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Egg chorion exploration of two important pests of family Noctuidae Latreille, 1809 from Himachal Pradesh (India) (Insecta: Lepidoptera)

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Abstract

Modern systematics uses taxonomy to reflect evolutionary history and in Lepidoptera, immature stage morphology is largely unknown for most species, but it has potential for classification and systematic studies as, Lepidoptera are common major and minor agricultural pests, the present study includes the examination of ultrastructure of Lepidoptera eggs of two pests, *Agrotis ipsilon* (Hufnagel, 1766) and *Spodoptera litura* (Fabricius, 1775) through scanning electron microscope (SEM), following a thorough investigation of the structural complexity of these tiny eggs, detailed ultrastructural descriptions, and comparative analyses, as well as identification keys, for important egg characters have been compiled, these findings will enrich the taxonomic database and update future pest management studies aimed at early potential pest detections at a much earlier stage of their life histories.
Keywords: Insecta, Lepidoptera, Noctuidae, aeropyles, *Agrotis ipsilon*, egg, micropylar rosette, micropyles, *Spodoptera litura*, ultrastructure, India.

**Exploración del corion de los huevos de dos importantes plagas de la familia Noctuidae Latreille, 1809 de Himachal Pradesh (India)
(Insecta: Lepidoptera)**

Resumen

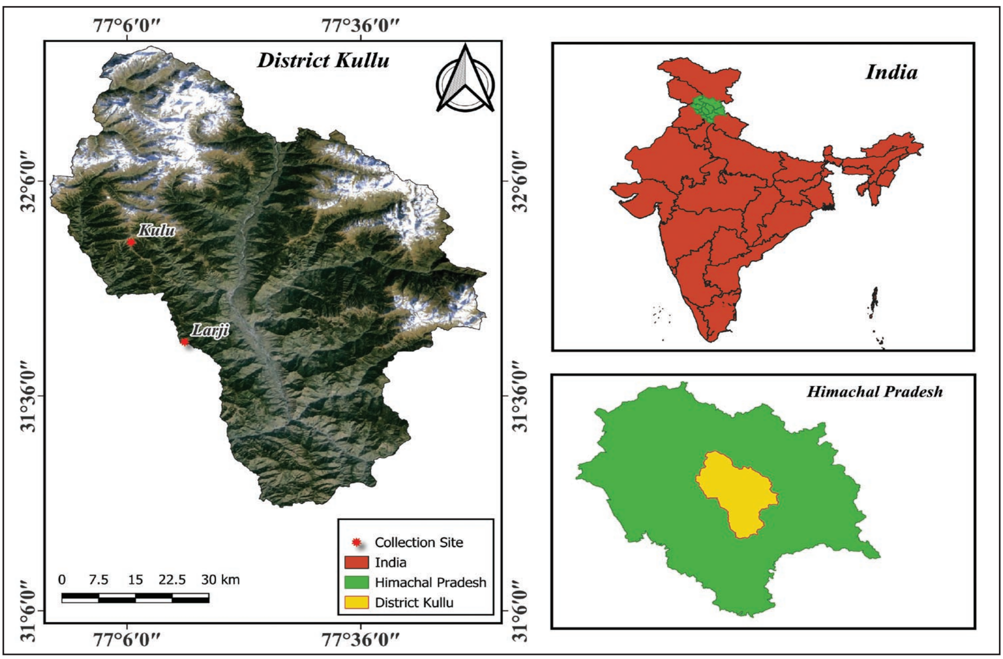
La sistemática moderna utiliza la taxonomía para reflejar la historia evolutiva y en Lepidoptera, la morfología del estado inmaduro es en gran parte desconocida para la mayoría de las especies, pero tiene potencial para la clasificación y los estudios sistemáticos, ya que, los Lepidoptera son plagas agrícolas comunes mayores y menores, el presente estudio incluye el examen de la ultraestructura de los huevos de Lepidoptera de dos plagas *Agrotis ipsilon* (Hufnagel, 1766) y *Spodoptera litura* (Fabricius, 1775) mediante microscopio electrónico de barrido (MEB), tras una minuciosa investigación de la complejidad estructural de estos diminutos huevos, se han compilado descripciones ultraestructurales detalladas y análisis comparativos, así como claves de identificación, para importantes caracteres de los huevos; estos hallazgos enriquecerán la base de datos taxonómica y actualizarán futuros estudios de gestión de plagas dirigidos a la detección precoz de posibles plagas en una fase mucho más temprana de sus historias vitales.
Palabras clave: Insecta, Lepidoptera, Noctuidae, aeropilos, *Agrotis ipsilon*, huevo, roseta micropilar, micropilos, *Spodoptera litura*, ultraestructura, India.

Introduction

There are various techniques for analyzing taxonomic characters, such as morpho-taxonomy, molecular taxonomy, behavioral taxonomy, and ecological taxonomy, among others. Identification of moths (particularly adults) always relied on characters such as wing maculation, wing venation, male and female genitalic characters and other features, but ultrastructural characters particularly of immature stages such as egg chorion patterns remain completely ignored for many years, even though such characters are equally important for identification. This research aims to fill a gap in taxonomic data and improve the recognition of species at the immature levels. Egg shell architecture has been shown to have taxonomic and phylogenetic significance in several Lepidopteran families Noctuidae (Salkeld 1984), Lycaenidae (Munguira et al. 2015), Danaidae (Kitching, 1985), Mnesarchaeidae (Kobayashi & Gibb, 1995), (Rougerie & Estradel, 2008), Pieridae (Llorente-Bousquets & Castro-Gerardino, 2007; Llorente-Bousquets et al. 2018; Hernández–Mejía et al. 2013), Nymphalidae (García-Barros & Martín, 1995; Freitas & Brown, 2004; Nieves-Uribe et al. 2015), Arctiidae (Kaleka et al. 2023a; 2023b), Lasiocampidae (Kaleka et al. 2023c) and others.

In present study, scanning electron microscope (SEM) was used to inspect and depict eggs of two important pest moths i.e., *Agrotis ipsilon* (Hufnagel, 1766) and *Spodoptera litura* (Fabricius, 1775). This comparative examination will enable the use of exo-chorionic characters in the systematics of the species under consideration, as well as aid in the formation of identification guides. Few important examples of this are the works of Hernández-Roldán et al. (2012) in Hesperiidae, Dolinskaya (2019) in Notodontidae, Munguira et al. (2012) in Lycaenidae and many others.

Both these species i.e., *Agrotis ipsilon* (Hufnagel, 1766), The Black Cutworm and *Spodoptera litura* (Fabricius, 1775), Tobacco Cutworm referable to ditrysian family Noctuidae are polyphagous pests. Morphological features such as egg chorion texture, polygonal cell types, presence of number of aeropylar and micropylar openings, and shape of micropylar rosette have been observed on the eggshells of both investigated species, and these characters must surely be of great taxonomic value at specific as well as generic levels in order to facilitate the identification tasks of moths at much earlier stages of their life histories, i.e., eggs, and such investigations must be expanded in order to improve and elevate the morphological classification of moths at an early stage of development. This article also provides differentiation keys for these two moth species based on examined egg characters.



Materials and Methods

In the month of April 2022, a collection tour was conducted in the locality of Larji (Kullu) (31.7251ºN, 77.2190ºE), District of Himachal Pradesh to collect moth eggs. The light trap method was used to collect and mate adult moths to obtain newly laid eggs. The female's freshly laid eggs were then picked with fine forceps and brushes. The collected eggs were preserved in 70% alcohol and glycerol in a ratio of 8:2; After obtaining eggs, the collected moths were killed, stretched, and preserved in fumigated and air-tight wooden entomological boxes for further identification.

For sample preparation and further SEM investigations methodology adopted by Kaleka et al. (2023) was followed:

Fixation: The sample material, namely the eggs, were fixed in 2.5% glutaraldehyde for at least one hour. The material was then immersed in phosphate buffer solution (PBS) with a pH of 7.4 and rinsed repeatedly for at least 15 minutes.

Dehydration: The eggs were dehydrated by moving them through a series of graded ethyl alcohol solutions (in 50% alcohol for 15 minutes, then 70% and 90% alcohol for 15 minutes each, and three changes in 100% alcohol for 10 minutes each).

Mounting and sputtering: The sample material was dehydrated properly before being mounted on aluminium stubs with double-sided adhesive carbon tape and sputter coated with a gold and platinum mixture.

Scanning: The sputtered egg samples were observed and studied using a SEM (JEOL) JSM-6510LV available at the Punjabi University's Sophisticated Instrumentation Centre in Patiala. The egg chorion, micropylar region, arrangement of micropylar rosette, aeropyles, and other external ultra-structural characters present on the eggshell were photographed.

Analysis: Different egg characters were identified from SEM micrographs using terminology proposed by Zolotuhin and Kurshakov (2009) and Dolinskaya (2019). The images were labelled using Adobe Photoshop CS6 software. Metric analyses of egg diameters were performed using ImageJ software.

Results

Agrotis ipsilon (Hufnagel, 1766) (Figures 1-6)

Egg Shape and Size: The egg of this noctuid moth species is spherical in its shape with 0.573mm diameter.

Egg Chorion: The egg chorion texture of this species is highly sculptured, with a total of 35 well defined ribs and ridges all over the egg chorion surface which are considered a species-specific character in noctuid moth eggs. The chorion is made up of rectangular and pentagonal shaped polygonal cells, starting write after the secondary petaloid cell (SPC) series and extending almost up to the base of the egg. The polygonal cells of the egg have well defined walls with clear and smooth base which imparts clear shape and boundaries to these polygonal cells.

Micropylar Region: The micropylar region of this egg is composed of micropyles which are present in a micropylar pit and which is again surrounded by a series of primary petaloid cells (PPC). The PPC are than encircled by a series of secondary petaloid cells, which are very different in their shapes, sizes, numbers, and texture. No, such visibly clear prominent transition zone is seen present between micropylar region and the general egg chorion surface of the egg.

Micropylar Rosette: The micropylar rosette on this egg is prominently present just in center at the anterior pole of the egg. The micropylar rosette is formed of a series of total 10 primary petaloid cells fashioned in a way that forms a flower shape around the micropylar pit. These PPC are double- walled towards the micropylar pit and up to one third of the entire cell shape. The base of these petaloid cells is slightly textured with prominent walls separating them from each other. The PPC are again seen surrounding by a series of 14 secondary petaloid cells, which are clearly not uniform in their shape and sizes. Micropylar rosette as observed is completely devoid of air- spaces or aeropyles.

Micropyles: The micropyles or also as they are called micropylar openings are observed in a well-defined square shaped micropylar pit in the very center of the micropylar rosette. The micropylar pit is clearly visible and holds four micropyles one at each corner of the square which are deep.

Aeropyles: Aeropyles or the aeropylar openings are the air-spaces present on the egg for the exchange of gases between the embryo and its environment. The aeropyles on this egg are observed present but they are limited to only four rows of the polygonal cells from where the micropylar rosette ends. These aeropyles are circular in their shapes as seen from above, but as they are present embedded into the walls of the polygonal cells, the exact shape they hold is cylindrical, each polygonal cell carries up to four to six aeropylar openings according to the shape of the polygonal cells. These air spaces are not uniformly present in each polygonal cell rather they are present in somewhat scattered manner, as no such clear pattern is visible in their actual arrangements. After four rows of the polygonal cells all the remaining egg chorion is completely devoid of the aeropyles.

Material Examined: India, Himachal Pradesh, Kullu, Larji, 2366 m, ♀, 20-VI-2022, Sainika Jallundhara, 2 eggs.

Distribution: Asia, Australia, Central and South America, Europe, Mexico, New Zealand, North Africa, Pacific Rim, and Southern Canada.

Spodoptera litura (Fabricius, 1775) (Figures 7-12)

Egg Shape and Size: The egg is ellipsoid in its shape when seen from above with somewhat swollen and flat base with a diameter of 0.439mm.

Egg Chorion: The general egg chorionic surface is highly sculptured and irregular with prominently visible ribs and ridges. The number of ribs present on the egg surface is considered as a species-specific character in Noctuid eggs. In the present species, the total number of ribs is 42-44. These ridges are countable up to the middle of the egg after which the egg sculpturing starts fading and becomes almost smooth towards egg base. The lateral sides of the egg are found to have some rectangular and squarish polygonal cells and their boundaries or walls are well defined with smooth texture-free base which gives every polygonal cell a clear and specific shape.

Micropylar Region: The micropylar region of this egg is present in the very center of the top anterior end of the egg. The micropylar area shows distinct and clearly visible structures such as micropylar rosette having micropylar openings in a small micropylar pit and series of primary and secondary polygonal cells. No, such visibly clear prominent transitional zone is present between micropylar region and the general egg chorion surface of this egg.

Micropylar Rosette: The micropylar rosette is placed at anterior pole of the egg and is composed of nine primary petal shaped cells or petaloid cells which provides a flower like appearance. These cells surround a small squarish micropylar pit in the center of the rosette which holds micropylar openings. The primary petaloid cells are further surrounded by an arrangement of total 16 secondary petaloid cells having slightly raised walls and a texture free base. These secondary petaloid cells are not uniform in their shapes and sizes. The PPC and SPC are completely devoid of any aeropylar openings.

Micropyles: These micropyles or also known as micropylar openings are present in center of the small squarish micropylar pit surrounded by series of PPC and SPC. The micropyles are four in number present one at each corner of the square pit.

Aeropyles: The rectangular and squarish polygonal cells are seen holding long and hollow cylindrical aeropylar structures on lateral sides of the egg. The aeropyles are present at each corner of the polygonal cell i.e., four aeropylar openings per cell are present all over the chorionic surface.

Material Examined: India, Himachal Pradesh, Kullu, Larji, 2366 m, ♀, 28-VI-2022, Sainika Jallundhara, 3 eggs.

Distribution: Australasia, Pacific Islands and Tropical & Temperate Asia.

Key to the studied species of family Noctuidae (Based on egg characters):

Egg chorion uniformly patterned with rectangular and pentagonal cells; 35 longitudinal ribs present all over the egg chorion; Micropylar rosette formed of 10 Primary petaloid cells and 14 Secondary petaloid cells; four to six aeropylar openings present on the walls of each polygonal cell ....*Agrotis ipsilon* (Hufnagel, 1766)

Egg chorion uniformly patterned with square and rectangular cells; 42-44 longitudinal ribs present

all over the egg chorion; Micropylar rosette formed of nine Primary petaloid cells and 16 Secondary petaloid cells; four aeropylar openings present on the walls of each polygonal cell..................................
...... *Spodoptera litura* (Fabricius, 1775)

Discussion

For the first time, the egg morphology, ultrastructure of egg chorion, and patterns in two species of Noctuid moths, *Agrotis ipsilon* (Hufnagel, 1766) and *Spodoptera litura* (Fabricius, 1775), were thoroughly investigated under scanning electron microscope. The external morphology or ultrastructure of the egg's chorion differs between the two studied species in terms of chorion sculpturing, the number of primary and secondary cells forming the micropylar rosette, and the number and shape of micropylar and aeropylar openings.

There have been very few studies on the aeropylar openings found on the egg chorion of lepidopteran eggs. Fehrenbach et al. (1987) tried to count the number of aeropyles on egg chorion in two Noctuid species, *Heliothis virescens* (Fabricius, 1777) and *Spodoptera littoralis* (Boisduval, 1833). *Heliothis virescens* (Fabricius, 1777) produced 50 aeropyles per egg, while *Spodoptera littoralis* (Boisduval, 1833) produced 400. The current study attempted to investigate the number and shape of aeropylar openings in the species under consideration. The number of aeropyles per polygonal cell present on the egg chorion has been recorded, and four to six aeropyles per polygonal cell have been observed in *Agrotis ipsilon* (Hufnagel, 1766) which remains limited to only four per cell in case of *Spodoptera litura* (Fabricius, 1775). The comparative ultrastructural details of the eggs of these two noctuid pest species have been summarized in Table 1 to simplify the similarities as well as differences between the two pests of same family. Through present investigations and based on studies carried out by Fehrenbach et al. (1987), on eggs of two noctuid lepidopteran pests namely, *Heliothis virescens* (Fabricius, 1777) and *Spodoptera littoralis* (Boisduval, 1833) we can conclude that the presence of ribs and ridges on the egg chorion as observed on all these species is a species-specific character and in present study too these are similarities between the two species when we consider the egg characters like shape of the polygonal cells, shape of the micropylar pit and number of micropyles.

Table 1. Comparative account of important ultrastructural egg characters investigated in the present study.

EGG FEATURES	STUDIED SPECIES	
	***Agrotis ipsilon* (Hufnagel, 1766)**	***Spodoptera litura* (Fabricius, 1775)**
EGG SHAPE	Spherical	Ellipsoid
EGG SIZE (DIAMETER)	0.573mm	0.439mm
CHORION TEXTURE	Highly Sculptured	Highly Sculptured
SHAPE OF POLYGONS	Rectangular & Pentagonal	Square & Rectangular
NUMBER OF AEROPYLES (PER POLYGONAL CELL)	04-06	04
NUMBER OF MICROPYLES	04	04
NO. OF PRIMARY PETALOID CELLS (PPC)	10 Petaloid	09 Petaloid
NO. OF SECONDARY PETALOID CELLS (SPC)	14 Petaloid	16
SHAPE OF MICROPYLAR PIT	Square-Shaped	Square-Shaped
TOTAL NUMBER OF RIDGES	35	42-44

In genus *Spodoptera* Guenée, Skudlik et al. (2005) carried out a detailed study on the eggshell ultrastructure of the eggs of *Spodoptera exigua* (Hübner, 1808). As far the shape of the egg is concerned, it is ellipsoid in *Spodoptera litura* (Fabricius, 1775) and spherical in *Spodoptera exigua* (Hübner, [1808]). In micropylar rosettes, 11 primary petaloid cells are involved in the arrangement in *Spodoptera exigua* (Hübner, [1808]) whereas nine primary petaloid cells form this arrangement in *Spodoptera litura* (Fabricius, 1775). They observed only single micropyle or micropylar opening in *Spodoptera exigua* (Hübner, 1808) and 4 micropylar openings are present in the present examined species i.e., *Spodoptera litura* (Fabricius, 1775). In the present study, the general chorionic surface is observed with same pattern of polygons and ridges giving an appearance of honeycomb as found in *Spodoptera exigua* (Hübner, [1808]).

For identification and differentiation of discrete taxa, superficial morphological attributes such as general coloration, ornamentation of the head, thorax, and abdomen, wing maculation, wing venation, and particularly, male, and female genitalic features are traditionally used. Based on current and relevant previous important works, it is easy to conclude that the ultrastructural features are also noteworthy and can authenticate and strengthen the morpho-taxonomy. Additionally, these types of investigations can be carried out in regard to pest management programs and crop protection from potential pests such as both the currently studied species as well as other species which are harmful agricultural pests.

Spodoptera litura (Fabricius, 1775) is a pest that is highly polyphagous (EPPO, 1979). According to a CABI (2018) factsheet, larvae consume at least 120 plant species. Shekhawat et al. (2018) cite literature indicating that *Spodoptera litura* (Fabricius, 1775) feeds on 180 host species, whereas Shu et al. (2017) cite literature indicating that *Spodoptera litura* (Fabricius, 1775) feeds on 389 hosts. Hosts can be found in at least 40 plant families. In contrast, the black cutworm *Agrotis ipsilon* (Hufnagel, 1766) is widespread in many temperate and subtropical regions, where it is a major pest of field crops, vegetables, and grasses. These moths frequently lay their eggs on weeds near crop fields, after which the larvae move to the plants, cutting off seedlings and destroying them. Almost all vegetable plants, as well as alfalfa, clover, cotton, rice, and others, are preferred crops (Capinera, 2009). So, the proper egg chorion studies and then the identification keys based on the egg characters will allow us to identify these types of harmful pests at the early egg stage and the exact pest management programs can be formulated for them before the larval stages hatch and attacks the crop fields and so that, crop damage can be reduced to much lower levels.

It is justified to mention here again that these SEM inspections of the eggshells of these moth species will prove to be valuable approach when it comes to identification of moths at earlier stage of their life-histories i.e., egg. Such investigations can be helpful regarding discrimination of different taxa at family, generic or species levels and even for resolving out species complexes.

In current study, as discussed above significant differences were observed in egg chorions of both the species. These findings will surely help in recognizing species and improving morpho-taxonomy at the immature stage. Tables 1 summarize significant observations from present study.

At last, on basis of these investigations this study can surely set a solid foundation and contribute a suitable model for carrying out such investigations on Indian moths by upcoming researchers in field of taxonomy.

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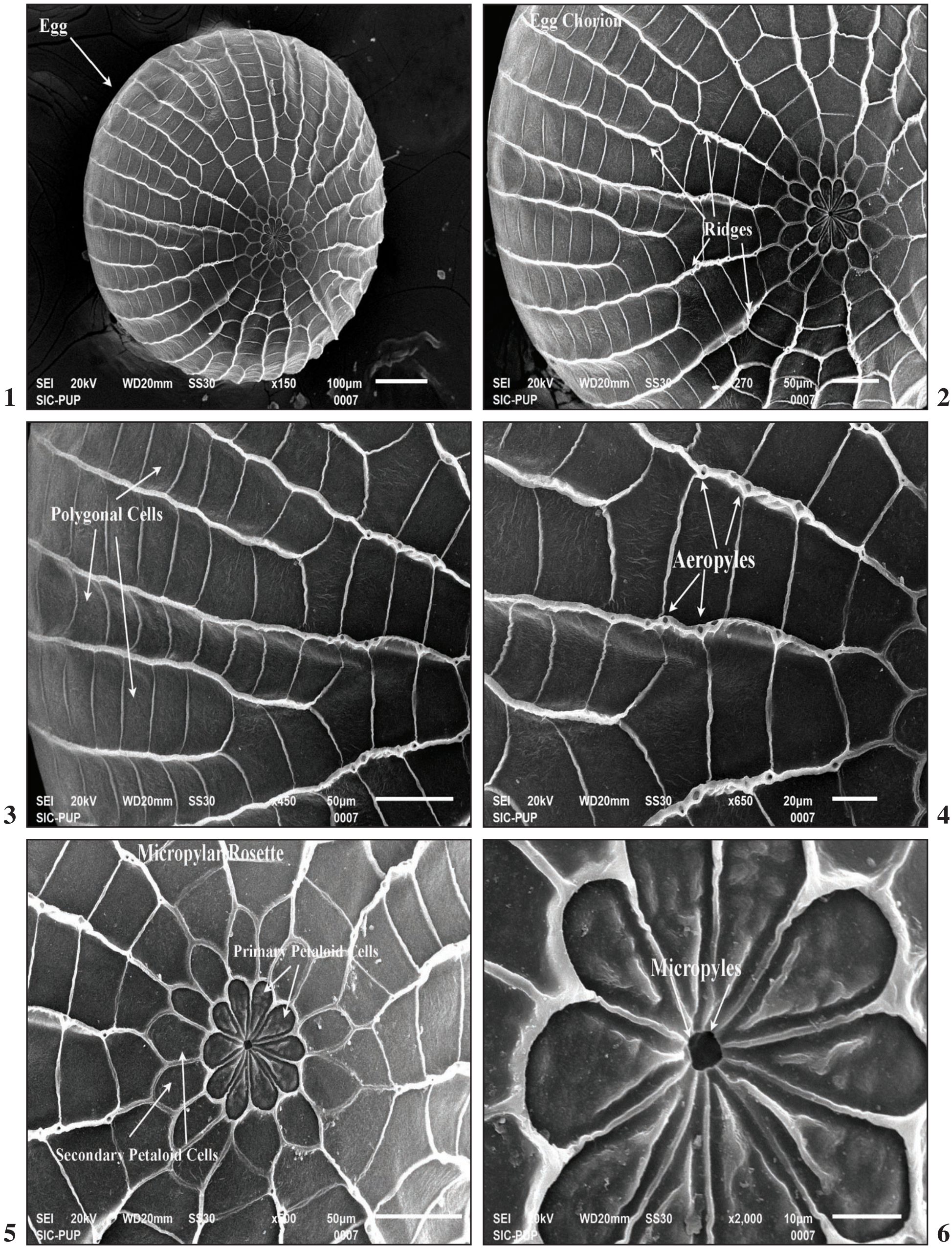
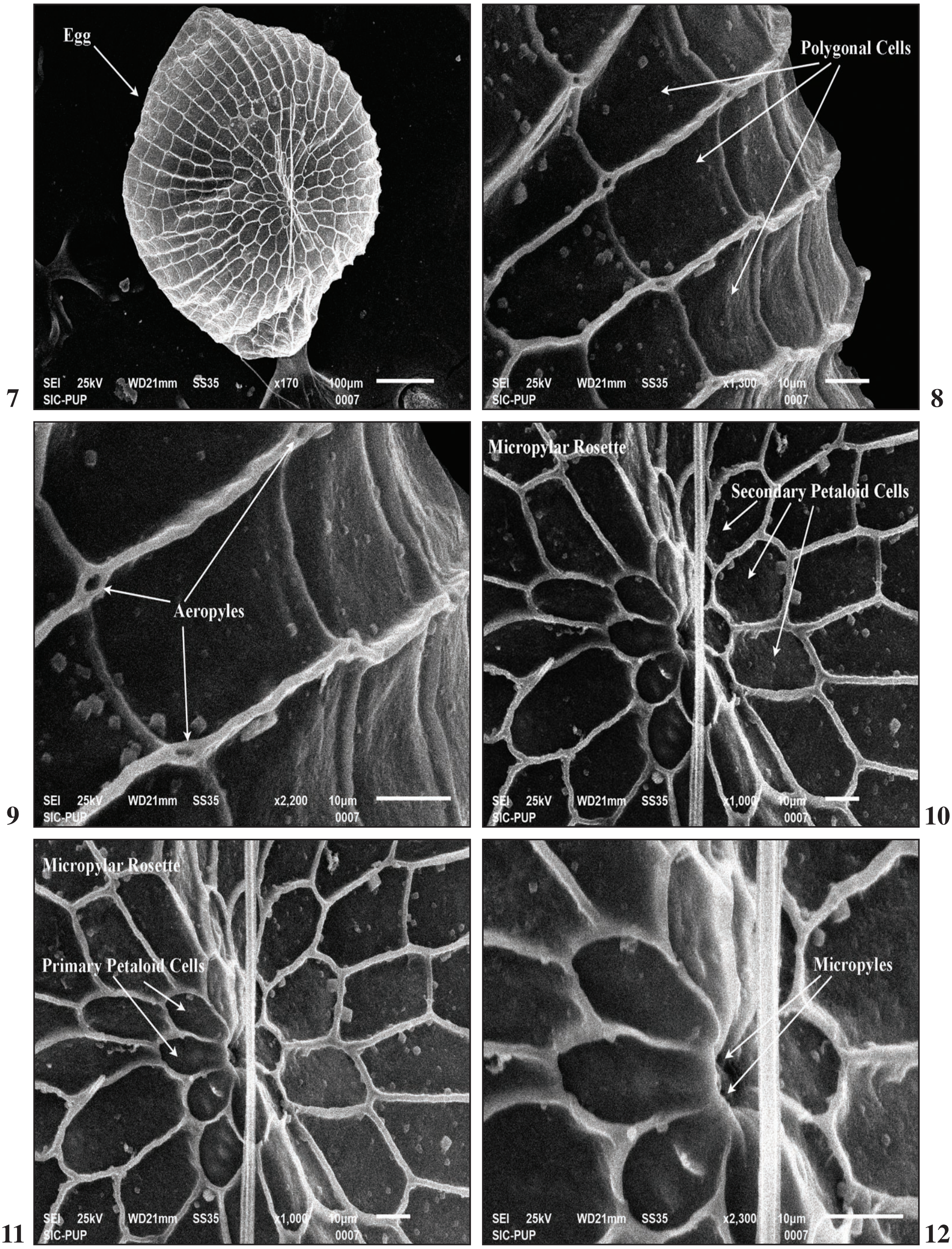


Figures 1-6. Egg of *Agrotis ipsilon* (Hufnagel, 1766): **1.** Dorsal view (Egg). **2-3.** Egg Chrorion. **4.** Aeropyles. 5-6. Micropylar Region.



Figures 7-12. Egg of *Spodoptera litura* (Fabeicius, 1755): **7.** Dorsal view (Egg). **8.** Egg Chrorion. **9.** Aeropyles. **10-12.** Micropylar Region.