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Ultrastructural observations on the lung of *Pelophylax* kl. *esculentus* tadpoles during development

V. CURCIO[1], R. MACIRELLA [1], M. TRIPEPI[2], & E. BRUNELLI [1]*

[1]*Department of Biology, Ecology and Earth Science, University of Calabria, Cosenza, Italy, and* [2]*Department of Biological and Chemical Sciences, East Falls Campus College of Life Sciences, Jefferson University, Philadelphia, PA, USA*

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Abstract
The anatomical organization of the lung in *Pelophylax* kl. *esculentus* tadpoles has been described in early and middle-staged tadpoles (Gosner stages 32 and 39). The lung's ontogenic development has been studied using light microscopy and both scanning and transmission electron microscopy. In the early-staged tadpoles, the lung is well vascularized, but it is provided with only two internal septa types and devoid of ciliated epithelium and lamellar bodies. The complete differentiation process is putatively reached in the middle-staged tadpoles (GS 39), and numerous septa of first, second, and third-order deeply protruding into the lung lumen could be recognized. The histological and ultrastructural features at this stage correspond to that described in the adults' lungs, but mucous secreting goblet cells could not be detected. Pneumocytes, with numerous apical microvilli, surround the growing network of capillaries and show a cytoplasm rich in dense bodies and few mature lamellar bodies. On the epithelial surface, a thin layer of mucous covering the underlying epithelium could also be seen. Neuroepithelial bodies, supposed to be involved in chemoreception, are organized in clusters on the first order septa and surrounded by cytoplasmatic processes originating by neighboring cells. The lung arrangement of *P.* kl. *esculentus* is compared with that of other Anurans in order to further elucidate the putative role of the lung in gas exchanges. The present studies reduce the information gap existing in the literature regarding lung morphology and development in amphibian larval stages, also contributing to the discussion on the onset of airbreathing respiration in tadpoles.

Keywords: *Lung, development, tadpoles, ultrastructure, Ranids*

Introduction

Due to their complex life cycle, amphibians exhibit a higher heterogeneity of the respiratory organs than any other vertebrate. Depending on their developmental stage, amphibians can use for gas exchange gills, skin, buccopharyngeal mucosa, and lungs (Duellman & Trueb 1986; Maina 2002). The wide range of habitats that amphibians have colonized also affects the respiratory system, considerable variation occurring among species, and in the different ecological and developmental conditions. Because of their compelling features, amphibians are considered useful model organisms in studies of the physiology of the cardiovascular and respiratory systems (Burggren & Warburton 2007).

In Anurans during the early larval stages, the skin and gills represent the main sites of gas exchange, and the role that lung plays in larval respiration has been discussed several times (Burggren & Just 1992; Burggren & Infantino 1994; Duellman et al. 2003). Ranids seem to have all three sites for gas exchange from the early developmental stages (Savage 1952), whereas, in *Xenopus laevis*, it has been shown that the presence of the lungs is not indispensable for survival through metamorphosis (Pronych & Wassersug 1994). Moreover, in *Xenopus laevis* and *Lithobates catesbeianus* (formerly *Rana catesbeiana*), preventing lung use severely affects metamorphosis and growth rates (Pronych & Wassersug 1994; Gdovin et al. 2006). In all instances, before the onset of the metamorphic climax, the skin

*Correspondence: E. Brunelli, Department of Biology, Ecology and Earth Science (DiBEST) - University of Calabria, 87036 Rende, Cosenza, Italy. Tel: +39 0984492996. Fax: +39 0984492986. Email: elvira.brunelli@unical.it

Vittoria Curcio and Rachele Macirella contributed equally to this work.

and gills' contribution decreases, and the lungs assume the primary role as a respiratory organ (Burggren & West 1982; Brunelli et al. 2004). Development, anatomy, and physiology of amphibians' gills and skin have been thoroughly investigated (Burggren & Just 1992; Brunelli & Tripepi 2005; Brunelli et al. 2007, 2009), but little is known about tadpole lung morphology. The tadpole respiratory system has been the subject of different studies relating to ventilation rates and development of lung use (Rose & James 2013; Janes et al. 2019); others have studied respiration in connection with feeding and locomotor performance (Hoff et al. 1999). Furthermore, all these studies mostly deal with three laboratory model species: the American bullfrog (*L. catesbeianus)*, the northern leopard frog (*L. pipiens*), and the African clawed toad (*Xenopus laevis*), with contributions from a few other (Janes et al. 2019).

The general anatomical arrangement of the lungs in amphibians is rather constant, and they appear as sac-like paired organs highly vascularised. On the contrary, the internal surface arrangement is highly variable among the different orders. Anurans' lungs typically hold numerous septa of different lengths (i.e., first, second and third-order septa) deeply protruding into the lung lumen by separating internal air space in different sized alveoli (Goniakowska-Witalińska 1986). Notwithstanding the existence of this common organization of the lungs in Anurans, more or less evident differences can occur in both morphology and timing of lung development. In tadpoles living in lentic water, the lungs are filled shortly after hatching, thus integrating aquatic and aerial gas exchanges, while tadpoles inhabiting lotic water do not expand their lungs until just ahead metamorphosis (Duellman et al. 2003).

The edible frog *Pelophylax* kl. *esculentus* (formerly *Rana esculenta*), a natural hybrid of *Pelophylax ridibundus* (*Rana ridibunda*) and *Pelophylax lessonae* (*Rana lessonae*), is able to colonize various types of aquatic environments and tolerates a broad range of ecological conditions (Voituron et al. 2005; Capula 2006). The structure of the lung of *P.* kl. *esculentus* has been previously studied in the adult (Tei et al. 2004), but nothing is known about the lung morphology and development in tadpoles.

Since there is a lack of knowledge regarding the lungs' ultrastructure in larval amphibians, this study analyzes the morphological arrangement of this organ in *P.* kl. *esculentus* larvae. This study aims to describe the histological and ultrastructural changes taking place during lung development in early and middle-staged tadpoles, corresponding to Gosner stages 32 and 39 (Gosner 1960) and further elucidate the putative role of the lung in gas exchanges.

Materials and methods

Study organism

European water frogs (*Pelophylax* sp.) represent a large species complex with several, widespread lineages (Dufresnes et al. 2017). In Central and Southern Italy (Sicily, islands of Elba and Giglio included), this group is represented by hybridogenetic complex *P.* kl. *hispanicus* (Capula 2006; Mori et al. 2013) that originates from hybridization between *P. bergeri* (genotype BB) and a recently recognized monophyletic lineage, *P.* n. t. 1 (genotype EE) that appears to be extinct as such (Dubey & Dufresnes 2017). The *Pelophylax hispanicus* complex, formerly known as *Rana esculenta* complex (Frost et al. 2006), inhabit a wide range of aquatic habitats (rivers, swamps, lakes and marshes) often located in agro-ecosystems and woodlands. It is still rather common in Italy, but it appears to be threatened by changes in agricultural practices and both destruction and degradation of breeding sites.

Animals collection

All tadpoles (n = 20) were obtained from naturally fertilized egg clutches in natural populations during the breeding season. Three clutches were collected from wetland ponds situated in a protected natural area close to Cosenza (Calabria, Southern Italy) and transported to the laboratory. Eggs were maintained in 50 L aerated tap water filled-tanks; water was replaced every 3–4 days as needed and maintained at 22 ± 1°C. Free-swimming tadpoles were fed boiled organic spinach *ad libitum* three times a week. Waste and debris were removed daily using a fine mesh net. Throughout the experiment, water quality parameters (pH, conductivity, temperature, and dissolved oxygen) were recorded daily, and the photoperiod was kept at 12:12-h light:dark cycle. During the whole experimental period, animals exhibited no signs of disease or stress.

Animals and tissue preparation

Morphological examination under a stereomicroscope (Leica MZ APO equipped with Canon camera) allowed tadpoles to be assigned to a developmental stage based on the presence of distinctive morphological features, according to Gosner (1960). To avoid any influence of body size, all tadpoles were selected to be similar in length and body mass.

Studies have been carried out using *Pelophylax* kl. *esculentus* tadpoles during either an early (GS 32) and a middle (GS 39) stage of larval development,

as defined by their external anatomy. The first group, representing an early stage, was characterized by the absence of forelimbs and the presence of a well-developed head and trunk. A slight indentation of the foot-paddle margin, on the dorsal side, marked the development of the 4th and 5th toes. The second group, corresponding to a more advanced stage of development, was characterized by the presence of completely separated toes and the appearance of sub-articular tubercles on the inner surface of the toes as light patches. The inner metatarsal tubercle became a small oval outgrowth.

Ten larvae for each developmental stage were sacrificed using an overdose of the anesthetic MS-222 (tricaine methanesulfonate, Sigma-Aldrich Chemicals Co., St. Louis, MO, USA). The removal and dissection phases were performed, while the specimens were kept covered continuously with a fixative solution of 3% glutaraldehyde in phosphate buffer. Lungs were carefully excised before being subjected to aldehyde fixation (glutaraldehyde at 3% in phosphate buffer, 0.05 M, pH 7.5) and post-fixation in osmium tetroxide (1% in the same buffer). After dehydration, in graded ethanol, samples for scanning electron microscope (SEM) observations were subjected to the progressive substitution of ethanol with hexamethyldisilazane, removed by complete evaporation, coated with gold in an Emitech K550 ion sputter unit, and then examined under a Zeiss DSM 940 SEM.

The samples used for transmission electron microscope (TEM) analysis were soaked in propylene oxide and embedded in Epon-Araldite. Semithin sections (1–2 μm) were stained according to the technique described by Humphrey and Pittman (1974) (methylene blue, azure II, and basic fuchsine) and observed by a light microscope Leitz Dialux 20 EB. Ultrathin sections were treated with uranyl acetate in ethanol and lead citrate, coated in Edwards EM 400 and then examined with a Zeiss EM 900 electron microscope.

All procedures for animal handling and tissue removal were carried out according to the Ethical Committee's recommendations and under the supervision of authorized investigators. Animal research procedures were approved by the Institutional Animal Care and Use Committee (permit number 2011/0002086).

Results

Observations under the stereomicroscope revealed in tadpoles of *P.* kl. *esculentus* a general structure of the lungs similar to that of other Anuran species. Two pairs of lungs, with comparable dimensions, originate from the buccopharyngeal cavity and extend posterodorsolaterally to the pronephros. Each lung consists of a sac-like structure surrounded by a visceral pleura with a wider proximal region, connected to the pharynx and a progressively smaller distal part. The pulmonary wall is rich in chromatophores and folds to form internal septa (Figure 1(a)) that increase the respiratory surface.

Early-staged tadpoles (GS 32)

Observed under SEM, after removal of the pleura, the lung of early-staged tadpoles (Gosner stage 32), appears as a delicate and spongy sac, conically shaped (Figure 1(b)). The lung surface is made by a simple squamous epithelium (mesothelium) and shows a smooth appearance in its cranial portion, whereas in the distal portion assumes a polygonal arrangement. Slightly protruding polygons of different sizes represent the alveoli and are surrounded by numerous capillaries that run along their margins. It is also possible to recognize the pulmonary vein that extends along the major axis in the medial region of the lung (Figure 1(b)). Observation of the inner surface reveals the presence of septa of different sizes (i.e., first and second-order septa) that depart from the internal wall and delimit the air space of alveoli (Figure 1(c)).

Histological observations have shown that the lung wall is made up of connective support, covered by a thin layer of mesothelial cells (Figure 1(d)), while the innermost layer is constituted by epithelial cells. In the connective intermediate layer, smooth muscle fibers and numerous chromatophores could be seen. The innermost layer consists of respiratory epithelium (pneumocytes). The folds protruding towards the lung's lumen are thin extensions of different lengths and thicknesses (i.e., septa of the first and second-order) and are covered by respiratory epithelium. The first order of septa dilates distally to form a round or oval apical tip in which a capillary and bundle of smooth muscle fibers, elastic fibers can be distinguished (Figure 1(d)).

By TEM observation, the lung's external wall appears to be formed by a layer of elongated mesothelial cells that present a wide, flattened nucleus sometimes indented and/or lobed, surrounded by little cytoplasm (Figure 1(e)). Mesothelium lays on a thin sheet of dense connective tissue (Figure 1(e,f)). The underneath layer of loose connective tissue contains melanocytes numerous scattered elastic fibers are noticeable; also, connective cells and the smooth muscle are detected (Figure 1(f)).

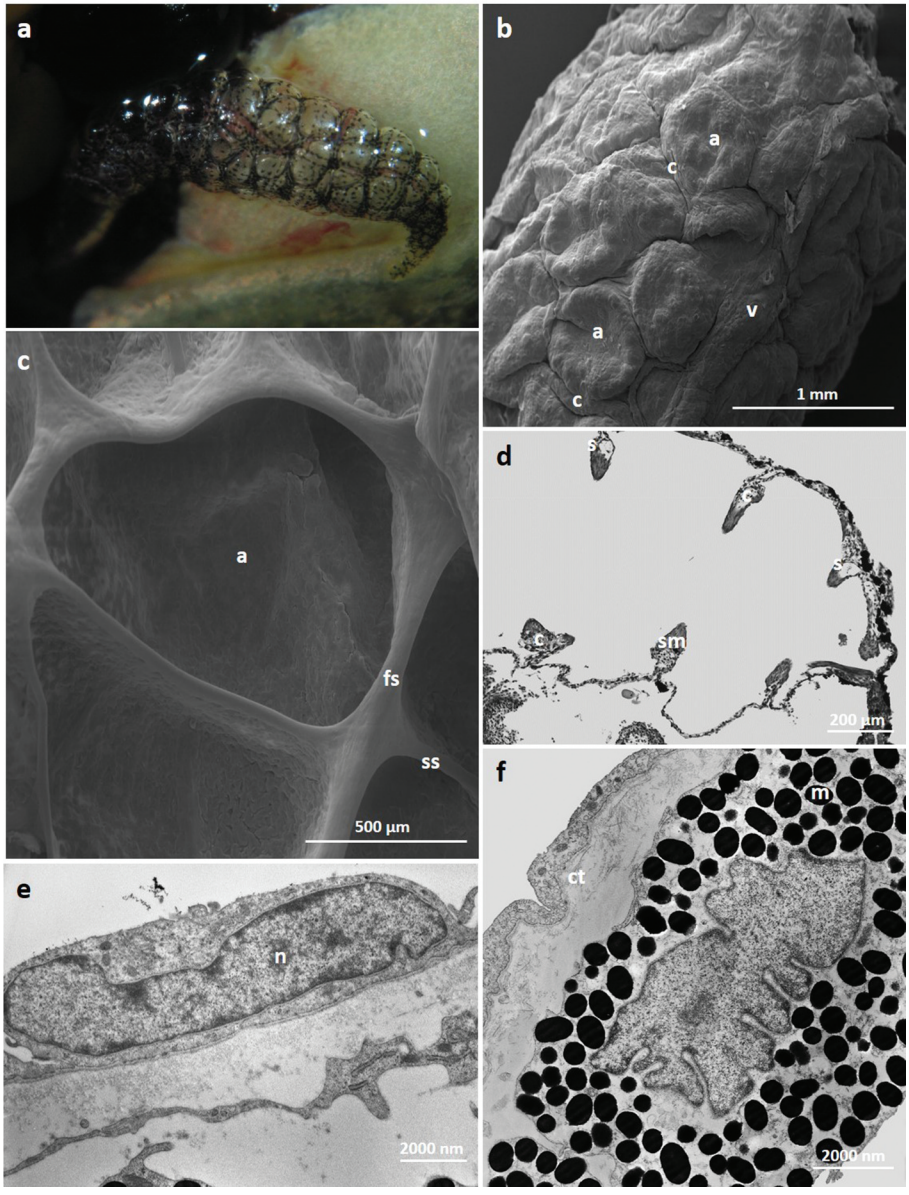

Figure 1. The lung of *P.* kl. *esculentus* tadpoles at early-stage (GS 32). (a) Stereomicroscope image showing the sac-like structure of the lung. The pulmonary wall is characterized by the presence of numerous melanocytes. (b) Under scanning electron micrograph, the lung surface is made by simple squamous epithelium; protruding polygons represent the alveoli (a) that are surrounded by capillaries (c); in the medial region the pulmonary vein (v) extends along the major axis. (c) Scanning electron micrograph of the inner surface; note the first (fs) and second-order (ss) septa delimiting the air space of alveoli (a). (d) Light microscope photograph showing the lung wall made by a layer of mesothelial cells, a connective intermediate layer, and an inner layer of epithelial cells. c = capillaries, s = septa, sm = smooth muscle. (e, f) Transmission electron micrographs of the external lung wall. (e) Detail of a mesothelial cell characterized by a wide and flattened nucleus (n). (f) Mesothelium lies on a thin sheet of connective tissue (ct), and a loose connective tissue containing melanocytes (m), elastic fibers, connective cells, and smooth muscle.

The respiratory epithelium is made by a thin layer of pneumocytes (i.e., respiratory cells) and blood capillaries (Figure 2(a)). Pneumocytes show an irregular shape and consist of a rather large cell body containing the prominent nucleus and irregular cytoplasmic processes that extend laterally overlaying the capillaries (forms the outer layer of the air–blood barrier) (Figure 2(a)). Several undifferentiated dense bodies could be recognized, scattered through the cytoplasm, and numerous short microvilli depart from the apical plasmalemma. Numerous electronclear vesicles are distributed in the cytoplasm of both respiratory and endothelial cells (Figure 2(a,b)). At the free surface, the

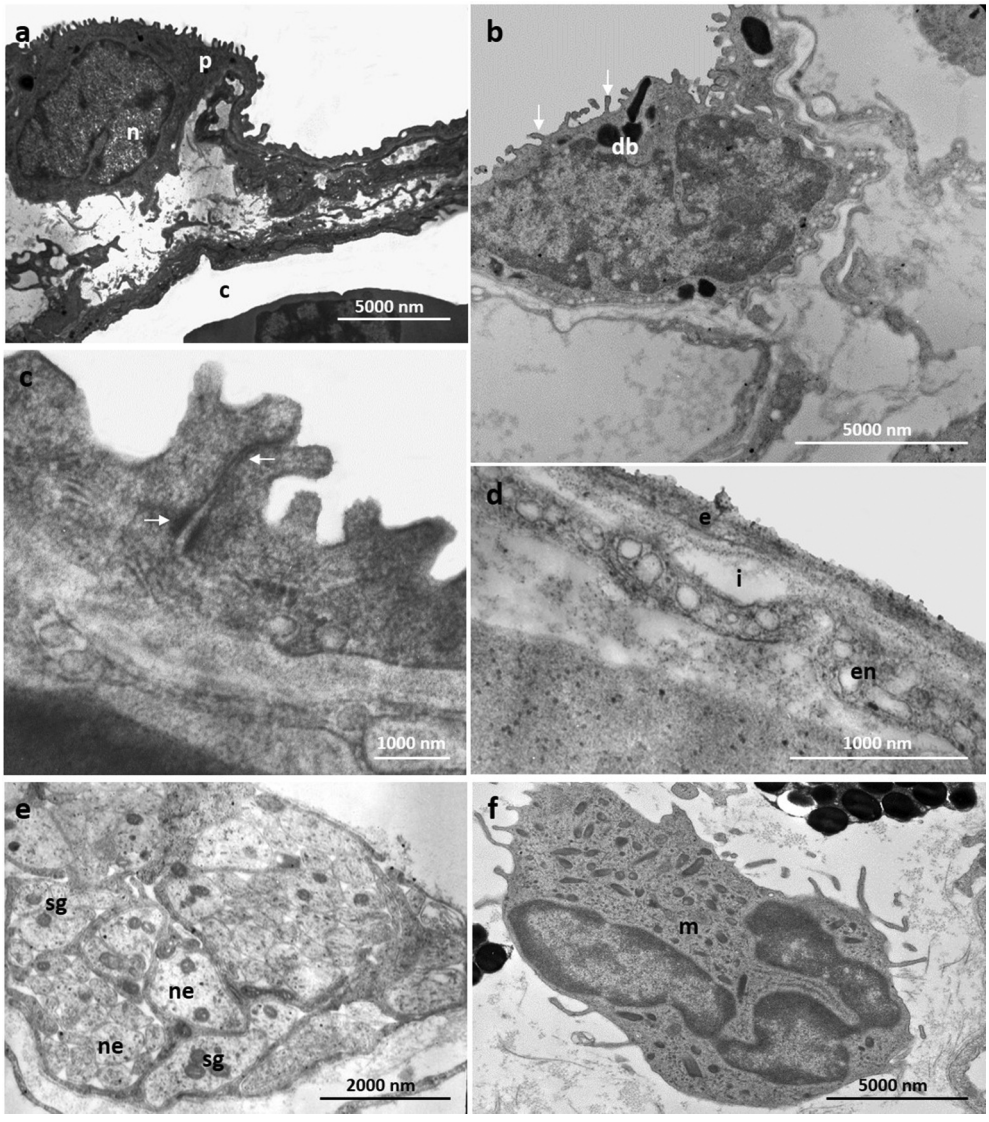

Figure 2. Transmission electron micrographs: early-staged tadpoles (GS 32). (a) Pneumocyte (p) showing a wide cell body filled with a large nucleus (n), and long cytoplasmic processes that extend laterally overlaying the capillaries (c). (b) Numerous dense bodies (db) are scattered through the cytoplasm of pneumocytes, and short microvilli (arrows) depart from the apical plasmalemma. Electron-clear vesicles are visible in the cytoplasm of endothelial cells and in the cytoplasm of adjacent pneumocytes. (c) Tight junctions and desmosomes at the free surface of pneumocytes (arrows). (d) The air-blood barrier is composed of an epithelial layer made by the cytoplasmic processes of pneumocytes (e), an intermediate layer (i) and, an inner layer of capillary endothelium (en). (e) Neuroepithelial bodies organized in clusters scattered on the first order septa. Neuroendocrine cells (ne) show a large nucleus, a reduced cytoplasm and numerous secretory granules (sg). (f) Detail of a macrophage (m) in the connective tissue.

pneumocytes are joined each other by tight junctions and desmosomes (Figure 2(c)).

In the air-blood barrier, it is possible to distinguish an epithelial layer made by the cytoplasmic processes originating from pneumocytes, an interstitial or intermediate layer composed by elastic tissue, collagen, and smooth muscle, and a layer of the capillary endothelium (Figure 2(d)).

Neuroepithelial bodies (NEBs) organized in clusters are located on the first order septa (Figure 2(e)). The NEBs are surrounded by thin cytoplasmatic processes originating by neighboring cells and are typically composed of two to six neuroendocrine cells (NEs) closely joined each other. NEs show a large ovoid nucleus and a clear cytoplasm in which microfilaments, vesicles, mitochondria, and numerous secretory granules (also called dense-core vesicles) are visible. Between the corpuscular cells, nerve endings and synaptic junctions are also recognized. Few macrophages could be seen scattered through the connective tissue (Figure 2(f)).

Middle-staged tadpoles (GS 39)

Under the scanning electron microscope, the lung surface is similar to that described for the early-staged tadpoles and each protruding alveolus could be recognized by its polygonal shape and well-defined margins (Figure 3(a)). From the inner wall of the lungs originate numerous folds, and it is possible to recognize first, second and third-order septa that separate the lung lumen into the alveolar spaces (Figure 3(b)).

With further enlargement, it is possible to observe the protruding capillaries in the internal lung surface, covered by the surrounding pneumocytes. Short microvilli cover the whole free surface of respiratory cells (Figure 3(c)). The surface of the primary septa is covered by a ciliated epithelium (Figure 3(d)).

Ultrastructural observations reveal that the thin lung wall is formed by an outer layer of mesothelial cells, an intermediated layer of loose connective tissue containing numerous melanocytes and an inner layer of pneumocytes (Figure 4(a,b)).

The ciliated cells, often distributed close to each other, exclusively occur on the tips of the first-order septa (Figure 4(c,d)). These cells are pleomorphic and show a rather ample luminal surface from which depart short microvilli that alternate with numerous long cilia (Figure 4(c,d)). The nucleus is large and usually located in the basal position. In the apical cytoplasm, basal corpuscles, mitochondria, and a Golgi complex occur along with dense bodies.

The respiratory epithelium is formed by pneumocytes equipped with numerous apical microvilli of different lengths on their free surface (Figure 4(e–g)). These cells show lung cytoplasmic processes that extend encircling capillaries. Connections among adjacent cells are achieved by tight junctions and desmosomes and

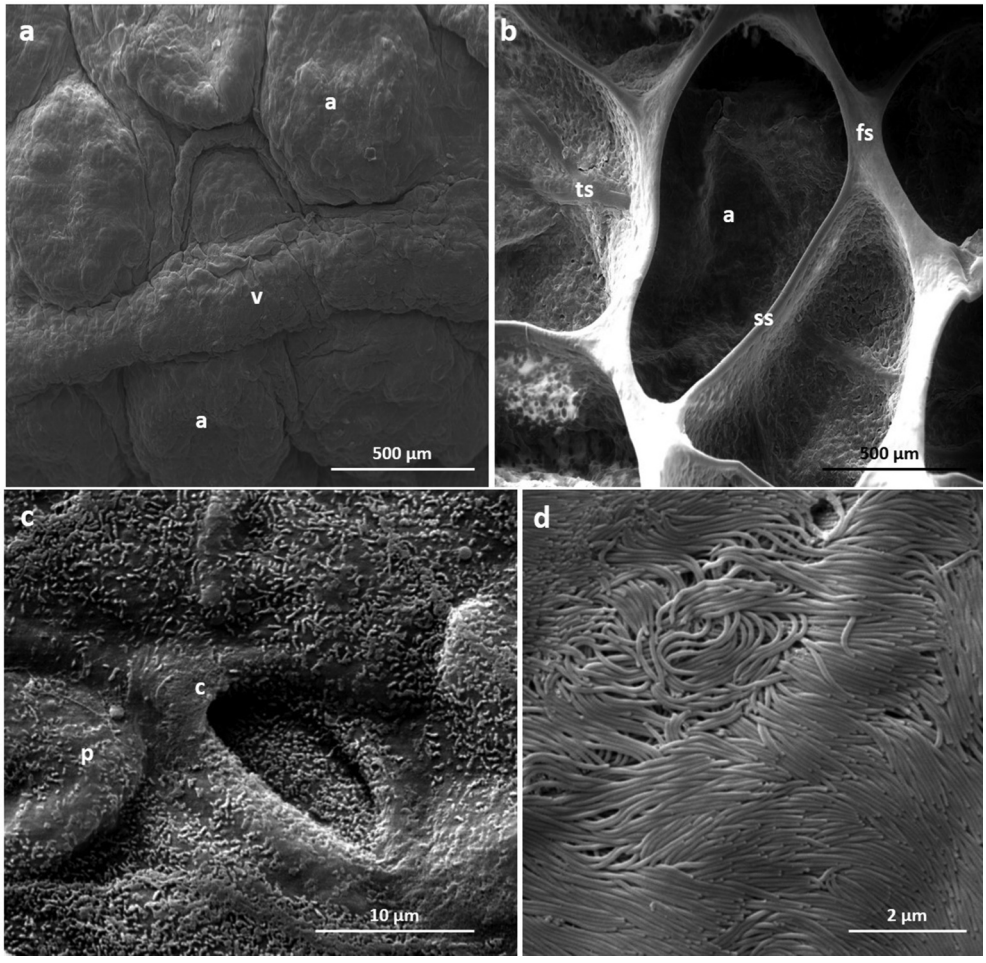

Figure 3. Scanning electron micrographs of the lung in middle-staged tadpoles (GS 39). (a) On the lung surface, the well-defined margins of protruding alveoli (a) are recognized; the pulmonary vein (v) is well distinguishable in the medial region. (b) First (fs), second (ss), and third-order (ts) septa separate the lung lumen into the alveolar spaces (a). (c) Protruding capillaries (c), covered by the cytoplasmic projections of surrounding pneumocytes (p), are visible on the internal lung surface; note numerous short microvilli departing from the apical surface of pneumocytes. (d) Ciliated epithelium at the apical endo of the primary septa.

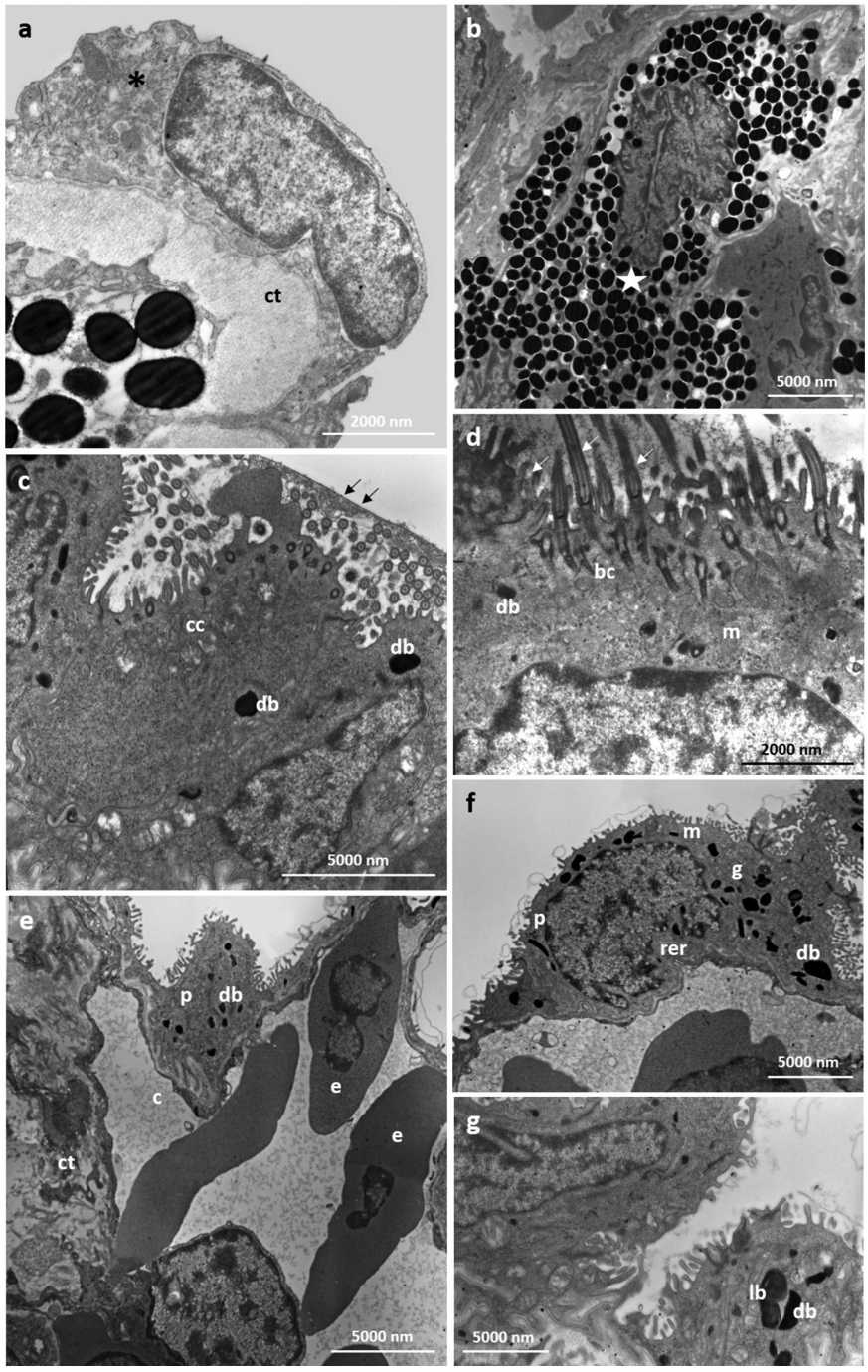

Figure 4. Transmission electron micrographs of the lung in middle-staged tadpoles (GS 39). (a) The lung wall is made by an external layer of mesothelial cells (asterisk) and an intermediated layer of loose connective tissue (ct). (b) Detail of a melanocyte in the intermediated layer of the lung wall (white star). (c) Ciliated cells (cc) at the apical tips of first-order septa; note the wide luminal surface from which depart numerous long tufts of cilia. A fuzzy coat and a thin lining film are visible on the epithelial surface (black star). db = dense bodies. (d) Detail showing short microvilli and long cilia (arrows); in the cytoplasm basal corpuscles (bc), mitochondria (m), and dense bodies (db) could be seen. (e) Pneumocytes (p) are characterized by lung cytoplasmic processes covering the capillaries (c). ct = connective tissue, e = erythrocyte, db = dense bodies. (f) Mitochondria (m), the Golgi apparatus (g), dense bodies (db), and the well-developed rough endoplasmic reticulum (rer) are visible in the cytoplasm of the pneumocytes (p). (g) Detail of dense bodies (db) and lamellar bodies (lb) in the cytoplasm of the pneumocytes.

occasionally by overlapping or interdigitating junctional complexes (Figure 4(e)). In the cytoplasm, numerous mitochondria, Golgi complexes, and a well-developed rough endoplasmic reticulum are often observed mainly in the apical portion of the cells. Numerous large dense bodies are noticed along with a small number of lamellar bodies characterized by the typical concentric array of lamellae (Figure 4(f,g)). Numerous electronclear vesicles are distributed in the cytoplasm of both respiratory and endothelial cells. At several points, it is possible to recognize a fuzzy coat made of a flocculent substance on its surface and a thin lining film (Figure 4(c,d)).

Macrophages sporadically occur on the respiratory epithelium (Figure 5(a)); in the cytoplasm of activated cells, partially phagocytized debris, lipid bodies, granules, and mitochondria are recognizable.

Neuroepithelial bodies (NEBs) arranged in clusters could be detected on both the primary and secondary septa (Figure 5(b)). Corpuscular cells show large nuclei with numerous invaginations and both electron-clear cytoplasmic vesicles and electron-dense bodies. In the basal portion of NEBs and between the corpuscular cells, afferent and efferent nerve fibers can be detected (Figure 5(c)).

Discussion

In the tadpoles of *Pelophylax* kl. *esculentus* lung anlage appears shortly after hatching, at a precocious stage of embryonic development (GS 19–20) when external gill filaments are well distinguishable at both sides of the head. As development proceeds, the lungs extend, epithelium begins to folds, and blood vessels develop surrounding the lung sac (Viertel & Richter 1999). In this study, we clearly show that the lung of early-staged tadpoles (GS 32) presents some rudimentary features such as the presence of only two types of internal septa (i.e., first and second-order septa), the absence of ciliated epithelium and lamellar bodies and the occurrence of a limited number of mature dense body. In contrast, the lungs are well vascularized, and neuroepithelial cells (NEs) are still detected, displaying the typical ultrastructural characteristics described in other Anuran species (Goniakowska-Witalińska 1981; Hermida et al. 2002). Although the role of NEs in vertebrate airways has not yet fully elucidated, it has been suggested that they perform a function of lung chemoreceptors and are sensitive to the content of O_2 and CO_2 (Goniakowska-Witalińska 1981, 1997).

The differentiation process through which the less specialized lung becomes a mature respiratory organ is putatively reached in the middle-staged tadpoles (GS 39). The histological and ultrastructural features of the lung at such stage corresponds to that described by Tei et al. (2004) in the adult of the same species and our observations confirm the presence of a general architecture similar to that previously reported for other Anuran species (Hermida et al. 1998 and references therein). The examination by both light and electron microscopy reveals the presence of numerous septa of first, second, and third-order deeply protruding into the lung lumen. The first order septa are characterized by ciliated epithelium located at their apical tips. However, even at this stage, we did not detect the mucous containing goblet cells typically distributed at the apical end of the first-order septa in the adult of numerous Anurans species, including *P.* kl. *esculentus* (Tei et al. 2004). Several authors observed that in species provided with few goblet cells or lacking of them, pneumocytes are rich in cytoplasmic vesicles in the subapical plasmalemma that contribute to mucous secretion (Goniakowska-Witalińska 1980a, 1986, 1980b; Hermida et al. 2002). Comparable cytoplasmic vesicles could not be recognized in *P.* kl. *esculentus* pneumocytes, which otherwise show typical ultrastructural features of Anurans' respiratory cells. Instead, numerous dense bodies at different stages of maturation (Goniakowska-Witalińska 1986) were scattered in pneumocytes' cytoplasm and few mature lamellar bodies. It has been suggested that both dense and lamellar bodies play a role in the storage of a surfactant substance released over the epithelium to form a monolayer film (Goniakowska-Witalińska 1986). Since in the middle-staged tadpoles, we releveled the presence of a thin lining film covering respiratory epithelium, it can be assumed that in *P.* kl. *esculentus*, the exocytosis of the surfactant is large enough, notwithstanding the low number of lamellar bodies. The intensity would probably increase at metamorphic stages, further reducing the surface tension at the air-epithelium interface.

In Anurans, the contribution of different sites to gas exchange was often inferred from their level of vascularization (Wassersug & Seibert 1975; Pan & Burggren 2010; Janes et al. 2019). Due to their supposed poor vascularization, the lungs of Ranids tadpoles are considered to negligibly contribute to basal gas exchange (Just et al. 1973). Nevertheless, here we clearly show the presence of an extensive network of capillaries surrounded by pneumocytes, which starts to develop at a precocious stage (i.e., GS 32) increasing in both number and size in the middle-staged tadpoles. As outlined by Wassersug and Seibert (1975), the amount of vascularisation by itself

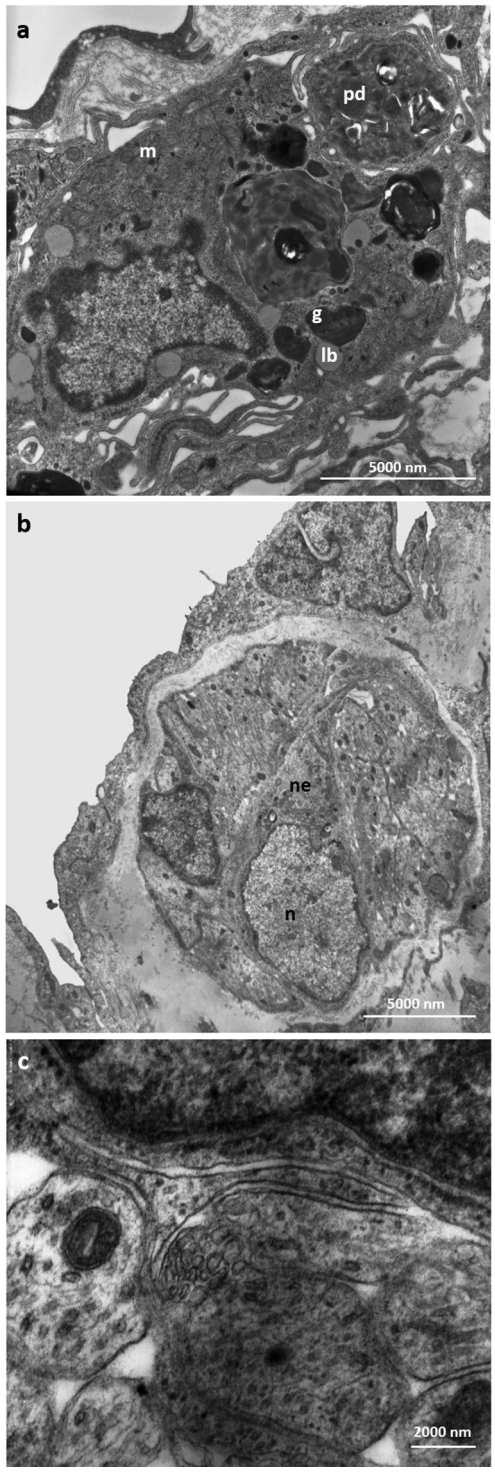

Figure 5. Transmission electron micrographs of the lung in middle-staged tadpoles (GS 39). (a) On the respiratory epithelium, activated macrophages are characterized by phagocytized debris (pd), lipid bodies (lb), granules (g), and mitochondria (m). (b) Neuroepithelial bodies are arranged in clusters and are composed of neuroendocrine cells (ne), showing large nuclei with numerous invaginations (n). Electron-clear vesicles and electron-dense granules are visible in the cytoplasm of neuroendocrine cells. (c) Detail of afferent and efferent nerve fibers between the neuroendocrine cells of the neuroepithelial bodies.

maybe not a measure for the contribution of the lung in active respiration, and other mechanisms would be involved in the regulation of gas exchanges.

The onset of breathing air may occur at different developmental stages (Rose & James 2013). *Xenopus laevis* tadpoles begin to use the lungs immediately after hatching while *Lithobates catesbeianus* and other Ranids airbreathing start in middle-staged tadpoles (GS 35–41) (Wassersug & Seibert 1975; Burggren & Just 1992; Ultsch et al. 1999). From the morphological and ultrastructural analysis, we have evidenced that lungs are largely incomplete in early-staged tadpoles and our results do not support the hypothesis of a role in respiratory exchanges. Instead, in middle-staged tadpoles, the lung shows an organization very close to that of the adult, clearly indicating that it is almost ready to assume a primary role as a respiratory organ.

Interestingly, a morphological arrangement of the tadpole' lungs similar to that observed in *P.* kl. *esculentus* has been previously described only in *Pseudis paradoxa* (GS 37), which showed well-developed internal septation and well-vascularized walls (Downie et al. 2009). The genus *Pseudis* possess unusual features among Anuran larvae, including the achievement of huge dimensions before metamorphosis, leading the authors to consider precocious lung maturation as another unusual trait unique for this species. However, the fact that in the large tadpoles of the American bullfrog, lung septation does not develop until metamorphosis (Atkinson & Just 1975), suggests that other factors than body dimension may be influencing lung development.

Moreover, in tadpoles, the relative contributions of gills, skin, and lungs to gas exchange may differ in different species and in the same species may change depending on aquatic oxygen availability (Crowder et al. 1998), temperature, metabolic demand and inter-species variability (Janes et al. 2019). Besides, it has been demonstrated that the tadpoles of the European water frogs are ecologically more oxygen-dependent than the other species belonging to the same genus (Plenet et al. 2000).

Although tadpoles usually breathe air, lung respiration is considered unnecessary for survival (Burggren & Just 1992; Pronych & Wassersug 1994; Ultsch et al. 1999) and the role of tadpoles airbreathing has been widely investigated resulting in the emergence of some recurrent hypotheses. It has been suggested that the onset of lung inflation contribute to lung development and would also be related to the outcomes on other functions such as buoyancy, locomotion, and feeding (Milsom 1990; Pronych & Wassersug 1994; Bruce et al. 1994; Crowder et al. 1998; Fejtek et al. 1998; Rose & James 2013; Janes et al. 2019).

This study clearly highlights the need of more investigation on different model species to better elucidate the relationship between lung morphology and its role as a gas exchanger. In fact, according to Janes et al. (2019), the study of respiratory development cannot be exhaustive if conducted on a small number of species. It is well acknowledged that Anuran larvae diversified into a wide range of ecomorphs (Roelants et al. 2011), and such enormous diversity reflects specific adaptations to the environment and their evolutionary history (Altig & Johnston 1989; Sherratt et al. 2018). Since the lungs' morphological and ultrastructural characteristics during ontogenesis are largely under-investigated, our study aims to reduce this gap by contributing to the discussion on the timing of tadpoles' lung respiration.

Disclosure statement

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

ORCID

R. Macirella http://orcid.org/0000-0003-4121-9837
E. Brunelli http://orcid.org/0000-0003-3669-1395

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