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How does the dengue vector *Aedes albopictus* survive through the winter season in University of Agriculture Faisalabad, Pakistan?

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Abstract: *Mosquitoes are considered as an important vector of various infectious diseases affecting humans, thus having great concerns in public health. The abundance of vector population is very important in the surveillance of diseases in the disease-endemic region. Over 3500 species of mosquitoes have been reported, have huge number of breeding and hiding places. Among them, tree holes are one of the most important breeding places and some are effective for their management. An entomological survey was conducted to estimate the larval populations of Aedes albopictus Skuse (Diptera; Culicidae) in tree holes. A total of sixty trees with tree holes were identified at different sites of the university campus. Dry soil samples were*

extracted from selected tree holes using a metallic spatula and subsequently transferred into water jars. Among the sixty tree holes surveyed, seven yielded positive results, hosting a sum of 277 larvae. All larval populations were identified as Ae. albopictus. The percentage of larvae from under study trees includes Mangifera indica (42%), Syzygium cumini (37%), Albizia lebbeck (13%), and Alstonia scholaris (8%). The examination of the collected population from the dry soil revealed that the eggs of Ae. albopictus undergo overwintering the dry period and subsequently hatch with the onset of favorable weather conditions, such as late spring and the monsoon period. These insights gleaned from the studies may serve as a catalyst for policy makers and stakeholders in the formulation of comprehensive and efficacious strategies for integrated mosquitoes control strategies to safeguard public health effectively.

1. INTRODUCTION

Dengue, malaria, filariasis, Zika, and chikungunya are one of the known important mosquito-borne diseases transmitted by the bite of infected mosquitoes (CDC, 2020). Every year, more than 390 million individuals face the risk of dengue infection, contributing to a staggering global tally over 100 million recorded cases (Bhatt *et al*., 2013). Since its inception, Pakistan has been plagued by mosquito-borne diseases. The inaugural occurrence of dengue hemorrhagic fever (DHF) was reported in Karachi in 1994 (Chan *et al*., 1995) and the transmitting vector responsible for the outbreak was identified as *Ae. aegypti* (Suleman *et al*., 1996). Succeeding the next year, a few instances of dengue infection emerged from the southern region of Pakistan in 1995 and from 2005 onward, dengue outbreaks have been documented across all regions of the country (Rasheed *et al*., 2012). A sum of 4231 dengue cases was reported from 3 populous provinces (Khyber Pakhtunkhwa, Sindh, and Punjab) of Pakistan, during that period, the vector expanded its reach from southern to the northern regions of Pakistan. Similarly, 2416 cases were reported in 2007. Up until 2009, the annual dengue cases fluctuated between 2000-3000, However, in 2010, a dramatic surge occurred with a staggering 17500 reported cases. Subsequently, 2011 proved to be even more dire, with a total of 40,810 reported cases from three main provinces of Pakistan. Notably, Lahore, the nation's second largest city, bore the brunt with 17,493 cases, constituting 42% of the total dengue cases. Additionally, Faisalabad reported 1149 cases during the same period (Rasheed *et al*., 2012). Moreover, Pakistan experienced dengue outbreaks in the consecutive years of 2017, 2018, and 2019, with recorded cases standing at 22,938, 3,204, and 24,547 respectively, (NIH report, 2020).

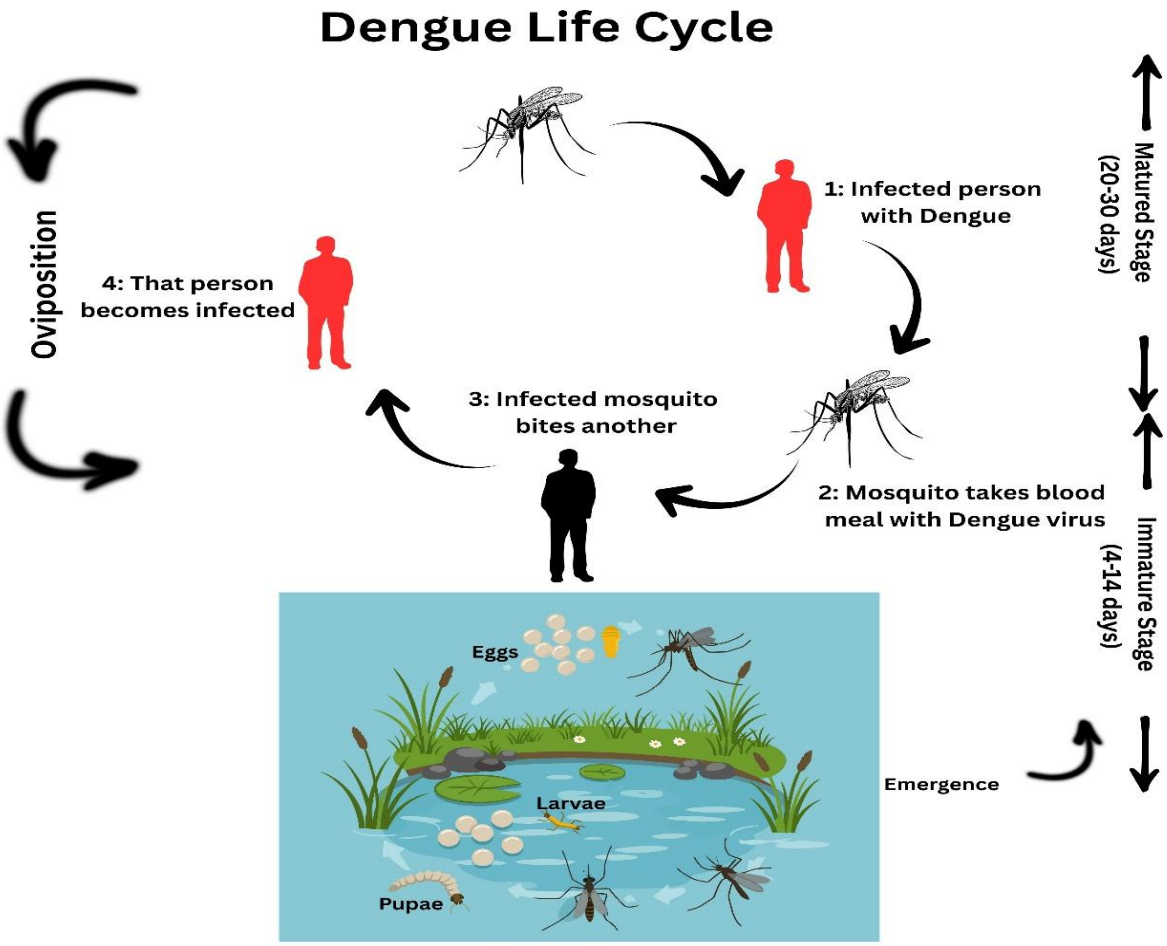
Tree holes being breeding sites for mosquito species are reported from Pakistan, Bangladesh, and India (Mehmood *et al*., 2017; Sultana *et al*., 2012; Selvan and Jebanesan, 2014). The aquatic environment of tree holes (phytotelmata) serves as a natural breeding ground for mosquitoes. The key phytotelmata includes three holes, pitcher plant, bamboo internodes, and axil water, are the rich sources of fauna (Kitching, 2000). Tree holes serve as a microhabitat, offering a natural setting conducive to the breeding of mosquitoes, particularly those belonging to the genus *Aedes* (Mangudo *et al*., 2015).

The ability of genus *Aedes* eggs to endure low temperatures inside the eggshell is referred to as cold hardiness, as discussed by Hanson (1991). The female mosquito of *Aedes albopictus* exhibit a preference for laying their eggs in a wide range of tree holes possessing suitable properties and observations have revealed the presence of eggs of *Aedes albopictus* collected from dry soil (Chowdhury *et al*., 2014). In addition to this, *Aedes albopictus* mosquitoes, found positive for dengue antigen, have been identified in tree holes (Joshi *et al*., 2006) this underscores the significance of tree holes as one of the most preferred breeding spots for the dengue vector. Given the scarcity of data on tree hole breeding mosquitoes in Pakistan, particularly in the city of Faisalabad, the present study was aimed to assess the mosquito population that persists in tree holes during the winter season. Understanding this population dynamics can provide insights and lead forward into the resurgence of mosquito populations in the upcoming seasons in endemic regions. For a public health perspective, this study will offer valuable insights into the population dynamics of dengue vectors, responsible for the transmission of associated diseases.

In the Punjab region of Pakistan, the dry winter season typically spans from mid-November and ended to mid-February. The monthly rainfall recorded over the last two winter seasons in Faisalabad shows 0.6 mm in November 2018, 0.7 mm in December 2018, 18 mm in January 2019, while 64 mm in February 2019, 5.3 mm in November 2019, and 0.7 mm in December 2019. Whereas 30.2 mm in January 2020 and 30.5 mm in February 2020. Consequently, a survey was conducted to investigate the overwintering of dengue vector during the dry winter season in the

Faisalabad, Pakistan. The lowest recorded temperatures during winter at University of Agriculture over the last three years were 4°C in 2017, 4°C in 2018, and 3.5°C in 2019.

Figure 1.1 Life Cycle of Dengue



2. Materials and Methods

Study Area A comprehensive entomological survey was undertaken within the premises of the University of Agriculture Faisalabad. Sample trees hosting tree holes were identified across various locations including the botanical garden, main road, D-ground, Horticulture fruit garden, football ground, agronomy garden and youngwala. (Table 1).

Table 1: Location, Tree species, Tree holes surveyed, positive tree holes and no of larvae in University of Agriculture Faisalabad, Pakistan

Location	Tree specie	No. of tree holes surveyed	No. of positive tree holes	No. of *Aedes albopictus* larvae
Main road	*Mangifera indica*	6	2	64
	Syginium cumin	5	1	26
	Alstonia scholaris	3	0	0
	Terminalia arjuna	1	0	0
Botanical garden	*Mangifera indica*	3	1	52
	Syginium cumin	4	0	0
	Alstonia scholaris	7	0	0
	Pterospermum acerifolium	1	0	0
	Tamarix indica	1	0	0
	Ficus religiosa	1	0	0
D-ground	*Syginuum cumin*	1	1	77
	Alstonia scholaris	2	0	0
	Terminalia arjuna	2	0	0
Yongwala	*Dalbergia sissoo*	5	0	0
	Vachellia nilotica	2	0	0
Football ground	*Mangifera indica*	4	0	0
	Syginium cumin	2	0	0
	Albizia lebbeck	1	1	36
Agronomy farm	*Alstonia scholaris*	3	1	22

Horticulture fruit garden	*Mangifera indica*	2	0	0
	Syginium cumin	3	0	0
	Morus alba	1	0	0

Table 2: Enlist positive tree species with associated physical characteristics of tree holes and no. of *Aedes albopictus* population.

Positive tree holes species	Diameter/Depth of tree holes (cm)	Height of tree holes (cm)	No of *Aedes albopictus* larvae
Mangifera indica	7/18	112	20
Mangifera indica	11/28	125	44
Mangifera indica	14/24	145	52
Syginium cumin	10/25	118	26
Syginium cumin	14/27	165	77
Alstonia scholaris	10/21	129	22
Albizia lebbeck	12/22	140	36
Total			277

Collection and Processing of Sample

In November, a comprehensive survey was conducted to assess all the 60 tree holes within the study area. The height range of tree holes varied, ranging from ground level to 7 feet. Upon inspection, it was observed that all the tree holes were devoid of water and contained mixture of decaying organic matter. Prior to sample collection, the inner walls of the tree holes were gently rubbed to maximize eggs retrieval. Dry soil accompanied by organic debris, was systematically collected from each tree hole using a metal spatula and carefully sealed in plastic bag for safe

transportation to Dengue Vector Research Laboratory, Department of Entomology, University of Agriculture Faisalabad.

2.1 Rearing and Identification of the Sample

The samples collected were transferred into plastic jars filled with tap water. These containers were then incubated in the laboratory under optimal conditions of temperature (26°C), and humidity (60%) for a period of 15 days. After 2-3 days, the mosquito eggs hatched, and the emerging larvae were carefully separated using a pipette. Each positive tree hole population was reared separately in a designated mosquito rearing cage for subsequent identification. To aid in species identification, slides were prepared using Hoyer's solution. The prepared slides were examined under a compound microscope, and the larvae were identified based on characteristic features described in taxonomic keys by Rueda (2004). The comb scales without subapical spines at eighth segment of abdomen are the identification of *Ae. Albopictus* species are at larval stage. Fig 2.1: microscopic image of abdomen of *Ae. Albopictus.* Further identification was also done at adult stage. Fig: 2.2 microscopic image of adults showed narrow median longitudinal white strips as described in Pictorial Keys for the identification of mosquitoes associated with Dengue Virus Transmission by Rueda (2004).

Figure 2.2 Microscopic and macro images of dengue vector *Aedes albopictus*

Table 2 results showed that tree holes diameter, depth and height greatly influenced the egg laying behavior of *Ae. albopictus*. During the survey, Ae. albopictus found utilizing smaller holes for oviposition (table 2), minimum 7cm to maximum 14cm diameter with different trees characteristics like height and depth of holes. The height of tree holes ranged from 112 cm to 165 cm.

The lowest abundance of Ae. albopictus eggs were collected at 4.5 m above ground. Traps at this elevation were more exposed and tended to lack foliage cover (Figure 2B) possibly resulting in lower levels of leaf-litter deposition and relative-humidity, factors known to adversely affect Ae. albopictus activity.

Table 3: Regression Summary

Multiple R	R square	Adjusted R	Standard Error
0.956017	0.913969	0.827939	8.405449

Table 3 shows the regression summary of tree holes characteristics and larvae of *Ae. albopictus.* The multiple R is a Pearson correlation coefficient value indicates the strong linear relationship between multiple factors like diameter, depth and height of tree holes with number of eggs laid by female mosquitoes. Table 4 showed the analysis of variance. Significance F value 0.041 is below

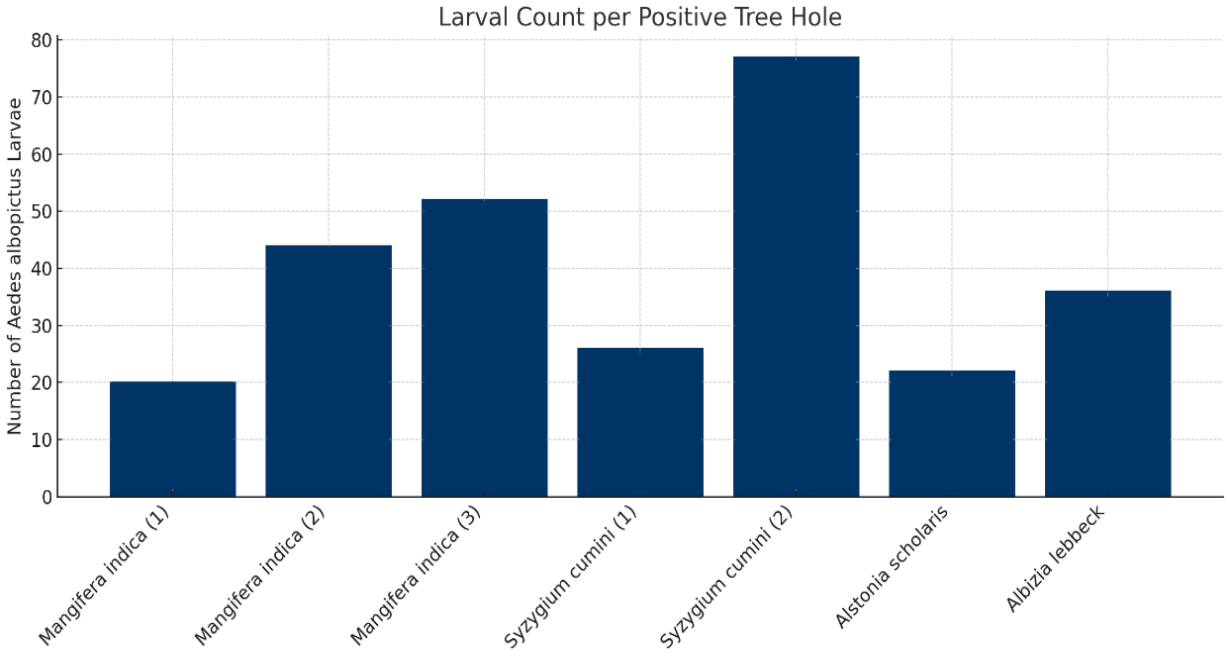
the alpha rejects the null hypothesis and highlights the significant relationship between number of eggs and physical attributes of tree holes.

Table 4: Analysis of Variance

ANOVA	D. F	SS	MS	F	Significance F
Regression	3	2251.76	750.5865	10.62378	0.041715
Residual	3	211.9547	70.65158		
Total	6	2463.714			

According to Chowdhury *et al*., (2014) reported that tree hole sizes have no influence on egg laying behavior of Aedes species as 8 tree holes size range 1-12.5 cm found positive with Ae. albopictus species. while 7 tree holes with size more than 65 cm found positive which means there are many other factors which affect the oviposition behavior of Ae. albopictus. A total of 1365 Aedes species larvae was reported from tree holes and 1096 were Ae. albopictus and 269 were other Aedes species and not even a single larva of Ae. aegypti was reported in their study. Muller *et al*., (2012) also studied that tree holes diameter size and depth ranges from 5-22 cm and 20-125 cm respectively found positive with Ae. albopictus.

3. RESULTS : The dry soil was collected from seven distinct locations within the University of Agriculture Faisalabad campus, and findings are presented in table 1. The findings



indicate that a total of seven tree holes tested positive for the presence of *Aedes albopictus* eggs. Specifically, three tree holes of *Mangifera indica*, two of *Syzygium cumini*, and one each of *Alstonia scholaris* and *Albizia lebbeck* were found to be positive. The corresponding larvae counts includes 116 no. of larvae from *Mangifera indica*, 103 from *syzygium cumini*, 36 from *Albizia lebbeck*, and 22 from *Alstonia scholaris*. Moreover, the distribution of larvae varied across different locations, with the percentage including 32% main road, 28% D- ground, 19% botanical garden, 13% football ground, and 8% agronomy garden. All the larvae emerged from dry soil, which was also enriched with decaying organic matter (Humus), providing an ideal environment for microbial growth. These eggs appeared to have been laid during recent rainy or monsoon season and subsequently entered a state of diapause due to the low temperatures as Lacour *et al*., (2015) suggested, initiating egg diapause at the right time is crucial for synchronizing development with seasonal circumstances. The highest incidence of diapause in the field occurred approximately one month before the minimum temperature dropped below 15°C.

Figure 1.1: Larva count per positive tree hole.

Hanson and Craig (1994) further substantiated that eggs of *Aedes albopictus* exhibit variable level of cold hardiness according to their geographical zones as temperate zone strain of *Ae. albopictus* demonstrate greater cold hardiness compared to central strains from Florida and tropical regions. Interestingly, tropical Asian strain and Brazilian strain of *Aedes albopictus* display comparable level of cold hardiness. Service (1965) investigated the ecological relationship between the tree hole breeding mosquitoes and their habitat, noting that eggs of *Aedes* species can endure the entirety of the dry season in a state of diapause. This observation aligns with the findings of present study, where eggs collected from tree holes were found within dry soil, providing further evidence of resilience of *Aedes* species eggs during dry periods.

All the larvae identified from dry soil of tree holes were confirmed to be *Aedes albopictus*, with no presence of *Aedes aegypti* detected. Given that *Aedes aegypti* is also a major vector and significant cause of dengue fever, this absence suggests a potential preference of *Aedes aegypti* to

avoid tree holes laying their eggs. This inference aligns with the observations made by the Barruad (1934) as documented as the author of the Fauna of British India: Ceylon and Burma.

Aedes albopictus, an urban mosquito species, predominantly inhibits areas surrounding human habitations. Its propensity for laying eggs in both natural and artificial containers not only possesses a nuisance through human biting but also elevates its potential as a vector for the transmission of arboviral diseases. Therefore, Female *Aedes albopictus* mosquitoes prefers to deposit their eggs in tree holes to ensure the continuity of their progeny. Among the various tree species, *Mangifera indica* emerges to be most favorite tree as breeding site for female mosquito as stated by Sathe and Jagtap (2009), who observed the maximum number of genera of *Aedes* mosquito preferred *Mangifera indica*. The prevalence of positive tree holes hosting *Aedes* population can be estimated based on our study's results, where approximately 12% surveyed tree holes were found positive with *Aedes* population as presented by Chowdhury *et al*., (2014), who reported 12% *Aedes* positive tree holes in the city of Dhaka Bangladesh.

Furthermore, the presence of *Ae. albopictus* showed that other species were more likely to also be present in tree holes during the rainy season, as reported by Sultana *et al*. (2012); Babar *et al*. (2021); Rasheed and Zaidi (2023). Therefore, it is expected that the eggs of other species could not resist the cold winter temperature. On the contrary, Kramer (2020) claimed that sub-zero and zero temperature exposure to eggs could not limit the viability of *Ae. albopictus* and *Ae. aegypti*. He reported that 3 adults of *Ae. aegypti* emerged after 6 days of exposure at -2°C, similarly to 1 adult of *Ae. albopictus* emerged after 1 day of exposure at -6°C.

4. DISCUSSION

Dengue fever and dengue hemorrhagic fever are the major health concerns to Pakistan since 2000. Due to lack of awareness about the breeding habitat it is spreading to all parts of the country and not only affects the lives of communities in big cities but also to small towns. The dengue vector could only survive at temperature above 20° C therefore it is impossible to persist the dry winter season in Pakistan. The winter season is from mid-November to mid-February and temperature ranges from 0°C to 24°C. Therefore, it overwinters the harsh cold in the form eggs in tree holes as this study indicates that tree holes are the possible diapause sites for *Ae. albopictus* eggs which is the main vector of dengue and chikungunya transmission in Pakistan. If this study

expands to the whole city, we could estimate the population of the dengue vector that overwinters in the holes of different trees and helps in the resurgence of mosquitoes in late spring and also this population provides a baseline for the proliferation of the vector and spreading of disease in the district. As no effective control strategy has been implemented for the management of tree hole breeding mosquito, this habitat can impede the progress of vector control measure programs.

As this study covered a limited area and a smaller number of trees, extensive research is required to better understand species breeding in tree holes and their management strategy. Further investigation is required to test the vertical transmission of the dengue virus through tree holes. It is necessary to practice integrated vector management practices (IVMP) at the University of Agriculture, Faisalabad, for better surveillance and management of these mosquito breeding sites.This scientific note will be very useful for vector control specialists and public health professionals to better understand and plan integrated mosquito control strategies.

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