

VERTICAL OVIPOSITION PREFERENCES OF THE ASIAN TIGER MOSQUITO,
Aedes albopictus (DIPTERA: CULICIDAE), IN TEMPERATE NORTH
AMERICA

by

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ABSTRACT OF THE THESIS

Vertical oviposition preferences of the Asian tiger mosquito, *Aedes albopictus* (Diptera: Culicidae), in temperate North America

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As a hyper-aggressive mosquito that is also a public health threat *Aedes albopictus* (Skuse) (Diptera: Culicidae), the Asian tiger mosquito, is a major priority for control efforts. We examine one aspect of *Ae. albopictus* biology: oviposition height. Field-based research in an urban habitat was conducted to determine if a height preference exists for this species. Larval and egg counts showed a significant preference for oviposition at ground level (0 m) heights compared to heights of 1, 2, 3, or 4 m ($P < 0.01$). An experiment conducted under semi-field conditions further supported our conclusion of oviposition preference at ground level ($P < 0.001$), and further defines the search image needed by mosquito control personnel when dealing with this invasive species.

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INTRODUCTION

In New Jersey and at Rutgers mosquitoes have been under study for well over 100 years, starting with the work of John B. Smith in the early 1900's and continuing on until today. Organized mosquito control originated in New Jersey in 1912 and has continued since working to efficiently control these insects that can be dangers to human health. As such, mosquito biology has been studied extensively both locally and around the globe. One aspect of mosquito biology that has been heavily studied is oviposition habits and how those behaviors influence other aspects of species biology.

Mosquito species show a remarkable range of oviposition behaviors, from generalist to specialist. Generalist behaviors can range from adult females laying eggs at or near the water line in moist soil, to specialist behaviors such as those shown by *Trichoproson digitatum* (Rondani). In this species, adult females lay their eggs on the surface of water in discarded fruit husks and hold the egg raft between their legs until larvae hatch to help protect them from predation (Lounibos and Machado-Allison 1987). Other mosquito species show a specialization in the type of habitat they will use, an extreme example being the genus *Deinocerites* in which all species utilize the burrows of land crabs to lay their eggs and complete their life cycle (Adams 1971). While these extreme behaviors are interesting biological curiosities, there are differences in oviposition between more typical species that effect their life cycle and importance.

In broad terms, oviposition behaviors can be broken down into four different types of behaviors. A specialist behavior is exhibited by mosquitoes in the tropical genera *Mansonia* and *Mansonioides* in which the female lays an egg raft that is then attached to vegetation below the water line (Lounibos and Linley 1987). Females may also lay their eggs singly directly on the water surface. Mosquitoes that utilize this behavior generally

will hover over the water while ovipositing and not directly land on the surface. A third more common behavior is exhibited by *Culex* along with other genera where females land on the water surface and lay eggs grouped together in an egg raft. And finally, females may lay their eggs singly at or near the water line on a moist substrate. This behavior is exhibited by many *Aedes* and *Psorophora* species.

Oviposition behavior exhibited by *Aedes* species requires specialized behaviors and egg structures that may not be present with other types of oviposition. Since these eggs are not laid directly on the water's surface, they must be desiccation resistant. This allows the eggs to dry at least partially without harming the developing embryo. In some species such as *Aedes vexans* (Meigen), a species that can be a significant nuisance in New Jersey, eggs have been shown to survive multiple years in large numbers (Gjullin and Yates 1946). This desiccation resistance allows the females to lay their eggs at the edge of large floodplains in great numbers where the eggs will survive until conditions are appropriate for hatching. Other species however, such as *Aedes japonicus japonicus* (Theobald) are not able to survive the same conditions. In nature, *Ae. j. japonicus* utilizes water filled containers as it's larval habitat, and lays eggs around the edge of the water. Williges et al (2008) found in colonizing this species that its eggs were prone to desiccation unless kept under moist conditions while the embryo developed. This restricts the potential oviposition sites that the species can use, and forces different behaviors in searching for a suitable site.

Oviposition site selection is a crucial component of the life history for all mosquitoes and a critical factor in the survival and population dynamics of a species. There are oviposition sites for mosquito species covering almost all possible aquatic

habitats, from large open floodplains to both natural and artificial containers. While many different species may utilize similar habitats types, within a species oviposition site selection stays consistent (Bentley and Day 1989). In our research, we examined one aspect of site selection and oviposition behavior; oviposition site height. Research on multiple species has shown height to be an important factor in choosing an oviposition site, and can lead to competition between different species in utilizing similar habitats (Sinsko and Grimstad 1977, Scholl and DeFoliart 1977).

The focus of this research project was the invasive mosquito species *Aedes albopictus* (Skuse). Native to Southeast Asia, *Ae. albopictus* has expanded its range throughout Africa, Europe, the Middle East, and North America (Benedict et al. 2007, Rochlin et al. 2013). In the United States, it was first discovered in Texas in 1985 (Sprenger and Wuithiranyagool 1986), but the species has since spread to 36 states (Enserink 2008). In New Jersey, the species was found in coastal Monmouth County in 1995 (Crans et al. 1996) and is now established in nearly all of the state (Farajollahi and Nelder 2009). As *Ae. albopictus* has expanded its range across the USA, populations have become associated with arboviruses such as Cache Valley, eastern equine encephalitis, Jamestown Canyon, LaCrosse, and West Nile (Armstrong et al. 2013). Along with these native arboviruses, *Ae. albopictus* is also a vector of exotic pathogens such as chikungunya, dengue, and yellow fever. Recent epidemics of chikungunya in the Indian Ocean region were driven by *Ae. albopictus* and caused nearly 250,000 human cases on La Reunion Island and more than a million probable cases in India (Bessaud et al. 2006, Chretien et al. 2007). These outbreaks demonstrate the effects on public health if local populations of *Ae. albopictus* become infected with an exotic arbovirus. Additional

research on the biology and ecology of this species is needed to improve the management of this invasive pest.

Researchers have examined the oviposition habits of *Ae. albopictus* and documented oviposition in both natural and artificial containers (Estrada-Franco and Craig 1995). Recently, Dieng et al. (2003) found container size to affect oviposition site selection; larger containers were more attractive oviposition habitats. Further research in Louisiana has shown oviposition at heights up to 7 m, but most eggs were found at ground level to 3 m (Schreiber et al. 1988). Amerisinghe and Alagod (1984) found *Ae. albopictus* oviposition at 3.5 and 6 m, though the species showed a preference to oviposit at ground level in Sri Lanka. In Singapore, Liew and Curtis (2004) showed *Ae. albopictus* is able move vertically through a 60 m high apartment building, ovipositing at all heights. This situation differs greatly from local habitats but does show *Ae. albopictus* with an ability to oviposit at great heights. Recent work in Florida showed *Ae. albopictus* preferred to oviposit at 1 m heights as opposed to 6 m in suburban habitats; however, no difference in oviposition height preferences was observed in sylvatic habitats (Obenauer et al. 2009). They attributed this variation in oviposition behavior to a potential decrease in habitat availability thus leading to more opportunistic oviposition behavior.

These studies mentioned above show similar oviposition preferences, but were conducted in tropical and subtropical climates within the range of *Ae. albopictus*. Nawrocki and Hawley (1987) estimated the range limit of *Ae. albopictus* to occur between 0°C and -5°C and Benedict et al. (2007) showed a similar range limit using an ecological niche model. In both studies, New Jersey is at or near the northern edge of this species proposed range and has a more temperate and variable climate than in areas

where prior research has occurred. Changes in temperature and precipitation affect populations of *Ae. albopictus* with cooler, wetter regions like New Jersey likely to have lower adult production (Alto and Juliano 2001). Competition from other species such as *Ae. j. japonicus* also affects oviposition site selection in New Jersey (Bartlett-Healy et al. 2012). As *Ae. albopictus* replaces *Aedes triseriatus* (Say) in habitats across New Jersey, *Ae. j. japonicus* has increased its abundance (Rochlin et al. 2012) and could affect the behavior of *Ae. albopictus*. Considering these observed differences in behaviors such as oviposition height and interspecies competition across the range of *Ae. albopictus*, it is important to examine all aspects of *Ae. albopictus* behavior to determine if this trait remains consistent at this northern edge.

Aedes albopictus uses a broad range of oviposition sites in both natural and artificial containers which makes control based on source reduction ineffective (Rochlin et al. 2013). To increase the efficacy of control measures, a clearer picture of the *Ae. albopictus* oviposition behavior is necessary. Focusing on how high oviposition occurs instead of habitat type or size will benefit surveillance and control measures by reducing the area that needs to be searched or treated during an inspection. Our research attempts to clarify *Ae. albopictus* vertical oviposition in the temperate climate of New Jersey and compares this behavior to observed preferences along its range.

MATERIALS AND METHODS

Between August and October of 2008 and July and October of 2009, a field study using two sites in Trenton, New Jersey, USA (40° 13' N, 74° 44' W) was conducted. The sites were selected based on *Ae. albopictus* abundance as observed during routine vector surveillance efforts and requests for service related to *Ae. albopictus* by the Mercer County mosquito control program. The sites were located in slightly wooded and shaded areas in an urbanized residential neighborhood and located approximately 300 m apart. Within each site, sample trees (*Quercus* and *Acer* spp) appropriately (30 m) tall were used for the study. These trees are the dominant species in our study site and were chosen based on accessibility and ease of use for sampling and not for reasons related to the species biology of *Ae. albopictus*. During 2008 ten trees were sampled, five in each site; however, 2009 sampling took place on seven trees total after three were destroyed during seasonal storms. Test oviposition habitats were constructed using black, 500 ml plastic containers affixed to the north-facing side of the main tree trunk (Steinly et al. 1991). Containers were placed at heights of 0, 1, 2, 3, and 4 m. All containers were placed in the field for two weeks before collections began and were filled with oviposition media consisting of 200 ml water, 1 g of oak leaves, and 1 ml of bacterial inoculum from previously existing *Ae. albopictus* habitats to increase their attractiveness. Holes were drilled in the oviposition containers above the 200 ml level to maintain consistent water levels and ensure oviposition substrate was available. Water levels were maintained twice a week as necessary.

Field collections were conducted using seed germination filter papers (Anchor Paper, St. Paul, MN, USA), placed in each container; 7 cm of paper was exposed above the water as an oviposition substrate. Egg papers were collected twice a week and larvae

were reared following the protocols in Nelder et al. (2010). Briefly, egg papers were submerged in 2 liters of dechlorinated tap water (1 paper per enamel tray) containing 0.15 g of lactalbumin/Brewer's yeast (1:1 ratio by mass), and eggs were allowed to hatch at 27°C under a 16:8 photoperiod. Egg papers with unhatched eggs were removed from trays after 24 hr to ensure larvae were of similar age. Larvae were fed finely ground Lab Diet© (Purina, St. Louis, MO) and allowed to develop to fourth instar before identification to species.

The number of eggs oviposited at each container height and the number of fourth instar larvae developing from each container were recorded. Using the number of eggs alone to determine results was confounded with multiple species potentially utilizing the same container type in our study site. Rearing larvae and identifying to the species level using definitive larval morphology restricted our data to *Ae. albopictus*. However, as hatch rates may differ depending on conditions experienced by eggs before hatch (Vitek and Livdahl 2006) larval identification alone was also insufficient. Results from both egg and larval populations were analyzed using Tukey's method of multiple comparisons. Egg and larval datasets were log transformed before analysis. Statistical analysis was done using the R software package (<http://www.R-project.org>).

An analysis was also performed to determine the effect of date (by week) on the number of eggs oviposited at each height to highlight any seasonal component to oviposition by *Ae. albopictus*. Each tree was treated as a separate replicate and the number of eggs were transformed at each height into a proportion oviposited per tree. If a tree was missing a sample from a given height, the tree was removed completely from this analysis. Using this method, data from 2008 created an incomplete dataset due to

missing containers, so only data from 2009 were used. After being transformed to proportions between 0 and 1, data was further transformed by an arcsin square root to normalize data before any statistical testing. A linear regression comparing the proportion of eggs laid at 0 m and total eggs laid by date was also performed using this same dataset.

Observations of behavior in the field were verified by an experiment conducted between August and September 2011. A portable insect cage 0.6 m wide by 0.6 m long by 4.5 m high was constructed and utilized for controlled oviposition at heights of 0 or 4 m. To obtain gravid *Ae. albopictus*, a temporary colony was established from eggs collected in Trenton during 2011 and reared as before. Adults were housed in 0.61 x 0.61 x 0.61 m cages with 10% sucrose solution on cotton wicks, for 4 days post-emergence before blood feeding to permit mating. Restrained guinea pigs were used to blood feed females (Rutgers University Animal Use Protocol #86-129) Adults remained in colony 48 hr post-feeding to allow for egg development.

The 4.5 m cage was placed into a wooded and shaded site in New Providence, NJ, and two oviposition containers lined with germination paper as before were set in the cage at ground level. Twenty gravid females were released into the cage and allowed 24 hours to oviposit before being removed along with both oviposition containers. The cage was immediately set up again using oviposition containers with germination paper at 4 m and another twenty gravid females were released and allowed 24 hours to oviposit before being removed. This process was repeated five times between August and September 2011, using 48 hr periods chosen to ensure similar weather conditions for each repetition. The total number of eggs collected by height was analyzed using the two sample t-test.

RESULTS

Sampling the two field sites resulted in the collection of a total of 43,213 eggs. Of the 15,605 larvae reared and identified, 99.92 % were *Ae. albopictus*. Other species identified were two *Ae.j. japonicus* and 13 *Ae. triseriatus* larvae. A significant effect of container height was found. Ground level oviposition habitats produced an average of 28.4 (n=134) larvae per container while 1, 2, 3, and 4 m containers had 16.3 (n=119), 12.8 (n=103), 11.8 (n=78), and 9.2 (n=81) larvae per container, respectively (Fig. 1a). The difference between ground level and all other heights was significant ($P < 0.01$).

Analysis of egg data also showed a significant effect of container height on the number of eggs. Ground level oviposition habitats produced an average of 98.0 (n=141) eggs whereas 1, 2, 3, and 4 m heights had 54.0 (n=149), 38.4 (n=139), 30.4 (n=134), and 23.9 (n=141) eggs respectively (Fig. 1b). The difference between ground level and all other heights was significant ($P < 0.01$).

Using egg collection date from 2009 as described earlier to determine the effect of date on oviposition preference showed the effect of container height was significant ($P < 0.001$), as was the effect of height by date ($P < 0.02$).

This 2009 dataset was also used in a linear regression comparing eggs oviposited at 0m and total eggs oviposited across the course of the season (Fig. 2). An effect of collection date was found on total eggs, with significantly less oviposition at the end of our sampling period ($P < 0.001$). We also found a significant effect on the proportion of oviposition at 0 m over time with a higher proportion of eggs at 0 m as the season progressed. A significant negative correlation was found between total eggs laid and eggs laid at 0m ($P < 0.05$, r^2 : 0.223).

Data from controlled-release experiments conducted in 2011 showed a strong preference for oviposition at 0 m with a significant difference between heights ($P < 0.001$). Oviposition papers from 0 m contained an average of 184.3 (n=10) eggs, whereas 4 m papers contained an average of 58.9 (n=10) eggs.

DISCUSSION

We attempted to clarify one area of species biology and behavior by examining the vertical oviposition preferences of *Ae. albopictus*. Prior research has shown they prefer ground level oviposition in some habitats (Obenauer et al. 2009); however, that study also examined oviposition infusions and the viability of ovitraps at differing heights in both urban and sylvatic habitats with several species of mosquito. By limiting our choices of habitat, infusion, and species, our aim was to further clarify oviposition height behavior in *Ae. albopictus* in our region. Using field populations, we have shown that *Ae. albopictus* prefers to oviposit low to the ground at the northern-most edge of its range.

This preference for ground level oviposition was shown in our New Jersey populations by examining the total number of eggs present at each height, and the total number of *Ae. albopictus* larvae reared from collected eggs. Both methods of analyzing our dataset were required due to the potential of mosquito eggs to hatch at different rates between species and within broods of the same species (Livdahl and Edgerly 1987). Analyzing egg and larval data allowed us to account for any difference in egg hatch rates between sites and between species to obtain a full picture of all species present at our sites. Our data show that this precaution was not necessary in our local habitat because *Ae. albopictus* is dominant and other container-inhabiting mosquito species are nearly absent. Another potential aspect of this field oviposition behavior relates to the time required to find a suitable site. It is possible that search time required to find a site is a driving factor in ground level oviposition and not height alone, however that was beyond the scope of this project.

Our research also examined oviposition height bias by providing gravid females an oviposition habitat at single heights. Removing the choice of height allowed us to

examine whether *Ae. albopictus* requires lower level oviposition habitats, or if it will readily utilize canopy height habitats if no others are available. The significant effect of height in the number of eggs oviposited that was observed in field experiments is strengthened by our observed effects in a semi-field environment. Whereas the effect observed was significant, from the standpoint of practical mosquito control and public health, significant oviposition still occurred at non-preferred heights. This suggests that although this species has a preference to oviposit at ground level heights, it is not a biological requirement.

Both results confirm previous research conducted on this species; however, prior work examined vertical oviposition at the same time as species competition for microhabitats (Obenauer et al. 2009, 2010). Our work expands on those ideas by removing any effects of interspecies competition, and by confirming the behavior in a different climate. Specifically earlier research took place in subtropical areas, whereas our studies were conducted in a temperate continental climate. As climate differences and change have been shown to affect pathogen and vector behavior (Gould and Higgs 2009) and were crucial in recent outbreaks of chikungunya transmitted by *Ae. albopictus* (Paupy et al. 2012), a complete understanding of *Ae. albopictus* biology at the northern edge of its range is necessary to optimally assess human risk.

We examined *Ae. albopictus* behavior across the mosquito season to determine if there is a change in oviposition height preference over time and as local populations approach diapause. Toma et al (2003) have shown changes in oviposition behavior and hatch rate correlated with change in temperature as the mosquito season progressed, which could lead to behavioral changes in our local area at the northern-most edge of the

species range. Any change in behavior could become an important factor for control personnel trying to reduce local populations. We confirmed an oviposition preference across time, with an increase in oviposition at ground level habitats near the termination of the mosquito season in temperate North America. This preference may be due to multiple factors including time of year and overall mosquito populations. We observed a significant correlation between the total number of eggs in a habitat, and eggs at ground level as well as a significant decrease in total eggs as the season progressed. Both of these factors could decrease the pressure on local populations to use less preferred heights as oviposition habitats especially at the termination of the mosquito season. This suggests that source reduction efforts by control personnel may be affected by both total mosquito populations and time of year when attempting to control this species. Future work is needed to determine whether seasonality and diapause or overall population is more important in determining oviposition height. This will allow more targeted control and source reduction measures.

Our observations may help control personnel to keep a consistent search image throughout the year while still effectively reducing local populations. A source reduction program undertaken to remove preferred habitats should result in greater use of non-preferred habitats, and therefore reduced fitness of the population. However, mosquitoes that use non-preferred height habitats could reduce the effectiveness of typical control measures targeted towards ground level. Research currently underway is refining methods of area-wide applications of larvicides such *Bacillus thuringienseis* variety *israelensis* and methoprene to reduce the impact of this potential behavior (Fonseca et al. 2013). If the larvicides are shown to effectively treat ground and canopy oviposition

habitats, concerns with source reduction programs changing the oviposition behaviors of *Ae. albopictus* may be reduced.

In the short term however, surveillance and control of this invasive mosquito will depend on inspections and treatments on a smaller scale by mosquito control professionals. Our research will allow inspectors to better identify potential larval habitats for *Ae. albopictus* and target treatments more effectively, to reduce the nuisance and public health issues caused by this hyper-aggressive species.

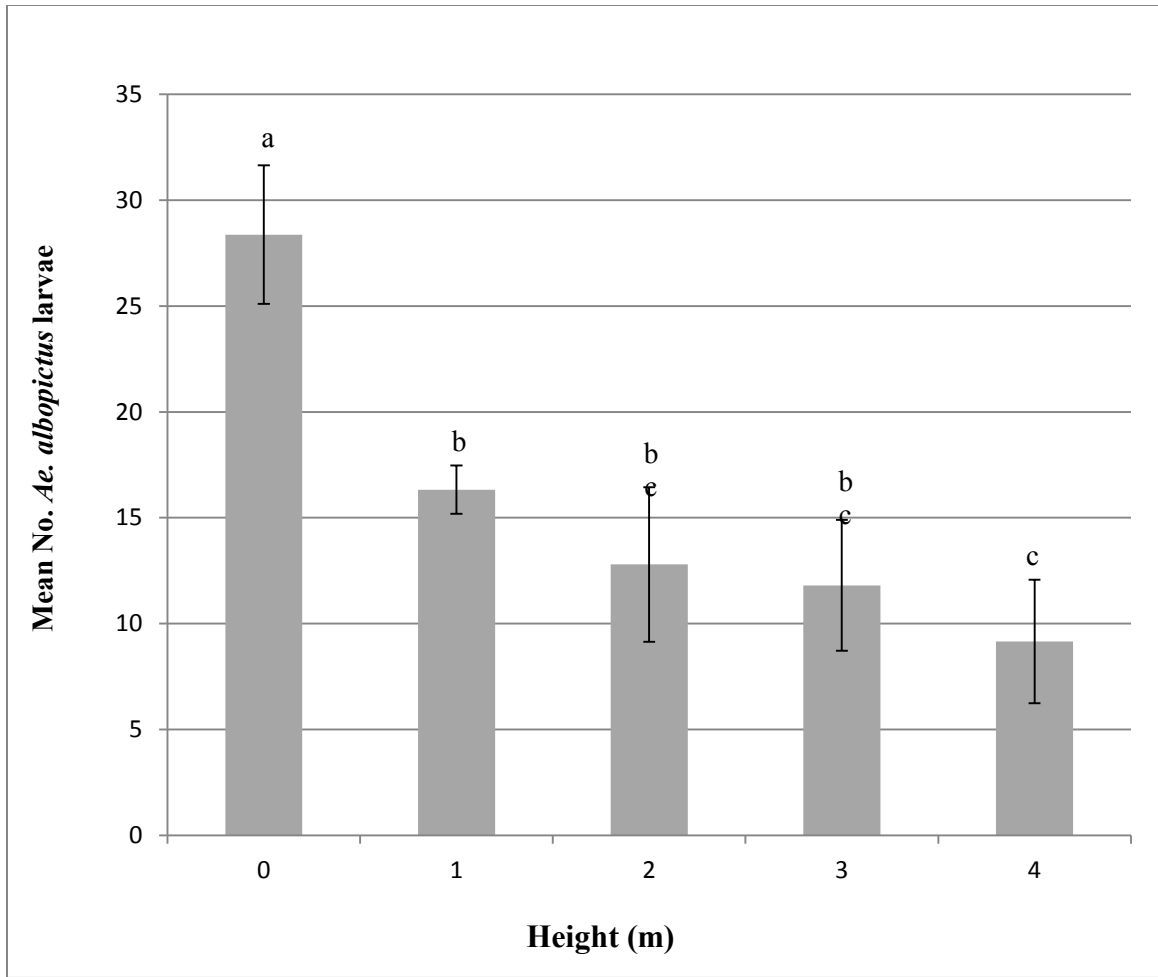


Figure 1a: Average number of *Ae. albopictus* larva collected from each container height under field conditions during 2008 and 2009. Letters above bars indicate statistical grouping ($\alpha = 0.05$).

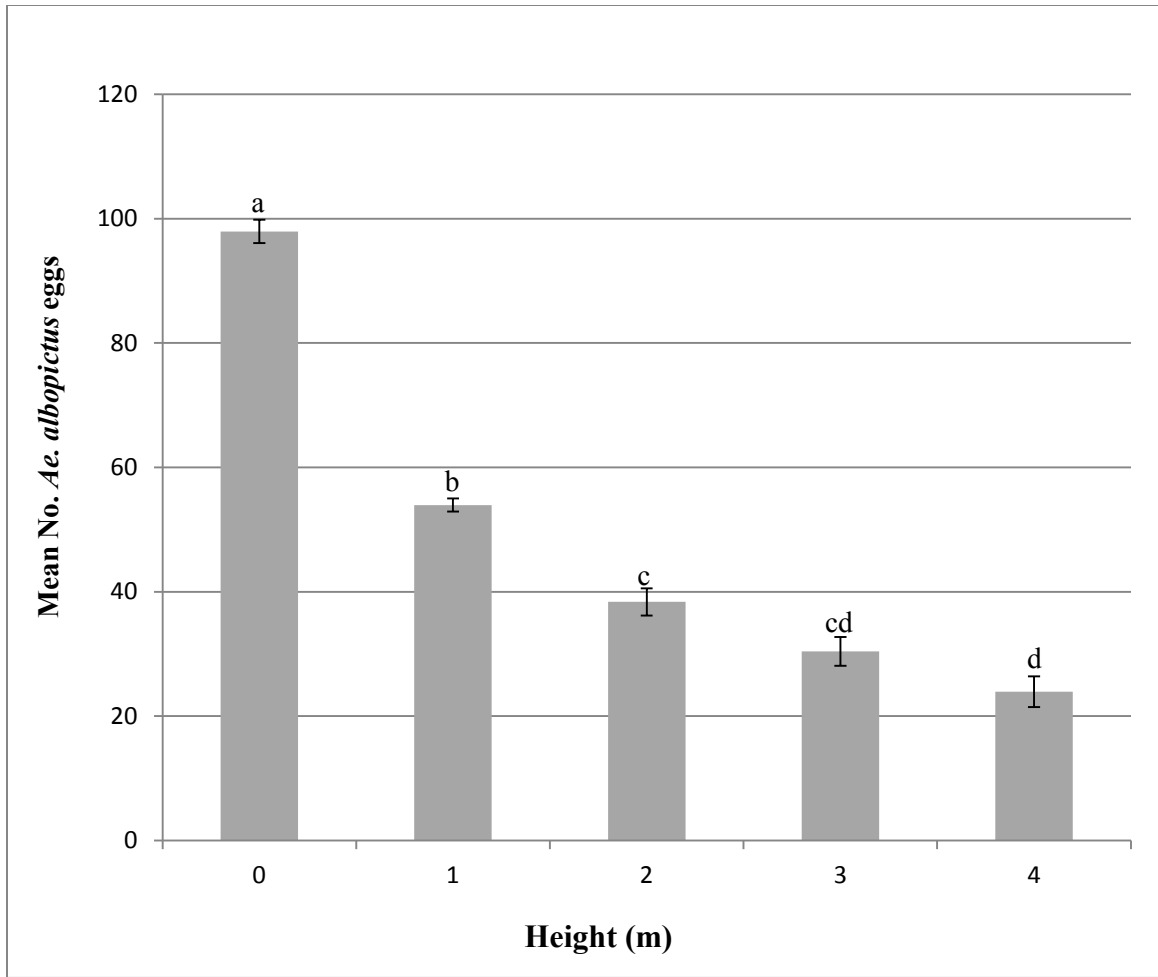


Figure 1b: Average number of eggs collected from each container height under field conditions during the summer of 2008 and 2009. Letters above bars indicate statistical grouping ($\alpha = 0.05$).

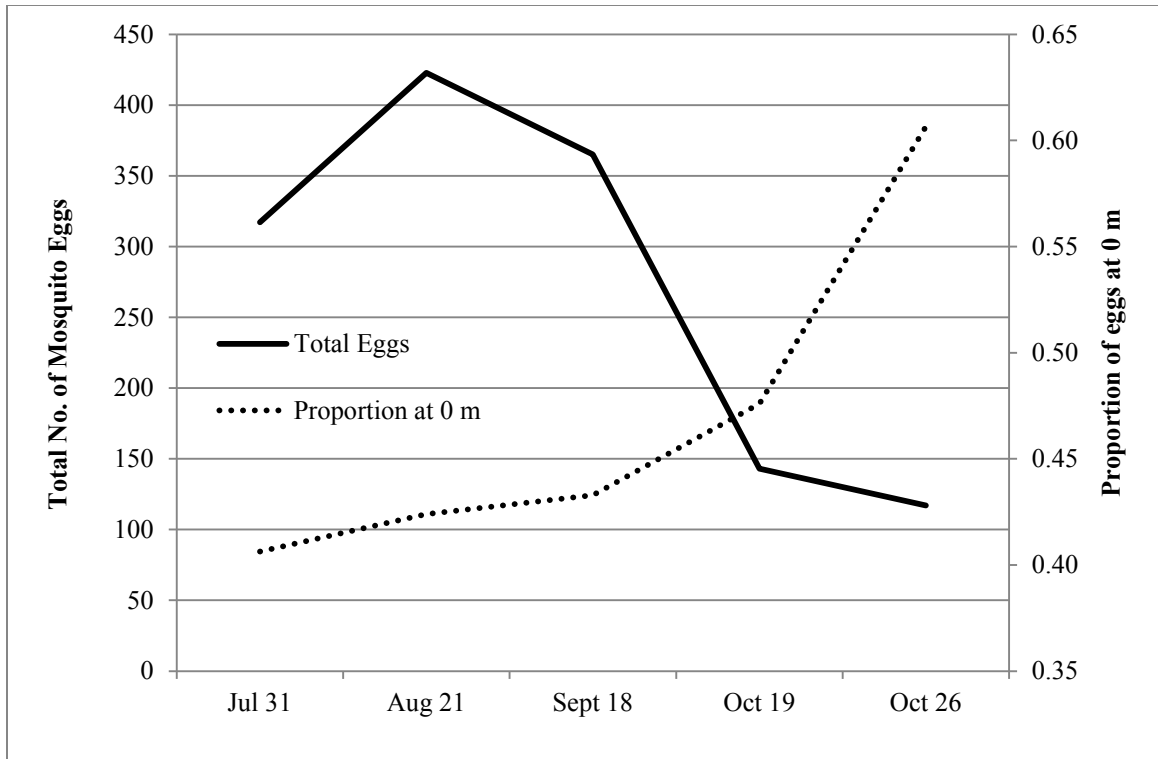


Figure 2: Proportion of eggs oviposited at 0 m compared to the total number of eggs during the summer of 2009.

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