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Book lung development in embryos of the cobweb spider, *Parasteatoda tepidariorum* C. L. Koch, 1841 (Araneomorphae, Theridiidae)

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ABSTRACT

Light and transmission electron microscopy were used to study the development of book lungs in embryos of the spider *Parasteatoda tepidariorum*. There is a bilateral cluster of temporary lamellae that form just posterior to the second opisthosomal (O2) limb buds. These lamellae are replaced by advanced embryo (AE) book lungs that continue into postembryonic stages. Results herein agree with earlier suggestions that the O2 limb buds become the AE book lungs. Each O2 limb bud merges with the ventral surface of the O2 segment, where the limb bud/book lung is internalized by covering with epidermis. A strand of tissue (entapophysis) from the epidermis at the posterior opisthosoma provides precursor cells for the book lung lamellae, and possibly entapophysis cells induce limb bud cells to align and produce lamellae. Electron micrographs show the different modes (I–III) of lumen formation. The result is a spiracle, atrium and alternating air and hemolymph channels. A hypothesis is presented for the role of precursor cell polarity in producing the planar tissue polarity of the channels. Some type of apical/apical affinity results in air channels, while basal/basal affinity results in hemolymph channels. Strong basal/basal affinity is likely as opposed cells in hemolymph channels extend basal processes that span the channel and start pillar trabeculae that continue in postembryonic stages.

1. Introduction

As reviewed earlier (Farley, 2008, 2010, 2011, 2012, 2015), since the late 19th century there is the hypothesis that the book lungs of arachnids are derived from aquatic ancestors with book gills like those in the horseshoe crab. The book gills are external and extend posteriorly from opisthosomal branchial appendages while the arachnid book lungs are internal in the opisthosoma and extend anteriorly from a spiracle and atrium. There has been much speculation about how the internalization of external lamellate structures may have occurred. The present investigation is part of a comparison of the development of book gills (Farley, 2010, 2012) and book lungs in scorpions (2005, 2008, 2011) and spiders (2015). The intent is to compare the cellular formation of these structures to provide information for studies of gene expression and organogenesis and for hypotheses about evolutionary relationships.

As shown in earlier studies, there are substantial differences in the formation of book gills (Farley, 2010, 2012) and scorpion (Farley, 2008, 2011) and spider (Farley, 2015) book lungs, although there are some similar cellular processes. For spider book lungs, the epithelial precursor cells must have the basic capabilities for proliferation, migration, alignment, secretion, channel formation and clearance and apicobasal polarity. For the latter feature, the basal surface has nutrient transfer with hemolymph while the apical surface has air channel development followed by cuticle and trabecular formation for stabilizing the air channel walls (Farley, 2015). The embryo epidermal cells have polarity and can probably develop a broad functional repertoire, but they are initially on the external surface. For the spider species of this investigation, there are several routes by which external epidermal cells might become precursors for the internal book lungs, but results show a likely role for opisthosomal entapophyses (internal tissue strands).

1.1. Initial temporary (IT) book lungs

Spider book lung development is complicated by the formation of a bilateral cluster of temporary lamellae in early embryos just posterior to the second opisthosomal (O2) limb buds. These lamellae (furrows, saccules) are open to the outside and have been observed externally with the scanning electron microscope (SEM) in embryos of *Cupiennius salei* (Wolff and Hilbrant, 2011) and

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Parasteatoda tepidariorum (Mittmann and Wolff, 2012). The IT book lungs are replaced by a pair of advanced embryo (AE) book lungs that eventually develop a spiracle. The AE book lungs continue development in the postembryonic stages and are probably more water-conserving (Farley, 2015).

The IT book lungs were not usually included in earlier histological studies of spider embryos (Kishinouye, 1891; Simmons, 1894; Purcell, 1895, 1909; Montgomery, 1909; Janeck, 1909; Ivanić, 1912; Kassianow, 1914; Versluys and Demoll, 1923; Dawydoff, 1949; Farley, 2015). They may have been absent or missed with the histological procedures used, and there are probably species differences in the expression of IT lamellae. In the present study, no chemical pretreatment was used to remove or permeate embryonic membranes before fixation, so there is better preservation of cell and lamellar structures in light and electron micrographs. This has enabled some clarification of the development of AE book lungs and the role of the O2 limb buds.

The IT book lung consists of several lamellae and one or more larger, lateral lamellae called the pulmonary sac (Wolff and Hilbrant, 2011; Mittmann and Wolff, 2012). In embryos of *C. salei* (Wolff and Hilbrant, 2011), those structures become deeper and more prominent as the embryo develops. In embryos of *P. tepidariorum*, the IT lamellae are less evident and possibly more internalized when viewed from the outside with SEM (Mittmann and Wolff, 2012). The cellular steps in the development of the IT lamellae are still unknown, but the prominent lamellae in *C. salei* appear to be invaginations of the external hypodermis (Wolff and Hilbrant, 2011). In both species, the IT lamellae are within the O2 segment and clearly posterior to the base of the O2 limb bud and not an outgrowth of it. At least in some embryos, part of the IT book lung may be covered by the O2 limb bud.

1.2. Advanced embryo (AE) book lungs

At the book lung site in embryos of *C. salei*, Damen et al. (2002) observed expression of *apterus-1* and *-2* and a striped pattern of *pdm/nubbin*. The latter gene was also expressed in the horseshoe crab opisthosomal segments that give rise to the genital operculum and book gills. Also in embryos of *C. salei*, Pechmann et al. (2010) observed stripes indicating *engrailed-1* expression at the book lung site. These were considered to be indicative of developing lamellae within the O2 limb bud. Results herein are in agreement with the hypothesis that the O2 limb bud becomes the AE book lung that continues in postembryonic stages.

In the transition from IT to AE book lungs, the former disappear, and as viewed with SEM, the book lung site becomes a small swelling with initially no spiracle or opening to the outside (Wolff and Hilbrant, 2011; Mittmann and Wolff, 2012). There is forward movement of the book lungs as yolk is moved from the prosoma to the opisthosoma in the inversion (stage 11; Mittmann and Wolff, 2012). Eventually a spiracle is formed at the posterior end of each of the bilateral AE book lungs as they continue to enlarge and increase the number of lamellae in advanced embryo and postembryonic stages (Farley, 2015).

Light and electron micrographs provide some information about the cellular processes of lamellar formation (Farley, 2015) and the structure of lamellae and space holders in adult spiders (Berteaux, 1889; Moore, 1976; Hexter, 1982; Reisinger et al., 1990, 1991; Felgenhauer, 1999; Schmitz and Perry, 2000; Scholtz and Kamenz, 2006; Foelix, 2011; Brunelli et al., 2015). In the present investigation, the focus is on the embryonic stage, and results support the earlier observations that there are similar cellular processes in the formation of book gills and book lungs (Farley, 2008, 2010, 2011, 2012, 2015). A striking example is the pairing of precursor cells from opposite walls of the hemolymph channels in book gills and book lungs, and these cell pairs become the pillar trabeculae that help hold the channel walls in place.

2. Materials and methods

2.1. Experimental animals

A culture of *P. tepidariorum* was maintained with procedures like those described earlier for this species (Mittmann and Wolff, 2012; Farley, 2015). Egg sacs and newly hatched spiderlings were kept in small petri dishes at 25–30 °C. Juveniles and adults were maintained at 22–24 °C in individual plastic containers (280 ml) with paper on the bottom. A partially cut open bottom of a styrofoam cup was provided for cover, web attachment and support for a small pad of moist paper. The spiders were fed, containers cleaned and the papers changed once each week. Spiderlings were given a flightless strain of freshly killed fruitflies (*Drosophila hydei*) and/or small crickets while juveniles and adults were given freshly killed crickets of larger size.

The embryonic staging sequence of Mittmann and Wolff (2012) for the species of this investigation was used to estimate the stage of development of the specimens used herein.

2.2. Histology

For preservation of cellular structure, no prior chemical treatment was used to remove embryonic membranes before fixation. Eggs were removed from egg sacs; to get some indication of the stage of development, eggs were examined with light transmitted from below the dissecting microscope. A fine pin was inserted into the prosoma or posterior opisthosoma, and each embryo with inserted pin was immersed in Bouin's fixative. After 12–24 h, the embryos were washed in spider physiological saline (Schartau and Leidescher, 1983), removed from the pins, dehydrated in a graded sequence of ethanol and embedded in paraffin. Sections (8 μm) were stained with Harris' hematoxylin and eosin Y. Bouin's fixative, staining solutions and procedures were obtained from American Master Tech Scientific, Lodi, CA.

2.3. Electron microscopy

A fine pin was inserted into the prosoma of advanced embryos as they were just starting to hatch as indicated by the partially opened embryonic membranes at the anterior end (Mittmann and Wolff, 2012). Each pinned embryo was immersed in a drop of glutaraldehyde fixative, where fine forceps were used to remove abdominal embryonic membranes and much yolk tissue. The dissected specimens, still pinned through the prosoma, were maintained in fixative for 12–24 h at 2–4 °C. The fixative consisted of 2% glutaraldehyde and 0.1 M sodium cacodylate. Tissues were washed in a buffer solution consisting of 0.1 M sodium cacodylate, 0.05% calcium chloride and 0.23 M sucrose. The pinned embryos were postfixed (12–24 h) in 2% osmium tetroxide, 0.1 M sodium cacodylate, 0.05% calcium chloride and 0.14 M sucrose. The tissues were washed again in buffer solution, the pins were removed and the embryos were dehydrated in a graded series of acetone. The tissues were embedded in Spurr's (1969) plastic, modified for a new replacement component (Ellis, 2006). The fixatives, wash solutions and physiological saline are each about 400 mOsm, as recommended for embryonic tissues (Hayat, 2000).

Semi-thin and ultrathin sections were cut on a RMC MT-X microtome (Boeckeler Instruments). The latter sections were collected on grids, stained with alcoholic uranyl acetate and lead