. These results suggest either prey naïveté or reduced vulnerability of these species to dragonflies compared to Chaoborus sp. Other potential mechanisms for the observed patterns are discussed.

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Introduction

Organisms with complex life cycles rarely provision young; thus, the ability of adult females to choose high-quality oviposition sites is crucial for offspring success. Females of many taxa can detect cues of site quality such as resource availability (Singer, 1986), the degree of intra- or inter-specific competition (Almohamad et al., 2010), and the presence of predators (Andrade et al., 2016), and may choose the sites that maximize offspring success (Sih, 1986; Blaustein et al., 2004; Andrade et al., 2016). Predation pressure can strongly influence oviposition site choice by gravid females (Sih, 1986; Kerfoot and Sih, 1987), particularly for aquatic insects that occupy ‘container’ habitats. These containers can be natural (e.g., tree holes, bromeliad tanks) or artificial (e.g., tires, cemetery vases) and are commonly occupied by a variety of micro- and macroinvertebrates, including insects with aquatic juvenile stages. Mosquitoes commonly colonize these habitats, and some species show strong preferences regarding oviposition site choice (Blaustein et al., 1995).

Mosquito oviposition in response to a predation risk is thought to be a result of co-evolution between mosquito prey and predators (Kerfoot and Sih, 1987). Differences in evolutionary exposure to specific predators among mosquito species may cause differential responses to cues (i.e., chemical or physical) of predator presence (Blaustein et al., 1995). Further, for many mosquito species, there is a tradeoff between competitive ability and vulnerability to predation that can produce diverse oviposition responses to the presence of predators ranging from strong avoidance (Vonesh and Blaustein, 2010) to neutral (i.e., lack of response) (Andrade et al., 2016); in some cases, mosquitoes may even prefer sites holding predators (Albeny-Simões et al., 2014).

In addition to predation, the physical container characteristics may also influence mosquito oviposition site choice. Container size, surface area and depth may indicate water permanence, whereby larger containers have lower risk of drying during the larval development period (Reiskind and Zarrabi, 2012; Segev et al., 2011; Burrone et al., 2007). Larger containers may also have higher resource capacity (Harrington et al., 2008), and several

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species have been shown to prefer containers with characteristics indicative of greater water holding capacity (Wong et al., 2011; Torrisi and Hoback, 2013). However, larger containers can also support higher abundances of all organisms (Connor and McCoy, 2001), including competitors and predators (Sunahara et al., 2002), and predators typically have longer development times and may preferentially occupy larger containers (Sunahara et al., 2002). Mosquitoes may avoid ovipositing in larger containers to reduce the risk of predation for offspring (Reiskind and Zarrabi, 2012). It is also expected that for species with rapid larval development, container size likely does not influence oviposition preferences to the same extent as in species with longer development periods.

We used a field experiment to evaluate the oviposition preferences based on container size in two, locally common Culex species (Culex eduardoi and Culex sp. j). We then assessed the relationships between container size and the abundances of common predators. We hypothesized that predator abundances would be highest in larger containers, and that Culex females would avoid ovipositing in larger containers due to high predator abundances.

**Methods**

**Study area**

This experiment was conducted at the Floresta National de Chapecó (27°06′39″S; 52°45′11″W) in Guatambú, Santa Catarina, Brazil. The vegetation is composed mainly of mixed ombrophilous forest and lies within the Atlantic forest biome, with a total area of 1604.35 ha. The area has humid subtropical climate with an average annual rainfall around 1600 mm and average monthly temperatures of 22°C in the summer (28.9°C max) and 10.4°C in the winter (4.4°C min). Previous observations revealed a diverse micro- and macroinvertebrate community occupying artificial container inhabitants, with over thirteen taxa and at least 15 species. The majority of mosquitoes that colonize containers belong to the genus Culex, where the most dominant mosquito species is C. eduardoi, followed by Culex sp. j (Albeny-Simões, pers. obs.).

**Experimental design**

Experimental microcosms (black plastic containers) were established along four, 300-m individual transects. Each transect contained 1-L (n = 8), 15-L (n = 4), 100-L (n = 2) and 200-L (n = 1) containers placed 20 m apart. Because the smallest containers (1 L) are expected to be more variable over time, we included a higher number of container replicates for smaller containers (n = 8 per transect, 24 total). We then collected 8, 4 and 2 sub-samples from the 200-L (n = 4), 100-L (n = 8) and 15-L (n = 16) containers, respectively. The position of containers along the transects was designed to keep a minimal distance between same-sized containers and to ensure roughly equal probability of adult female mosquito encounter with the various container sizes. This resulted in a distance of 40, 80, 160, and 320 m between the 1-L cups, 15-L buckets, 100-L and 200-L barrels, respectively, along each pair of contiguous transects (Fig. 1). In order to assure colonization by microbiota, 0.3 g/L of field-collected, mixed-species, previously dried leaf litter was added to each container. We used tap water to fill the containers to 70% maximum volume. The water level was not manipulated thereafter, and the containers were permitted to undergo natural colonization by invertebrates. After a 15-day colonization period, the containers were sampled every two weeks for 105 days (n = 7 sampling periods).

![Fig. 1. Numbers of eggs rafts number as a function of sampling days and container size. The 100 and 200-L barrels are represented by closed triangles and closed circles, respectively. The open circles represent the 1-L barrels, and asterisks represent the 1-L cups. The bars represent standard error. Different letters indicate different means among the treatments.](image)

**Egg raft sampling**

Egg rafts were searched for by direct observation of the water surface in containers. Using a white plastic spoon, all surface egg rafts were collected from the water surface and stored individually in Eppendorf tubes. Each container was inspected for 10 min. The egg rafts were then transported to the Entomology Laboratory at the Community University of the Chapecó Region in Chapecó, SC, Brazil. The egg rafts were inspected under a stereoscopic microscope, and those already hatched were not considered in the analysis. For species identification, the rafts were placed individually in 250-ml Nalgene® plastic containers holding 100 ml of tap water. After hatching, the larvae were reared to fourth instar by a single feeding with 0.02 g of TetraFin® gold fish flakes. For larval identification, the fourth-instar larvae were mounted on slides and identified following the methods described by Forattini (1965).

**Predator sampling**

The predators in the containers were sampled by sieving all water content from half of the 1-L cups every sampling period (i.e., destructive sampling) and by sub-sampling the other container sizes. The 15-L buckets were sampled using a coffee filter made with cloth, and the 100 and 200-L barrels were sampled using a conical, 52 cm height × 18 cm radius plankton net. The predators collected in the samples were placed in a 500-ml plastic container and fixed in 70% ethanol for later identification to the lowest possible taxonomic level.

**Data analysis**

We perform a repeated-measures ANOVA to evaluate the effects of container size, sampling day and the interaction on the number of Culex egg rafts (both species) and those of C. eduardoi and Culex sp. j individually. We also tested for effects of predator abundances of the two most common taxa (dragonfly and Chaoborus sp. larvae) combined, then for the effects of these taxa individually. We then used AIC-based model selection (Table 3) to determine the importance of the main treatment effects (container size and sample period) and important predatory taxa (dragonfly larvae and Chaoborus sp.) both combined and separated on the numbers of egg rafts of both mosquito species (combined and separately). For each analysis, the response variable was tested for normality using a Shapiro–Wilk test. Non-normal data were log-transformed in order to adjust the data distribution. All statistical analyses were carried out using the free software R (R Development Core Team, 2014).
Table 1
Repeated-measures ANOVA indicating the effects of site, time and site: time interactions on the abundance of Culex egg rafts, the two main predators combined, and individual effects of dragonflies and Chaoborus sp.

<table>
<thead>
<tr>
<th>Abundance</th>
<th>Size (df=1.3)</th>
<th>Sample day (df=1.6)</th>
<th>Size: day (df=3.6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F-value</td>
<td>p-value</td>
<td>F-value</td>
</tr>
<tr>
<td>Culex egg rafts</td>
<td>16.95 &lt;0.001</td>
<td>4.44 &lt;0.001</td>
<td>3.57 &lt;0.001</td>
</tr>
<tr>
<td>C. eduardoi</td>
<td>21.45 &lt;0.001</td>
<td>1.18 0.31</td>
<td>–</td>
</tr>
<tr>
<td>Culex sp. 1</td>
<td>4.13 &lt;0.05</td>
<td>1.98 0.06</td>
<td>–</td>
</tr>
<tr>
<td>Predators (Total)</td>
<td>16.43 &lt;0.001</td>
<td>9.49 &lt;0.001</td>
<td>–</td>
</tr>
<tr>
<td>Dragonflies</td>
<td>15.46 &lt;0.001</td>
<td>6.97 &lt;0.001</td>
<td>–</td>
</tr>
<tr>
<td>Midges</td>
<td>29.64 &lt;0.001</td>
<td>0.98 0.43</td>
<td>–</td>
</tr>
</tbody>
</table>

Results

A total of 78 egg rafts were sampled during the entire experimental period. The sampled egg rafts yielded on average 10.3 mosquito larvae. Twenty-four percent was identified as Culex sp. 1 (21 egg rafts) and the remaining 76% belonged to C. eduardoi (57 egg rafts). We sampled 775 predators divided among 5 taxa. Chaoborus sp. and dragonflies (Odonata: Libellulidae) were the most abundant taxa with 224 and 542 individuals, respectively. The remaining 9 individuals included Dysticidae, Hydrophilidae (both Coleoptera) and Toxorhynchites sp. (Diptera: Culicidae).

Effects of container size and sampling day on Culex oviposition

Container size, sampling day and the size × day interaction significantly affected the total number of oviposited eggs rafts (Table 1). Egg raft abundance peaks on the 75th sampling day, mostly in 100-L barrels and 15-L buckets (Fig. 1). On the 15th, 45th, 90th and 120th sampling days, the 15-L buckets contained the highest numbers of egg rafts (Fig. 1). We then analyzed the effects of the model variables on C. eduardoi and Culex sp. 1 separately, and found an interaction between container size and sampling day for C. eduardoi. However, Culex sp. 1 was affected only by container size (Table 1) and was never found in the 200-L barrels and 1-L cups (Fig. 2).

Effects of container size and sampling day on predator abundance

The total predator abundance per liter was strongly affected by size and day (Table 1). The two main predators were more abundant and statistically equally abundant in the 15-L buckets, 100 and 200-L containers beginning on the 45th sampling day, and slightly increased until the last sampling day. In contrast, the 1-L cups had low predator abundance per liter during the entire experimental period (Fig. 3). The two most abundant predatory taxa (dragonflies and Chaoborus sp.) when analyzed separated from the main dataset were strongly and oppositely affected by size and the size: day interaction (Table 1). While dragonfly abundance was highest in the 15-L buckets followed by 100-L barrels, Chaoborus sp. larvae preferentially colonized 200-L barrels and were rarely found in 1-L container and 15-L buckets, and showed low abundance in 100-L barrels (Fig. 4).

Effects of the main predators on Culex oviposition

The total numbers of predators (dragonflies + Chaoborus sp.), did not significantly affect C. eduardoi and Culex sp 1. oviposition in the experimental containers (Table 2). However, when we analyzed the presence/absence of dragonfly and Chaoborus sp. larvae separately, we found an effect of dragonflies on overall numbers of Culex egg rafts (Table 2). No eggs were found in the 1-L cups. The barrels (100 and 200-L), which held high abundances of Chaoborus sp. larvae, received statistically equivalent numbers of egg rafts as the predator-free barrels. Interestingly, 15-L buckets holding dragonfly larvae receive statistically more egg rafts than the predator-free containers (Fig. 5).

Discussion

Our results did not support our hypothesis that Culex would lay more eggs in the smallest containers to avoid predation (Fig. 1). However, the results show that fewer egg rafts were laid in the 200-L barrels and 1-L cups compared to the 100-L barrels and 15-L buckets. Although they did not statistically differ from 100-L barrels, the 15-L capacity buckets contained the highest numbers of egg rafts on four out of seven sampling days. Chaoborus sp. larvae had low abundance per liter in the 15-L buckets and the 1-L cups and showed significantly higher abundance per liter in the 200-L barrels compared to the other container sizes (Fig. 4). Culex sp. 1 egg rafts were not found in the 200-L barrels. Predator avoidance behavior has been commonly described for Culex species (Vonesh and Blaustein, 2010; Andrade et al., 2016). For example, predator avoidance oviposition behavior has been documented in Culex (an Atlantic forest species) in response to chemical cues of Toxorhynchites thobaldi (Diptera: Culicidae) predatory larvae (Andrade et al., 2016). This suggests that adult Culex sp. 1 females may be able to detect environmental indicators of predator presence inside the oviposition containers (Bentley and Day, 1989). Our experimental design did not rule out the possibility that egg predation impacted our counts of egg rafts over the

Fig. 2. Numbers of Culex sp. 1 (left) and C. eduardoi (right) egg rafts as a function of sampling day and container size. The 100 and 200-L barrels are represented by closed triangles and closed circles, respectively. The open circles represent the 15-L buckets, and asterisks represent the 1-L cups. The bars represent standard error. Different letters indicate different means among the treatments.
The 100 and 200-L barrels are represented by the closed triangles and closed circles, respectively. The open circles represent the 15-L buckets, and asterisks represent the 1-L cups. The bars represent standard error. Different letters indicate different means among the treatments.

The 100 and 200-L barrels are represented by the closed triangles and closed circles, respectively. The open circles represent the 15-L buckets, and asterisks represent the 1-L cups. The bars represent standard error. Different letters indicate different means among the treatments.

Experimental period; however, dragonfly larvae are well-known to associate with the bottom portion and sides of water-holding containers, occupying a space that is far from surface egg rafts (Moore et al., 1994); and dragonfly larvae have not been reported as mosquito egg predators in the literature. There is also no indication that Chaoborus sp. consume mosquito eggs. Instead, the preferred food sources of Chaoboridae, in addition to larvae of mosquito and other organisms (e.g., copepods), are zooplankton and phytoplankton. The described feeding behaviors of these taxa therefore suggest that the abundance of Culex egg rafts reflects adult female oviposition preferences instead of post-colonization processes such as egg predation.

The mean predator abundance per liter was affected by sampling day and container size, with no statistical difference among the 15-L buckets and 100 and 200-L barrels (Fig. 3). The number of C. eduardoi egg rafts did not statistically differ among 15-L buckets and the 100 and 200-L barrels (Fig. 2). This suggests that C. eduardoi females do not respond to the higher occurrence of predators in the 100 and 200-L barrels (Fig. 3) when choosing oviposition sites (Fig. 2). The ability of a prey species to recognize predators is a product of co-evolution (Sih, 1986; Kerfoot and Sih, 1987). Predator–prey coexistence is thus contingent upon the ability to share the same environment with natural enemies (King and Hastings, 2003), and this is often facilitated by behavioral changes in prey after the detection of predation risk (Blaustein et al., 1995).

C. eduardoi females oviposited into containers where the abundance of dragonflies per liter was high. Dragonfly larvae display a "sit and pursue" predatory behavior in which prey are captured by chance due to proximity (Pierce, 1988; Gavin et al., 2007). Culex larvae and pupae are known to be fast swimmers and to spend more time resting at the surface portion of the water column (Andrade et al., 2016) compared to other genera (Roberts, 2017; Brackenbury, 2001), and these behaviors may result in lower vulnerability to predation. For this reason, dragonfly larvae might not pose a threat to C. eduardoi, as this species may be more able to escape predation compared to other taxa. This may facilitate co-existence and lead to lack of oviposition avoidance by Culex females into containers with dragonfly larvae.

### Table 2
Repeated-measures ANOVA indicating the effects of the presence/absence of the two main predators combined, and dragonfly larvae and Chaoborus sp. individually on numbers of Culex egg rafts (combined and individually).

<table>
<thead>
<tr>
<th>Numbers of egg rafts</th>
<th>Total predators</th>
<th>Dragonflies</th>
<th>Phantom midge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F-value</td>
<td>p-value</td>
<td>F-value</td>
</tr>
<tr>
<td>Total Culex</td>
<td>3.33</td>
<td>0.06</td>
<td>5.42</td>
</tr>
<tr>
<td>Culex eduardoi</td>
<td>1.62</td>
<td>0.20</td>
<td>3.01</td>
</tr>
<tr>
<td>Culex sp. 1</td>
<td>1.45</td>
<td>0.22</td>
<td>1.69</td>
</tr>
</tbody>
</table>

### Table 3
AIC-based model selection criteria. The model (with or without interaction) was chosen based on the lowest AIC value, and the best models for each of the response variables are represented in bold.

<table>
<thead>
<tr>
<th>Response variable</th>
<th>No interaction model (AIC)</th>
<th>Interaction model (AIC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culex egg rafts</td>
<td>180.53</td>
<td>169.23</td>
</tr>
<tr>
<td>C. eduardoi egg rafts</td>
<td>98.51</td>
<td>90.60</td>
</tr>
<tr>
<td>Culex sp. 1</td>
<td>112.46</td>
<td>–81.38</td>
</tr>
<tr>
<td>Predators (total)</td>
<td>681.38</td>
<td>658.73</td>
</tr>
<tr>
<td>Dragonflies</td>
<td>619.23</td>
<td>622.33</td>
</tr>
<tr>
<td>Midge</td>
<td>41.77</td>
<td>67.39</td>
</tr>
</tbody>
</table>

Akaike information criterion (AIC) is an estimator of the relative quality of statistical models for a given set of data.

*Fig. 3.* Predator abundance per liter as a function of sampling day and container size. The 100 and 200-L barrels are represented by the closed triangles and closed circles, respectively. The open circles represent the 15-L buckets, and asterisks represent the 1-L cups. The bars represent standard error. Different letters indicate different means among the treatments.

*Fig. 4.* The abundance of dragonfly larvae per liter (top) and midge (Chaoborus sp.) larvae per liter (bottom) as a function of sampling day and container size. The 100 and 200-L barrels are represented by the closed triangles and closed circles, respectively. The open circles represent the 15-L buckets, and asterisks represent the 1-L cups. The bars represent standard error. Different letters indicate different means among the treatments.

*Fig. 5.* Total number of Culex egg rafts number as a function of dragonfly larvae (predator) presence/absence in the experimental containers. The 100 and 200-L barrels are represented by the closed triangles and closed circles, respectively. The open circles represent the 15-L buckets, and asterisks represent the 1-L cups. The bars represent standard error. Different letters indicate different means among the treatments.
On most sampling days, Chaoborus sp. larvae abundance per liter was highest in the 200-L barrels (Fig. 4) and were concentrated on the median and surface part of the water column (pers. obs.). Among the life history strategies of mosquitoes to enhance offspring success, gravid females may avoid laying eggs in harsh (e.g., predator-rich) environments (Blaustein et al., 1995; Eitam et al., 2002; Silberbush and Blaustein, 2008; Andrade et al., 2016), or offspring may reduce predation risk by altering their behavior when exposed to predators or cues of predator presence (Kesavaraju and Juliano, 2004; Kesavaraju et al., 2011; Andrade et al., 2016).

The absence or reduced number of Chaoborus sp. larvae per liter in smaller-sized containers (1-L cups and 15-L buckets) (Fig. 3) may be explained by the behavior of adult females choosing larger containers, which theoretically have greater food availability (other mosquito species or other animal taxa) (Connor and McCoy, 2001), or due to dragonfly predators occupying these containers and depleting Chaoborus larval populations. Competition between predators at upper and intermediate trophic levels can also benefit prey populations by decreasing predation pressure on prey at lower levels (Strong, 1992; Costa and Vonesh, 2013; Albeny-Simões et al., 2015).

Our results collectively suggests that Culex sp. 1 avoid oviposition in the largest containers, and that C. eduardoi spread their egg rafts among intermediate and large containers. As we predicted, container size was positively correlated with predator abundance (per liter) for Chaoborus sp. larvae; however, dragonfly larvae in this study occupied all containers, with the lowest abundances and most frequent absence in the smallest containers. The abundance per liter of both Culex species was affected by the sampling date, and intermediate-sized containers (15 and 100-L) were preferred by dragonflies.

There are several ways in which container size may affect adult mosquito oviposition choices. First, larger containers can be indicative or greater resource availability (Connor and McCoy, 2001), which should positively influence larval development. Second, larger water bodies may indicate greater water permanence and reduced risk of habitat drying. Mosquitoes may perceive physical characteristics of a habitat through tactile, olfactory or visual cues (Bentley and Day, 1989; Beehler et al., 1993) related to surface area or water depth (Reiskind and Zarrabi, 2012), color or material (Bartlett-Healy et al., 2012), water surface reflectance (Harrington et al., 2008) or container height (Obenauer et al., 2009), among other characteristics. Different mosquito species show different oviposition preferences based on container characteristics that are indicative of predation risk (Sunahara et al., 2002), which is positively correlated with habitat size (Juliano, 2009). Our experimental design does not allow us to discern which cues related to container size may have influenced the observed patterns of oviposition; however, multiple container characteristics are likely used by mosquitoes when processing information about potential breeding sites (Bentley and Day, 1989).

The results from this study contribute to our understanding of how predators affect mosquito colonization processes in the natural environment. Understanding the relationships between physical and biological conditions in aquatic habitats and the resulting mosquito oviposition site choice is of fundamental importance for our general understanding of how habitat characteristics influence mosquito colonization and spatial and temporal distributions under natural conditions. Our results suggest that Culex oviposition preferences are mediated by container size and sampling period, perhaps through the influence of these factors on the presence and abundance of predators. Further studies aimed are needed to evaluate how aquatic habitat characteristics (e.g., predators, nutrient levels, physical aspects) influence mosquito oviposition choice and to better characterize how the environmental and physical characteristics of breeding habitats influence mosquito occurrence patterns and resulting larval abundances.

Conflicts of interest
The authors declare no conflicts of interest.

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