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Moulting of insect tracheae captured by light and electron-microscopy in the metathoracic femur of a third instar locust *Locusta migratoria*

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ABSTRACT

The insect tracheal system is an air-filled branching network of internal tubing that functions to exchange respiratory gases between the tissues and the environment. The light and electron-micrographs presented in this study show tracheae in the process of moulting, captured from the metathoracic hopping femur of a juvenile third instar locust (*Locusta migratoria*). The images provide evidence for the detachment of the cuticular intima from the tracheal epithelial cells, the presence of moulting fluid between the new and old cuticle layers, and the withdrawal of the shed cuticular lining through larger upstream regions of the tracheal system during moulting. The micrographs also reveal that the cuticular intima of the fine terminal branches of the tracheal system is cast at ecdysis. Therefore, the hypothesis that tracheoles retain their cuticle lining at each moult may not apply to all insect species or developmental stages.

1. Introduction

The insect tracheal system functions to exchange respiratory gases between the tissues and the environment (Wigglesworth, 1965). The pathway for oxygen begins with its diffusion into the spiracle openings, where it is then delivered in the gas-phase along an internal system of successively smaller tracheal tubes (Fig. 1), the terminal branches of which are commonly referred to as tracheoles. Oxygen diffuses across the tracheole walls and into the surrounding tissue where it is consumed by mitochondria during aerobic respiration (Whitten, 1972; Wigglesworth, 1965, 1983).

The maximum rate that oxygen can be delivered along the tracheal system is at least equal to the maximum rate it is required for aerobic metabolism, because even during heavy exercise there is little accumulation of anaerobic products (Beenakkers et al., 1981; Sacktor, 1976; Worm and Beenakkers, 1980). The tracheal system also keeps pace with increasing oxygen demands of the insect throughout ontogeny, and we now have a good understanding of the developmental processes involved. The basic plan of the tracheal system is laid out during embryogenesis when major tracheae arise from epithelial sacs composed of tracheal precursor cells (Ghabrial et al., 2003; Manning and Krasnow, 1993). In *Drosophila* at least, the embryonic growth of major tracheae occurs solely by cell migration and elongation (Samakovlis et al., 1996), and it is not until egg hatching that the terminal tracheoles really begin to develop in the juvenile instar (Ghabrial et al., 2003; Manning and Krasnow, 1993). Impressively, the tracheoles react and grow toward signals produced by oxygen-starved cells (Guillemin et al., 1996; Jarecki et al., 1999; Wigglesworth, 1954). Experiments performed mostly on *Rhodnius* have shown that when a major trachea is severed, or if a metabolically active organ is implanted into the insect body, nearby tracheoles are pulled by epidermal filaments to service the hypoxic tissue, and if the insect is then given the opportunity to moult, the intrusion and proliferation of tracheoles is even more extensive (Locke, 1958a; Manning and Krasnow, 1993; Wigglesworth, 1953, 1954, 1959, 1977).

The growth and proliferation of hypoxic-seeking tracheoles is not the only mechanism insects use to enhance oxygen delivery capacities during development. Insects also significantly increase the size of the tracheal system with each moult. This is achieved through the synthesis of new tracheal tubes that are cemented onto the system, and by a substantial increase in the volume of existing tracheae (Keister, 1948; Locke, 1958b; Manning and Krasnow, 1993; Wigglesworth, 1954, 1973, 1981). Existing tracheae increase in size as the epithelial cells divide and multiply (Ryerse and Locke, 1978; Wigglesworth, 1981), although in *Drosophila* larvae it might solely be due to changes in cell shape and size (Madhavan and Schneiderman, 1977; Manning and Krasnow, 1993). Either way, the diameter of the tracheal wall increases, and as this occurs the cuticular intima becomes detached from the epithelial layer in a process known as apolysis

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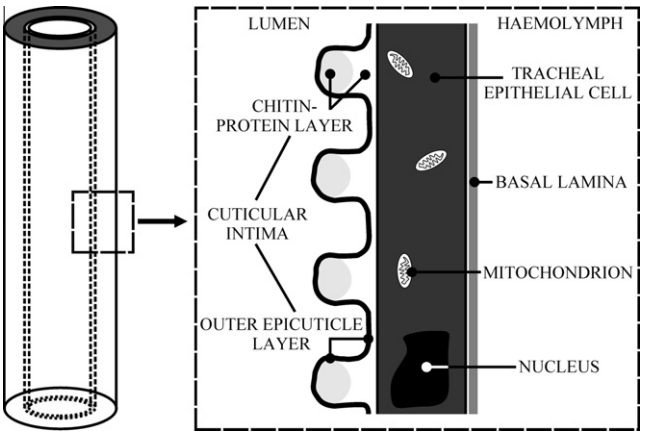

Fig. 1. Diagram of an insect trachea showing the outer epicuticle layer, the chitin–protein matrix layer, the epithelial layer, and the basal lamina (after Locke, 1957; Wigglesworth, 1965). In smaller tracheae, the thickness of the respective layers is much reduced, and the chitin–protein complex is often absent. In small terminal tracheae (tracheoles) the epithelial layer may also be absent.

(Wigglesworth, 1973). The exuvial space between the new wall and the shed cuticular intima fills with moulting fluid (Jungreis, 1974; Passonneau and Williams, 1953), and a new cuticle begins to line the new and larger tracheae (Ryerse and Locke, 1978). The old cuticular intima is then withdrawn through the tracheal system towards the spiracles where it is shed along with the rest of the exuviae at ecdysis. To complete the moulting process, moulting fluid is resorbed by the epithelial layer (Lensky and Rakover, 1972) and the lumen of the new and more extensive tracheal system fills with air (Keister, 1948; Locke, 1958b; Ryerse and Locke, 1978; Wigglesworth, 1981).

Although the process of tracheal apolysis and ecdysis is fairly well established, there is confusion over whether or not the cuticular intima of the tracheoles is shed along with the rest of the tracheal system at moulting. Apparently, tracheoles in *Rhodnius* remain unchanged throughout their lifecycle (Wigglesworth, 1973), and in *Bombyx*, the tracheoles also appear to retain their cuticle with each moult, at least in the first three larval stages (Matsuura and Tashiro, 1976). However, earlier studies on *Sciara* (Keister, 1948) and *Drosophila* (Whitten, 1957) using phase-contrast optical microscopy found that the cuticular intima of tracheoles is shed in the early larval stages, but not in the final larval or pupal moults. Keister (1948) suggested that partial moulting might take place in complex tracheal systems, especially when tracheoles penetrate the surface of other cells, and complete moulting might only occur in insects with relatively simple tracheal systems.

High magnification electron micrographs of tracheoles in the process of moulting have never been captured and published, and so the early claims made by Keister (1948) and Whitten (1957), that in some insects at least, the tracheoles do in fact shed their cuticular lining remain unsubstantiated. The purpose of the current paper is to present a series of light and electron-micrographs captured from the metathoracic femur of a third instar migratory locust *Locusta migratoria* that provide unequivocal evidence that some tracheoles shed their cuticle lining at moulting.

2. Materials and methods

The locust reported in the current study was a single third instar gregarious-phase nymph, 3–4 days post-moult, of the species *L. migratoria*. It was sourced from a breeding colony at the University of Adelaide, Australia, where locusts were reared in a large terrarium at 33 ± 1 °C, with a relative humidity of ~30%, under a 12:12 h light–dark cycle, with *ad libitum* access to seedling wheatgrass and wheat germ. When the locust was taken from the colony, it displayed no apparent visual indication that it was in the initial stages of moulting, and was simply part of a much larger cohort of approximately 20 locusts processed for electron microscopy.

The procedure used to dissect and fix locust tissue was modified based on the methods of previous electron microscopy studies of insects and spiders (Biserova and Pfluger, 2004; Hartung et al., 2004; Kohnert et al., 2004; Schmitz and Perry, 2002a,b). Initially, the locust was fasted for 6–10 h before it was cold anaesthetised for 20 min in a refrigerator at 4 °C. Both metathoracic femurs were carefully removed with angled spring scissors and then a razor was used to slice each femur along the transverse plane into six equal length tissue pieces. The femur pieces were immediately immersed into a chemical fixative solution of 2.5% glutaraldehyde and 2% formaldehyde in 0.2 M phosphate buffer with pH 7.4, and left overnight in a refrigerator at 4 °C. On the second day, each piece was given a series of buffer rinses; the first rinse was 20 min followed by four, 60-min rinses. Then there were three, 20-min distilled water rinses and then the tissue was placed into a 1% aqueous solution of osmium tetroxide for secondary fixation and left overnight at room temperature. On the third day, each piece was given five, 20-min rinses in distilled water and then placed into a 2% aqueous solution of uranyl acetate and left overnight at room temperature. On the fourth day, the pieces were given four, 20-min rinses in distilled water and then dehydrated in ethyl alcohol in 10% incremental steps starting from 50% to 80%, each for 20 min. The tissue was then further dehydrated in consecutive, 20-min immersion in 90% ethanol (2×), 100% ethanol (2×), and finally pure propylene oxide (2×). Following dehydration, samples were incrementally infiltrated with embedding resin (Durcupan, Fluka, Switzerland) at ratios of 3:1, 2:2, 1:3 (propylene oxide:resin) each for a duration of 60 min, and left overnight at room temperature in pure resin. On the fifth day, each piece was aligned longitudinally and in a proximal to distal orientation in individually labelled embedding moulds where they were covered with pure embedding resin and left to polymerise in a 70 °C oven for 48 h.

One metathoracic femur was then randomly selected for light microscopy analysis. Twelve parallel, transverse and equidistant, 1-μm thick sections were cut proximally to distally along the leg using 8 mm glass knives and an ultramicrotome (EM UC6, Leica Microsystems, Germany). The sections were placed onto glass slides, stained with toluidine blue, rinsed with distilled water, and air-dried. Each section was viewed under an optical microscope (BX51, Olympus, Germany) and tracheae were photographed with a mounted digital colour camera (ColorView III, Soft Imaging System, Olympus, Germany) which generated 24-bit 2576 × 1932 pixel resolution images.

The remaining femur was used for transmission electron microscopy. Six equidistant 70-nm vertical sections were cut proximally to distally along the leg at systematically random angles around the vertical plane using a diamond knife (Diatome, Switzerland) and ultramicrotome. Sections were placed onto 3 mm copper mesh grids, stained with lead citrate, and viewed with a transmission electron microscope (CM 100, Philips, The Netherlands) where 16-bit 1280 × 1024 pixel resolution images of tracheae were captured with a mounted digital camera (MegaView II, Soft Imaging System, Olympus, Germany).

3. Results and discussion

3.1. Description of moulting tracheae

The moulting of the tracheal system begins with the formation of a new and larger tracheal wall around the existing wall (Fig. 2A),