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# A possible effect of the phenoloxidase level on developmental stages of *Aedes aegypti* (Diptera: Culicidae), a vector of dengue and chikungunya in Thar desert, India

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**ABSTRACT**

Phenoloxidase (PO) plays an important role in various physiological functions in insects, e.g., cuticular sclerotization, egg tanning, wound healing and immune response against pathogens and parasites. This study reports on the PO activity in laboratory reared and field collected mosquitoes *Aedes aegypti*, showing its decline from the larval to pupal and adult stages. The field collected specimens had a significantly higher PO level than their laboratory reared counterparts. The PO activity in the field collected larvae was found to be higher than in the corresponding laboratory reared specimens. The rate of pupation among the field collected mosquitoes was significantly higher than among the laboratory reared mosquitoes at different holding periods. Similarly, the PO activity in the field collected pupae was higher than in the laboratory reared ones. A higher rate of adult emergence was recorded in the field collected material at 24h and 48h of holding. Therefore, a positive correlation between the production and regulation of PO and the rate of molting has been suggested.

KEYWORDS: *Aedes aegypti*, mosquitoes, phenoloxidase, larvae, molting, pupation.

## INTRODUCTION

Dengue virus is responsible for the onset of dengue fever, which can be exacerbated by development of Dengue Hemorrhagic Fever and Dengue Shock Syndrome. These conditions pose a global health problem, with approximately 3.9 billion people being at risk of contracting the disease. It is estimated that 285–528 million cases occur all over the globe every year (WHO 2015). The disease prevails throughout the tropical and subtropical zones and is endemic to South-East Asia, the Pacific, East and West Africa, the Caribbean and the Americas. India witnessed her first dengue outbreak in 1996 in Delhi, followed by regular surges of cases every year (Rathore *et al.* 2017). Dengue fever is one of the most deadly and difficult-to-treat tropical diseases in Rajasthan including the desert part of India. The Thar desert is situated in the Rajasthan State and dengue fever is endemic to the region (Rathore *et al.* 2017). The principal vector of dengue fever in India is mosquito *Aedes aegypti* (Linnaeus, 1762) (Diptera: Culicidae).

The outbreak of chikungunya, a disease caused by the chikungunya virus was first recorded in 1963 in Kolkata (Sarkar 1966). The virus emerged again in 2006 after a gap of 32 years and caused an explosive outbreak affecting 13 states in the country including Rajasthan (Cecilia 2014). The primary vector of the chikungunya

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virus in India is also *Ae. aegypti*, which is abundant in India (Kalra *et al.* 1997), including the desert part of Rajasthan (Tyagi & Hiriyan 2004).

Reduction of the vector population is currently the only way to prevent dengue and chikungunya in the absence of a commercially available vaccine or effective clinical treatment. Fluctuations of the vector population density depend on the biology of the vector and are influenced by climatic conditions. The biology and life cycle of the vector need to be thoroughly studied before any control strategy is applied. During their development and growing, mosquitoes undergo periodic molting (Chang 1993; Reynolds & Samuels 1996). This process includes activation of dermal cells, epidermal cells separation and secretion of molting fluids. Besides, the enzymes, proteases and chitinases produced by epidermal cells during molting period are deposited in the molting fluid in between the old cuticle and the epidermis (Dziadik-Turner *et al.* 1981; Samuels & Reynolds 1993; Samuels & Paterson 1995; Reynolds & Samuels 1996). This process entails the complete replacement of the entire exoskeleton of immatures, including digestion, resorption and recycling of the inner, more pliable layers and shedding off the outer one. During several hours after molting, the dermal cells secret and transport the phenoloxidase to epidermal layers. Finally, phenoloxidase participates in the oxidation of phenolic compounds to produce quinones (Cerenius & Soderhall 2004).

The objective of the present study was to establish the level of the phenoloxidase activity in different stages of the laboratory reared and field collected *Ae. aegypti*. The study was also aimed to establish whether the level of the phenoloxidase activity has any correlation with the molting process and the life cycle of *Ae. aegypti*.

## MATERIALS AND METHODS

### Sources, collection and rearing of field-collected *Aedes aegypti*

Eggs, larvae and pupae were collected from breeding sites in the Bamba Mohalla area near the Jodhpur City. The breeding sites were represented mainly by cemented tanks. Immatures of *Ae. aegypti* were collected with dippers, along with the water and substrate. Field-collected larvae and pupae were transported to the laboratory in ambient condition for rearing and further experiments. Larvae were reared in enamel trays filled with source water and substrate at an initial density of 200 individuals per tray in semi-natural condition. Their diet constituted of food particles in water, as well as on the substrate. No additional nutrients were added to the containers with the field collected larval populations. Immatures were grown up to adults in 0.6 $m^3$ cages as described by Ansari *et al.* (2000).

### Colonization of laboratory reared *Aedes aegypti*

The laboratory reared *Ae. aegypti* colony used in this study originated from mosquitoes collected from Bamba Mohalla, Jodhpur. The immature stages of *Ae. aegypti* were collected mostly from unused cemented water containers. The F-1 progeny of the field collected mosquitoes were used for the maintenance of further

generations. The colony was established in 2009. In the insectary, adult *Ae. aegypti* colony was maintained at a photoperiod of 10:14 (light/dark) hours, temperature 28 ± 1 °C and relative humidity 75 ± 5 %. Adults were allowed to mate freely in cages (0.6 $m^3$). Larvae were provided a mixture of yeast powder and dog biscuit (1:2) as food at 24-h intervals. Females were given blood meal at weekly intervals and were also supplied with 10 % glucose solution (Ansari *et al.* 2000). Soaked raisins and 10 % glucose solution were provided to males. The adults were kept in the same cages for successive generations.

**Survival assay**

The rate of pupation, pupal survival and adult emergence was calculated for the laboratory reared and field collected larvae. Groups of 50 larvae of each type were kept in enamel bowls at the same conditions as above and were examined at 24-h intervals. Dead larvae and pupae were removed before counting. The number of pupae developed at 24-h intervals was tallied. The experiments were run in triplicates. For the evaluation of the adult emergence percentage, batches of 50 pupae were put in separate cages. Adults emerged on each day were counted. Un-emerged pupae were discarded after day four. The rate of pupation was calculated as follows:

$$\text{Rate of pupation on day } x = \frac{\text{Number of pupae formed on day } x \times 100}{\text{Total number of live larvae on day 5}}$$

**Phenoloxidase assay**

Phenoloxidase is known to initiate hydroxylation of monophenols (tyrosin) to diphenol (dihydroxyphenylalanine [DOPA]) compound. The DOPA compound is then readily oxidized by phenoloxidase to their respective quinine (dopaquinone, dopaminoquinone), which spontaneously converts to dopachrome. The production of dopachrome was estimated as increase in optical density of reaction mixture at 420 nm wavelength by the modified method of Cornet *et al.* (2009).

The phenoloxidase activity was measured quantitatively at different developmental stages of mosquitoes. Homogenates of larvae, pupae and 3-day old adults were used to assess the phenoloxidase activity. Each larva, pupa and adult were put separately into a new 1.5 ml micro-centrifuge tube with 30 µl chilled grinding buffer (0.112 M Tris-acetate buffer, pH 7.0). The samples were homogenized using sterilized plastic grinder and centrifuged at 15,000 rpm in a refrigerated centrifuge machine (Prime R Biofuge, Heareus) for 15 min at 2 °C. The supernatant was taken out by pipetting and kept in 1.5 ml fresh and labeled micro-centrifuge tubes, and the residue was discarded along with the tube.

A 10 µl portion of the supernatant was used for the protein estimation and rest was kept for the phenoloxidse assay. A 15 µl sample homogenate of each type was mixed with 500 µl of substrate buffer (200 mM tyrosin in 50 mM sodium phosphate buffer, pH 6.8) in a 1.5 ml micro-centrifuge tube. The reaction mixture was

briefly spinned (Eppendorf, Mixmate) to settle down drops of the reaction mixture from the wall of the tube and incubated in water bath (Yamato-300) for 2–2.5 hrs at 30 °C in darkness.

Once incubation was over, 250 µl of the incubated reaction mixture was transferred to a round bottom 96 well micro titer plate and the production of dopachrome, i.e. the phenoloxidase activity, was read at 420 nm wavelength on ELISA Reader (Microscan-MS5608A).

**Statistics**

Mean and standard deviations and differences among mean values were determined by *t* test at P=0.05 using the MS Excel software.

## RESULTS

The first analysis of the phenoloxidase (PO) activity was made for the laboratory reared mosquitoes. The PO activity per 1 µl homogenate of the 4$^{th}$ stage larva after 24-hour incubation was 3.96, 5.64 and 6.42 higher than those of the pupae, female and male mosquitoes respectively (Tables 1–3). The PO activity subsequently declined nearly two-fold in pupae and four-fold in adults compared to the 4$^{th}$ stage larva (Table 2). Females exhibited a higher PO activity compared to males (Table 3).

The second analysis of the PO activity was made for the field collected mosquitoes. In the 4$^{th}$ stage larvae after 24 hours of incubation, the PO activity per 1 µl of homogenate was 5.58, 7.97 and 9.36 higher than in the pupae, females and males respectively (Tables 1, 2, 3). Females showed a higher level of the PO activity than males. When comparing the PO activity between the laboratory reared and field collected mosquitoes, the latter had significantly higher (*t* test, P=0.05) PO activity than the former. The PO activity per 1 µl homogenate (pooled activity) in the field collected larvae was 1.42 times higher than in their laboratory reared counterparts. Similarly, the PO activity in the field collected pupae (pooled activity) was 1.48

**Table 1.** The level of phenoloxidase per 1 µl homogenate in larvae of laboratory reared and field collected mosquitoes.

| Hours after 4$^{th}$ stage | Laboratory reared | Field collected |
|---|---|---|
| 24 | 7.50±1.26 | 10.68±2.43 |
| 48 | 7.24±1.35 | 10.34±1.26 |
| 72 | 6.73±1.05 | 9.42±1.41 |
| 96 | 6.17±0.23 | 8.98±0.75 |
| 120 | 5.81±0.74 | 8.36±0.62 |

**Table 2.** The level of phenoloxidase per 1 µl homogenate in pupae of laboratory reared and field collected mosquitoes.

| Hours after pupation | Laboratory reared | Field collected |
|---|---|---|
| 24 | 3.54±0.42 | 5.10±0.77 |
| 48 | 2.61±0.71 | 3.82±0.15 |
| 72 | 1.93±0.28 | 3.09±0.28 |

**Table 3.** The level of phenoloxidase per 1 µl homogenate in adults of laboratory reared and field collected mosquitoes.

| Sex | Laboratory reared | Field collected |
|---|---|---|
| Female | 1.86±0.45 | 2.71±0.38 |
| Male | 1.08±0.14 | 1.32±0.06 |

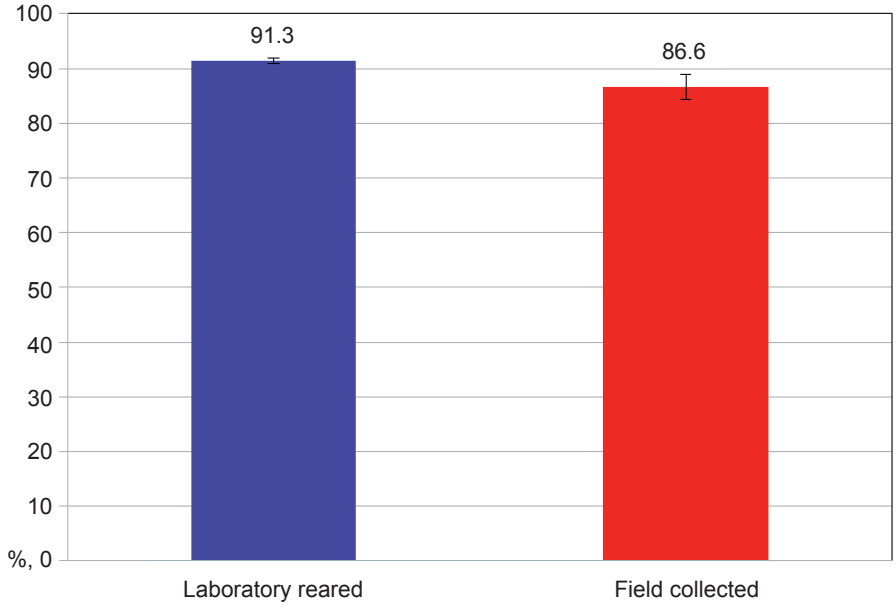


**Fig. 1:** Percentage of the 4th stage *Ae. aegypti* larvae pupated after 5 days of retention.

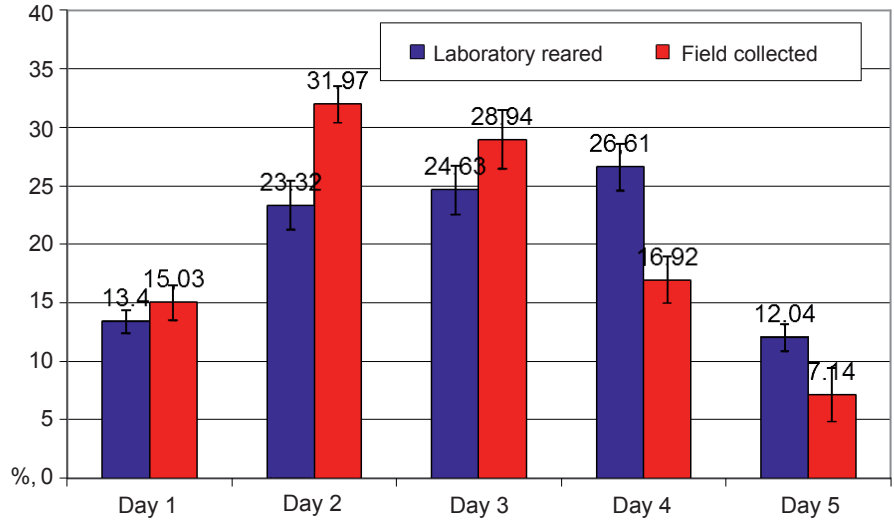


**Fig. 2:** Daily rate of pupation of the 4th stage *Ae. aegypti* larvae.

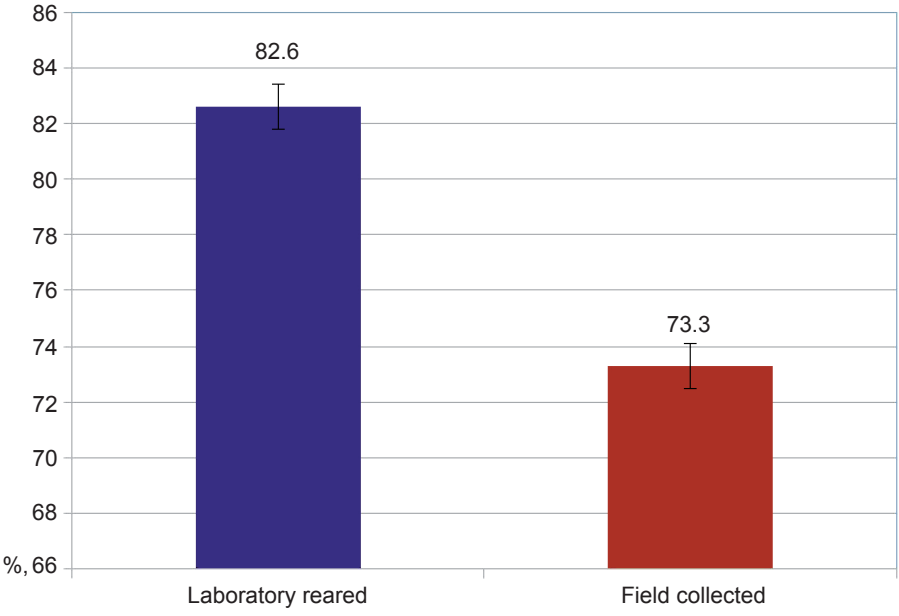


**Fig. 3:** Total percentage of emerged adults from laboratory reared and field collected pupae.

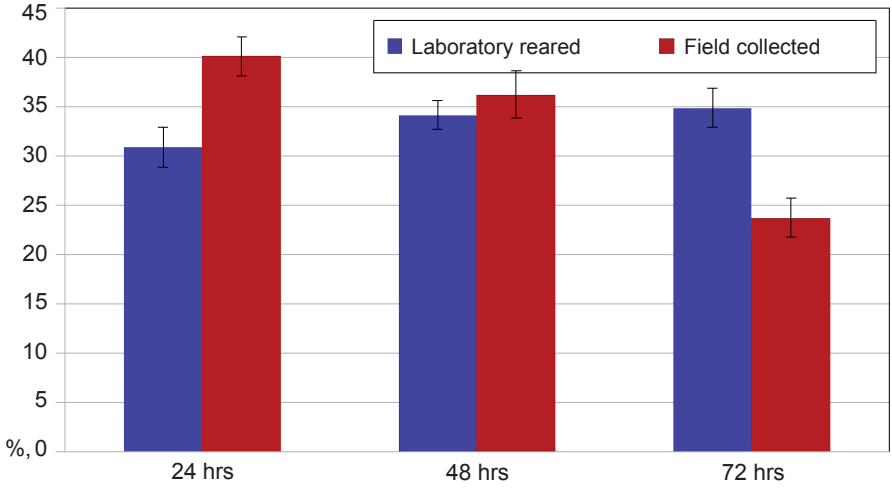


**Fig. 4:** Rate of emergence of *Ae. aegypti* adults after 24, 48 and 72 hours.

times higher compared to that in the laboratory reared ones. The PO activity (at 24 hours) in field collected larvae, pupae, females and males were 1.42, 1.44, 1.45, 1.22 times higher than in the laboratory reared mosquitoes. The PO activity in larvae of both types was significantly (*t* test, P=0.05) higher than in all other stages.

A total of 86.6±4.7% of the field collected larvae pupated, whereas 91.3±2.4% of larvae pupated among the laboratory reared mosquitoes (Fig. 1). However, the rate of pupation of the field collected larvae were significantly (*t* test, P=0.05) higher than that of the laboratory reared larvae at 24 h, 48 h and 72 h. The rate of pupation in the laboratory reared larvae was higher at 96 h and 120 h of holding (Fig. 2). A total of 82.6±0.57% pupae originated from the laboratory reared mosquitoes emerged as adults, whereas among the field collected mosquitoes 73.3±0.5% pupae emerged as adults (Fig. 3). A higher rate of adult emergence was recorded in the field collected pupae at 24 and 48 hours (Fig. 4).

## DISCUSSION

In arthropods, phenoloxidase plays an important role in cuticular sclerotization and in defense against pathogens and parasites (Jiang *et al.* 1998; Hillyer & Christensen 2005). This enzyme is also vital for tanning and melanization of the epidermal layer. The results of the present study suggest that phenoloxidase is linked to the development of the mosquitoes and play a cohesive role in molting. Phenoloxidase enhances the molting process in mosquitoes, which results in a higher rate of pupation and adult emergence recorded in the present study. The level of phenoloxidase was higher in the field collected larvae and 75.94% of them

pupated within 72 hours, whereas, only 61.36 % of the laboratory reared larvae turned into pupate. A seemingly higher number of pupated larvae in the laboratory reared group at 96 h and 120 h is due to the decreased number of the field collected larvae. Similarly, the phenoloxidase level was higher in the field collected pupae and 76.25 % of them, compared to 65.01 % of laboratory reared ones, emerged as adults in 48 hours. Wang *et al.* (2013) observed that polyphenoloxidases belong to the group of the key enzymes in molting, which are closely related to ecdysone; the expression of phenoloxidase in silkworm was higher during molting stage and decreased once the process completed. Likewise in the present investigation, the phenoloxidase activity decreased in each subsequent developmental stage. The percentage of individuals that underwent molting was higher in the laboratory reared mosquitoes, which could be explained by controlled conditions of rearing with no possible infestation. Cornet *et al.* (2013) reported that the level of active phenoloxidase in females was significantly higher than in males. The female reproductive life is made up of lengthy gonotrophic cycles, each consisting of a period of host seeking, followed by blood feeding, egg maturation and oviposition. For females, their longevity depends on the level of their immune response and ability to maintain their reproductive fitness. Whereas males feed exclusively on nectar (Clements 2000), which is mostly bacterial and fungi free (Nepi *et al*. 2009), females are exposed to a wide array of pathogens that include viruses (West Nile and encephalitis), protozoan (*Plasmodium*, *Trypanosoma*) and metazoan (*Wuchereria*) parasites. This could explain the difference in the phenoloxidase level between males and females, also observed in the present study.

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**Conflict of interest**

The authors do not have any conflicts of interest and all have equally contributed to this study and review.