

NEW ASPECTS OF ULTRASTRUCTURAL STUDIES ON GILL FILAMENTS AND SPIRACLE DEVELOPMENT OF *RANA RIDIBUNDA* TADPOLESGamal A. Bekhet^{1,2*}¹Department of Zoology, Faculty of Science, University of Alexandria, 21511, Egypt²Department of Biology, Faculty of Science, King Faisal University, P.O. Box 1759 Al Hufuf 31982 Al Hassa, Saudi Arabia

ABSTRACT: The pattern and sequences of development of the gill apparatus and operculum, in the marsh frog *Rana ridibunda* via different stages have been analyzed and studied using scanning electron microscopy. The external gills (or transient gills) of the *Rana ridibunda* tadpole (stage 18) develops as two bulges followed by formation of upper and lower folds (stage 20). The ultra structural organization of the gill filaments undergoes, conspicuous variations in number and shape. Otherwise, variation in the types density and orientation of epidermal ciliated cells on the gill filaments. The number of filaments is 3+1, 3+2, 5+3, 6+4 and 8+5 at stages 21, 22, 23, 24 and 25 respectively. The shape is either flattened, or finger like -structure. Ciliation are of three types, ciliated cells (CC), microvillated cells (MV) and microridgid "pavement" cells (MR). The new findings in our result are elongated flattened or rounded extensions on epidermal filament surface and transverse cellular bridge connect some microridged cells (MR). Ciliation is few, intermediate, dense or very dense depending on the developmental larval stages. The orientation of the ciliated cells is at right angles or parallel to the long axis of filaments. The external gills are completely hidden from the right to the left side and have largely atrophied followed by formation of internal gills (stages 23-26). The transient gills covered by operculum shifted to the left resulting in an asymmetrical sinistral spiracle that migrated medially, covered with mucus then become empty at premetamorphic stages.

Novelty: Novel structural elements identified during gill development and regression in the marsh frog *Rana ridibunda* that consist of finger like- protrusions, distributed on the epidermal surface of the secondary gill filaments and located between ciliated and microridged cells (Figure 6). We suggest these are microfilaments rebranched from the secondary filament to increase the respiratory surface areas. Also a cellular bridge observed above the microridged cells of epidermal surface of the gill filaments (Figure 7c arrow heads). Although, Orton (1953) categorized anuran, s larvae depending on the spiracular opening of the operculum as anuran tadpoles with spiracle in a median position or in some tadpoles the spiracle in a lateral position (sinistral) others with paired spiracles as pipidae and lepidobatrachus, discoglossidae, bufonidae and microhyla orientalis ranidae and otophryne. While in *Rana ridibunda* tadpoles of the present study ,we found the two positions , firstly a lateral position (sinistral) in early larval stages (Figure 9a-d). Then it migrated into a median spiracle in late stages (Figure 9 e-f). Tadpoles larvae of *Rana ridibunda*.

Key words: *Rana ridibunda* -External Gills –Operculum-Spiracle –Internal Gills –microridges –Atrophy - Ciliation.

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INTRODUCTION

In the anuran tadpoles, the respiratory apparatus represented by the gills which are one of the major sites of gas exchange together with skin and operculum. The gills of Anuran tadpoles has been the subject of different studies relating to anatomy and gill ultrastructure in different species [1] larval gill morphology has also been used in studies of evolutionary relationships [2-7]. Moreover, others have studied gill apparatus in connection with blood circulation or with respect to different aspects involving pharyngeal organs. The respiratory gill apparatus of Amphibia Anura is characterized by two distinct moments of development, the terms external and internal gills do not reflect the actual positions in the embryo prior to the closing of the opercular fold. All gills and their anlagen are external, the terms outer and inner generally apply to Gosner 23, 24. Apart from their position relative to the branchial arch ,the fundamental differences lies in the fact that the internal gills persist, whereas the transient gills atrophy at the time of the closing the opercular fold. Viertel (1991) [8] considered the terms persistent and transient gills to be more exact than outer and inner gills. Persistent gills develop along the ventral and transient epidermal gills develop along the ventrolateral parts of branchial arch I-IV. The ultrastructural organization of the gills undergoes, during the course of larval development, conspicuous modifications that take it from a relatively simple organ to a more complex structure protected by an operculum. There are two kind of cells on the epidermal surface of the gill filaments of *Rana dalmatina* mainly microridged "pavement "cells and a few ciliated cells [5]. While Some authors [1,4,9,5,7] found three types of cells on the epidermal surface of gill filament mainly microvillated cells ,microridged cells and ciliated cells and the density of ciliation is few ,intermediate ,dense or very dense. The low temperature and high oxygen pressure stimulate the growth of shorter gills while the high temperature and high CO₂ pressure stimulate the longer gills [10-13].Gill regression begins when the operculum grows over the persistent gills . Although the regression of the internal gills of anurans under hormonal control is well known, the regulating loss of the external embryonic gills is not known, [14,15].The regression occurs either by lost or it can be resorbed. If it lost the gill filaments appeared as broken freely in the water whilst if it resorbed, the filaments size would decrease [16, 5] The resorption of gill are already known in some teleosts [17,18] According to physiological studies, the principal site of ionic exchange and equivocal is the gill chamber [19]. The presence of specialized cells in the transient gills of *R. cancrivora* adapted to different levels of [1] .So the morphological differences in the different species of Amphibia Anura related to environmental conditions. The regression of external gills depended on the larval stages. Metamorphic stages lost gill circulation and losed transient gills more rapidly than premetamorphic stages. In a stable environment the gradual loss of external gills is therefore not surprising. Orton' s categorized anuran' s larvae depending on the spiracular opening of the operculum and the cornified oral apparatus. The operculum develops as a fold that begins laterally and shortly extends across the ventrum . the operculum grows posteriorly for varying distances before fusion with the body wall fusion begins laterally and progresses medially leaving the spiracle in a median position in tadpoles in some tadpoles the spiracle in a lateral position (sinistral) others with paired spiracles as pipidae and lepidobatrachus , discoglossidae , bufonidae and microhyla orientalis ranidae and otophryne tadpoles [21-23] . The marsh frog, *Rana ridibunda*, has a widespread distribution in Kingdom Saudi Arabia. Although no report have been published on the ultrastructural development of the gill apparatus in *Rana ridibunda* and their ontogeny. Therefore the present study was undertaken to work out the ultrastructural development of the gill apparatus in the successive developmental stages of the tadpoles of marsh frog *Rana ridibunda*. The ultrastructural analysis of the morphology of *Rana ridibunda* during premetamorphic and metamorphic stages can be of great interest and value in the future studies of development of Ranidae in the context of ecology, phylogeny and evolution. The ontogeny based on the description of ultrastructure of external gills used for identification of heterochronic processes and origin of Ranidae larvae.

MATERIALS AND METHODS

Ribbons of fertilized eggs from couples of the available *Rana ridibunda* were collected from breeding sites from natural sites near the city of Al-Hassa (Easten region, K.S.A.) using a close-mesh net. The egg ribbon was collected in separate masses. All procedures for animal handling and tissue removal were carried out according to the recommendations of the Ethical Committee .Before hatching the desired stages were selected and examined .After hatching, the larvae were daily fed on a meal of boiled spinach daily, transported to the laboratory and kept in plastic bowls filled with water of the same pond where they are collected, containing some aquatic plants or boiled spanish. The water was renewed periodically every two days and kept at room temperature under a natural light/dark cycle until they reached the desired stages needed for experimental work, according to the normal table of [24].

The larval stages were used for the present study namely, stages number 18-27. Five larvae at each stage were selected for the experiment and examined under light microscope for selected the healthy specimens. For scanning electron microscope examination ,the specimens were fixed in a 2-3% Glutaraldehyde solution for 3-4 h at room temperature, then washed in 0.1 M phosphate buffer for three 15 min. Next, specimens were dehydrated in a graded Ethanol series as follows: 35%, 50%, 70%, 80%, 95%; three changes at 100% for 15 min each, and a final wash in acetone for 5 min. Specimens were dried in CO_2 , mounted on aluminium stubs and sputter coated with gold. Features of external and internal gill anatomy were examined and photographed using a scanning electron microscope (Jeol) attached to a computer. Terms used to describe features of the gill apparatus are derived from [2,3].

RESULTS

Development of the External Gills

The transient gills (external gills) of the *Rana ridibunda* tadpole, start as two bulges posterior to the adhesive organs(AG) and optic lobes at stage 18 .The two bulges by stage 20,elongated and branched into two folds, upper and lower folds (UF &LF) which originate the gill filaments (Fi). At stage 20,from each fold arise a primary filament (PFI) that rebranch to form secondary filaments(SFi) also three gill filaments originated on the upper fold and two on the lower fold (Figure 1a,b). In Figure 1a-c, scanning electron micrographs of the external gills development of *Rana ridibunda* during the early stages of ontogeny stage 20a- 5 external gill filaments arised from (3) from upper and (2) lower folds b- Enlargmed gill filaments showing branching of primary and secondary filaments and density of epidermal cells c-Epidermal gill filament surface showing the three types of cells Ciliated cells (CC) polygonal, Microvillated cells (MC) and Microridgid cells (MR).

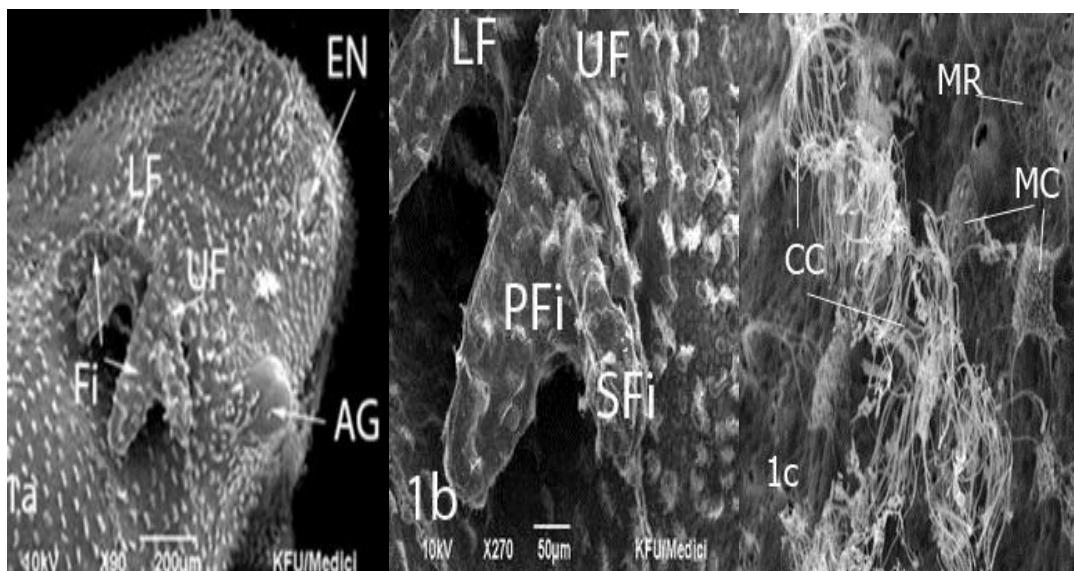


Fig-1

By observation the ultrastructural of the epidermal surface of gill filaments ,we examined the gill filaments shape is flattened or round and there is a variable lengths .The ciliation of the epidermal surface of gill filaments represented by three types of cells mainly ciliated calls(CC) which are very abundant characterized by their elongated cilia filled the whole cell, moderate microvillated cells (MV) showed polygonal structure and very fine cilia scattered all over the cell and few of microridgid "pavement" cells (MR) which are large cells devoid of obvious cells. The orientation of cilia of gill filament is at right angle to the long axis of filaments (Figure 1c).

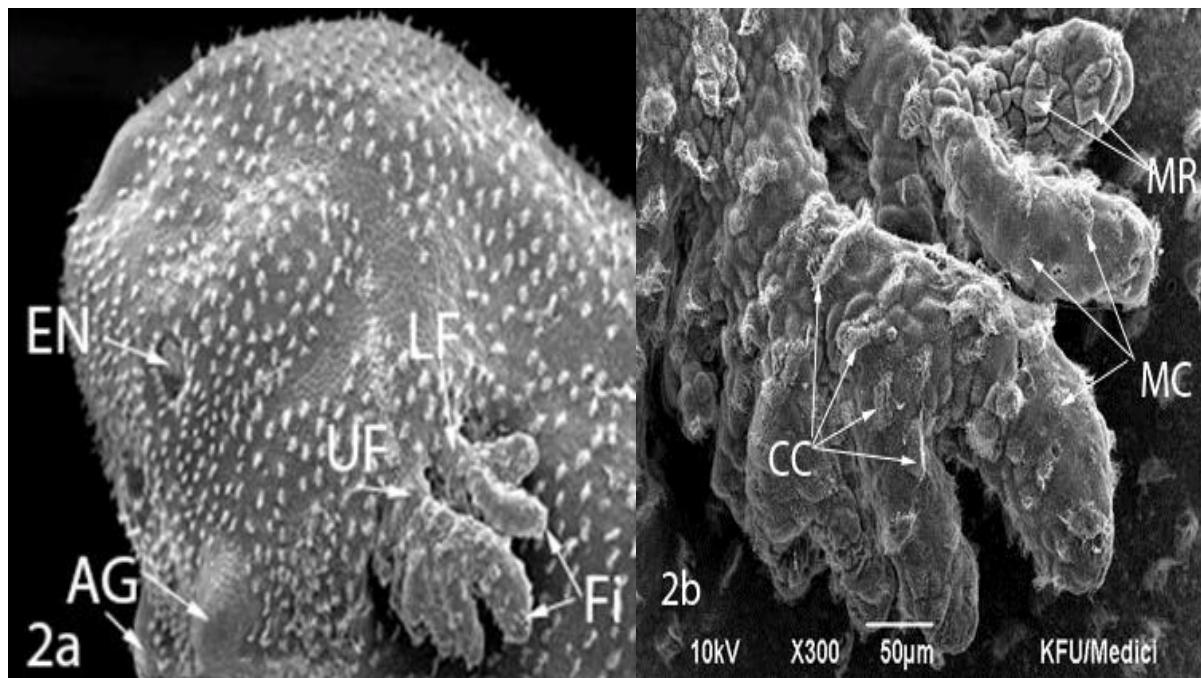


Figure 2a-b: (a) Scanning electron micrographs showing rounded filaments at stage 21, four on the upper fold and 2-3 short on lower fold. (b) Ciliation with relatively few (CC) and abundant of (MR) and moderate (MC).

At stage 21 (Figure 2a) there are four gill filaments arise from the upper fold and three from the lower fold, the epidermal surface occupying the same three types of cells as in stage 20 but the ciliation vary in the density, the microridged cells are very abundant than the ciliated cells and the orientation of cilia is parallel to the long axis of gill filament (Figure 2b).

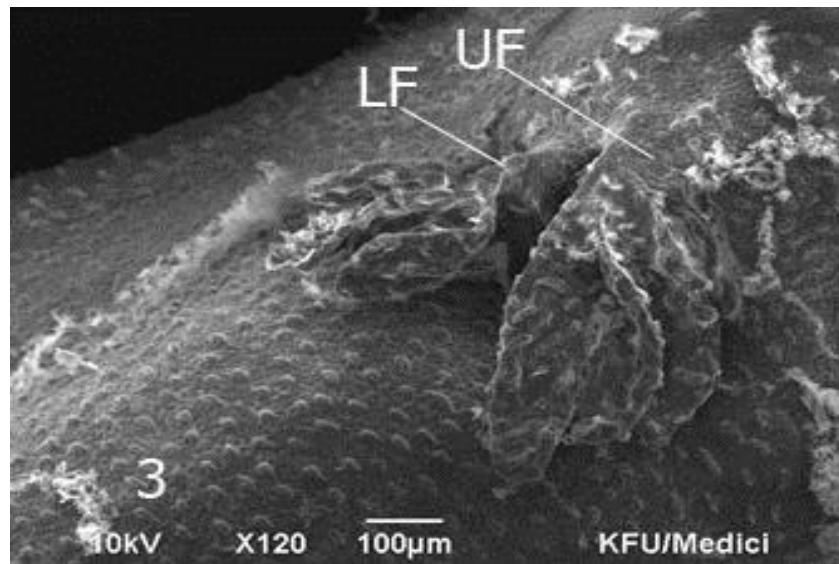


Figure 3: Scanning electron micrographs, Stage 22 with relatively elongated, flattened filaments FIVE on the upper fold and three on lower fold.

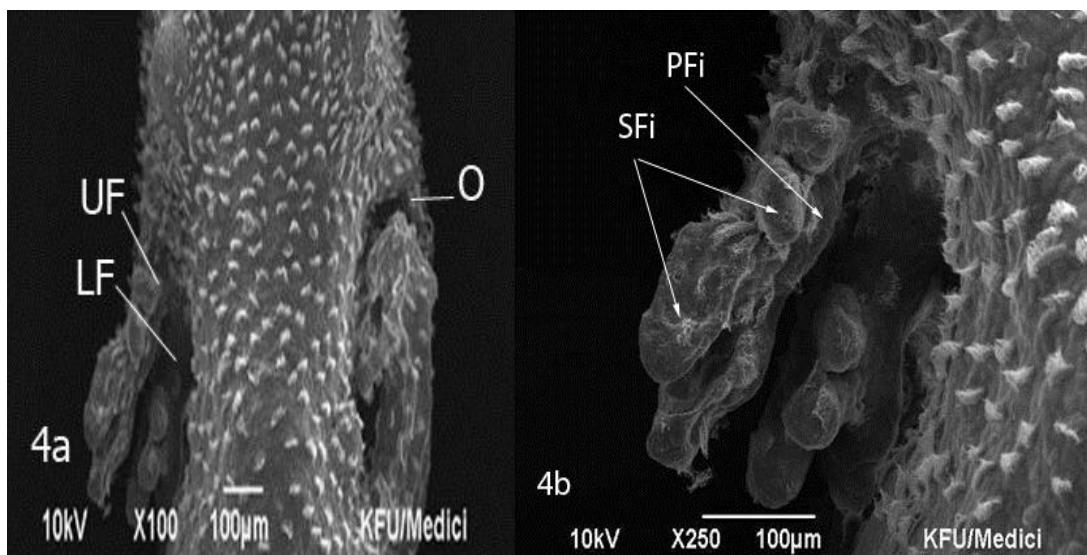


Figure 4a-b: (a) Scanning electron micrographs of the external gills, stage 23, are covered by operculum (O) (b) showing six elongated, rounded filaments on the upper fold and four on the lower fold, with intermediate ciliated cell density.

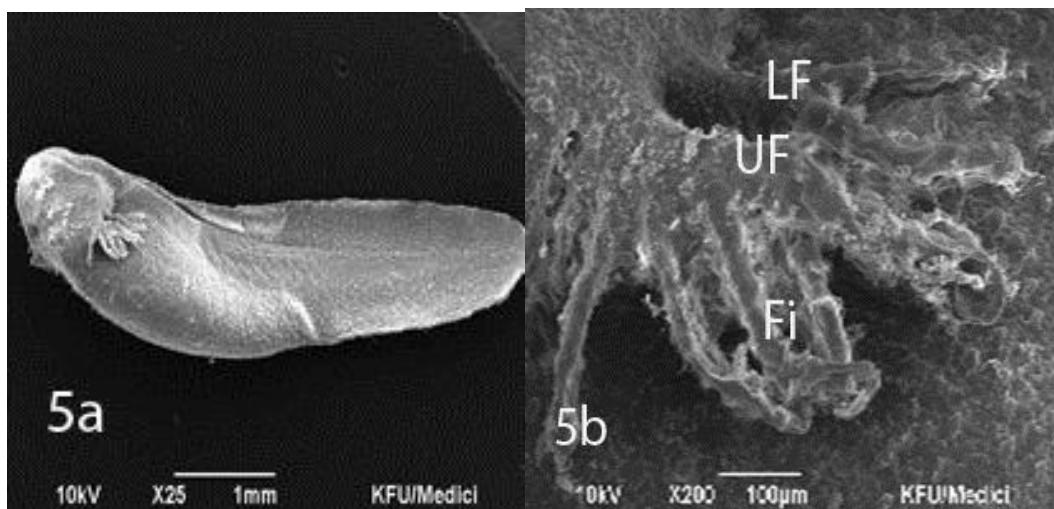


Figure 5a-b: Scanning electron micrographs (a) stage 24, the gills filaments with maximum length (b) Eight filaments (Fi) from the upper fold and five from the lower fold, with marginal dense ciliation.

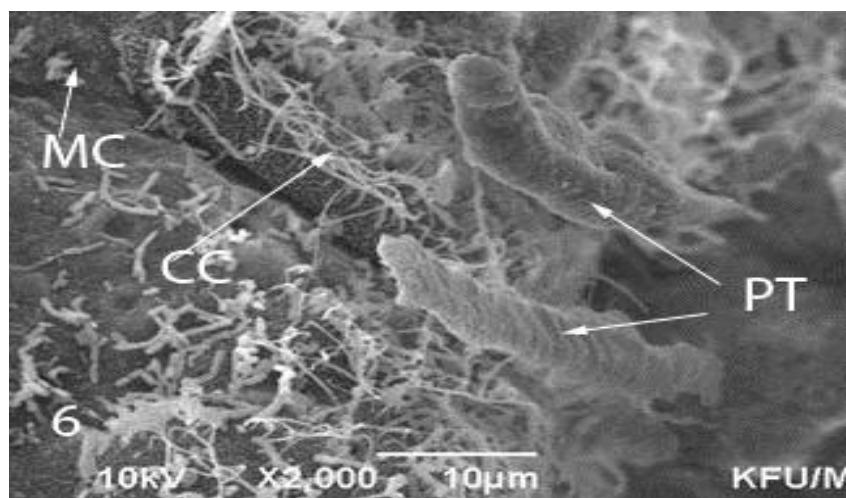


Figure 6: Scanning electron micrographs at stage 24 of tadpoles *Rana ridibunda* showing formation of finger like-structures (PT) on the epidermal surface of the gill filaments (may be microfilaments).

Consequently, the number of gill filaments increased into five and three at stage 22 (Figure 3), followed by six and four at stage 23 (Figure 4a&b) and finally filaments reached to its maximum number which is eight and five at stage 24 (Figure 5a&b). Ciliation is examined with three cell types as that of the preceding developmental stages. Our result observed a new finding in the epidermal surface of gill filament at stage 24, elongated microfilament like-structures protruded from the epidermal surface of the secondary gill filament, the shape of these structures is either flattened or finger like-structure with rounded end. The apical part containing cusps like that of marginal papillae of oral disc shapes (PT, Figure 6).

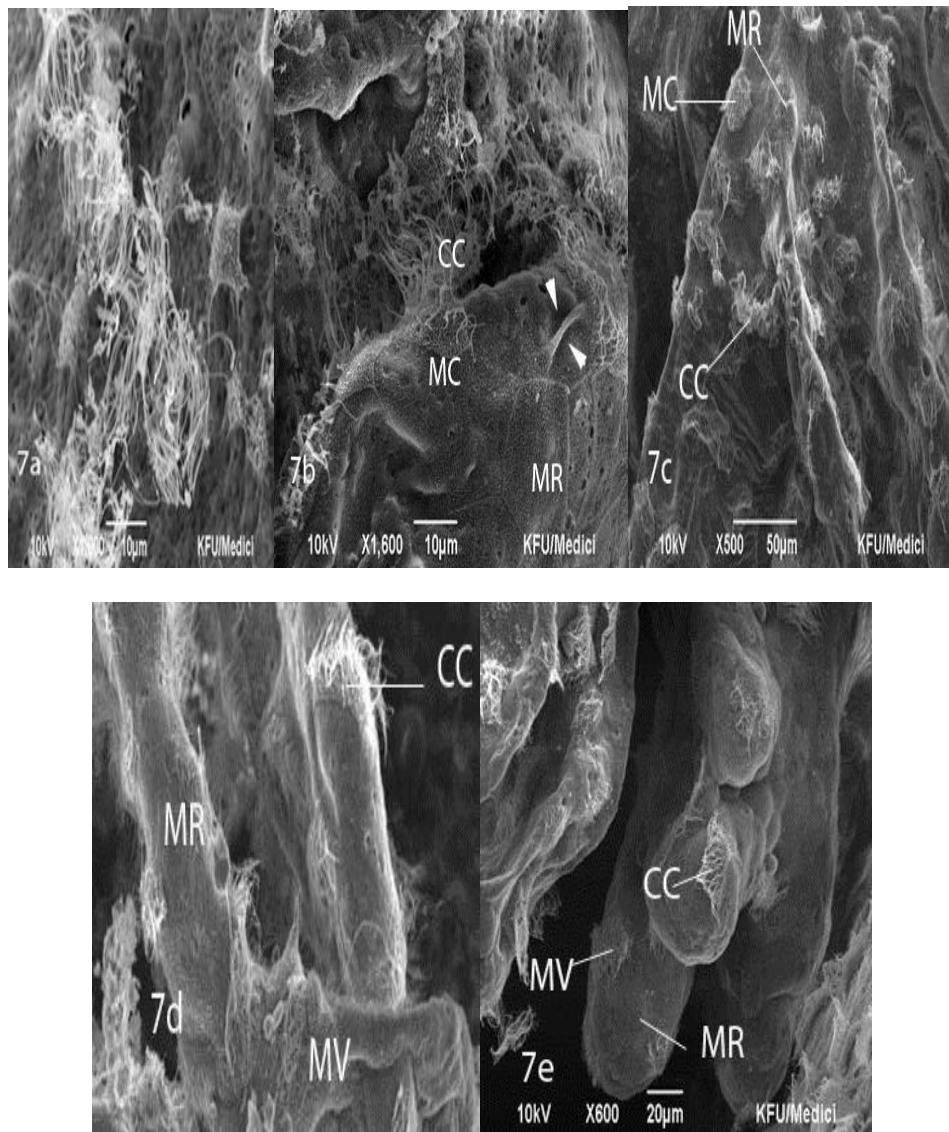


Figure 7a-e: Scanning electron micrographs at a high resolution revealed the density and the orientation of the ciliated cell on external gill filament of *Rana ridibunda*: (a) highly dense in stage 20. (b) Dense in stage 21. Note a cellular bridge connect microridged cells (arrow heads). (c) Intermediate dense in stage 22. (d) Low dense in stage 23. (e) Very low dense in stage 24. (The ciliated cells oriented at right angle to the long axis as in (7a,b,d) or parallel to the long axis as in (7c, e)).

The variation of the density of ciliated epidermal cells is few, intermediate, dense or very dense depending on the successive developmental larval stages as shown in (Figure 7a-e). Also it was observed in Figure 7b stage 23 a transverse cellular bridge found above the microridged cells (MR).

Development of the Operculum

The operculum begins with formation of two skin folds at stage 18 till stage 21, just lies anterolateral to the external gills. Each fold covers the basal part of the external gills (Figure 8a).

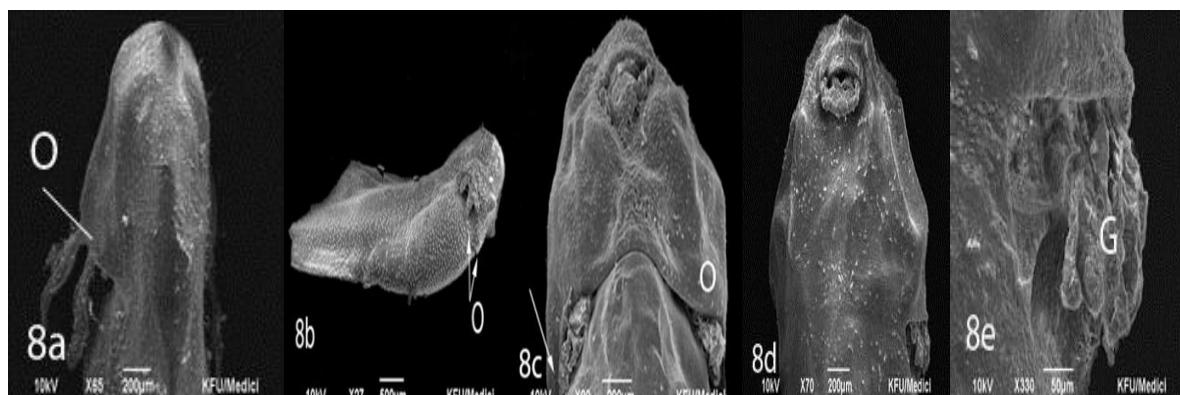


Figure 8a- e: Scanning electron micrographs showing development of the operculum of *Rana ridibunda* and atrophy of external gills: (a) Stage 20 lateral opercular fold. (b) Transverse opercular folds fused with the body ventrally. (c) Right base of external gill firstly start to cover by operculum (arrow). (d) Right external gills completely covered by operculum while the left one still persist. (e) Enlarged left external gill showing the atrophy process of gill filaments.

After that at stage 22, they grow and extend ventrally forming curved fold below the gills, followed by a transverse straight in structure that covers most of the gills (Figure 8b). Consequently, at stage 24 the fold completely covered the right external gills (Figure 8c&d) then the left gills. The process of atrophy of internal gill is cleared in Figure (8e).

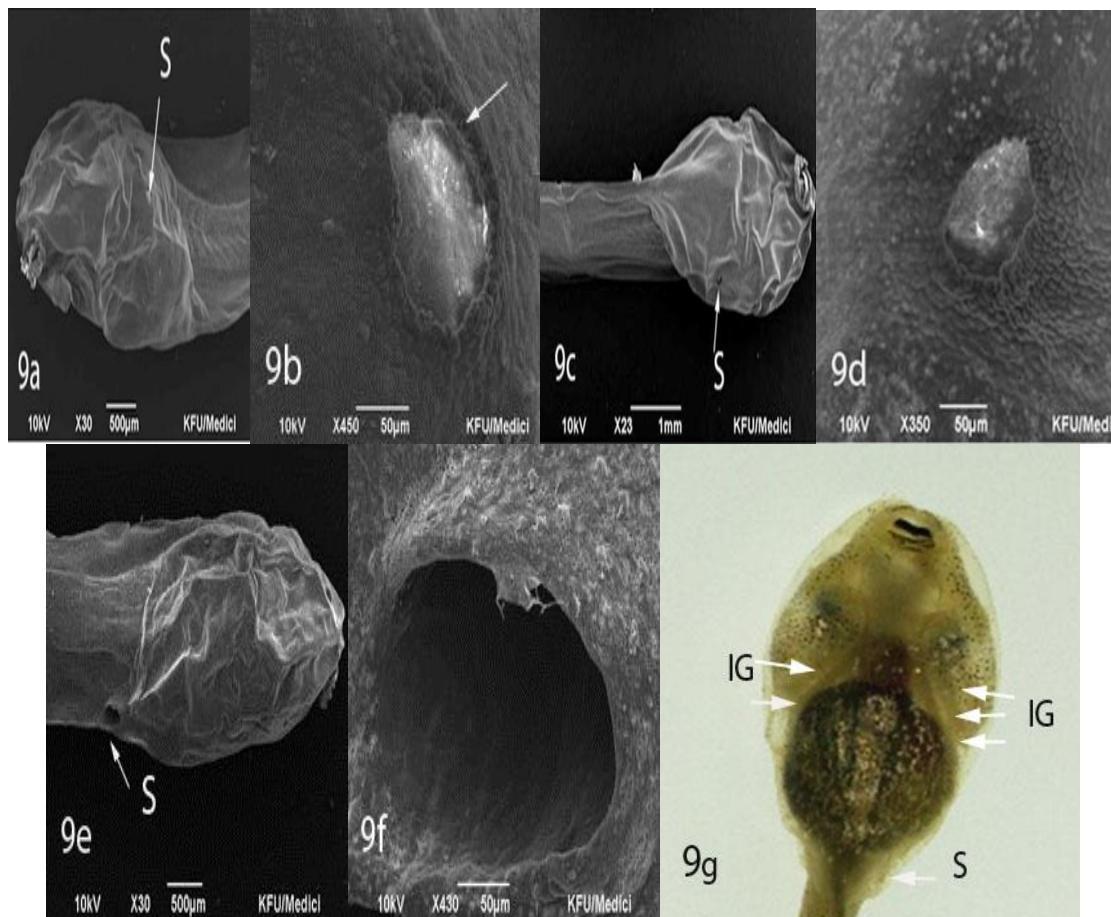


Figure 9a-g: Scanning electron micrographs showing positions of spiracle and internal gills of *Rana ridibunda*: (a) Dorsolateral sinistral spiracle (S) stage 26. (b) Spiracular cavity filled with mucus (arrow) (c) Invaginated sinistral spiracle. (d) The spiracular cavity with low mucus. (e) Orientation of spiracle from sinistral to median position. (f) Enlarged median spiracle with tubular empty cavity. (g) Completely atrophy of external gills and formation of internal gills (white arrows, IG).

The anterolateral and median straight folds are fused together forming the future spiracular opening just located ventrolaterally on the left side ("S"sinistral) (Figure 9a) .The internal cavity of spiracle appeared filled completely with mucus secreted from mucous glands situated among ciliated cells (Figure 9b), then it became partially filled with mucus(Figure 9c&d). At stage 27 the spiracle shifted to the median side, surrounded with thick wall and empty of mucus (Figure 9e&f). After atrophy of the external gills, the internal gills (persistent) are developed inside the trunk region (arrows in Figure 9g).

DISCUSSIONS

The organization of the external gills or transient gills during the initial phases of development brings to mind the characteristics of Anuran larvae and they are transitory embryonic structures. Observed with an SEM, the gill filaments are diverse in their shapes, number and length at anatomical and ultrastructural levels .The shape of gill filament of *Rana ridibunda* displays either a flattened shape or finger like –protrusion with rounded margin [25,9,6,7] . The total number of gill filaments is increased throughout the developmental larval stages (i.e., 5,7,8,10 and 15 at stages 20, 21,22,23and24 respectively) this coincide with that of [6,7], otherwise the number of gill filaments in Ranidae are lower than in bufonidae, [25] on the contrary in Microhylid genera *Cophixalus* and *Oreophryne*,as the development is direct, the external gills are not formed [9].Consequently, the gill filaments are also vary in the orientation and density of cells on the surface of gill filaments. In the present study we observed three types of epidermal cells mainly ciliated cells(CC), microvillated cells(MC),and microridged (pavement cells MR), also the same cells reported by [1,25] .While [5] found two types cells on the epidermal surface of external gills of *Rana dalmatina* (ciliated cells and pavement cells). With SEM the epithelial surface appears to be formed by irregular polygonal cells, with clear margins characterized by the presence of numerous ciliated cells , moderate microridged cells and few microvillated cells which often seem in an rectangular shape .The microridges in the late stages, represent the most frequent cellular type. In the present study, the organization and arrangement of gill apparatus of the tadpoles of *Rana ridibunda* was similar with that reported in the other ranidae and pond-dwelling salamanders [26,5,27,28].

On the contrary, in axolotl *Amystoma mexicanum*,it was different than that of our results [29].The transient gills of the anuran tadpoles appear at the early stage of embryonic development and display a general organization similar to that of the gills of urodea [28]. The anatomical organization of the *Rana ridibunda* tadpoles corresponds to that of other species of Orton's larval type IV (1953). Also the gills originate from the visceral arches III, IV and V and are formed by primary filaments from which secondary filaments start, similar to that described for *Rana catesbeiana* and *Rana delamatina* [5]. The filaments have rapid development and just as rapidly they come against the phenomenon of regression this is also coincide with reports of [18]. Our new finding by the ultrastructural analysis has demonstrated the presence of superficial elongated, microfilament like-structures protruded from the surface of secondary gill filament at stage 24, the shape of these structures is flattened or finger like-structure with rounded end. The apical part containing cusps like that of marginal papillae of oral disc shapes. (PT, Figure 6). We suggest that, they may be microfilaments of secondary gill filament (Figure 6). Also it was observed new finding the presence of a transverse cellular bridge connect between the microridges (MR) in (stage 23, Figure 7b).During the development of the operculum, In our result it was concluded that the transient structures covered by the developed operculum and regression soon occur by atrophy of the external gills gradually, starting by the disappearance of the right external gills (Figure 8d), followed by the left one at stage (Figure 8e). This result coincide with [20,30-33,3]. The two lateral folds joined in posteromedial ventral region forming the median spiracle (Figure 9e) as in *microhyla orientalis* tadpoles [3,22] *Bufo bomina* [23]. Although [32,7] found a contrast observation where both sides of external gills are covered by the transverse operculum fold by a symmetrical process ,then fold joined in posteromedial ventral region forming the median spiracle. The transient gills become persistent gills inside the trunk region (Figure 9g). Also new finding is the orientation of position of spiracle in tadpole larvae of *Rana ridibunda* from dorsolateral sinistral spiracle (Figure 9a) to median spiracle, during stages 24-27 in the same species (Figure 9e) .The spiracle is closed with mucus (Figure 9b) which disappeared gradually with progress of larval development (Figure 9d).The ultrastructural analysis of the morphology of *Rana ridibunda* during premetamorphic and metamorphic stages can be of great interest and value in the future studies of development of Ranidae in the context of ecology ,phylogeny and evolution. The ontogeny based on the description of ultrastructure of external gills used for identification of heterochronic processes and origin of Ranidae larvae.

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