

Behavior, Chemical Ecology

Comparative Skip-Oviposition Behavior Among Container Breeding *Aedes* spp. Mosquitoes (Diptera: Culicidae)

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Abstract

Container *Aedes* mosquitoes are the most important vectors of human arboviruses (i.e., dengue, chikungunya, Zika, or yellow fever). Invasive and native container *Aedes* spp. potentially utilize natural and artificial containers in specific environments for oviposition. Several container *Aedes* spp. display ‘skip-oviposition’ behavior, which describes the distribution of eggs among multiple containers during a single gonotrophic cycle. In this study, we compared individual skip-oviposition behavior using identical eight-cup testing arenas with three container *Aedes* species: *Aedes aegypti* (Linnaeus), *Aedes albopictus* (Skuse), and *Aedes triseriatus* (Say). We applied the index of dispersion, an aggregation statistic, to individual mosquitoes’ oviposition patterns to assess skip-oviposition behavior. *Aedes aegypti* and *Ae. albopictus* utilized more cups and distributed eggs more evenly among cups than *Ae. triseriatus* under nutritionally enriched oviposition media (oak leaf infusion) conditions. When presented with a nutritionally unenriched (tap water) oviposition media, both *Ae. aegypti* and *Ae. albopictus* increased egg spreading behavior. *Aedes albopictus* did not modify skip-oviposition behavior when reared and assessed under fall-like environmental conditions, which induce diapause egg production. This study indicates specific oviposition site conditions influence skip-oviposition behavior with ‘preferred’ sites receiving higher amounts of eggs from any given individual and ‘non-preferred’ sites receive a limited contribution of eggs. A further understanding of skip-oviposition behavior is needed to make the best use of autodissemination trap technology in which skip-ovipositing females spread a potent larvicide among oviposition sites within the environment.

Key words: bet-hedging, container habitat, autodissemination

Invasive and native container *Aedes* mosquitoes are important vectors of mosquito-borne viruses (Lounibos 2002). Anthropophilic invasive *Aedes* mosquitoes include *Aedes aegypti* L. and *Aedes albopictus* (Skuse), which are the main vectors of dengue, chikungunya, and Zika virus in tropical and subtropical regions worldwide (Bonizzoni et al. 2013, Leta et al. 2018). Native container *Aedes* mosquitoes are also important vectors of disease, with *Aedes triseriatus* (Say) the primary vector of La Crosse virus within the United States (Miller et al. 1978, Borucki et al. 2002). Most container *Aedes* species mosquitoes will readily take

bloodmeals from human hosts, making them effective vectors of human pathogens (Lounibos 2002). *Aedes triseriatus* remains a concern for invasion into Europe due to the presence of similar, favorable larval habitats as in its native range in the United States, which facilitated the invasion of another container utilizing native North American mosquito, *Aedes atropalpus* (Coquillett), in the late 1990s (Medlock et al. 2012, Schaffner et al. 2013). The worldwide success of invasive container *Aedes* mosquitoes is associated with the laying of desiccation-resistant eggs in small water-filled containers such as natural tree holes, rock pools,

tires, or other artificial containers easily transported by human activity and often found around human habitation (Bentley and Day 1989, Lounibos 2002, Juliano and Lounibos 2005, Reiter 2007, Day 2016).

The term ‘skip-oviposition’ was coined to describe the oviposition behavior of *Wyeomyia smithii* (Coquillett) female mosquitoes distributing eggs among multiple pitcher plants (Mogi and Mokry 1980). Skip-oviposition now broadly describes behavior in which a female mosquito distributes eggs among multiple oviposition sites during a single gonotrophic cycle (Mogi and Mokry 1980, Harrington and Edman 2001, Day 2016). *Aedes aegypti*, *Ae. albopictus* and *Ae. triseriatus* exhibit skip-oviposition behavior, i.e., egg spreading, in laboratory studies (Corbet and Chadee 1993, Trexler et al. 1998, Davis et al. 2015, Swan et al. 2018). Skip-oviposition behavior likely contributes to the global invasion success of *Aedes* spp. mosquitoes through desiccation-resistant eggs that can survive international trade (Hawley 1988, Lounibos 2002). The propensity to skip oviposit is not well studied despite its importance in *Aedes* vectors’ biology.

Container choice due to the quality of oviposition sites, instead of propensity to skip-oviposit, is a repeating focus for skip-oviposition studies. Implicitly, these studies use skip-oviposition behavior as a tool for assessing habitat preference by comparing counts of eggs in one container versus others (Corbet and Chadee 1993). Oviposition site ‘quality’ is a variable definition that includes but is not limited to differences in available nutrition, conspecific/antispesific larval, conspecific/antispesific eggs, local site environment, container size, or site color (Bentley and Day 1989). Here, we define ‘quality’ as correlating with adult mosquitoes’ production from a larval habitat (Bentley and Day 1989). Oviposition site nutrient diversity influences the quality of the larval habitat for *Ae. albopictus* and *Ae. triseriatus* and determines adult development (Reiskind et al. 2009, Reiskind and Zarrabi 2013). The presence or absence of conspecific individuals affects the quality of a site, modifying the oviposition behavior of both *Ae. aegypti* and *Ae. albopictus*. *Aedes albopictus* displays a preference for quality oviposition sites with uncrowded larval conditions (Davis et al. 2015). Similarly, *Ae. aegypti* individuals prefer to aggregate eggs in oviposition cups containing lower conspecific egg density (Chadee et al. 1990, Nazni et al. 2016). The type of oviposition container can also function as a quality measure in skip-oviposition studies. *Aedes aegypti* avoids clear plastic cups in comparative studies with artificial and natural containers, while preferring black plastic cups. (Reiskind and Zarrabi 2013, Oliva et al. 2014). The previous examples focus on oviposition site choice when there is a difference among multiple containers. However, there is a lack of information on the underlying instinctive behavior or propensity of skip-oviposition choice without differences in container quality.

Few studies specifically address fundamental behavioral mechanisms associated with risk-aversion egg-spreading in container *Aedes* species mosquitoes. A consistently observed behavior from previous skip-oviposition studies is individual *Ae. aegypti* and *Ae. albopictus* aggregate a substantial proportion of eggs into a ‘favorite’ cup even without intended differences in habitat quality (Oliva et al. 2014, Santos de Abreu et al. 2015). Individuals from both species display this behavior by aggregating 40% or more of their eggs in one cup when presented with a universally identical multiple oviposition cup environment (Oliva et al. 2014, Davis et al. 2015, Santos de Abreu et al. 2015). *Aedes aegypti* and *Ae. albopictus* respond to an increase in the number of oviposition sites by increasing cup occupation and subsequently decreasing

average egg contribution to each cup (Santos de Abreu et al. 2015, Swan et al. 2018). These observations suggest *Ae. aegypti* and *Ae. albopictus* potentially conduct more skip-oviposition egg clutch events than available oviposition sites, hence a corresponding increase in cup occupation with the addition of more cups. Furthermore, *Ae. aegypti* spreads eggs more evenly than *Ae. albopictus*, even as both species utilize the same number of oviposition sites (Rey and O’Connell 2014, Swan et al. 2018). There is no indication either age or gonotrophic cycle modifies skip-oviposition behavior as individual *Ae. aegypti* females consistently spread eggs through five gonotrophic cycles (Oliva et al. 2014). However, there is evidence that physiological condition affects behavioral mechanisms. *Aedes aegypti* held 3 d post-bloodmeal without an oviposition site, e.g., forced egg retention, significantly reduced skip oviposition behavior when presented with a multiple oviposition site test arena (Chadee 1997).

Skip-oviposition is a targeted behavior for control using larvicide autodissemination traps, potentially controlling *Ae. aegypti*, *Ae. albopictus*, and possibly additional container *Aedes* species (Buckner et al. 2017). Control is achieved through the placement of larvicide autodissemination stations into the environment, allowing skip-ovipositing container species of mosquito to spread the larvicide throughout the natural habitat. However, the ecology of skip-oviposition demonstrated in the laboratory environment is poorly understood in natural settings. It is not easy to measure how multiple site variables affect any given mosquito species’ individual oviposition choices. Larger experimental arenas provide a closer approximation to natural environments, allow bigger containers, but compound the difficulty of retrieving individuals and oviposited eggs. Therefore, a greater understanding of the individual’s behavioral mechanisms associated with skip-oviposition at smaller scales may provide insights into how to enhance container mosquito control.

Our work is the first study to report a single gonotrophic cycle skip-oviposition pattern of three container *Aedes* species mosquitoes in identical conditions. We hypothesized these three species have different propensities to skip-oviposit, with the more urban species, *Ae. aegypti* spreading their eggs the widest, *Ae. albopictus* intermediate, and *Ae. triseriatus* the least spread. We also hypothesized these propensities are affected by the quality of habitat and the physiological state of the individual ovipositing female. The first series utilized a known oviposition attractant associated with larval growth, oak leaf infused water, to measure how assessed species spread or aggregated eggs in a nutritionally attractive condition. We predicted skip-oviposition behavior in urban adapted *Ae. aegypti* and *Ae. albopictus* would result in greater egg spreading among containers as compared to *Ae. triseriatus*. The second series used tap water as a nutritionally unattractive condition to assess oviposition behavior changes compared to the oak leaf infusion condition. In this series, we predicted all species would increase in the number of cups utilized and reduce the number of eggs contributed to any individual container. Previous laboratory assessments of *Ae. albopictus* found this species spreads eggs in summer conditions and aggregates eggs under fall conditions (Fonseca et al., 2015). Therefore, we also assessed diapause egg laying induced individual *Ae. albopictus* to assess skip-oviposition under fall conditions with the predicted species response of decreased egg spreading behavior. These assessments measured skip-oviposition behavior under universal oviposition conditions in individual eight-cup testing arenas, allowing for comparisons across species and conditions.

Materials and Methods

Mosquito Colonies

We reared colonies of *Ae. aegypti*, *Ae. albopictus*, and *Ae. triseriatus* in a temperature-controlled room located in the North Carolina State University (NCSU) Biological Resources Facility (BRF). Our laboratory *Ae. aegypti* colony was derived from field material collected in Florida and Arizona in 2013–2014, kept separate for ≈ 10 generations than hybridized. This hybrid strain was used at F_{18} – F_{19} . The *Ae. albopictus* colony is from locally collected populations in Wake County, North Carolina, started in 2014. Individuals from field-collected *Ae. albopictus* eggs were introduced into the colony each summer to reflect a current field population during the months of collection (April–October, 2014–2019). *Aedes triseriatus* was recently colonized in our laboratory (2018) from local populations in Wake, New Hanover, and Nash Counties, North Carolina, with additional material added in summer of 2019 to the F_4 generation from 2018. We maintained both *Ae. aegypti* and *Ae. albopictus* colonies in Bugdorm-1 adult cages (Insect Rearing Cage, Dimensions: W30 cm $\times$ D30 cm $\times$ H30 cm, MegaView Science Co., Ltd., Taiwan). *Aedes triseriatus* require larger collapsible adult cages (Dimensions: 46 cm $\times$ 46 cm $\times$ 46 cm, Bioquip Products, California) for long-term breeding success; however, we reared single generations of *Ae. triseriatus* in Bugdorm-1 cages for these skip-oviposition studies.

Aedes aegypti, *Ae. albopictus*, and *Ae. triseriatus* mosquitoes were hatched and reared in identical environmental conditions: 27°C, 14:10 light:dark photoperiod, and average relative humidity of 52% monitored by data loggers (Model DS1923-F5# iButton, Maxim Integrated Products, Wisconsin). For colony maintenance and experimental testing, we hatched eggs by submerging egg papers from each species in 1 liter of tap water in modified Rubbermaid Egg Keeper (Rubbermaid Inc., North Carolina, US. Modified) larvae rearing trays containing 0.6 g ground fish food (Wardley Pond Pellets, Hartz Canada Inc., Ontario, Canada). Two days post-hatching, we separated larvae into new rearing trays at a concentration of 100 larvae per tray with the same food concentration. Upon development, we collected each species pupa in small emergence cups and placed them in adult cages. Each cage contained a single sugar feeder allowing *ad libitum* feeding with a 10% sucrose solution in a 120 ml lidded cup with a 100 mm filter wick (Tidi Products Neenah, Wisconsin) extending 25 mm outside of the cup. Adult female mosquitoes were fed weekly with defibrinated bovine blood (HemoStat Laboratories, California) in hog casing (The SausageMaker, Inc., New York) covered small Petri dish. A hand warmer (HeatMax Hothands, Kobayashi Consumer Products LLC, Georgia) warmed the bovine blood dish for 10 min at which point the dish was inverted and placed on the top of the adult cage. An oviposition cup lined with heavy weight seed germination paper (Anchor Co., St. Paul, MN) was placed in the adult cages to collect eggs for laboratory experiments and colony propagation.

Experimental Procedures

We used recently emerged non-parous adult females in the experimental series and Bugdorm-1 cages as adult mosquito skip-oviposition testing arenas for all species. Testing arenas contained eight black plastic 473 ml oviposition cups, each filled to 250 ml with oviposition media and a sugar feeder. Oviposition media was either nutritionally enriched media (2 g/liter oak leaf infusion) or nutritionally unenriched media (tap water). Each oviposition cup contained a single 7 cm $\times$ 25 cm strip of oviposition paper (seed germination paper) to measure egg distribution. Cup placement was

identical for each trial cage, beginning with cup A in the front left corner of the test arena, then placing cups clockwise around the arena, ending with cup H. Mosquitoes were provided sugar *ad libitum* from a centrally located sugar feeders containing a 10% sucrose solution.

We conducted skip-oviposition behavioral trials in two incubators (Model DR-36VL, Percival, Perry, IA), each holding twelve test arenas. Incubators were set to the temperature and photoperiod as required for each specific experimental trial. Relative humidity remained high, $87\% \pm 2.5\%$, throughout each trial, due to the large volume of oviposition media per incubator.

Oviposition media for the skip-oviposition experimental series included a nutritionally ‘enriched’ media (oak leaf infusion) and a nutritionally ‘unenriched’ media (tap water). Previous experiments indicate that enriched oak leaf infusion (OLI) is an attractive oviposition water for *Ae. aegypti*, *Ae. albopictus*, and *Ae. triseriatus* mosquitoes (Trexler et al. 1998, Ponnusamy et al. 2010). A single 90 liters batch of concentrated 16 g/liter OLI reduced variation in oviposition media used for subsequent skip-oviposition experiments. To make 90 liters of OLI, we used a 32-gal Rubbermaid trash bin (Rubbermaid Inc., North Carolina) filled with 90 liters of tap water and 27 g (0.3 g/liter) of brewer’s yeast (M.P. Biomedicals, cat no. 903312, Fisher Scientific), and egg albumin (Fisherbrand Albumin from eggs (Powder) cat no. A388-500, FisherChemical), 27 g (0.3 g/liter) (Obenauer et al. 2009, Reiskind and Janaïro 2018). We used locally collected dried willow oak leaves (*Quercus phellos*) in a fine mesh cloth bundle immersed in the water mixture. We have successfully used this infusion for larval development studies in our laboratory (Reiskind and Janaïro 2018). A smaller trash bin held down the willow oak leaf bundle. The infusion was incubated at room temperature for 5 d, then one-quart Ziploc plastic bags each received a 250-ml aliquot of OLI. The 16 g/liter OLI aliquots froze at -20°C in six-quart plastic containers to prevent media leakage from the Ziploc bags. The day before skip-oviposition experiments, thawed 16 g/liter OLI aliquots mixed with tap water created a working concentration of 2 g/liter OLI oviposition media. Similarly, we incubated tap water in a water cooler at room temperature one day before skip-oviposition trials serving as unenriched oviposition media. This approach allowed us to conduct experiments across time with identical infusion, except for the time spent frozen, thus minimizing the error associated with comparing across experiments in time.

We assembled test arenas the morning of mosquito blood-feeding, provisioning each with oviposition cups and media sugar feeders. Post-blood-feeding, we aspirated female mosquitoes from adult rearing cages into small transport containers, observing each mosquito to ensure they contained a bloodmeal. For trials, numbered test arenas received an individual mosquito randomly assigned via the random() function in Microsoft Excel (2016) (Microsoft Corporation, Seattle, WA). Trials began once we placed the blood-fed mosquitoes into test arenas and incubators.

We collected mosquitoes and oviposition papers after 6 d. Mosquitoes were frozen at -20°C , then dissected for wing length measures and egg retention assessment. We took measurements from the right-wing of each female mosquito, using the left-wing if the right-wing was damaged, and measured the distance from the axillary incision to the end of the R_{4+5} vein to the nearest 0.001 mm, excluding the fringe scales with a microscope camera and software (Model MU1803-CK, AM Scope, California) (Armbruster and Hutchinson 2002). We dissected each mosquito for egg retention following wing removal. Finally, after a 2-h drying period, we counted each oviposition paper for eggs. Six

Ae. albopictus females are excluded from correlations by wing length due to damaged wings; however, we included them in other analyses.

Aedes Species Skip-Oviposition Assessment: Three Experimental Series in Assessed Skip-Oviposition Behavior in Container *Aedes* Species

Aedes species comparison

The first series of experiments assessed differences in skip-oviposition behavior between *Ae. aegypti* ($n = 36$), *Ae. albopictus* ($n = 60$), and *Ae. triseriatus* ($n = 36$) using 2 g/liter OLI oviposition media and incubators set to 27°C and 14:10 photoperiod. Each of the eight oviposition cups contained equal amounts of 2 g/liter OLI oviposition media to assess differences in container *Aedes* species response.

Unenriched oviposition media assessment

The second series of experiments assessed skip-oviposition behavior changes when the oviposition media is unenriched (tap water) for *Ae. aegypti* ($n = 12$), *Ae. albopictus* ($n = 12$), and *Ae. triseriatus* ($n = 12$) as compared to enriched (2 g/liter OLI) oviposition media. In this assessment, we maintained the incubators at 27°C and a 14:10 photoperiod. The eight oviposition cups in each test arena contained an equal volume of tap water.

Egg diapause induced *Aedes albopictus* assessment

The third series of skip-oviposition experiments utilized *Ae. albopictus* species mosquitoes and 2 g/liter OLI oviposition media in all test arena oviposition cups. This experiment compared individual *Ae. albopictus* ($n = 24$) reared and assessed in a ‘summer-like’ condition (27°C and 14:10 photoperiod) and a ‘fall-like’ condition (21°C and 10:14 photoperiod), which is an environmental condition known to produce diapause egg-laying adult females (Lounibos 2002, Fonseca et al. 2015). We only used *Ae. albopictus* females for this series of trials as this species is known to lay diapausing eggs, whereas *Ae. aegypti* is not known to enter diapause and *Ae. triseriatus* diapauses during egg and larvae stages, creating a logistical difficulty in producing diapause egg-laying adult females. *Aedes albopictus* in each condition were hatched for synchronized adult emergence to assess both conditions simultaneously. The females in the ‘fall-like’ diapause egg condition were collected at 11 d post-blood-feeding due to the longer development time induced by colder environmental conditions.

Skip-Oviposition Metrics

We assessed skip-oviposition behavior with two metrics comparing between species and treatment combinations. The first metric counted occupied oviposition cups, which is the primary method of skip-oviposition comparison in previous studies (Chadee 1997, Trexler et al. 1998, Oliva et al. 2014, Davis et al. 2015, Santos de Abreu et al. 2015, Swan et al. 2018). For the second metric, an aggregation statistic known as the index of dispersion provided a single useful statistic assessing skip-oviposition differences (Fisher et al. 1922, Young and Young 1998, Walker 1999). Walker (1999) recommended a modification from the original Fisher (1922) formulation for more complicated data sets resulting in an *I.D.* score scaled from zero to one. The following formula calculates the index of dispersion:

$$\text{Index of dispersion} = \frac{K(N^2 - \sum f^2)}{N^2(K - 1)}$$

K is the number of oviposition cups, N is the total number of eggs, and f is the number of eggs in each oviposition cup. We simplified the Walker 1999 formula for *I.D.* as follows:

$$\text{Index of dispersion} = \frac{K - \frac{\text{variance}(f)}{\text{mean}(f)^2}}{K}$$

The simplified formula is easier to calculate and produces identical *I.D.* scores. The index of dispersion allows for a more sophisticated statistical analysis as it creates a single index value that accounts for both cup occupancy and an individual's per cup contribution of eggs.

Oliva et al. (2014) described a method for assessing skip-oviposition patterns within groups of mosquitoes by ordering oviposition cups by the number of eggs each cup received during assessment. We used this methodology to order each mosquito's oviposition cups from the highest egg count (Cup 1) to the lowest egg count (Cup 8). Then we calculated the average egg contribution for the ordered cups 1 through 8 for each group (i.e., species/treatment combination) and the standard error of the mean (SEM) around each ordered cup. Finally, we calculated the *I.D.* for each species/treatment combination from the mean number of eggs per ordered cup, as previously described for individual mosquito assessments (Oliva et al. 2014).

Data Analysis

Data analysis was conducted in R 3.6.2 (R Core Team 2017) using the PMCRmplus package (Pohler 2019) and produced all graphs using the ggplot2 package (Wickham 2009). Of the 192 individual *Aedes* mosquitoes assessed for skip-oviposition behavior, 151 mosquitoes completed skip-oviposition trials. We excluded analysis of the 41 mosquitoes who failed to complete skip-oviposition assessment (26 who retained eggs and 15 who failed to produce eggs). The skip-oviposition cup occupation and *I.D.* score distributions are not normal; therefore, it requires a nonparametric Kruskal–Wallis test to assess differences in skip-oviposition behavior between species occupied cups and *I.D.* A *post hoc* multiple comparisons Mann–Whitney *U* test with the Benjamini & Hochberg adjustment following a significant Kruskal–Wallis test compared groups using the pairwise.wilcox.test() function in R (Mann and Whitney 1947, Benjamini and Hochberg 1995).

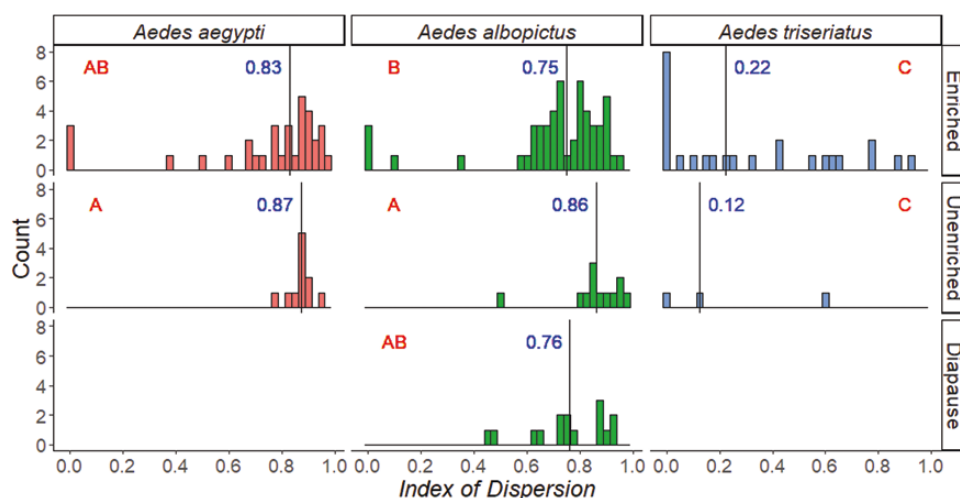
Results

Aedes aegypti and *Ae. albopictus* under enriched (OLI) oviposition media conditions occupied more cups and displayed a greater *I.D.* than *Ae. triseriatus* (cup occupation: KW $\chi^2 = 47.13$; $df = 6$; $P < 0.001$, ID KW $\chi^2 = 49.57$; $df = 6$; $P < 0.001$; Table 1). A *post hoc* multiple comparison analysis found no statistically significant egg spreading (*I.D.*) between the *Ae. aegypti* group and the *Ae. albopictus* group (*I.D.* MW, $P = 0.0583$). Additionally there was no difference in cup occupation (MW, $P = 0.3909$; Table 1). We found the assessed groups of *Ae. aegypti* and *Ae. albopictus* utilized a median of six cups in our experimental trials. *Aedes albopictus* increased egg spreading in response to unenriched oviposition media treatment as compared to enriched (OLI) oviposition media (*I.D.* MW, $P = 0.0043$) but did not increase cup occupation (Table 1). Conversely, *Ae. aegypti* increased cup occupation in response to the unenriched oviposition media treatment (MW, $P = 0.0264$) but did not increase in *I.D.* (Table 1).

Distributions of skip-oviposition reveal a proportion of individuals from each species aggregated all eggs in one container under

Table 1. Adjusted *P*-values for post hoc Mann–Whitney *U* test with Benjamini & Hochberg adjustment for multiple comparisons following the Kruskal–Wallis test

Skip-oviposition Treatment comparisons	Index of dispersion KW $\chi^2_6 = 49.57$; $P < 0.001$	Oviposition cups KW $\chi^2_6 = 47.13$; $P < 0.001$
Enriched (OLI) Media		
<i>Ae. aegypti</i> – <i>Ae. albopictus</i>	0.0583	0.3909
<i>Ae. aegypti</i> – <i>Ae. triseriatus</i>	0.0001	0.0001
<i>Ae. albopictus</i> – <i>Ae. triseriatus</i>	0.0001	0.0001
Unenriched (Tap Water) Media		
<i>Ae. aegypti</i> – <i>Ae. albopictus</i>	0.9279	0.1699
<i>Ae. aegypti</i> – <i>Ae. triseriatus</i>	0.0144	0.0166
<i>Ae. albopictus</i> – <i>Ae. triseriatus</i>	0.0205	0.1007
Enriched vs Unenriched Media		
<i>Ae. aegypti</i>	0.2624	0.0264
<i>Ae. albopictus</i>	0.0024	0.2186
<i>Ae. triseriatus</i>	0.7409	0.8292
Diapause Inducing Conditions		
<i>Ae. albopictus</i>	0.6444	0.2043

**Fig. 1.** Histograms displaying the distribution of skip-oviposition behavior scores (I.D.) for *Ae. aegypti*, *Ae. albopictus*, and *Ae. triseriatus* in response to enriched (2 g/liter OLI) and unenriched (tap water) oviposition media. Additional histogram of *Ae. albopictus* skip-oviposition (I.D.) response to diapause egg-laying inducing conditions with enriched (2 g/liter OLI) oviposition media. The index of dispersion is the calculated score specific to an eight-cup test arena. An I.D. score of 0.0 represents complete egg aggregation in one cup, whereas an I.D. score of 1.0 represents equal spreading of eggs among all eight cups. The black vertical line highlights the median I.D. score per individual for each distribution, shown by the number next to the black vertical line. Letters in the upper left (*Ae. aegypti* and *Ae. albopictus*) or upper right (*Ae. triseriatus*) indicate significant ($P < 0.05$ or lower) differences between assessed groups by Mann–Whitney *U* test adjusted for multiple comparisons.

enriched (OLI) oviposition media treatment (Figs. 1 and 2). A significantly higher proportion of *Ae. triseriatus* individuals aggregated eggs in one cup ($\chi^2 = 11.02$; $df = 2$; $P = 0.004$) as compared to *Ae. aegypti* and *Ae. albopictus*. Under unenriched oviposition media conditions, no *Ae. aegypti* or *Ae. albopictus* aggregated all eggs in a single cup (Figs. 1 and 2). *Aedes albopictus* under diapause and non-diapause inducing conditions did not change distributions in cup occupation and ID score (Cup MW, $P = 0.2043$, I.D. MW, $P = 0.6444$; Table 1).

Cups were reordered by each individual mosquito's egg contribution from highest to lowest, allowing us to average the number of eggs in each cup groups by species/treatment group (Table 2). The average number of eggs per group produced an estimate of egg contribution (in percentage) per cup from the total remaining eggs for each species/treatment combination group using the average I.D. and total eggs (Table 2). *Aedes aegypti* and *Ae. albopictus* under enriched (OLI) oviposition media conditions demonstrated a higher

estimated percentage of eggs contributed per cup (43% and 48%, respectively) from the remaining total eggs than unenriched (tap water) oviposition media condition, which reduced the estimated egg contribution in both species (33% and 34%, respectively). In contrast, *Ae. triseriatus* under the OLI treatment contributed a much higher estimated percentage (75%) of eggs to each reordered cup group, tending to focus eggs in only a few cups.

When looking at the populations of assessed *Aedes* spp. a significantly larger proportion ($\chi^2 = 32.89$; $df = 2$; $P < 0.001$) of the *Ae. triseriatus* mosquitoes retained eggs (35.4%) as compared to the *Ae. aegypti* and *Ae. albopictus* groups. A majority (73%) of all tested *Aedes* mosquitoes who failed to develop eggs were *Ae. albopictus*, however, *Ae. albopictus* also comprising 50% of all tested mosquitoes. We found no proportional difference in failure to develop eggs among the three *Aedes* species ($\chi^2 = 2.76$; $df = 2$; $P = 0.2521$).

Aedes mosquito wing length did not influence skip-oviposition behavior for any species and treatment combinations (*Ae. aegypti*/

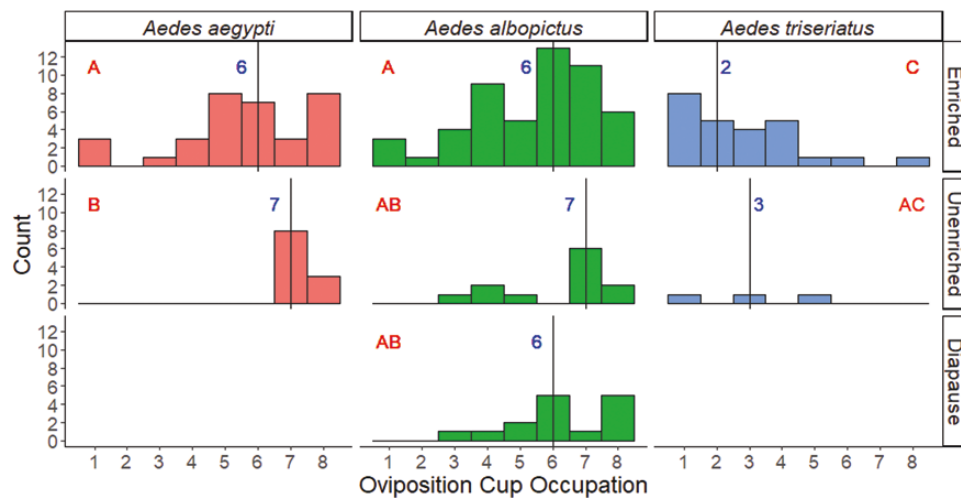


Fig. 2. Histograms displaying the distribution of skip-oviposition behavior scores (cup occupation) for *Ae. aegypti*, *Ae. albopictus*, and *Ae. triseriatus* in response to enriched (2 g/liter OLI) and unenriched (tap water) oviposition media. Additional histogram of *Ae. albopictus* skip-oviposition (cup occupation) response to diapause egg-laying inducing conditions with enriched (2 g/liter OLI) oviposition media. Oviposition cup occupation lists the number of cups occupied cups per individual. The black vertical line highlights the median cups occupied per individual for each distribution, shown by the number next to the black vertical line. Letters in the upper left (*Ae. aegypti* and *Ae. albopictus*) or upper right (*Ae. triseriatus*) indicate significant ($P < 0.05$ or lower) differences between assessed groups by Mann-Whitney U test adjusted for multiple comparisons.

OLI treatment I.D.: $t = -1.28$; $df = 31$; $P = 0.209$, *Ae. aegypti*/tap water treatment I.D.: $t = -0.79$; $df = 9$; $P = 0.452$, *Ae. albopictus*/OLI treatment I.D.: $t = -1.26$; $df = 46$; $P = 0.215$, *Ae. albopictus*/tap water treatment I.D.: $t = 0.98$; $df = 10$; $P = 0.348$, *Ae. albopictus*/diapause treatment I.D.: $t = 0.93$; $df = 11$; $P = 0.371$, *Ae. triseriatus*/OLI treatment I.D.: $t = 0.70$; $df = 23$; $P = 0.488$, *Ae. triseriatus*/tap water treatment I.D.: $t = -0.54$; $df = 1$; $P = 0.683$). An individual's total eggs did not influence skip-oviposition behavior in *Ae. aegypti* and *Ae. triseriatus* regardless of treatment (*Ae. aegypti*/OLI treatment I.D.: $t = 0.89$; $df = 31$; $P = 0.3819$, *Ae. aegypti*/tap water treatment I.D.: $t = 0.18$; $df = 9$; $P = 0.864$, *Ae. triseriatus*/OLI treatment I.D.: $t = 1.22$; $df = 23$; $P = 0.235$, *Ae. triseriatus*/tap water treatment I.D.: $t = -24.15$; $df = 1$; $P = 0.026$). However, under enriched (2 g/liter OLI) oviposition media and unenriched (tap water) oviposition media treatments *Ae. albopictus* displayed a slightly positive correlation between total eggs and skip-oviposition behavior as individuals with more eggs displayed a higher I.D. ($t = 2.14$; $df = 50$; $P = 0.038$ and $t = 2.29$; $df = 10$; $P = 0.045$, respectively). In contrast, there was a slight negative correlation in the same comparison for egg diapause induced *Ae. albopictus* ($t = -2.23$; $df = 13$; $P = 0.044$).

Discussion

This study compared the skip-oviposition behavior of *Ae. aegypti*, *Ae. albopictus*, and *Ae. triseriatus* under laboratory conditions. Our skip-oviposition behavioral experiments used small cage test arenas with eight identical oviposition sites, similar to previous studies (Chadee et al. 1990, Chadee 1997, Trexler et al. 1998). The resulting assessed skip-oviposition behavior found urban adapted *Ae. aegypti* and *Ae. albopictus* spread eggs more than forest preferring *Ae. triseriatus* under identical oviposition conditions (Barker et al. 2003, Reiskind et al. 2017). The differences in egg spreading behaviors are not clear between *Ae. aegypti* and *Ae. albopictus*. A direct species comparison suggests these two species uses a similar number of oviposition cups. We could not show that *Ae. aegypti* spread their eggs more evenly among cups than *Ae. albopictus*, although increased replication of this comparison may yield significant differences. *Aedes*

aegypti and *Ae. albopictus* both increased egg spreading behavior in response to a nutritionally unenriched (tap water) oviposition media. The media 'quality' comparison supports the idea that *Ae. aegypti* and *Ae. albopictus* aggregate more eggs under 'preferred' conditions, increasing egg spreading under 'non-preferred' conditions. *Aedes triseriatus* simply retained eggs in response to the nutritionally unenriched oviposition media treatment, resulting in a sample size that was too small to make substantiated inference about modifications in *Ae. triseriatus* skip-oviposition behavior.

Aedes albopictus females were previously found in laboratory settings to spread eggs under 'summer-like' conditions and aggregate eggs in 'fall-like' conditions in response to containers with conspecific eggs (Fonseca et al. 2015). These results suggest *Ae. albopictus* should aggregate their eggs in fewer cups under 'fall-like' conditions, i.e., lower temperature and shorter day length. However, we could not support the third hypothesis as *Ae. albopictus* did not modify skip-oviposition behavior under egg diapause inducing 'fall-like' conditions. Fonseca et al. (2015) assessed groups of gravid *Ae. albopictus* as opposed to individuals, which could explain the difference between studies. This study does not account for the effect of multiple mosquitoes ovipositing in the same environment. Additionally, Fonseca et al. (2015) only allowed 5 d for oviposition by diapause egg laying induced gravid *Ae. albopictus*, this study found *Ae. albopictus* require at least 11 d for all individuals to complete a gonotrophic cycle under diapause-inducing conditions, 21°C and a 10:14 photoperiod. The previous study utilized mosquito material sourced at the northern range of the species, where rapid evolution has selected for a more robust diapause response (Lounibos 2002, Urbanski et al. 2012). We used mosquitoes from North Carolina and therefore the lack of support for diapause induced changes in behavior may also have a genetic component. Direct comparison between southern and northern populations in the United States would help resolve this question. Comparing native *Ae. triseriatus* in fall-like and summer-like conditions could also provide additional insight into the effects of diapause on oviposition behavior, however, we were not able to conduct this experiment here.

Table 2. Skip-oviposition pattern for *Aedes aegypti*, *Aedes albopictus*, and *Aedes triseriatus* in response to enriched (2 g/liter OLI) oviposition media, unenriched (tap water) oviposition media, and diapause inducing conditions

Species treatment	Total Eggs (SEM)	Cup 1 (SEM)	Cup 2 (SEM)	Cup 3 (SEM)	Cup 4 (SEM)	Cup 5 (SEM)	Cup 6 (SEM)	Cup 7 (SEM)	Cup 8 (SEM)	I.D.	Percent estimate
<i>Aedes aegypti</i> Enriched OLI Media (n = 33)	70.27 (3.89)	31.48 (3.09)	15.12 (1.50)	10.3 (1.07)	6.33 (0.67)	3.54 (0.51)	2.09 (0.43)	0.91 (0.26)	0.48 (0.17)	0.82	43%
<i>Aedes aegypti</i> Unenriched Media (n = 11)	103.91 (7.9)	34.58 (3.33)	24.17 (1.87)	18.5 (1.73)	11.42 (1.56)	6.83 (1.57)	5 (1.47)	3 (1.16)	0.42 (0.34)	0.90	33%
<i>Aedes albopictus</i> Enriched OLI Media (n = 52)	85.77 (3.39)	43.15 (2.26)	18.86 (1.27)	10.75 (0.89)	5.91 (0.59)	3.55 (0.45)	2 (0.35)	1.11 (0.28)	0.19 (0.08)	0.77	48%
<i>Aedes albopictus</i> Unenriched Media (n = 12)	109.27 (5.54)	38.54 (2.77)	25.64 (2.26)	17.27 (2.27)	11.55 (1.59)	7.36 (1.13)	5.36 (1.03)	3.09 (0.61)	0.45 (0.25)	0.89	34%
<i>Aedes albopictus</i> Diapause Induced (n = 15)	108.2 (6.58)	55 (6.43)	22.93 (2.84)	13.2 (0.86)	7.93 (0.99)	4.47 (0.83)	2.33 (0.60)	1.13 (0.46)	0.8 (0.36)	0.77	48%
<i>Aedes triseriatus</i> Enriched OLI Media (n = 25)	84.28 (4.99)	62.92 (5.47)	11.52 (2.48)	4.44 (1.30)	2.56 (0.92)	0.84 (0.49)	0.56 (0.39)	0.36 (0.32)	0.04 (0.04)	0.46	75%
<i>Aedes triseriatus</i> Unenriched Media (n = 3)	69.33 (2.08)	60.33 (14.4)	5.67 (7.37)	2 (2.64)	1 (1.73)	0.33 (0.57)	0 (0)	0 (0)	0 (0)	0.27	87%

The presented data are the average egg totals with the standard error of the mean (SEM) for each oviposition cup reordered from highest to lowest egg totals for each treatment and species combination. The calculated index of dispersion is from cup averages. The percent estimate shows the estimated proportion of eggs deposited per oviposition cup to achieve the calculated I.D.

The index of dispersion provides a sensitive single variable for statistical analysis adaptable for the multiple permutations of skip-oviposition studies in which individual mosquitoes aggregate or spread eggs during a single gonotrophic cycle. Previous studies used occupied cups to assess skip-oviposition differences (Trexler et al. 1998, Santos de Abreu et al. 2015, Swan et al. 2018). As the *I.D.* statistic is sensitive to egg distribution differences, it is well suited for comparative analysis between species and oviposition treatments, and should be used in any future comparative studies. Additionally, the index of dispersion could potentially assist with the development of a skip-oviposition behavior predictive model.

This study provides insight into the component mechanisms of skip-oviposition between container *Aedes* species mosquitoes. When a gravid female mosquito reaches the first oviposition site, she assesses the site and proceeds to lay no eggs, all of her eggs or a proportion of eggs (Day 2016). In *Ae. aegypti* and *Ae. albopictus*, only a small percentage from this study (9% and 5.7%, respectively) aggregate all their eggs into a single container. Chadee et al. (1990) found a similar result in *Ae. aegypti* females reporting 10% aggregated all eggs in one container with 200 ml nutritionally unenriched water. In our study, a significant percentage (32%) of *Ae. triseriatus* aggregated all eggs in the same container. Trexler et al. (1998) found a similar result, with 70% of tested *Ae. triseriatus* occupying 1 or 2 oviposition sites. These data suggest individual container *Aedes* mosquitoes lay an average proportion of eggs per oviposition event based on her current egg total (Table 2). It is unknown what mechanism allows the mosquito to ‘feel’ done and move on to another site. Another relatively unknown factor is how many skip-oviposition events occur during a normal gonotrophic cycle. The results suggest *Ae. aegypti* and potentially *Ae. albopictus* skip-oviposit at a higher rate than measured due to high *I.D.* distribution, which is toward the maximum of occupying all eight containers available (Fig. 1). Previous studies support this assessment as cup occupation increases in response to increases in cup number, but few studies have utilized more than eight cups in a test arena (Santos de Abreu et al. 2015, Swan et al. 2018). An eight-cup oviposition test arena may not be enough sites to accommodate egg spreading of *Ae. aegypti*; therefore, one could potentially identify a measurable difference in skip-oviposition between *Ae. aegypti* and *Ae. albopictus* by increasing the number of cups in oviposition assessments.

One complication is the potential for cup re-visitation during skip-oviposition assessments. Chadee et al. (1990) found *Ae. aegypti* avoided ovipositing into containers that received eggs from the first gonotrophic cycle during her second gonotrophic cycle. It is relatively unknown in a natural environment if individual mosquitoes visit the same container multiple times during a gonotrophic cycle. We found that re-visitation does occur in a preliminary skip-oviposition assessment using sensor-equipped oviposition cups. In this assessment, a single gravid *Ae. albopictus* visited two sites eight times, moving back and forth between cups (unpublished observation). Oviposition re-visitation remains an open question, as this proof-of-concept test was unable to confirm egg laying occurred during any given visitation, as the sensors only counted the mosquito’s movement into and out of the equipped cups.

It is relatively unknown how container *Aedes* spp. distribute eggs among multiple containers. Does the mosquito oviposit in an ordered manner contributing eggs to containers from high amounts to low amounts, or is the behavior more disorganized? Previous studies suggest a ‘favorite’ oviposition cup that *Aedes* species contribute a higher proportion of their eggs (Oliva et al. 2014, Davis et al. 2015, Santos de Abreu et al. 2015). If we assume, a ‘favorite’ cup represents a mosquito’s initial oviposition site, the next cup should then contain

the next most numerous egg total. An oviposition pattern is revealed by grouping oviposition cups by organized egg totals from highest to lowest (Table 2). When organized using this method, we found a specific skip-oviposition pattern, which suggests each species contributes a proportion of their eggs depending on habitat ‘quality’ (Table 2). As the gonotrophic cycle continues, this mosquito visits more oviposition sites with fewer and fewer eggs to contribute to provide to any particular container. Previously, this method suggested *Ae. aegypti* have a specific skip-oviposition pattern showing a ‘favorite’ cup and an order to the subsequent egg contributions (Oliva et al. 2014). In identical oviposition habitat conditions as presented in this study, the proportion of eggs laid per skip-oviposition event is assumed to be consistent. The assumptions of the ‘favorite’ cup as initial oviposition site and proportional egg contributions based on habitat ‘quality’ in subsequent oviposition events provide structure to observed oviposition patterns in *Ae. aegypti*, *Ae. albopictus*, and *Ae. triseriatus*. We note that this insight remains an assumption without supporting data from a direct observational skip-oviposition study. Further observational studies are required assess a specific pattern to skip-oviposition behavior in container *Aedes* spp.

This study provides additional insight into skip-oviposition behavior, unearthing aspects of this behavior that are not well understood. Future studies should include direct observation technology to document each skip-oviposition event accurately. We believe current technology is sufficient to directly observe skip-oviposition behavior by creating assessment arenas with camera-equipped oviposition. A study directly observing skip-oviposition has yet to be conducted due to the technological difficulties of developing and testing such a design. To assess the extent of this behavior in a controlled environment, we propose to assess other container *Aedes* species mosquitoes. For example, *Aedes japonicus* (Theobald) is a recent invasive mosquito, now found throughout the eastern United States, and the Australian *Aedes notoscriptus* (Skuse) has invaded California (Kampen and Werner 2014, Peterson and Campbell 2015). Continuation of skip-oviposition assessments furthers understanding of this behavior as an adaptive bet-hedging trait potentially assisting with the invasion of new habitats or urban/human dominated environments. Human activities produced container habitats favorable to recent successful invasions container mosquitoes (Lounibos 2002). Indeed, comparing invasive populations of these species to native populations in their ancestral range could provide evidence for the importance of skip-oviposition as a selected trait in invasions. Finally, future studies should account for container numbers when assessing skip-oviposition behavior. While logistically challenging, accurate assessments may require larger test arenas with higher numbers of oviposition sites; our data suggests a limitation in *Ae. aegypti* and *Ae. albopictus* skip-oviposition behavior due to an eight-cup oviposition arena.

Autodissemination traps take advantage of container *Aedes* species mosquitoes’ propensity to oviposit in multiple containers (Chism and Apperson 2003, Devine et al. 2009). Traps such as the In2Care coat an ovipositing mosquito with larvicide, i.e., pyriproxyfen (Buckner et al. 2017). Treated mosquitoes then distribute larvicide to additional oviposition sites through skip-oviposition behavior, achieving mosquito control with repeated larvicide distribution to previously untreated larval habitat. This method allows for larvae control in cryptic oviposition sites, typically not easy to find or treat. Skip-oviposition studies, such as ours, provide insight into an individual’s behavior as they interact with a multiple oviposition site environment. These data emphasize the need to understand species-specific behaviors in targeting control methods. Our results indicate autodissemination

traps would not be as effective on *Ae. triseriatus* control compared to *Ae. aegypti* or *Ae. albopictus*. Furthermore, our data suggest it is possible to develop a species-specific predictive model for trap placement in a generalized setting, potentially enhancing control strategies in using this technology for container mosquito control. Our study also suggests oviposition media plays a vital role in the number of eggs spread among containers, with a low-quality environment facilitating the spreading of more eggs. Autodissemination trap oviposition media quality should be further assessed as the goal is twofold: be the first container visited during a mosquito's gonotrophic cycle and limit the number of eggs laid in the trap. A combination of these traits would facilitate the maximum effectiveness of this control method. Overall, a more in-depth understanding of the species propensity to skip-oviposition will enhance the autodissemination trap's effectiveness and broaden this emerging control technology's usefulness.

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