

Study of egg laying pattern and hatching line formation of *Culicoides peregrinus* Kieffer and *C. oxystoma* Kieffer (Diptera, Ceratopogonidae), vector of bluetongue virus

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ABSTRACT: Gravid *Culicoides* females play an effective role in the propagation of the midge population which is ultimately associated with the outbreak of the disease. The high abundance of the vectors *Culicoides peregrinus* Kieffer and *C. oxystoma* Kieffer (Diptera, Ceratopogonidae) has been recorded in West Bengal, followed by *C. fulvus* Sen and Das Gupta, *C. innoxius* Sen and Das Gupta and *C. anophelis* Edwards. The habitat of these vector species was reported from semi-aquatic muddy areas mixed with dung, but the species-specific micro-habitat remains unknown. The gravid females influenced by various cues like physicochemical parameter of habitat (i.e., pH, salinity, bacterial influence) to assess suitable oviposition site to optimize survival of progeny. To avoid competition, gravid female can express different oviposition strategies. The study focused on the timing and differences in egg laying pattern with initiation of hatching line in both species in laboratory condition. Oviposited eggs and the hatched eggs were examined by scanning electron microscope (SEM) to observe the changes in exochorion. The results revealed that both species showed crepuscular oviposition activity but *C. peregrinus* laid eggs in cluster and *C. oxystoma* oviposited singly. The arrangement of ansulae on egg surface and hatching pattern of eggs in both species was different. The egg laying and hatching pattern seems to be species specific which may be used as taxonomic marker. © 2025 Association for Advancement of Entomology

KEY WORDS: Ansulae, crepuscular observation, hatching line, SEM, exochorion, physicochemical parameter

INTRODUCTION

Culicoides sp. are the smallest haematophagous flies, with many species acting as biological vectors of pathogens of medical and veterinary importance (Meiswinkel *et al.*, 2004). They transmit pathogens such as bluetongue virus (BTV), epizootic haemorrhagic disease virus (EHDV), and African horse sickness virus (AHSV). The World Catalogue

on *Culicoides* documented 1347 extant species within this genus, based on adult morphology (Borkent and Dominiak, 2020). The relative abundance of *Culicoides* vectors within geographic areas would determine the likelihood of disease outbreaks. The gravid females are responsible for the propagation of the *Culicoides* midge population (Meiswinkel *et al.*, 2004). Commonly, crepuscular or nocturnal activity, i.e., peak activity of

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Culicoides species with low light intensity, was recorded by Mellor *et al.* (2000). Borkent (2014) reviewed the availability of information about immature and noted the need for more information for immature compared to adult midges. The midges *Culicoides peregrinus* Kieffer and *C. oxystoma* Kieffer showed high abundance in West Bengal followed by *C. fulvus* Sen and Das Gupta, *C. innoxius* Sen and Das Gupta, *C. actoni* Smith and *C. anophelis* Edwards (Harsha *et al.*, 2020; Mandal *et al.*, 2022; Kar *et al.*, 2022). The habitat of these vector species was reported from semi-aquatic muddy areas mixed with dung, but the species-specific habitat remains unknown (Uslu and Dik, 2010). However, the information about the suitable oviposition site selection parameters of these *Culicoides* spp. remains unknown. Despite the economic importance of the genus, descriptions of the eggs are available for only 47 of the extant species of *Culicoides* (Borkent, 2014). The outer surface of the eggs is covered with exochorion as a protective layer (Hinton, 1981; Harsha *et al.*, 2017a, b). Some small structures were noticed on the surface of *Culicoides* eggs by Hill (1947), Jobling (1953) and Becker (1961). Becker termed this structure as ansulae, which are likely to be involved in egg adhesion with the substrate. Kariya *et al.* (1989) first documented the grouping of these species based on their egg morphological similarities based on papillae and assumed that they shared similar habitats with similar morphological features of eggs. On the egg surface of *C. oxystoma*, papillae arranged in rows and the average diameter of each papilla was 1.25µm and in *C. peregrinus* papillae arranged randomly, measured 3.23µm. Previous studies have been recorded on the ultrastructure of the egg surface of some *Culicoides* spp. (Day *et al.*, 1997; Harsha *et al.*, 2017a, b). Mean length of *C. peregrinus* egg measured 489.36µm and mean width 51.28µm and in *C. oxystoma*, mean length measured 290µm and width 41µm. This study recorded the oviposition period and the egg laying pattern of the two vector species, *C. peregrinus* and *C. oxystoma*. This study aimed to determine the ultrastructural variation during hatching line formation and initiation of hatching line by light microscope and scanning electron microscope (SEM).

MATERIALS AND METHODS

Live adults were collected from the cattle shed using a UV LED light trap. Then *C. peregrinus* and *C. oxystoma* were kept in oviposition vials individually. These oviposition vials (n=100 for each species) were prepared with wet cotton and filter paper placed on them. Then vials were transferred to the Environmental Test Chamber (ETC) (at 26±1°C, RH 75%, 13L:11D) and observed up to 48-72 hrs at every 6-hr interval. To detect the egg-laying period, filter paper was changed every 6 hrs under a stereomicroscope (Olympus Magnus MS24). Eggs were counted on filter papers and kept in ETC for hatching. The egg laying time was considered as 0 hr, and few eggs were preserved for every 8 hr up to hatching. The eggs (n=10 for every hr) were prepared for scanning electron microscopy (SEM). After preservation in glutaraldehyde solution (2%) at a freezing temperature for 24 hr, and washed in PBS solution (0.2 M, pH 7.4). Eggs were treated with 1% osmium tetroxide, followed by ethanol dehydration and critical point drying. Specimens were then placed on stubs covered with sticky carbon tape coated with gold-palladium and examined for SEM (Model Sigma 300, Carl Zeiss) at the University Science Instrumentation Centre, The University of Burdwan (Turner and Mahowald, 1976; Abubekurov and Mullens, 2017). A few eggs were mounted on slides following Wirth and Marston (1968) to observe changes in egg from egg laying to hatching.

RESULTS AND DISCUSSION

The time of egg laying was recorded for both species. These both species have shown their activity at dawn and dusk period. Egg laying of *C. peregrinus* began around midnight, and this time was considered to be 0 hr. Within 48 hr, at every 6-hr interval, the laid eggs on filter paper were counted. This data demonstrates that the maximum egg-laying events recorded at night, and during the daytime, no egg-laying recorded. *Culicoides oxystoma* began laying eggs near dusk, and it continued until dawn. At the day time, no laid eggs were observed. The pattern of egg-laying was also observed in both species. *Culicoides peregrinus*



Fig. 1 Egg laying pattern of (a) *Culicoides peregrinus* and (b) *C. oxystoma*; (c) unfertilized egg (light appearance egg), fertilized egg (dark appearance egg) of *C. peregrinus*

commonly laid eggs in clutch (Fig. 1a). Each clutch consisted of 2-8 eggs. Sometimes, 10-12 eggs were also recorded. But *C. oxystoma* laid eggs individually (Fig. 1b). Just after oviposition, the eggs appeared light in appearance; then they turned dark (Fig. 1c). Noh *et al.* (2021) also observed this melanization of oviposited eggs in *Drosophila melanogaster*. The eggs from laid to hatching were mounted on slides for both species and the initiation of the hatching line, the appearance of the larval head during hatching and eggs after hatching were observed under the light microscope (Zeiss, Axioscope 5). The figures for eggs are presented in (Fig. 2, 3)

After that the eggs of every 8-hr interval examined in SEM and the terminology was followed by Harsha *et al.* (2017b) and Abubekrov and Mullens (2017). The external morphology of *C. peregrinus* eggs as shown in Fig. 4, is laterally compressed, dorsally curved and tapered at both ends at the time of oviposition. The range of length was 454-538 μm and width 42-57 μm . Eggs surface bear pillar-shaped ansulae and these were randomly arranged (Fig. 4a). The changes of ansulae every 8 hr are shown in Fig. 5. The ansulae became depressed (Fig. 5g, h) and the eggs became narrower (Fig. 4b). The hatching line was initiated from the mid of the eggs and it extended at anterior end (Fig. 4c, d).

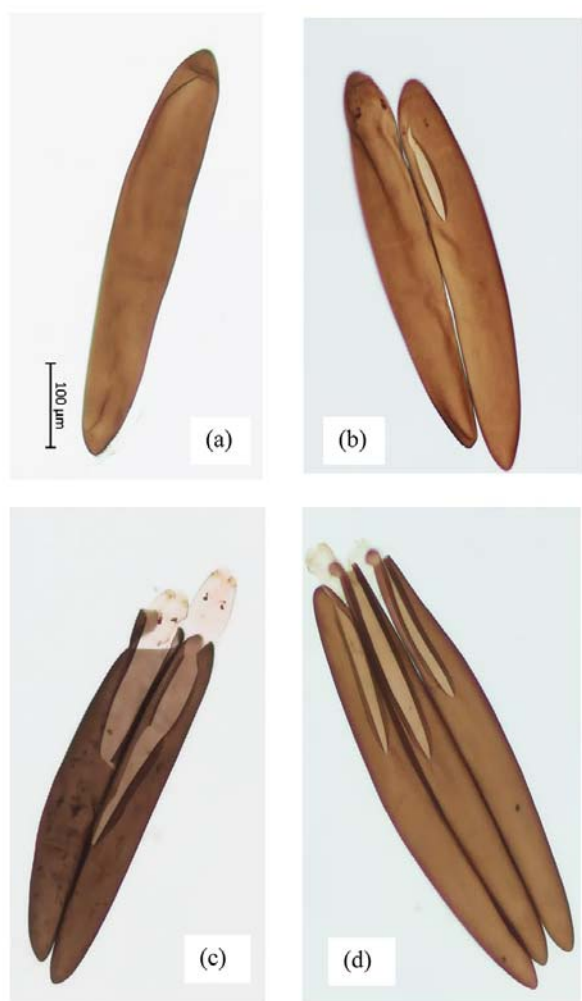


Fig. 2 *Culicoides peregrinus* eggs (a) oviposited egg (b) appearance of hatching line (c) protrusion of larva (d) eggs after hatching

The range of external length *C. oxystoma* egg was 262-354µm and width 34-62µm. The eggs are straight, gradually tapering posteriorly. The ansulae of the chorion on the egg surface were arranged in longitudinal rows (Fig. 4f). The ansulae were depressed with the progression of time (Fig. 6g). The hatching line of *C. oxystoma* eggs was initiated anterior portion of the eggs (Fig. 4g, h).

Monitoring of vector *Culicoides* midges is a method to consider population and management strategies in risk areas. *Culicoides* of medical and veterinary health importance require assessing the risk areas of disease outbreaks. According to Purse *et al.* (2015), these vectors generally have peak activity,

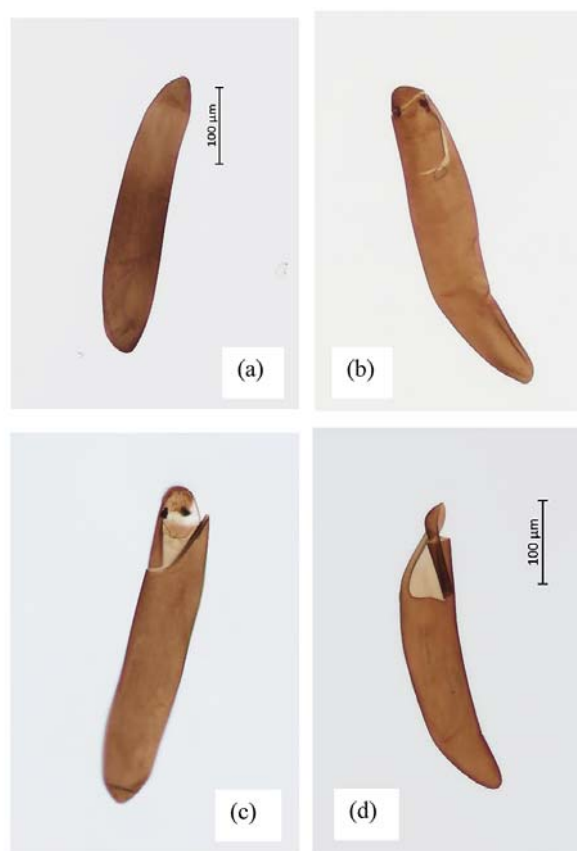


Fig. 3 *Culicoides oxystoma* eggs (a) oviposited egg (b) initiation of hatching line (c) larval protrusion (d) hatched egg

usually around dawn and dusk. The diel periodicity of several *Culicoides* species has been described worldwide, and it occurs during diurnal, nocturnal, and crepuscular periods depending of species. However, their periodic activity remains poorly known due to the absence of laboratory colonies of these vector species (Mellor *et al.*, 2000; Sanders *et al.*, 2012; Erram and Burkett - Cadena, 2021). The nocturnal activity of *C. peregrinus* was examined using an aspirator, while *C. oxystoma* and *C. fulvus* Sen and Das Gupta showed crepuscular activity (Bellis *et al.*, 2004; Kar *et al.*, 2022). Harsha *et al.* (2020) recorded similar activity periods for these *Culicoides* spp. This study showed that both species' active maximum egg-laying period was nocturnal. According to Mellor *et al.* (2000), it may be assumed that this nocturnal activity would adapt to the exposure of low risk and also benefit from low temperature with high

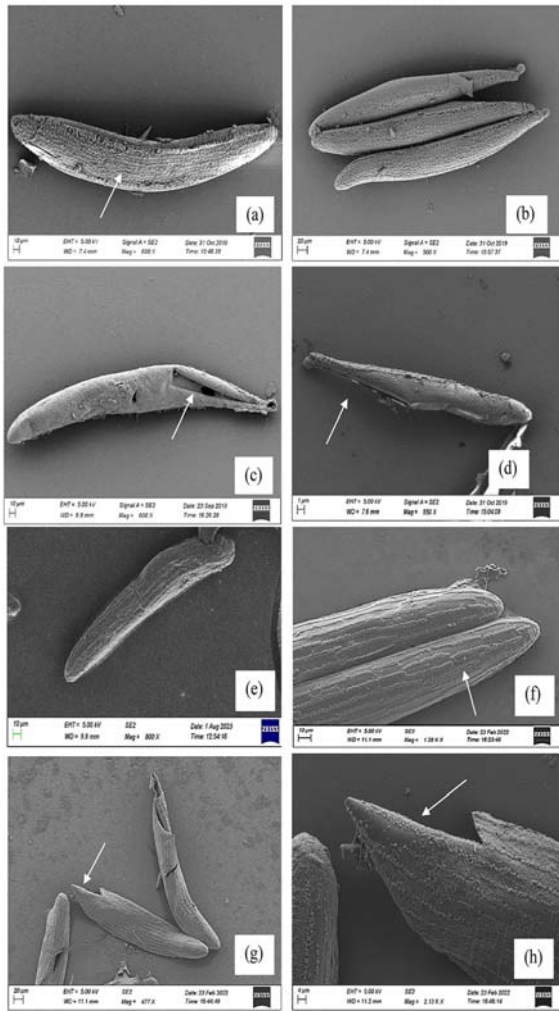


Fig. 4 Egg morphology with arrangement of ansulae and hatched eggs with hatching line of using SEM of *Culicoides peregrinus* (a-d) and *C. oxystoma* (e-h)

relative humidity of the habitat at night. The initiation of the hatching line for both species was different (Fig. 4). According to Pereira *et al.* (2006), the initiation of the hatching line was indicated by a small protrusion on the eggshell at the anterior pole, and hatching was completed at that point. The hatching occurs by the repetitive and synchronized movement of the larval head and the egg buster. These egg busters initiate small fissures in the chorion of the shell. Previously, some studies reported the ultrastructural morphology of chorionic tubercles (also referred to as hairlike papillae, ansulae) of eggs of *Culicoides* spp. The

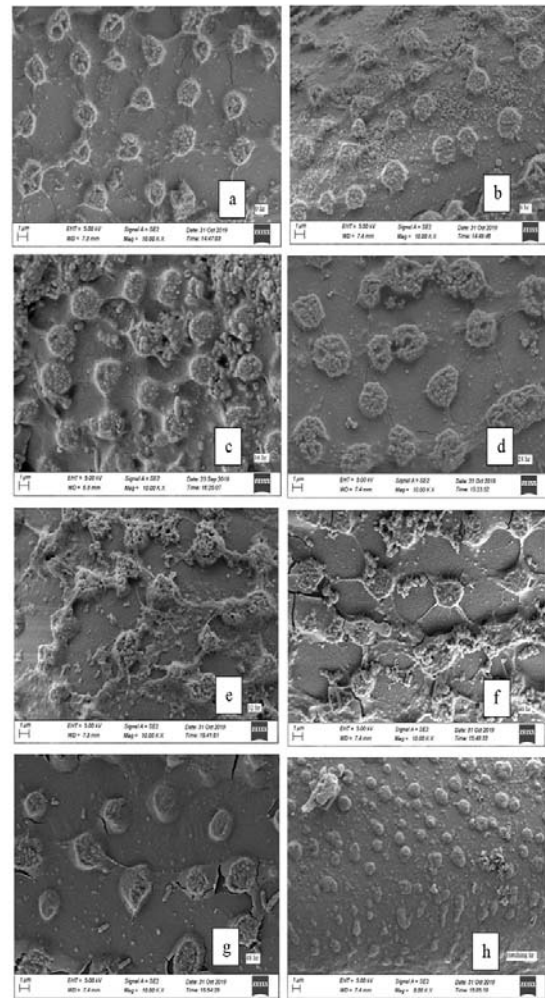


Fig. 5 Changes of ansulae of egg exochorion of *C. peregrinus* at 8 hr intervals from oviposited egg to hatched egg (a: 0 hour (oviposited eggs), b: 8hr, c: 16hr, d: 24hr, e: 32hr, f: 40hr, g: 48hr and h: hatched egg)

arrangement of ansulae is varied with the species of *Culicoides* such as these ansulae are arranged in rows in *C. circumscriptus* Kieffer, *C. gejjelensis* Dzhaferov, and *C. imicola* Kieffer (Day *et al.*, 1997); and in the egg of *C. arakawae* Arakawa, *C. oxystoma*, *C. punctatus* Latreille, *C. sumatrae* Macfie, and *C. actoni* Smith (Kariya *et al.*, 1989). These are randomly scattered over the egg surface of *C. brevitarsis* Kieffer (Campbell and Kettle, 1975), *C. maculatus* (Shiraki) (Kariya *et al.*, 1989), and *C. variipennis* (Coquillett) (Nunamaker *et al.*, 1987). The absence of ansulae was reported in the eggs of *C. molestus* (Skuse) and *C. brookmani*

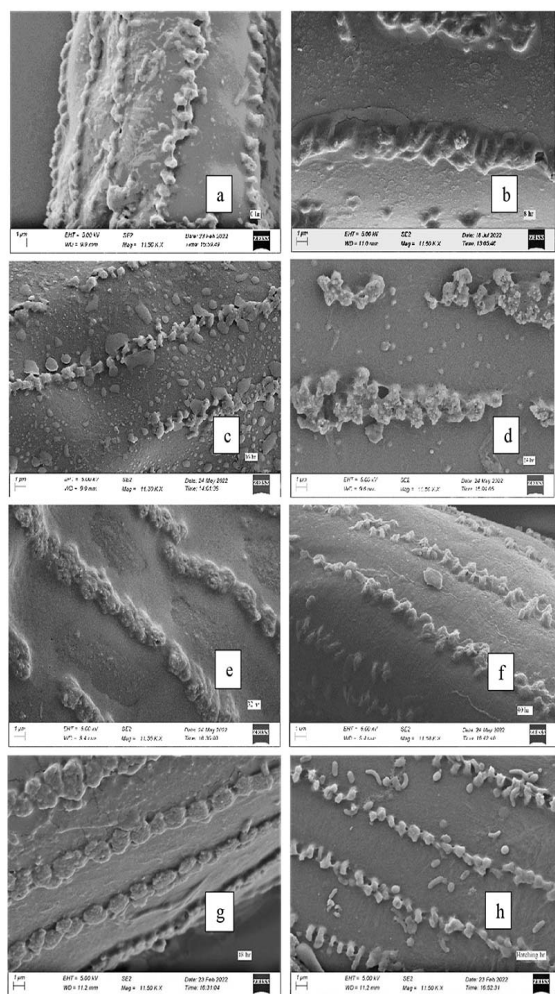


Fig. 6 Changes of ansulae of egg exochorion of *Culicoides oxystoma* at 8 hr intervals from oviposited egg to hatched egg (a: 0hr (oviposited eggs), b: 8hr, c: 16hr, d: 24hr, e: 32hr, f: 40hr, g: 48hr and h: hatched egg)

Wirth; it may be associated with their semi-aquatic habitat (Cribb and Chitra, 1998; Breidenbaugh and Mullens, 1999). Whereas species like *C. brevitarsis*, *C. imicola*, *C. circumscriptus* possess long ansulae that breed in semi-dried habitats (Campbell and Kettle, 1975; Kariya *et al.*, 1989; Day *et al.*, 1997). The correlation of ansulae with their habitat and the respiratory structure of mosquito species was studied (Linley *et al.*, 1993). The breeding habitat of both species of *Culicoides* was associated with muddy areas mixed with cow dung. This taxonomic information about the

immature stages will be useful in determining the oviposition sites, identifying immatures, and establishing management strategies against these vector species.

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