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Chorion is a complex structure of protein and polysaccharide – a microscopical study in *Oxya hyla hyla* (Orthoptera: Acrididae) (Serville, 1831)

A. Shyam Roy, D. Ghosh

ABSTRACT
Oxya hyla hyla is a paddy field grasshopper and a major pest of rice. Here an attempt has been made to analyze and reveal the chemical composition with regard to protein and sugar moiety of chorion by microscopical techniques. From these observations using light and TE Microscopy it has come out that polysaccharide and protein were key ingredients of chorion. These components might be involved in chorion hardening and the amount of these components increased with the maturity of the chorion.

Keywords: *Oxya hyla hyla*, chorion, polysaccharide, protein, TEM.

1. Introduction
Insect eggs are characterized by eggshell or chorion, secreted by follicular epithelium which provides mechanical protection to the developing embryo [12]. Eggshell or chorion is a complex structure and composed of proteinaceous and organic molecules [15]. Earlier light microscopic studies on Orthoptera chorion were done by some researchers [5, 17, 18]. A light microscopic study of eggshell surfaces of 10 Indian Acrididae was undertaken by earlier researcher [9]. Secretion of the chorion layer by the follicle cells was established in different insects by TEM studies [10, 14]. Proteinaceous features of chorion was also established earlier [8]. The architecture of chorion is mainly due to presence of proteinaceous components [4]. Polysaccharides were also described as a key chemical present in *Lygus* [2, 11, 13] and in *Drosophila*. In Orthoptera, such study about the chemical composition of chorion are very few. Therefore the objective of the present study was to document the chemical composition mainly proteinaceous and polysaccharide compounds in *Oxya hyla hyla* chorion.

2. Materials and Methods
2.1 Egg collection:
Oxya hyla hyla (paddy grasshopper) were collected from the paddy fields in and around Agartala city. Mature eggs were dissected out and collected from the mature ovarian follicle, oviduct and after laying eggs were collected just after laying of the eggs before pod formation. These eggs were cleaned in 100 mM Tris-HCl buffer (pH-8) with brush.

2.2 Light microscopy
The ripe ovaries were dissected out by needle and forceps and kept in Ringer's solution. For living cell analysis dissected ovaries taken in ringer's solution were directly observed under LEICA Microscope (DM 1000) and subsequently photographs were taken.
For histochemical studies the dissected ovaries were placed in 4% Formaldehyde in a watch glass overnight for fixation. After fixation ovaries were transferred to distilled water and kept overnight for complete removal of fixative. Then ovaries were transferred in graded series of alcohol (30, 50, 70 and 90%) for one hour in each grade and in 100% for overnight to dehydrate. The dehydrated tissues were cleared in xylene and then transferred in xylene-paraffin mixture (60 ^{0}C) for overnight for diffusion of paraffin. The tissues were then transferred to full paraffin for two hours at 60 ^{0}C. Then embedding was done in full paraffin and allowed the paraffin to get solid. The prepared blocks were sectioned at 5 µm thickness by using a LEICA rotary microtome (LEICA RM 2125RT). Sectioned tissues were de-paraffinized with xylene and after re-hydration (100, 90, 70% and distilled water) stained with bromophenol blue and PAS method. Stained

sections were viewed under LEICA Microscope (DM 1000) and subsequently photographs were taken.

2.3. Transmission electron microscopy
For transmission electron microscopy eggs were fixed in 4% glutareldehyde for four hours then washed in 100 mM phosphate buffer (pH 7.2). The fixed eggs were dehydrated in graded series of ethanol (30, 50, 70, 90, 95 and 100%). The dehydrated eggs were passed through araldite CY212 embedding medium at 60 ^{0}C for 24 h. Sections were made by ultramicrotome of RMC. Sections were stained with uranyl acetate and lead citrate [13] and subsequently examined by JEOL 2100 Transmission Electron Microscope and photographs were taken.

3. Results and Discussion
3.1. Secretion of chorion from follicle cells
In follicle cell stage living cells of living egg of *O. hyla hyla*, it was clearly observed that the chorionic layer started forming and depositing beneath the covering of follicle cell layer. In grasshoppers it was found that the chorion was secreted by follicle cells [5]. From these pictures the follicle cell layer was also observed clearly. In case of *O. hyla hyla* the thickness of the chorionic layer varied from approximately 14.3 µm to 73.7 µm (Fig: 1). Highest thickness was found at the terminal end of posterior pole and lowest thickness was found at the anterior pole. At anterior pole and other part of the egg chorion had almost similar thickness. From these observations it appeared that in this stage, chorionic layer just had started to form and the formation and the thickness of the layer was not uniform throughout the egg covering. Thickness of depositing chorion at this preliminary stage of formation was different among anterior and posterior pole of this grasshopper's eggshell.

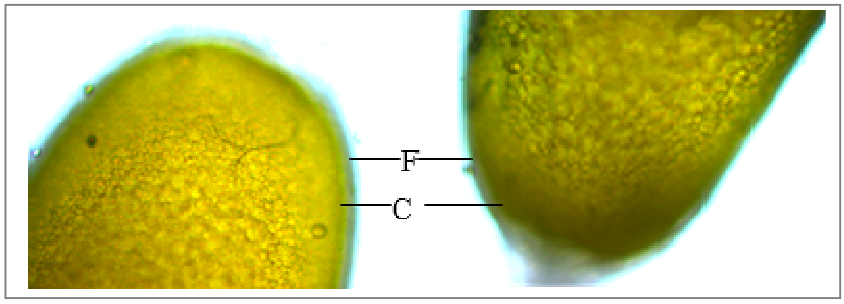


Fig 1: Bright field analysis of *O. hyla hyla* follicle stage egg in 10X magnification. Follicle cell layer (F), Chorion (C).

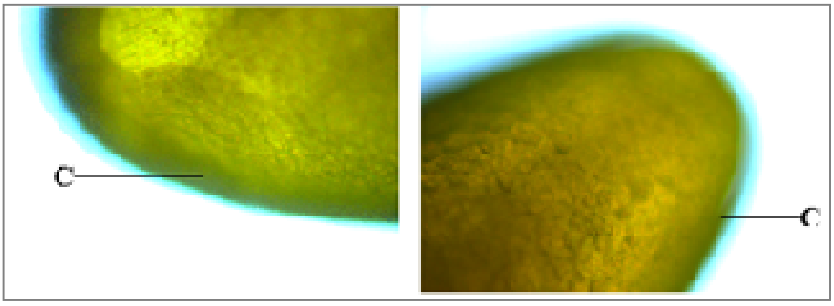
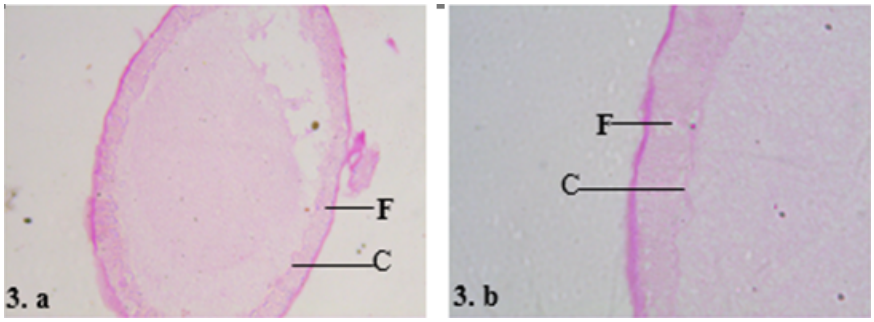


Fig 2: Bright field analysis of *O. hyla hyla* oviduct stage egg in 10X magnification. Chorion (C).

From living analysis of the oviduct stage egg of *O. hyla hyla* it was observed that at this stage thick chorion layer was formed. In *Oxya* the thickness of the chorion ranged from 14.2µm to 137 µm (Fig: 2). The highest thickness of the chorion layer was observed in the posterior pole. Same feature was also revealed by in other Acridid eggs [6]. Lowest thickness was found in the anterior pole. The thickness of the chorion was discontinuous and this feature was continued throughout the whole egg surface. With this preliminary observation and little sensitivity of the bright field microscopical technique it was inferred that the formation of chorion might had started from posterior pole or rate of secretion of chorionic layer by follicle cells was higher in this portion.



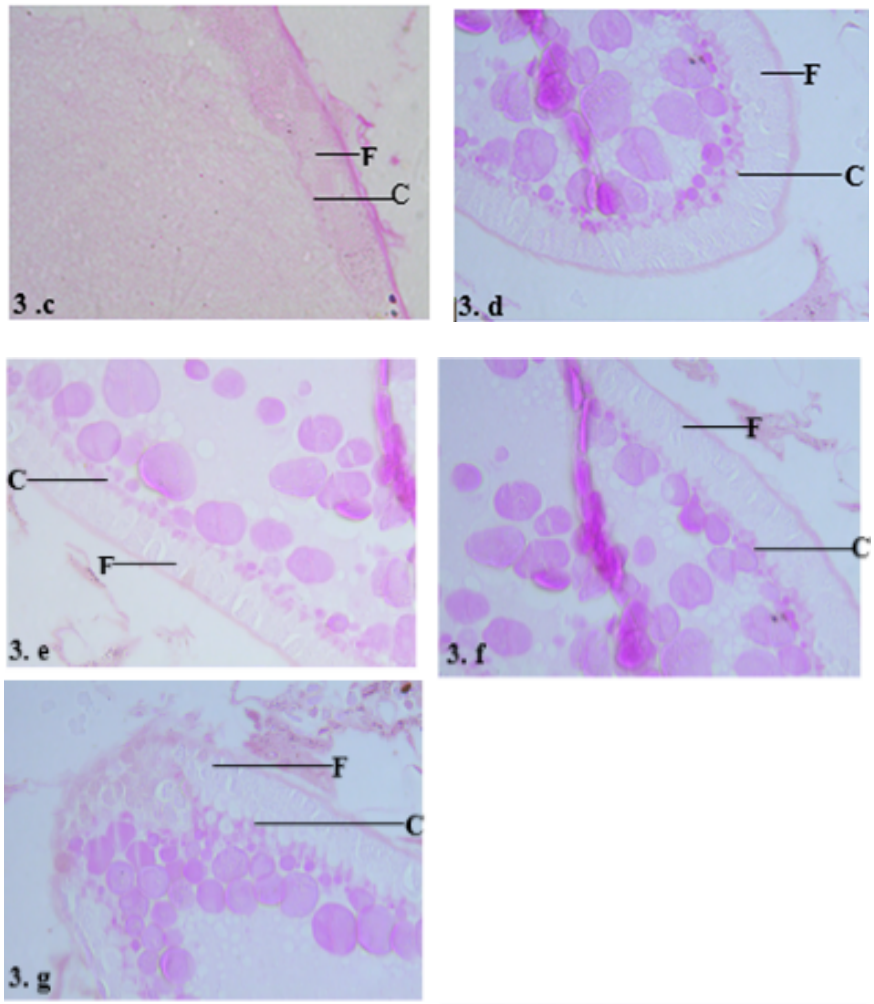
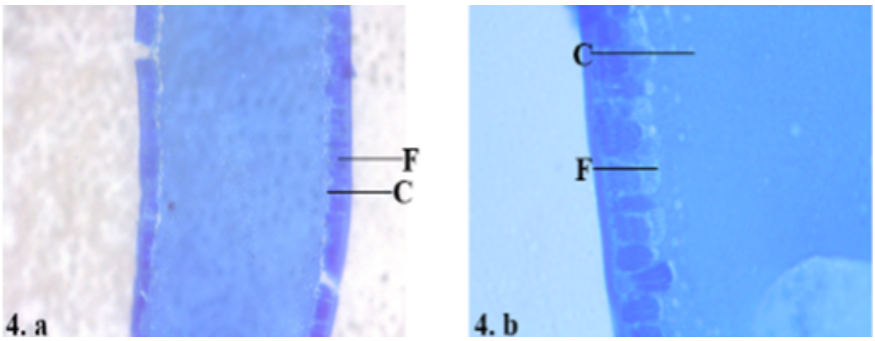


Fig 3: Photographs of PAS positive reaction of the section of *O. hyla hyla* oocyte taken in bright field. Follicle cells (F), Chorion (C) at different stages of growth. (Fig: 3. a in 40X magnification and Fig: 3.b- 3.g in 100X magnification).

When sectioned terminal oocyte was subjected to staining by PAS reaction and viewed under bright field microscope, it was observed that the chorion layer was intensely PAS positive and showed a pink colour. The follicular epithelium was altogether negative for PAS reaction.

In *O. hyla hyla* immature oocyte was slightly PAS positive, showing faint pink color. One layer of follicle cells was found and the thickness was 81.9 µm to111 µm (Fig: 3. a-c) and initial chorionic secretion was observed. In matured follicular stage egg follicle, cells were 2 to 3 layers of follicle and the thickness of the layer was 109 µm to 296 µm. Single follicle cells had 109 µm to 127 µm thickness and total thickness of follicular cell layer was 115 µm to 285 µm (Fig: 3. d-g). The chorionic secretion (C) had PAS positive reaction and showed pink colour. This result suggested that the chorion contained carbohydrate moiety which was PAS positive and suggested its histochemical nature like cuticle.

Chorion proteins have been analyzed in *Drosophila* and shown that eggshells contained a good amount of amino sugars [16]. So, it may be inferred from our present finding that the chorion of *Oxya* had either amino sugar or other sugars which were present and positive for PAS reaction. It was also evident from the results that the consistency of chorion was somewhat like cuticular protein which also contained a good amount of sugar moiety [1].



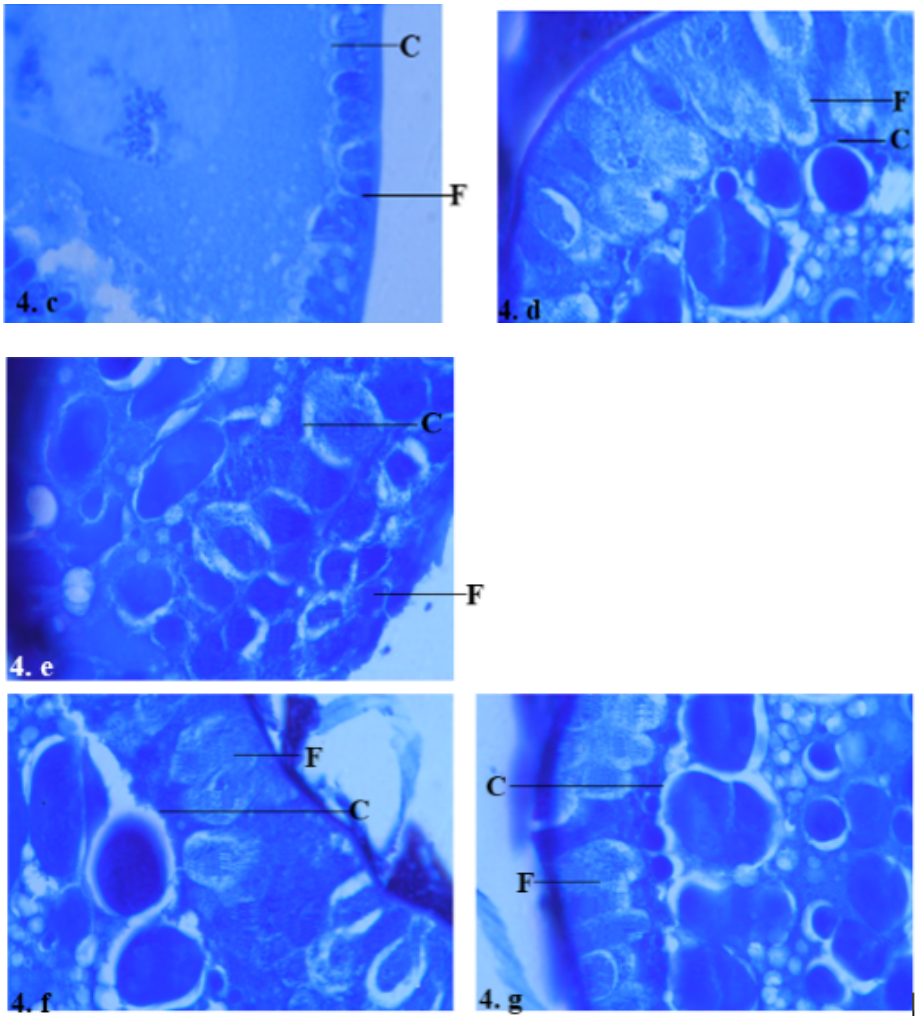


Fig 4: Photographs of Mercurry- bromopheol blue stain and section of *O. hyla hyla* oocyte taken in bright field. Follicle cells (F), Chorion (C). (Fig: 4.a in 40X magnification and Fig: 4.b- 4.g in 100X magnification).

Mercurry- bromophenol blue staining was done for observing the presence of proteinaceous compound of the egg of different maturation stages. It was observed that the whole section of matured egg stained deep blue and on the other hand it was comparatively light blue for the sections of immature eggs by this method.

Immature egg of *O. hyla hyla* showed a single cell layer of follicle cell, in some portions it was observed to be bilayered. Total thickness of the follicle cell layer was approximately 183 µm -375 µm in every side of the egg section (Fig: 4.a-c). The length of each follicle cell varied from approximately 76.2 µm – 309 µm. Little chorionic secretion was found in this stage. In comparatively matured eggs, follicle cell layer was three to four cells thick in every portion of the section and the thickness varied from 415 µm - 733 µm. Four to five layers of follicle cells were found at the posterior end. The length of each follicle cell varies from 120 µm – 408 µm. At this stage of maturity, it was observed that the secretion of chorionic layer (C) took deep blue stain and had begun to form discontinuously beneath the follicular cell layer (Fig: 4.d-g).

Staining was not homogenous for both insect eggs. The follicular cells showed uniform staining reaction with bromophenol blue in early stage of maturation and in mature one follicle cells showed slight variation. The chorion secretion at the periphery of the follicular cells took deep blue staining at the mature egg but this feature was minimum in case of immature egg. Development of grasshoppers egg have been studied with eosin-haematoxylin staining in earlier research [18]. This result strongly suggested proteinaceous nature of chorion and it also indicated that the formation of chorion started from the follicle cells when it was a single layer, but when the follicle cells became three to five layers thick maximum maturity in size and chorionic secretion took place.

3.2. TEM Analysis

Follicular stage of the egg for electron microscopic studies in case of *O. hyla hyla* were taken at the late stage of follicle development prior to entering into the oviduct. Here four distinct layers vitelline membrane (vm, ca. 0.36 µm), interchorionic layer (icl, ca. 0.27-0.36 µm), air layers (al, ca. 0.35-0.45 µm) and almost fully formed chorionic layer (Fig: 5) were observed. Same feature was also found in *Lygus* [13]. Here electron dense granules became coarser in the air layer which resembled with the findings of the light microscopic observation about the initiation of formation of polysaccharide components and proteinaceous structures. Inner most chorionic layer was also almost fully formed.

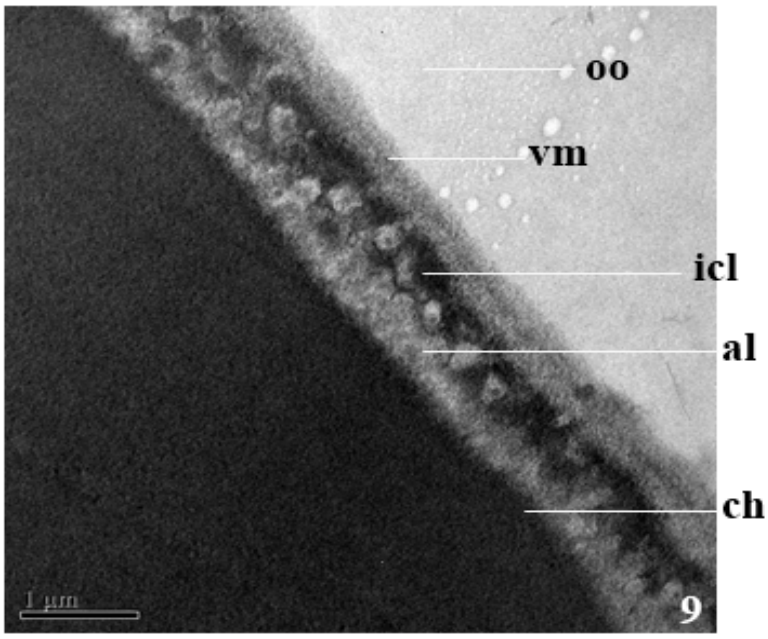


Fig 5: Transmission electron microscopic of follicle stage chorion with five layers in *O. hyla hyla.* Outer chorionic layer (ch), inter chorionic layer (icl), vitelline membrane (vm), Air layer (al), oocyte (oo). Scale bar= 1µm.

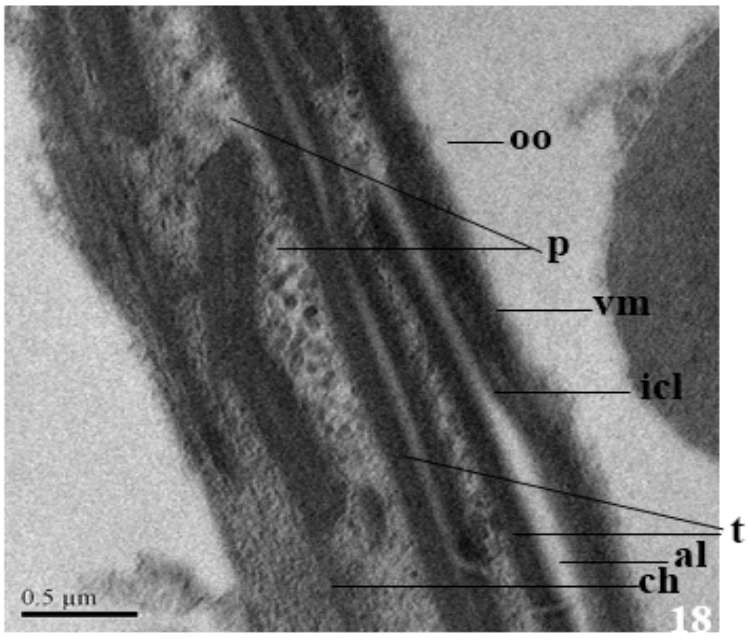


Fig 6: Oviduct stage of chorion maturation of *O. hyla hyla*. . Outer chorionic layer (ch), inter chorionic layer (icl), vitelline membrane (vm), Air layer (al), oocyte (oo), thread like structures (t), polysaccharide droplets (p). Scale bar= 0.5 µm.

In *O. hyla hyla* oviduct egg stage four layers were present the vitelline membrane (vm, ca. 0.1 µm), inner most chorionic layer (icl, ca. 0.05 µm), air layer (al, ca. 0.1 µm-0.3 µm), outer chorionic layer (ch, ca. 1.35 µm). Droplets of polysaccharides (p) were also present with protein deposition as thread like structures (t). Polysaccharide droplets were present in high amount at this stage. The length and the width of these thread like structures were 2.1 µm – 3.5 µm and 0.1 µm – 0.2 µm respectively (Fig.6).

In after laying stage, four layers were also present, the vitelline membrane (vm, ca. 0.18 µm), innermost chorionic layer (icl, ca. 0.18 µm), outer chorionic layer (ch, ca. 3.64 µm-4.55 µm) and air layer but the thickness was almost negligible (Fig:7). Outer chorionic layer became very much rigid with the condensation of the proteinaceous structures. Amount of polysaccharides droplets became little bit less at this stage and this might be because of condensation of polysaccharides with the protein substances. It could be inferred from this that the condensation of these protein and polysaccharide compound had taken part in the hardening of the chorion.

In the present study, in chorion of *O. hyla hyla*, wax layer was absent. Wax layer was also absent in eggshell of *Blattella germanica* [7]. In some other insects like *Acheta* and *Schistocerca*

(Orthoptera) there was also no wax layer [3, 10]. This characteristics could be considered as a unique feature of Orthopteran eggshell. Modifications of the thread like structures of proteins had occurred and that might be due to intercalation with droplets of polysaccharides. Presence of such phenomena was observed in development of *Lygus* chorion [13]. The dimensions of these proteinaceous structures in *O. hyla hyla* were about 1.82 µm-10.91 µm in length and 0.18 µm -0.55 µm in width. Space between these protein fibers were also observed. Thus from the results it may be inferred that in Orthoptera, specially in grasshopper, chorion is made up of large amount of protein substances and polysaccharide residues.

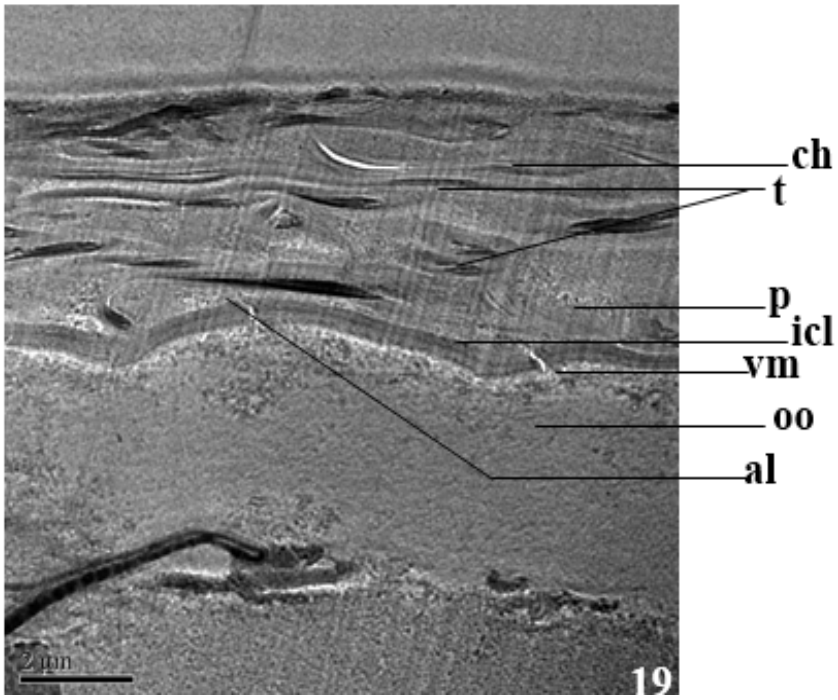


Fig 7: After laying stage of chorion maturation of *O. hyla hyla*. Outer chorionic layer (ch), inter chorionic layer (icl), vitelline membrane (vm), Air layer (al), oocyte (oo), thread like structures (t), polysaccharide droplets (p). Scale bar= 2µm.

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