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Scanning electron microscopic observations on the eggs of *Anopheles annularis* (Diptera: Culicidae)

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ABSTRACT: Accurate identification of *Anopheles* using different techniques is essential for having effective malaria control strategies. Present study describes the eggs of species belonging to subgenus *Cellia* of genus *Anopheles* collected from North India using scanning electron micrographs. *An. annularis* are sympatric with major malaria vectors like *An. culicifacies*, *An. stephensi* and *An. maculatus*. The eggs of the *Anopheles* species described so far are boat-shaped with rounded anterior and posterior ends. However, they can be easily distinguished by the shape of the deck present on the ventral (upper) surface of the egg. In *An. annularis* the deck is continuous along the length of the egg. The restricted deck in some species is due to presence of chorionic cells on the upper surface of the eggs. The floats (structure present on the lateral side of the eggs) in some *Anopheles* species are located in the middle of the egg and are limited to the lateral sides. In *An. annularis* the floats extends up to anterior and posterior frills(slight wavy structures present on both the ends of an egg). The eggs are compared with related species of genus *Anopheles*, notably *An. culicifacies*, *An. splendidus* and *An. stephensi*. It was found that *An. annularis* can be easily distinguished from other sympatric species by egg morphology study.
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KEY WORDS: scanning electron microscopy, *Cellia*, morphology.

Сканирующая электронная микроскопия яиц *Anopheles annularis* (Diptera: Culicidae)

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РЕЗЮМЕ: Правильная идентификация видов рода *Anopheles* с использованием современных технологий имеет большое значение для эффективного контроля и разработки стратегий регулирования малярии. Яйца нескольких видов комаров подрода *Cellia* и рода *Anopheles* собраны в северной Индии и описаны при помощи сканирующей электронной микроскопии. *An. annularis* — это симпатрический вид, обитающий на одной территории с большинством переносчиков малярии, таких как *An. culicifacies*, *An. stephensi* and *An. maculatus*. Яйца *Anopheles* из Старого Света описаны как лодкообразные с закругленными передним и задним концами. Эти яйца могут быть легко определены по форме верхней поверхности яйца («палубе» лодкообразного яйца). У *An. annularis* «палуба» имеется на всем протяжении яйца, в то

время как у других видов, «палуба» ограничена хориальными клетками, присутствующими на верхней поверхности яйца. Особые структуры — поплавки — у некоторых видов *Anopheles* обнаруживаются на боковых сторонах яйца, как в правило в центральной их части. У *An. annularis* поплавки выходят за пределы боковых сторон и простираются на переднюю и заднюю оборку. Проведено сравнение тонкой морфологии яиц у близких видов рода *Anopheles*: *An. culicifacies*, *An. splendidus* and *An. stephensi*. Сравнительный анализ показал, что тонкая морфология яиц *An. annularis* существенно отличается от таковой у близких видов.

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КЛЮЧЕВЫЕ СЛОВА: сканирующая электронная микроскопия, *Cellia*, морфология.

Introduction

In the family Culicidae, members of the genus *Anopheles* include some of the principal vectors of malaria, while others act as secondary vectors of local importance. Unfortunately, the majority of them exist in the form of species complexes of two or more sibling species, which are difficult to identify on the basis of their morphotaxonomic characters. This has necessitated the application of additional parameters for accurate identification of species, which is a prerequisite for understanding the ecological and genetic characteristic of the species and thus for developing effective means of population suppression and control of their malariogenic activity (Green, Miles, 1980; Rao, 1984; Green *et al*., 1985; Takai, Kanda,1986; Damrongphol, Baimai, 1989; Linley *et al*., 1993a; Ramirez *et al*., 1994; Reinert *et al*., 1997; Lounibos *et al*., 1997, 1999). The use of species-specific polytene chromosome banding pattern, inversion polymorphism, amino acid and enzyme estimations, and PCR-based genomic characterization are the outcome of these basic requirements. In some species the variations in the egg surface architecture has provided valuable additional information of taxonomic value. For example, the *type* form, *intermediate* form and variety *mysorensis* of *Anopheles stephensi* have been successfully distinguished on the basis of chorionic features of their eggs (Subbarao *et al*., 1987; Chaudhry, Gupta, 2004; Tyagi *et al*., 2017). Those concerned in the control of malaria have for long paid special attention to the egg stage as differences between the eggs of closely allied species are often more marked than the differences in the larval and adult stages and it is through the availability of the eggs in the natural breeding places that it becomes possible to predict the number of species and races prevalent in an area. Regardless whether the eggs have been deposited or retained in the ovaries, they bear distinctive markings when observed under light microscope, phase contrast microscope and scanning electron microscope (Hinton, 1968). The first recognized complex of species of *Anopheles (Anopheles) maculipennis* was elucidated on the basis of differences in the egg morphology (Hackett, Missiroli, 1935). Since then, this source has been successfully applied to describe relationships in a number of anopheline species complexes and their geographical population (Linley *et al*., 1993b, 1995, 1996; Tyagi *et al*., 2016). However, these studies have been so far restricted to the eggs of the *Anopheles* species belonging to the New World. The present paper reports the SEM study of eggs of *An. annularis*, which are among the *Anopheles* species prevalent in the Old World. *Anopheles stephensi* (Chaudhry, Gupta, 2004; Tyagi *et al*., 2017), *An. culicifacis* (Chaudhry, Gupta, 2003; Tyagi *et al*., 2016) and *An. fluvitilis* (Sehrawat, 2014) are few *Anopheles* species inhabiting this part of the world on which similar kind of studies have been carried out so far. In addition to facilitating species identification, the comparisons of egg structure

among the closely related species act as source of interpreting developmental and evolutionary origins of these characters and their functional significance (Lounibos *et al*., 1997).

Materials and Methods

Adult forms of *Anopheles annularis* were collected during early morning hours from cattle sheds in the village Bela Dheyani near Nangal, 110 km NW of Chandigarh (30°44′N, 76°53'E). All the specimens were individually identified by using identification keys (Christophers, 1933; Wattal, Kalra, 1967; Rao, 1984) and finally on the basis of features of the larval salivary polytene X-chromosomes. Each gravid female was individually kept in a vial, where it was allowed to oviposit on the moist filter paper; that took about 12–16 hrs. These eggs were gently lifted with the help of a camel hair brush and transferred to rearing bowls filled with distilled water, where they were allowed to embryonate for 20–30 hours at a temperature of 26–27 °C and relative humidity of 80±5%. 100–150 eggs from each female were first observed under an optical microscope, so as to make sure they were neither damaged nor hatched, and then processed further for SEM examination. For this purpose, they were dehydrated by following the standard procedure (Linley *et al*., 1993a). Accordingly, they were fixed for 1 hour in alcoholic Bouin's solution in vials. Complete dehydration was achieved by treatment with 80% ethanol (two changes of 10 minutes each) and then by increasing the ethanol concentration from 5 to 10%, with 10 minutes of treatment in each concentration. These dehydrated eggs were then placed on the stubs and sputter-coated with gold. Each egg was carefully examined under a Jeol-JSM 6100 scanning electron microscope and the viewing was completed as quickly as possible because they have a tendency to collapse within few hours. Micrographs of 10–20 eggs were examined from all the desired directions and magnifications, after which the measurements were taken for various parameters. For each dimension, the mean±standard error is given, usually basing on 10 measurements. If the number of measurements (n) was different, it is given in brackets.

Results

COLOR. Black.

SIZE. Mean length (±Standard Error (SE)): 440.051±7.211 μm (number of eggs (n)=10; range:420–500 μm).

Mean width (±SE): 151.044±1.737 μm (n=10; range: 145.5–168.27 μm).

OVERALL APPEARANCE. The egg of *Anopheles annularis* is boat- shaped in lateral view with rounded anterior and posterior ends (Fig. 1A, D). The deck is almost of same width throughout the egg length except for the portion where the frills terminate. The pointed and inward projecting ends of the frill narrow towards the deck (Fig. 2A). Floats constitute about 45% of the egg length and are not continuous with the end of the frill.

VENTRAL (UPPER) SURFACE. Deck continuous along the length of the egg and slightly narrow at both the ends of the middle region. Frill moderate in height and extends beyond the floats (Fig. 1A, B). The edges of the frill are undulated with grooved inner surface. Deck surface is completely covered by the presence of large, prominent irregularly formed tubercles which are interspersed with the smaller ones (n=10; mean diameter±SE of tubercles: 2.574±0.399 μm) (Fig. 2A, B, C, F). Three to four prominent lobed and elliptic tubercles are present at the anterior end of the deck (n=10; mean diameter ±SE:8.024±0.220 μm) while two similar tubercles are present at the posterior end (n=3; mean diameter±SE: 6.034±0.540 μm).

DORSAL (LOWER) AND LATERAL SURFACES. The dorsal chorionic cells are mushroom-shaped convex structure interconnected by bridges of nearly same width. The chorionic pores in between these cells are almost of same size but are lesser in number when compared to those eggs of other species (Fig. 2D, E). Floats occupying the lateral sides do not extend to cover the ventral surface and the number of ridges on the float varies from 16 to 19.

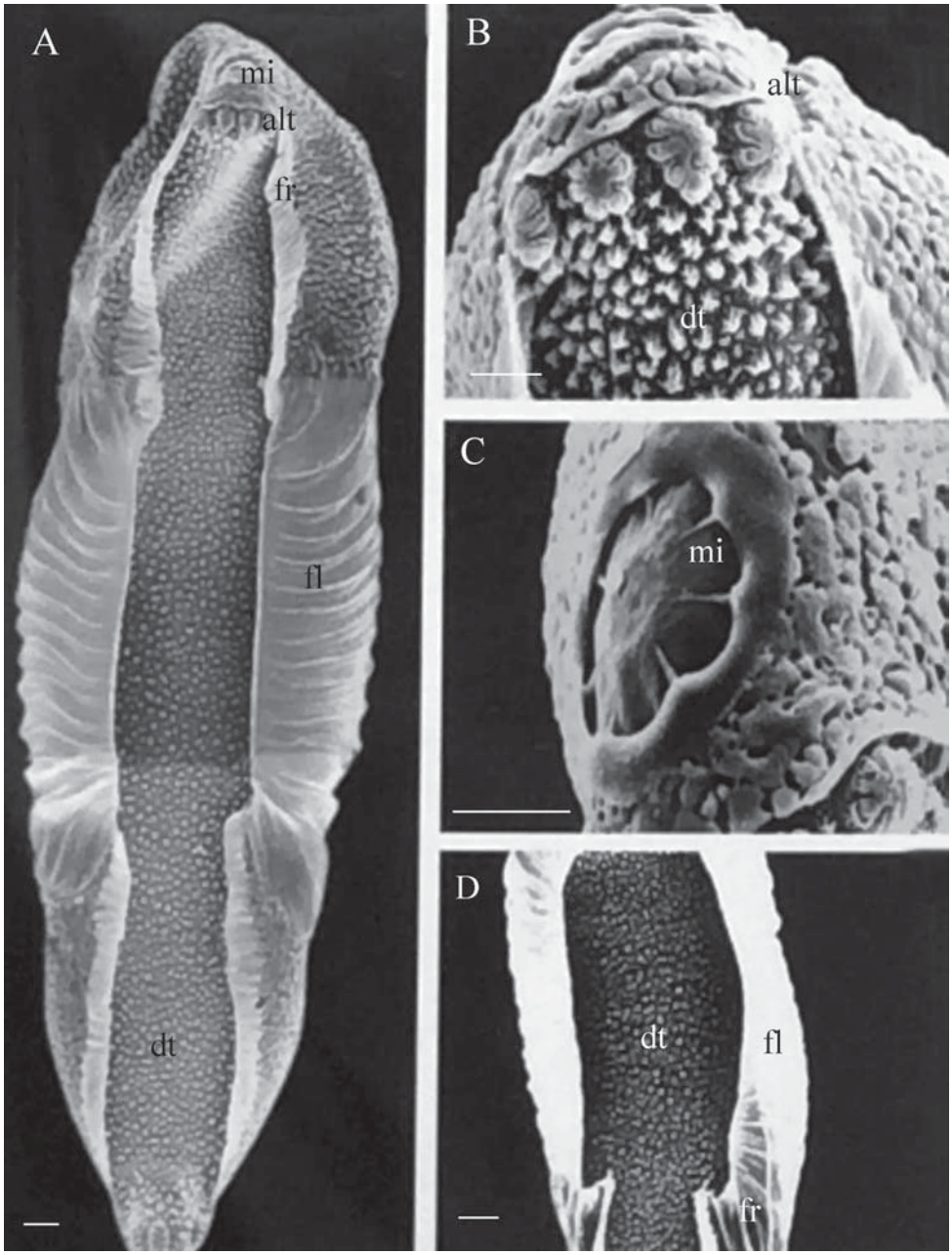

Fig. 1. Egg of *Anopheles annularis*. A — ventral (upper) view; B — anterior lobed tubercles; C — micropyle; D — attachment of floats with frill.

Abbreviations: alt — anterior lobed tubercles; dt — deck tubercles; fl — float; fr — frill; mi — micropyle. Scale bar: 10 μm.

Рис. 1. Общая морфология и детали тонкой организации яйца *Anopheles annularis*. A — вид с вентральной стороны, сверху; B — передние дольчатые бугорки; C — микропиле; D — место прикрепления поплавка и оборки.

Обозначения: alt — передние дольчатые бугорки; dt — бугорки «палубы»; fl — поплавок; fr — оборка; mi — микропиле. Масштаб 10 мкм.

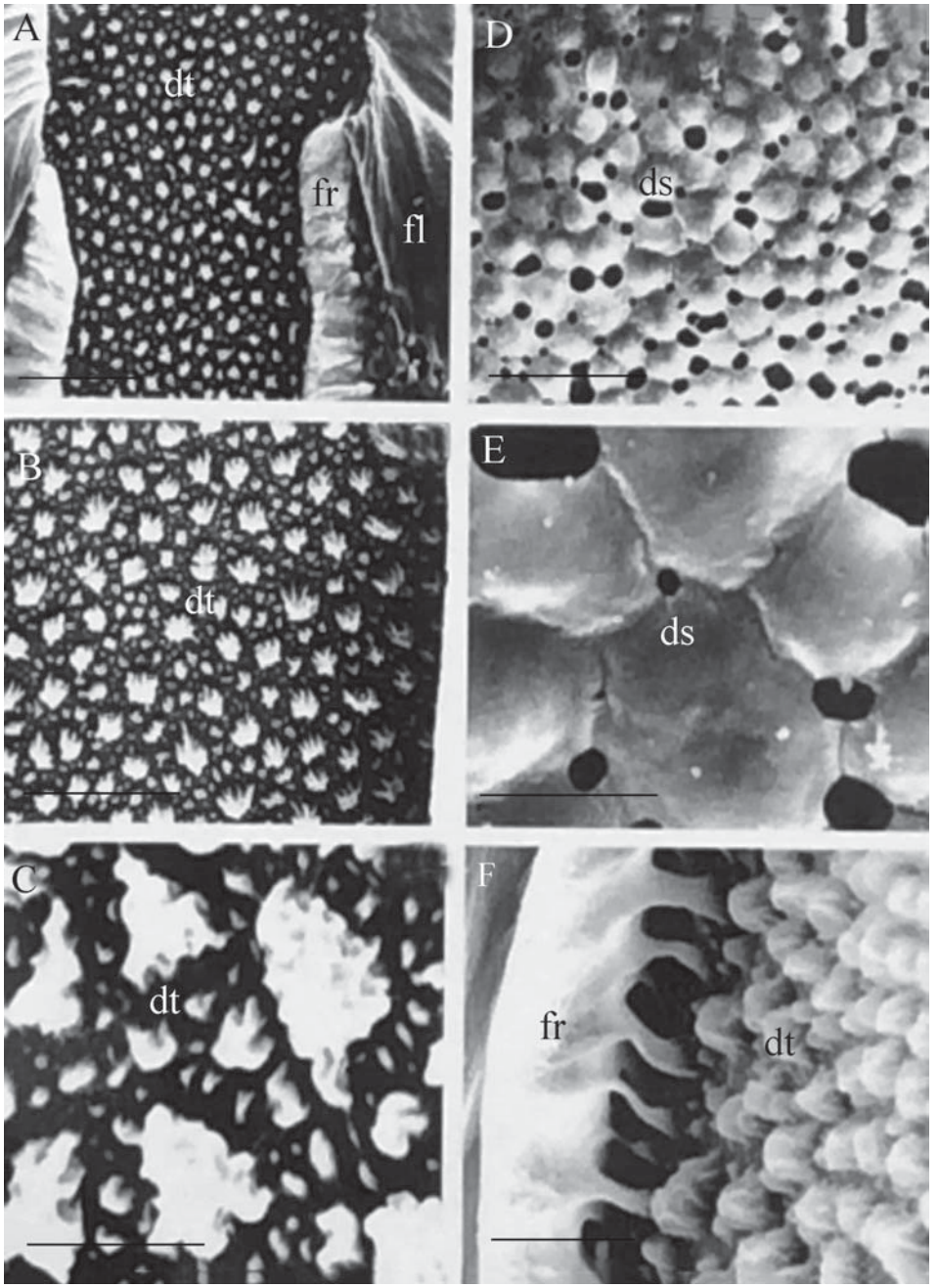

Fig. 2. Details of fine structure of egg of *Anopheles annularis.* A — deck tubercles; B, C — magnified deck tubercles; D — dorsal surface; E — magnified dorsal surface; F — frill and deck tubercles.
Abbreviations: ds — dorsal surface; dt — deck tubercles; fl — float; fr — frill. Scale bar: 10 μm.
Рис. 2. Детали тонкой морфологии яйца of *Anopheles annularis.* A — бугорки «палубы»; B, C — то же при большем увеличении; D — поверхность дорсальной стороны; E — то же при большем увеличении; F — место контакта оборки и «палубы» (видны бугорки «палубы» и выросты оборки).
Abbreviations: ds — дорсальная поверхность; dt — бугорки «палубы»; fl — поплавок; fr — оборка. Масштаб 10 мкм.

ANTERIOR END (MICROPYLE). The collar around the micropyle is irregular in shape and seven ridges from the collar towards the central orifice which is without any plug (n=2; mean diameter ±SE: 24.302±0.924 μm) (Fig. 1C).

POSTERIOR END**.** The posterior end is rounded as compared to anterior end and the ornamentation of the lateral surfaces is similar to that of the dorsal surface (Fig. 1A).

Discussion

The results of present SEM studies revealed that *An. annularis* can be distinguished from *An. culicifacies* (Chaudhry, Gupta, 2003), *An. stephensi* (Chaudhry, Gupta, 2004) and *An. splendidus* (Gupta, Chaudhry, 2005) on the basis of chorionic sculpturing. In addition to the ventral surface of all the four species which presented characteristic chorionic sculpturing, the length of deck, frill and floats were found to be species specific. *Anopheles culicifaces* could be distinguished from other three species by the features of dorsal plastron on the ventral surface which restricts the deck to anterior and posterior portions. The eggs of *An. stephensi* could be easily distinguished by being slipper-shaped and floats extending to the middle of the ventral surface. The eggs of *An. splendidus* and *An. annularis* were found to be similar in overall morphology, in accordance with the similarity found in the adults of both the species. However, they could be distinguished by position of floats and frill. In *An. annularis*, the frill extends slightly beyond the floats, whereas in *An. splendidus*, the beginning of floats and end of frill coincide. The present observations also revealed that the condition of the floats in *An. splendidus* and *An. annularis* was very different from the anopheline species studied so far. Very few species like *Anopheles* (*Nyssorhynchus*) *pessoai* and some members of *Anopheles gambiae* complex have the eggs which possess the floats occupying less than half of the total length of the egg (Causey *et al*., 1944; Lounibos *et al*., 1999). In species having restricted deck, like *An. culicifacies* and *An. fluviatilis* (Sehrawat, 2014), the floats extends almost throughout the length of the egg. Polymorphic eggs found in studies of *An. culicifacies* (Chaudhry, Gupta, 2003), *An. triannulatus, An. strode* and *An. punctimacula* (Rodriguez *et al.,* 2002) couldn't be detected during the present investigations. The presence of lobed tubercles at the anterior and posterior ends is a feature which is shared by all those species of the subgenera *Cellia*, *Anopheles* and *Kerteszia* of the genus *Anopheles* whose eggs have been studied for supplementing the data of taxonomic value. These points of chorionic homologies have also helped in differentiating the species belonging to these subgenera from those belonging to subgenus *Nyssorhynchus* (Lounibos *et al*., 1998, 1999). In the present study, the eggs of both the species were found to have lobed tubercles at the anterior and posterior end.

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