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Ultrastructural Changes in the Ovariole of *Isophya nervosa* Ramme, 1931 (Orthoptera: Tettigoniidae) and Egg Morphology

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Abstract

This study was conducted to assess the morphology of eggs and histology of the ovaries in female *Isophya nervosa* Ramme, 1931 (Orthoptera: Tettigoniidae). While the egg morphology of *I. nervosa* was studied and examined by a stereomicroscope, a light microscope, and a scanning electron microscope, respectively, the morphology and histology of the ovary of this species were studied and examined by a stereomicroscope, a light microscope, a scanning electron microscope, and a transmission electron microscope, respectively. We found that the adult female had two pairs of ovaries, lateral oviduct, common oviduct, and spermatheca. Morphological study of the ovariole revealed that it is categorized under panoistic type of ovariole which is divided into three regions, the terminal filament, the germarium, and the vitellarium. We also observed that the eggs in *I. nervosa* have an ellipsoidal shape and are brown in color. Three different layers such as extrachorion, exochorion, and endochorion were observed. When the egg morphology is examined, it is understood that the surface pattern of the egg and the features of the micropylar areas may be distinguishing characters at the subfamily level, in addition to known classical taxonomic characters.

Key words: female reproductive system, insect histology, scanning electron microscopy, staining, transmission electron microscopy

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Introduction

Orthopteran species are one of the groups with great taxonomic diversity. They are also considered as a marker group in biogeographical studies (Çıplak, 2004; Chobanov et al., 2017). *Isophya* is the second richest bush-cricket genus in the Orthoptera order, and most of the species are also endemic in the Balkans and Anatolia (Chobanov et al., 2017).

Isophya nervosa Ramme, 1931 (Orthoptera: Tettigoniidae) is a species that is widely distributed in and around the Anatolia region including Ankara, Kırşehir, Eskişehir, Kütahya, Çankırı, Karabük, Bolu, Sinop, and Kastamonu (Ünal, 2010; Mol et al., 2016). There are many studies on the taxonomy and systematics of this species (Ünal, 2005, 2010; Grzywacz et al., 2014; Mol et al., 2016; Chobanov et al., 2017). However, we could not find any publications related to the internal morphology of the species, to the best of our knowledge. We know that apart from the qualitative and quantitative distinction of taxonomic characters, the classification may be made by examining different characteristics such as internal and external morphology. Based on this idea, this study aimed to illuminate the ovariole structure of *I. nervosa*, which is a part of the female reproductive system, to indicate the changes that occur in the follicle development, and to reveal the characteristics of the mature egg.

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Studies related to the female reproductive system of insects reveal interspecific differences in the size and structure of the reproductive organs. Hence, the internal anatomy and morphology of insect provide important characters for their systematic position. It also helps to expand our knowledge of the reproductive biology of species (Miller, 2001; Opitz, 2003; Church et al., 2019, 2021).

In this paper, the ultrastructure of the ovariole and egg morphology in *I. nervosa* will be described. The similarities and the differences will be explained by comparing them with other closely related species.

Materials and Methods

Insects

Collecting adult individuals of *I. nervosa* by using a sweep net have been carried out in Kızılcahamam, Ankara Province, Turkey during 2017–2018. Insects were brought to the laboratory in small plastic containers (Size 5 lit.). Individuals of *I. nervosa* were maintained in the laboratory at temperatures ranging from 20 to 25°C for approximately one week before dissections. Female individuals of adult *I. nervosa* were maintained on a diet of oak leaves. The female reproductive system was removed in a physiological solution (sodium phosphate buffer, pH 7.2). The species were placed on the dissecting tray ventral side up. The exoskeleton's ventral side was cut from the head to the posterior end of the abdomen by using scissors. The cut sides were pulled apart and each side of the insect was pinned to the dissecting pan. While removing the female reproductive system, the

female genitalia at the end of the abdomen was held with forceps and whole female reproductive system was lifted up forward. Finally, the photos were obtained using a stereomicroscope (Olympus SZX7, Tokyo/Japan). Approximately 20 female individuals were used for light and electron microscopy studies. While all experiments for light microscope were carried out in the Microtechnique Laboratory, located in Gazi University Faculty of Science in Ankara Province, Turkey; all experiments for electron microscope were carried out in the Prof. Dr. Zekiye Suludere Electron Microscope Center, located in Gazi University Faculty of Science in Ankara Province, Turkey.

Light Microscopy Methods

The entire reproductive system and collected eggs were fixed for at least 24 h in a 10% formaldehyde solution. After fixation, the samples were transferred to tap water for 24 h, and thereafter dehydrated in an ascending ethanol series (50–70–80–90–100%, 1 h each). They were then embedded in paraffin. Serial sections of 5 and 7 μm were obtained using a Microm HM 310 microtome (Walldorf/Germany). The samples were stained with hematoxylin and eosin (H&E) (Avwioro, 2011) and Mallory's trichrome stain techniques (Mallory, 1900). The stained sections were examined using an Olympus BX51 light microscope (LM) (Tokyo, Japan) and photographed using an Olympus E330 camera (Tokyo, Japan). This method was used to obtain histological descriptions.

Scanning Electron Microscopy Methods

Extracted samples of *I. nervosa* were also used for scanning electron microscopy (SEM). Tissues were fixed in 5% glutaraldehyde for 24 h. The samples were rinsed three times with sodium phosphate buffer (pH 7.2) and then dehydrated in an ascending ethanol series (70–100%). The samples were dried using a critical point dryer (Polaron CPD 7501) (East Grinstead, London, UK) using CO_2. The samples placed on the stubs were coated with gold using the Polaron SC 502 (Uckfield, UK) coating device and examined using a JEOL JSM 6060 LV SEM (Tokyo/Japan) at 5–10 kV.

Transmission Electron Microscopy Methods

Other extracted samples of *I. nervosa* were used for transmission electron microscopy (TEM). They were fixed in 5% glutaraldehyde for 24 h. Subsequently, the samples were rinsed three times with sodium phosphate buffer (pH 7.2), post-fixed in 1% osmium tetroxide, dehydrated in an ascending ethanol series (70–100%), and then embedded in Araldite resin. Ultrathin sections were cut using Leica EM UC6 ultramicrotome (Wien, Austria) and contrasted with 1% uranyl acetate (prepared in water) and lead citrate (prepared in water) (Reynolds, 1963). The material was photographed in a JEOL JEM 1400 TEM (Tokyo, Japan) operating at 80 kV.

Results

Ultrastructure of the Ovariole

The general anatomy of the female reproductive system in *I. nervosa* consists of a pair of ovaries, the lateral oviducts to which the ovaries are attached, the common oviduct, and the spermatheca (Figs. 1a–1c) at the level of the two to six abdominal segments. The female reproductive structures are located dorsally to the alimentary canal.

The female reproductive system in *I. nervosa* has approximately 10–15 ovarioles in each ovary. In the ovary, while the color of the immature eggs is yellow (Fig. 1b), the mature ones look brown (Fig. 1c). Each ovariole terminates distally with a thin and slightly elastic filament called terminal filaments (Figs. 1a, 1b). Each ovariole is composed of a number of follicles. The follicle maturation along the length of the ovarioles is usually based on yolk deposition. Follicle maturation begins when the accumulation of yolk begins (Raikhel & Dhadialla, 1992; Swevers et al., 2005). Each follicle is separated from the previous and next follicle with interfollicular tissue (Figs. 2a, 2b).

The ovariole is divided into three regions, the terminal filament, the germarium, and the vitellarium (Fig. 1a). We determined the ovariole type as panoistic, as the germarium contains oogonia and primary oocytes (Wilde & Loof, 1973) and all germ cells differentiate into oocytes without nurse cells (Küpper et al., 2019). In the germarium region, there are oogonia with

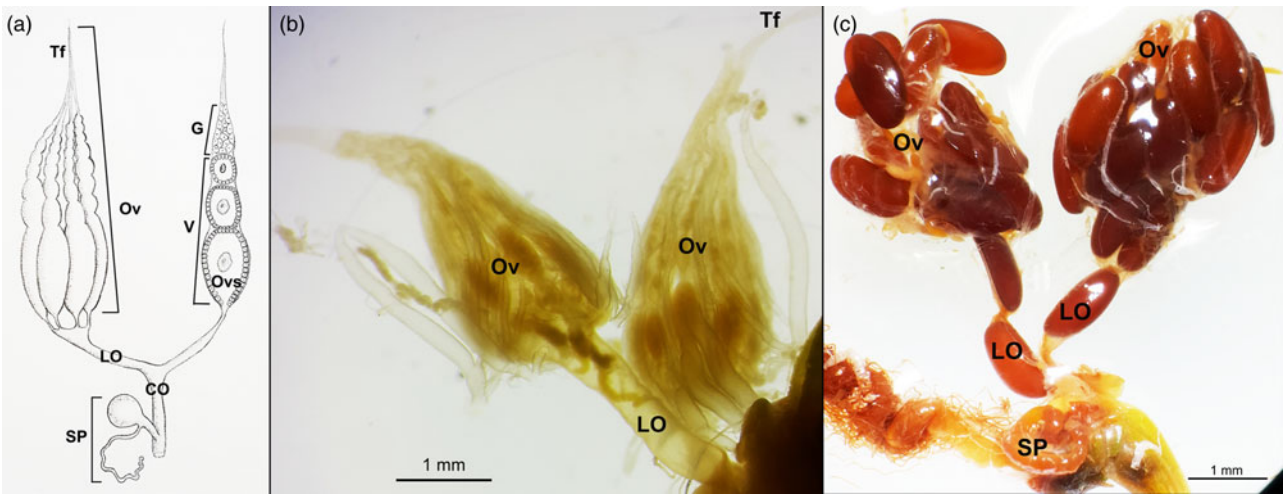

Fig. 1. (**a**) Schematic dorsal view of the female reproductive system in *I. nervosa* (illustrated by Damla Amutkan Mutlu). (**b,c**) Female reproductive system of *I. nervosa*. Tf, Terminal filament; Ov, ovary; LO, lateral oviduct; CO, common oviduct; G, germarium; V, vitellarium; Ovs, ovarioles; SP, spermatheca (Stereomicroscope images, Scale bar = 1 mm).

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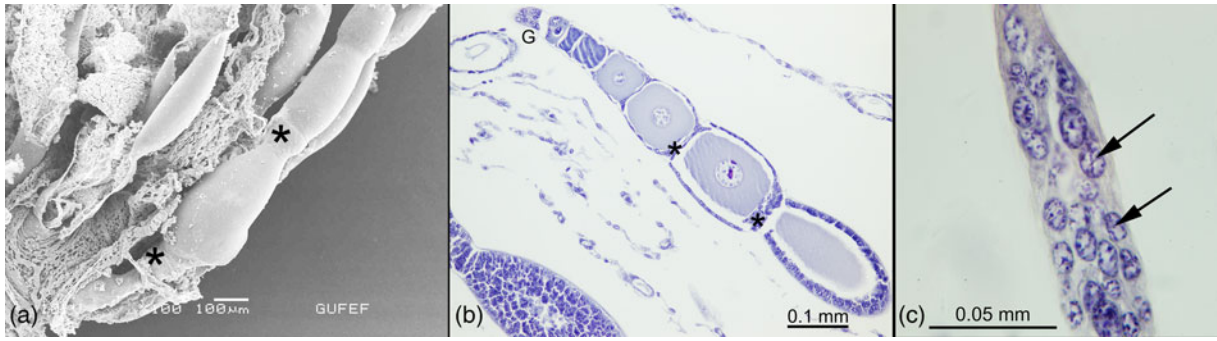

Fig. 2. (**a**) Scanning electron microscope image of the ovariole (scale bar = 100 μm). (**b**) Longitudinal section of the ovariole (LM, H&E, Scale bar = 0.1 mm). (**c**) High magnification of the germarium region (LM, H&E, Scale bar = 0.05 mm). G, Germarium; →, Oogonia; *, interfollicular tissue.

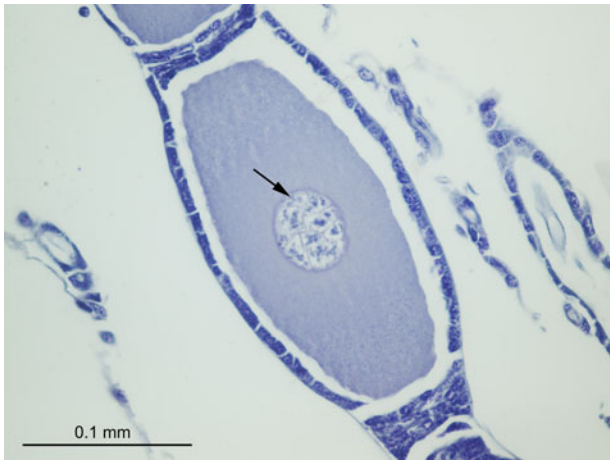

Fig. 3. Longitudinal section of the ovariole. Germinal vesicle (→) in the vitellarium was enveloped by a monolayer of follicular epithelial cells (LM, H&E, Scale bar = 0.1 mm).

many round nuclei that can undergo mitosis (Fig. 2c). Oogonia later transform into young oocytes. Young oocytes are replaced by mature oocytes while they progress throughout the ovary (along the vitellarium region) (Amutkan Mutlu, 2021; Amutkan Mutlu et al., 2021). Follicular epithelial cells initially show a single-layered cubic epithelium, but as follicle maturation continues, they begin to thicken (Figs. 3–8). Germinal vesicles (oocyte nuclei) are located in the center of the follicles during developmental stages (Figs. 6b, 8a). In the TEM images, enlargements are observed in the perinuclear space between the inner membrane and the outer membrane of the nuclei of the follicle epithelial cells in the vitellarium regions (Figs. 7, 8b).

Egg Morphology

In *I. nervosa*, the eggs that have completed their development are released to the external environment via the oviduct after they are fecundated with spermatozoa from the spermatheca. The oviduct serves as a delivery tube for mature eggs ovulated from ovaries to egg-laying sites. The mature eggs are released out with contractions of muscles in the oviduct walls in *I. nervosa* as other insects (Klowden, 2009). We did not observe any differences in egg morphology of those found in the oviduct and after oviposition.

The egg shape is ellipsoidal and bilaterally symmetrical, flattened toward the anterior pole, and the color is brown (Figs. 9a, 9b). The size of the egg was measured from a total of 25 eggs obtained from approximately 20 insects used in the study. It is approximately 3.61 ± 0.03 mm long and 1.25 ± 0.04 mm in diameter. There is a rib structure extending from the anterior pole to the posterior pole of the egg. Cracks occurred on the surface of the egg during drying. The micropyle region is anteriorly located on the egg (Figs. 9a, 9b). There are 8–12 micropyles, somewhat distributed circularly around the anterior end of the egg (Fig. 10a). It is seem that it is rather raised with respect to the egg surface. The proximal end of the micropyle is narrower than the distal one. The micropylar orifices are 5.85 ± 0.03 μm in diameter and are clearly seen at the proximal end of the

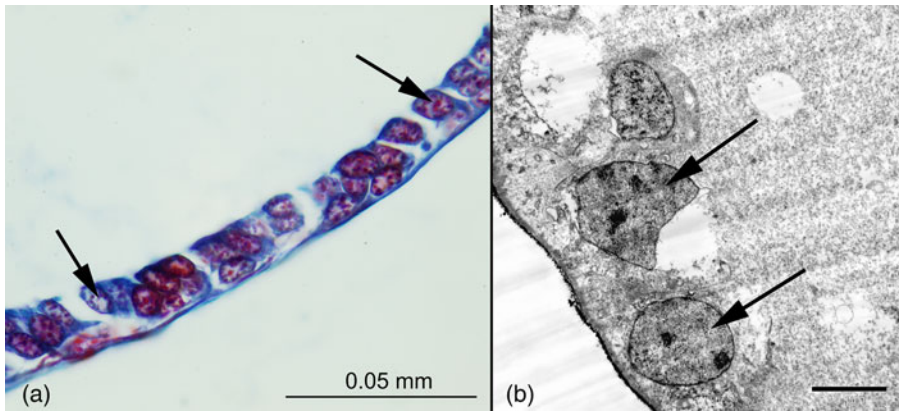

Fig. 4. Nuclei (→) of cells in the vitellarium were enveloped by a monolayer of follicular epithelial cells (**a**) LM, Mallory's trichrome. (**b**) TEM (Scale bar = 5 μm).

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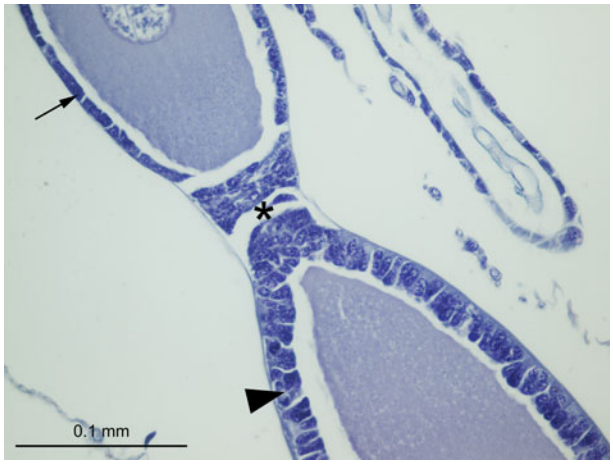

Fig. 5. Follicular epithelial cells that begin to thicken (►) in the vitellarium. Monolayer follicular epithelial cells (→) and interfollicular tissue (*) (LM, H&E, Scale bar = 0.1 mm).

micropyle (Fig. 10b). In SEM examinations, it is clearly seen that the pattern on the surface of the egg is polygonal or pentagonal in appearance. The lengths of the polygon sides are not equal (Figs. 11a, 11b). In the mature egg surface of *I. nervosa*, the surface pattern was clearly observed, and tubular air channels (aeropiles) are observed in some broken parts of the egg. There are exit spaces of air channels on the polygonal patterns. These air channels are associated with round-oval patterns of different sizes evident on the egg surface (Figs. 12, 13). The eggshell consists of three different layers. One of these layers is the outer extrachorion, the middle layer is columnar exochorion layer, and the other is the dense inner endochorion (Figs. 12, 13; Hartley, 1961; Viscuso et al., 1990; Yilmaz et al., 2012; Amutkan Mutlu et al., 2022). The columns of the exochorion have a height of approximately 3.23 ± 0.02 μm and a diameter of 1.25 ± 0.02 μm, and they have a sponge-like structure that is composed of small pores (Fig. 13). Pores in an average of 30 columns in the exochorion layer were counted and approximately 35 pores were observed on each column. The middle of these columns also contain air channels and these columns are directly connected to exit spaces of air channels on the polygonal patterns (Fig. 13). The endochorion has many thin air channels (Fig. 13).

Discussion

Ovaries, one of the parts of the female reproductive system, vary in number, shape, and location in the abdomen in most insect

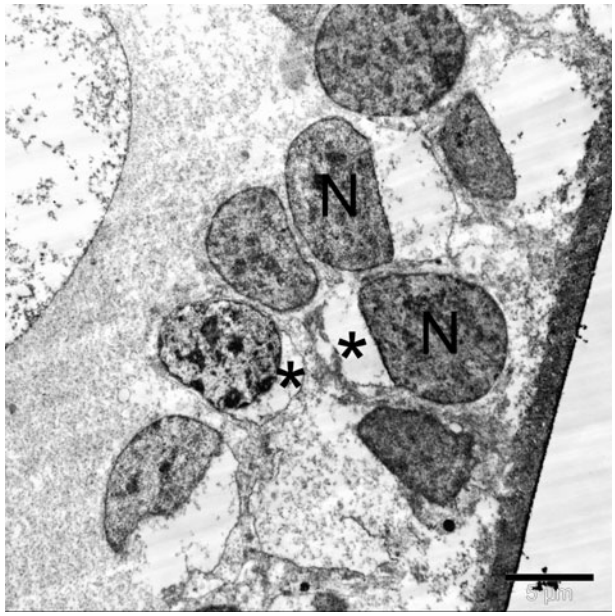

Fig. 7. The follicular epithelial cells that begin to thicken in the vitellarium. Enlargements (*) between the inner and outer membrane of the nucleus (N) (TEM, Scale bar = 5 μm).

species (Heming, 2018). Its location in the abdomen can be changed depending on the maturity of the female. It helps us locate the insect's reproductive system when it is dissected. In some studies, the positions of the reproductive systems of mature females in the abdominal cavity are indicated as follows. Ovaries extend around from the first to sixth abdominal segment in *Orthetrum sabina sabina* (Drury, 1770) (Anisoptera, Libellulidae) (Verma & Andrew, 2016). In *Pantala flavescens* (Fabricius, 1798) (Odonata, Libellulidae), ovaries extend around from the first to fifth abdominal segment (Prasad & Srivastava, 1961; Verma & Andrew, 2016). In *Baeacris punctulatus* (Thunberg, 1824) (Orthoptera, Acrididae), ovaries are around from the second to fourth abdominal segment (Michel & Terán, 2017). The ovaries in *I. nervosa* are approximately from the second to sixth abdominal segment. It can be said that the reproductive organs of the insects which are mentioned in this paper are at a medio-ventral position in the abdominal cavity.

The number of ovarioles in each ovary varies depending on the Orthoptera order (Chapman, 2013; Heming, 2018). When the different families belonging to the Orthoptera order are investigated, it is observed that there are about 5 to 10 ovarioles in species of

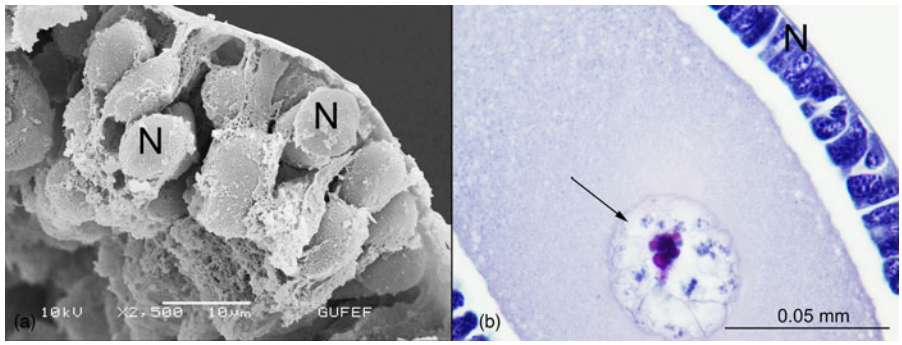

Fig. 6. The nuclei (N) in follicular epithelial cells that begin to thicken in the vitellarium. Germinal vesicle (→). (**a**) SEM (Scale bar = 10 μm). (**b**) LM, H&E (Scale bar = 0.05 mm).

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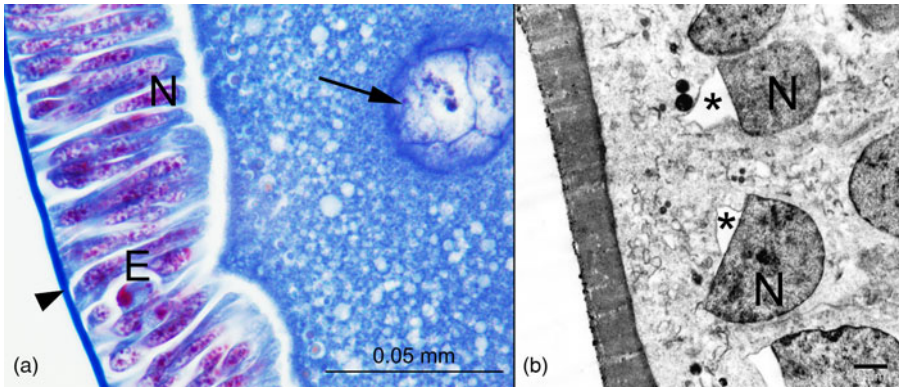

Fig. 8. The follicular epithelial cells (E) that are quite thickened in the vitellarium. Basal lamina (►), nucleus (N), germinal vesicle (→), enlargements (*) between the inner and outer membrane of the nucleus. (**a**) LM, Mallory's trichrome (Scale bar = 0.05 mm). (**b**) TEM (Scale bar = 2 μm).

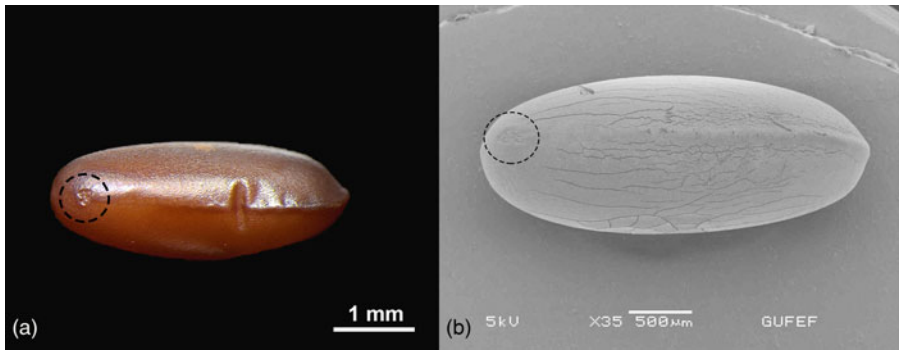

Fig. 9. The mature egg in *I. nervosa.* Micropylar region (◌). (**a**) Stereomicroscope image (Scale bar = 1 mm). (**b**) SEM (Scale bar = 500 μm).

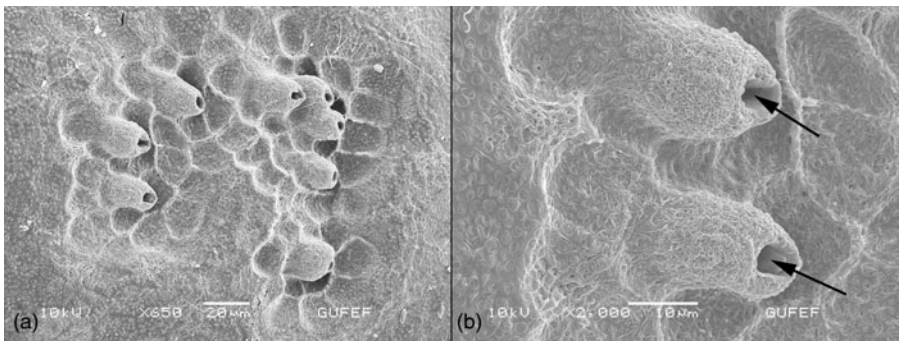

Fig. 10. (**a**) Scanning electron microscope image of the micropylar region of the mature egg (Scale bar = 20 μm). (**b**) High magnification of the micropylar orifices (→) (SEM, Scale bar = 10 μm).

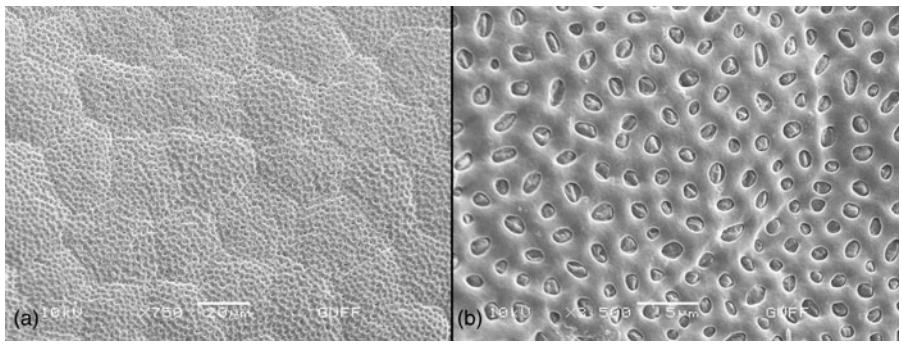

Fig. 11. (**a**) Scanning electron microscope image of the surface pattern of the mature eggs in *I. nervosa* (Scale bar = 20 μm). (**b**) High magnification of the surface pattern (SEM, Scale bar = 5 μm).

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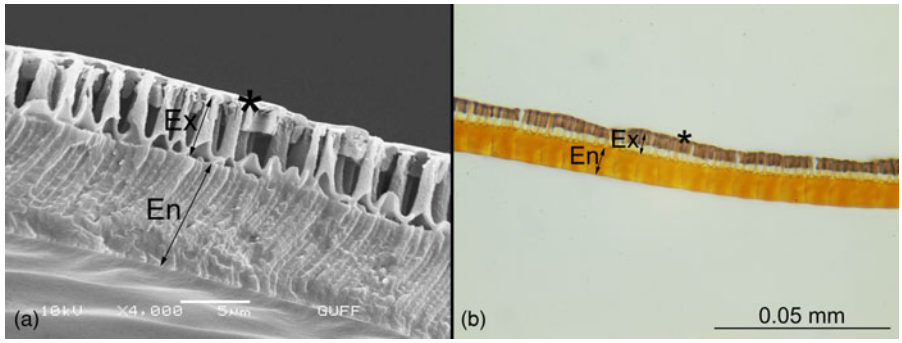

Fig. 12. (**a**) The cross-section of the eggshell. *, Extrachorion, Ex, exochorion, and En, endochorion layers. (**a**) SEM (Scale bar = 5 μm). (**b**) LM, Mallory's trichrome (Scale bar = 0.05 mm).

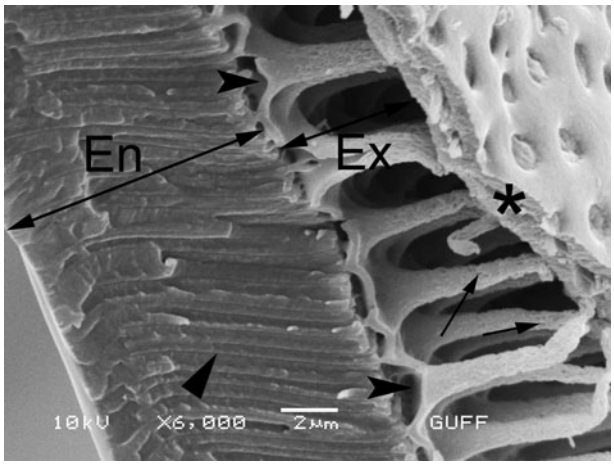

Fig. 13. Scanning electron microscope image of the cross-section of the eggshell. Extrachorion (*), exochorion (Ex) that have sponge-like structure that has small pores (→) and air channel (➤) inside the columns in the exochorion layer, and endochorion (En) layers with thin air channel (►) (Scale bar = 2 μm).

the Acrididae family (Chapman, 2013; Leather & Hardie, 2018; Amutkan Mutlu, 2021). This number varies from approximately 15 to 30 in those species belonging to the Tettigoniidae family. Species in the Gryllidae family possess between 150 and 170 ovarioles (Leather & Hardie, 2018). In *B. punctulatus* (Orthoptera, Acrididae), approximately 10 ovarioles were identified in each ovary (Michel & Terán, 2017). The ovary of *Pseudochorthippus parallelus parallelus* (Zetterstedt, 1821) (Orthoptera, Acrididae) have about 10–12 ovarioles (Amutkan Mutlu, 2021). *Metrioptera roeselii* (Hagenbach, 1822) (Orthoptera, Tettigoniidae) has approximately 25 ovarioles (Marrable, 1980). The number of ovarioles ranges from 60 to 64 in *Segestidea novaeguineae* (Brancsik, 1897) (Orthoptera, Tettigoniidae) (Solulu et al., 1998). 15–20 ovarioles were seen in *Poecilimon cervus* (Orthoptera, Tettigoniidae) (Polat, 2016), and this number varied from 9 to 12 ovarioles in *Poecilimon ataturki* Ünal, 1999 (Orthoptera, Tettigoniidae) (Amutkan Mutlu et al., 2021). Similarly, in *I. nervosa*, there are approximately 10–15 ovarioles in each ovary. Ovariole number is helpful in quantifying the reproductive potential in insects. The number of eggs laid is equal to the number of ovarioles or less than it (Bellinger & Pienkowski, 1985). It was also indicated that the variation in ovariole number is due to body size (Bellinger & Pienkowski, 1985). It has been stated that this change is related to the taxonomic position of the species within the order (Chapman, 2013).

Morphologically, some insects' species are reported to have four regions of ovariole including the terminal filament, the germarium, the vitellarium, and the stalk or calyx (Yamany, 2012; Mohamed et al., 2015). In *I. nervosa*, only three regions are distinguished including the terminal filament, the germarium, and the vitellarium, which was mentioned by Michel & Terán (2017), Amutkan Mutlu (2021), and Amutkan Mutlu et al. (2021).

While the follicular epithelial cells are cubic at the beginning of oocyte growth, during advanced vitellogenesis, they transform into cylindrical in *I. nervosa*. We observed that there was a perinuclear space enlargement in TEM images (Figs. 7, 8b).

The micropylar area is generally found at the posterior pole of the egg in some Tettigonidae species such as *Cyrtaspis scutata* (Charpentier, 1825), *Antaxius hispanicus* (Bolívar, 1887), *Decticus verrucivorus* (Linnaeus, 1758), *Phaneroptera nana* (Fieber, 1853), *Lluciapomaresius ortegai* (Pantel, 1896), *Lluciapomaresius panteli* (Navás, 1899), *Parasteropleurus perezii* (Bolívar, 1877), and *Ephippiger diurnus cunii* (Bolívar, 1877) (Sas et al., 2017). In *I. nervosa*, the micropylar area is posteriorly located on the egg. When compared to the previously studied species in the way of the location of the micropyles on the egg surface, it appears similar.

When the egg surface patterns are examined, the surface of the egg in *E. diurnus cunii* (Bolívar, 1877) have hexagonal and pentagonal follicular cells pattern. Nonetheless, follicular cells form hexagonal pattern that cover all the surface of the egg in *P. perezii* (Bolívar, 1877), *L. panteli* (Navás, 1899), and *L. ortegai* (Pantel, 1896) (Sas et al., 2017). The eggs of *P. cervus* have polygonal patterns in patches on their surface (Yilmaz et al., 2012). Despite that, no pattern was found on the egg surface in *P. nana* (Fieber, 1853) (Sas et al., 2017). When compared, *I. nervosa* with a polygonal, mostly pentagonal pattern on the egg surface is different from these previously studied species.

Insect eggshells are generally composed of an outer chorion and an inner vitelline membrane. The chorion is the thickest of all egg envelopes and is generally distinguished from different layers (Viscuso et al., 1990). For example, the chorion of the *Eyprepocnemis plorans* (Charpentier, 1825) (Orthoptera, Acrididae) egg consists of two layers: the exochorion and the endochorion. *Romalea microptera* Serville, 1831 (Orthoptera, Romaleidae) has two layers as the exochorion and the endochorion in eggshell (Hartley, 1961). Similarly, the eggshell of *Tettigonia viridissoima* (Linnaeus 1758) (Orthoptera, Tettigoniidae) has two layers. The wall of the egg in *Locusta migratoria* (Linnaeus, 1758) (Orthoptera, Acrididae) is reported as three layers: the extrachorion, the exochorion, and the endochorion (Hartley, 1961). These three layers were also identified for eggs of *P. cervus* (Orthoptera,

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Tettigoniidae) (Yilmaz et al., 2012). When compared previously studied species, the eggshell of *I. nervosa* has three different layers.

Conclusion

The egg morphology and the structure and morphology of the ovary, which is a part of the female reproductive organs in *I. nervosa*, are described in detail. Similarities and differences with other species were revealed. It is understood that the surface pattern of the egg and the features of the micropylar areas may be distinguishing characters at the subfamily level, at least when the species compared in this study are considered, in addition to the known classical taxonomic characters.

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Conflict of interest. The authors declare that they have no competing interest.

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