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Thoracic spiracular gill structure of *Lipsothrix* (Diptera, Limoniidae) in Britain described from scanning electron micrographs

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Abstract

Recent ecological surveys have resulted in rearing examples of the five British members of the genus *Lipsothrix* (Diptera, Limoniidae). These have provided samples for investigating the morphology of their thoracic spiracular gill by the use of scanning electron microscopy. This reveals unique characteristics for the five species available. The differing structures may relate to varying larval habitats. A species identification key for pupae is based on features of the plastron. Information from the juvenile stages supports the placing of *Lipsothrix* in the Limoniinae.

Key words: plastron, saproxylic, aquatic

Introduction

The thoracic spiracular gills of the pupae, often referred to as pupal horns (Fig. 1), of members of the genus *Lipsothrix* (Diptera; Limoniidae) were described in detail first by Hinton (1955) using light microscopy and some transmission electron microscope work. In *Lipsothrix* they achieve the greatest level of complexity of these structures to be found in the family (Hinton, 1967). These remarkable features of the pupae in which plastron structures are also present are found in a number of genera of the limoniids. The gills provide for gaseous exchange between the insect and its environment through fine structures of the plastron in the membrane. They function for both oxygen transfer while submerged and water retention when exposed to air (Hinton, 1955). The plastron lines (Hinton, 1957; p.113) are clearly visible using a scanning electron microscope, marked by rows of punctures (e.g., Fig. 2). Plastrons are found mostly in insect stages associated with well-aerated water but also prone to fluctuating levels (Hinton, 1981).

Later, Hinton (1967) was able to use a scanning electron microscope, then a newly invented machine, and could see more detail in order to better understand the structure (Fig. 18). His diagrammatic reconstruction based on these further observations accords very closely to the image produced by a more sophisticated modern machine (Fig. 17). He regarded the thoracic gills of *Lipsothrix* as the most complex of any such structures that were available for study. Several other

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genera of limoniid craneflies have more or less prominent spiracular gills. The genus *Gnophomyia* includes saproxylic species and are found under bark amongst fermenting sappy tissue of living or recently fallen trees. They are effectively semi-aquatic but in a situation protected from extremes of desiccation or flooding of their environment. Their thoracic gills are less complex than *Lipsothrix* and do not have a plastron.

The plastron was succinctly described by Hinton (1962) as a constant volume of gas held in position by hydrophuge structures that resist the entry of water under pressure. Thoracic gills with their plastron structures provide for respiratory function when immersed in water. The small area of the spiracle from which they arise prevents water loss when not submerged (Hinton, 1955). This is particularly well-expressed in the genus *Lipsothrix* and in this genus the gills evolved as a response to the semi-aquatic nature of the habitat in which the larvae develop and the changing water levels they experience during development. There are analogous structures in certain insects' eggs in those species that also are prone to periodic flooding (Hinton, 1962, 1981). After the initial investigations into plastron morphology (Hinton, 1955; 1967) no other studies have examined the structures in such detail although the genus has been the subject of ecological research (Dudley & Henderson, 1987). Recently, in order to define species and habitat conservation strategies for dead-wood insects, surveys conducted in Britain have concentrated on *Lipsothrix* species as representatives of water-logged or submerged saproxylic insect faunas (Godfrey, 2000, 2001a, 2001b, 2002, 2007; Hancock, 2002; Hewitt & Parker, 2004; Rotheray, 2000).

Lipsothrix species deposit their eggs in wet wood where larval development takes place (Brindle, 1967). Submerged or semi-submerged wood is a component of riverine habitats that is sensitive to disturbance or unsympathetic management (Dudley & Anderson, 1987; Godfrey, 2000; Hewitt & Parker, 2005). In *Lipsothrix* the characteristic thoracic spiracular gills of the pupae are useful in field surveys as they are a prominent and highly visible feature of the pupal exuviae, remaining visible after adults have emerged. They are more easily located than live adults and so good sizes of samples can be collected. As the species can be identified without the need to collect the adults this stage is free to mate and lay eggs. A method of acquiring distributional and abundance data based on pupal exuviae helps to conserve scarce species.

Method

Larvae or pupae were collected from wet wood that was either partly submerged, close to the margins of small streams and slow-flowing rivers or in associated seepages. Occasionally they can be found in wood lying within the splash zone of waterfalls. Varying sizes of wood were sampled from large logs to small branches and twigs. The larvae and pupae were found under loose bark or within tunnels close to the surface of the exposed soft-decayed wood. This situation is described in detail by Dudley & Anderson (1987). Specimens were kept alive within their wood samples and placed in clear plastic containers with perforated lids using the same technique as Hewitt & Parker (2005). Small pads of paper tissue in the containers were kept moist until adults appeared. Each emergent adult was removed and kept with its empty pupa, labelled to ensure accurate association of field data with the preserved specimens. Sites were located in Scotland, England and southwest France.

Species identifications were established from morphological features of the adults (Edwards, 1938; Coe, 1950). Dried pupal exuviae were selected for examination by scanning electron microscopy. Specimens mounted on stubs were gold-palladium coated before examination using a Jeol 6400 Scanning Electron Microscope. The areas of the thoracic spiracular gills selected for image production were the outer and inner rims at the midpoint of the gill and the membrane of the outer and inner surfaces below these rims. At each of these locations the magnification was at x400 or x500 (Figs 2–11). Details of the inner surfaces were viewed at x2500 or x3000 (Figs 12–17). For comparison, if specimens of the same species were available from different sources, other examples were examined also.

Results

The fieldwork resulted in the five known British species being collected and reared in captivity. These are *Lipsothrix ecucullata* Edwards, 1938, *L. errans* (Walker, 1848), *L. nervosa* Edwards, 1938, *L. nobilis* Loew, 1873 and *L. remota* (Walker, 1848). The thoracic spiracular gills bear surface details of a number of features that vary between the species. The inner concave and the outer convex surfaces differ from each other and are illustrated showing details of the rim structure and the adjacent membrane surface. The outer rims of the five species are shown in Figures 2-6. In *L. ecucullata* the rows of aeropyles marking the position of the plastron lines (Hinton, 1967) terminate in a tear-drop shaped thickening to the integument (Fig. 2). It is the only one of these species in which the rows of aeropyles are not continued down the outer convex face. *Lipsothrix errans* has its rim edge clearly defined by impressed pores. Below this the plastron lines continue in parallel rows that occasionally anastomose (Fig. 3). In *L. nervosa* (Fig. 4) the rim has a corrugated appearance due to raised plastron lines. The arrangement in *L. nobilis* differs in having the marginal pores below the rim not deeply impressed (Fig. 5). A combination of features can be seen in *L. remota* where the rim is defined by deeply impressed pores and the plastron lines form branches almost immediately with adjacent rows forming a mesh-like pattern on the surface (Fig. 6).

The inner rim of *L. ecucullata* shows plastron lines as short, well-spaced raised rows that expand apically creating finger-like shapes. The membrane beyond is covered with small raised spots (Fig. 7). A distinct rolled-rim effect is seen in *L. errans* beyond which the plastron lines extend for a short distance in short finger-like extensions. The membrane bears small clusters of short spines within a smooth but slightly undulating surface (Fig. 8). In *L. nervosa* (Fig. 9) the wide finger-like plastron lines around the rim are longer than the other species and bear transverse slits. Within the membrane are raised spots, similar to *L. ecucullata*. *L. nobilis* is similar to *L. ecucullata* but the finger-like rows over the rim are of varying length and the membrane immediately beyond is wrinkled and then bears elongated clusters of closely packed small spines (Figs. 10A, 10B). The plastron lines over the rim in *L. remota* are parallel, not terminating in finger-like shapes, below which the surface membrane bears a raised mesh-like pattern and is completely covered in spicules (Fig. 11).

A closer examination of the inner membranes shows the raised spots in *L. ecucullata* (Fig. 12), similar to *nervosa* (Fig. 14) but the latter are more densely packed. The groups of spines in *L. errans* are not associated with any other surface feature (Fig. 13) but in *L. nobilis* they surround sinuous slits in the surface (Fig. 15). The whole membrane in *L. remota* is covered by short, branched and stellate processes including the raised plastron lines forming a strong mesh-like pattern (Fig. 16).

Lipsothrix pupae are mobile under bark or within tunnels in the surface layers of water-logged wood in which the larvae fed. In addition to the physical function of the plastron structures there are behavioural responses by pupae to immersion. We have observed *L. remota* moving forward to the mouth of the tunnel in which they lie and protrude the thoracic gills which then open out. This allows full gaseous exchange to take place when submerged in water. If the water level is reduced to below the level of the tunnel the pupae withdraw again and the gills fold over the anterior part of the pupa, closing off the tunnel exit. This will assist with preventing excess water loss during exposure. Upon emergence the pupae move to the surface of the wood and after the adult has flown away the empty pupal exuvium is left protruding from the substrate as evidence of the former occupation.

Discussion

Having described the thoracic spiracular gills of these *Lipsothrix* species some consideration can be given to their functional morphology. As mentioned above, the ability in at least one species of the pupae to move in response to flooding or drying out indicates a behavioural reaction to the respiratory need. Also, there are clear differences in spiracular gill morphology as revealed by the scanning electron microscope. This variation in structural complexity might be explained if the species were exposed to differing risks of flooding or occupy partly submerged habitats. Present field data do provide some information to support this. *L. remota* is ubiquitous in wet wood lying in the water in stream margins. *L. nobilis* occurs usually in larger pieces of wood, actually in streams or rivers and in backwater channels. Both *L. ecucullata* and *L. errans* are found in valley-side wet woodland floors where there are seepages and small streams. These may be considered more prone to risks of drying out. In these cases their reduced rows of aeropyles and lack of openings within the membrane surfaces as shown (Figs 7–8) would serve as protection against excess water vapour loss. Alternatively, it could be argued that in the high-rainfall upland areas of north-western Britain where *L. errans* and *L. ecucullata* occur, the sloping wet woodland floor is a relatively stable environment that neither floods nor dries out and so for this reason they are less complex. By contrast, *L remota* and *L nobilis* occur in situations prone to sporadic inundation in flood events and this might explain the adaptive behaviour noted above in *L remota* pupae as well as the greater number of openings in the membrane surface, enabling greater gaseous exchange in flood situations. Currently, there is insufficient information on the location of *L. nervosa* larval substrate to relate the plastron structure in that species to risks of flooding or degrees of submergence.

A relationship between plastron structure and habitat was mentioned by Hinton (1976) with reference to *Simulium* species (Diptera, Simuliidae). In observing the differing abilities to withstand experimentally induced flooding of the plastron under pressure, he suggested these varying capacities might relate to the flooding risk for these species in nature. However, detailed morphological differences of the plastron structures were not indicated for the *Simulium* species mentioned in this context. Our investigation into the microscopic complexity of *Lipsothrix* shows consistent variation in plastron structures between species that may relate to fluctuating habitat requirements.

Note on taxonomic positioning of *Lipsothrix*

Until recently *L. nobilis* was referred to as *nigristigma* Edwards, 1938 in many works; the synonymy was formalised in Stary (2007) after earlier investigation by Godfrey (2001). Several aspects of the juvenile stages support the additional proposal by Stary (2007) in moving *Lipsothrix* from the Chioneinae (= Eriopterinae) to the Limoniinae, based on adult characters. The larvae have distinct ventral creeping welts with minute spicules (Brindle, 1967; Alexander & Byers, 1981) that are a feature of the Limoniinae but absent in the Chioneinae. The phylogeny of the Tipuloidea based on immature stages (Oosterbroek & Theowald, 1991) also shows that *Lipsothrix* in relation to the Eriopterini (*sensu lato*) and Limoniini is clearly embedded within the latter, based on several morphological features of the head capsule, amongst other characters. In the Limoniidae pupae that have large thoracic gills with plastron structures are found only in other limoniines such as *Geranomyia*, *Antocha* and *Dicranomyia*. Hinton (1968) considered the spiracular gills to have evolved independently in these four groups although at the time *Lipsothrix* was placed in the Eriopterini, as then defined. The transfer of *Lipsothrix* does not alter his statement that these represent four separate invasions of fresh or sea water. He showed that structural details in the gills and their plastron are not homologous. Features of the immature stages serve to reinforce the relatively new position of *Lipsothrix* as a limoniine cranefly.

Key to pupal exuviae based on features of the plastron visible in scanning electron microscope images

The characters of the thoracic gills used in this key are on both inner and outer rims and surfaces at the mid-point. It is possible to see most of these features with a light microscope. Care should be taken if examining specimens with transmitted light because the plastron lines from the inner face can show through to the outer face and vice versa. It is better to use reflected light, if possible.

1. Outer face below rim with straight plastron lines terminating in tear-dropped raised area (Fig. 2) .... *ecucullata* Edwards
- Outer face below rim with plastron lines that anastomose, joining together and rejoining, over the outer face 2
2. Inner surface with scattered spots and no spicules; finger-like plastron lines elongate and bearing transverse slits (Figs 9 & 14).. *nervosa* Edwards
- Inner surface with spicules; plastron lines on rim with circular openings (Figs 8, 10A, 10B & 11) 3
3. Spicules covering whole inner surface (Fig. 11) ....*remota* (Walker)
- Spicules in distinct groups (Figs 8, 10A & 10B).. 4
4. Inner surface with a few small spicules, not associated with any openings in the integument; plastron lines over rope-like rim terminating in short finger-like processes (Fig. 8 & 13) .... *errans* (Walker)
- Inner surface with numerous spicules grouped around small slits in the membrane; rim with finger-like plastron lines below which the membrane is transversely wrinkled (Figs 10A, 10B & 15).. *nobilis* Loew

Acknowledgements

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FIGURE 1. Thoracic gill of *Lipsothrix errans* (Walker), pupal exuvia; from Old Close Wood, Cumbria, England, 1 April 2004, S.Hewitt. Scale bar: 0.1mm (100 mu).

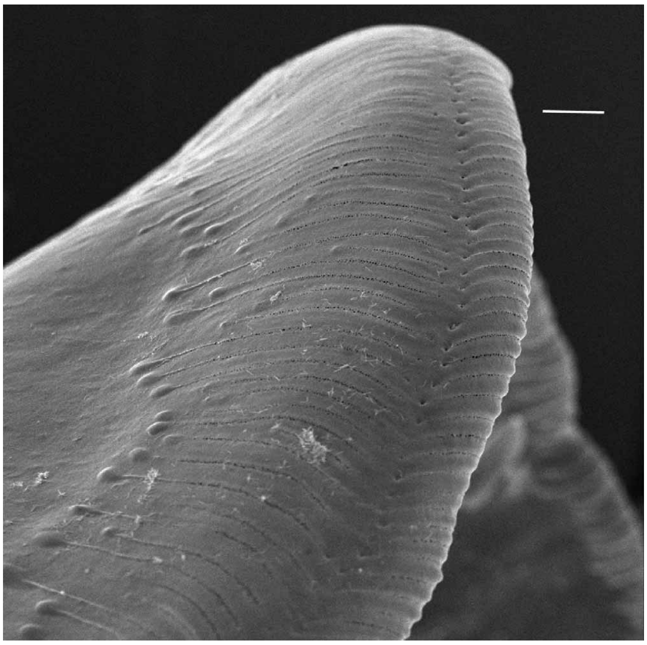

FIGURE 2. Thoracic gill of *Lipsothrix ecucullata* Edwards, outer rim (x500); from Invershin, Sutherland, Scotland, 14 April 2000, E.G. Hancock. Scale bar: 20 mu.

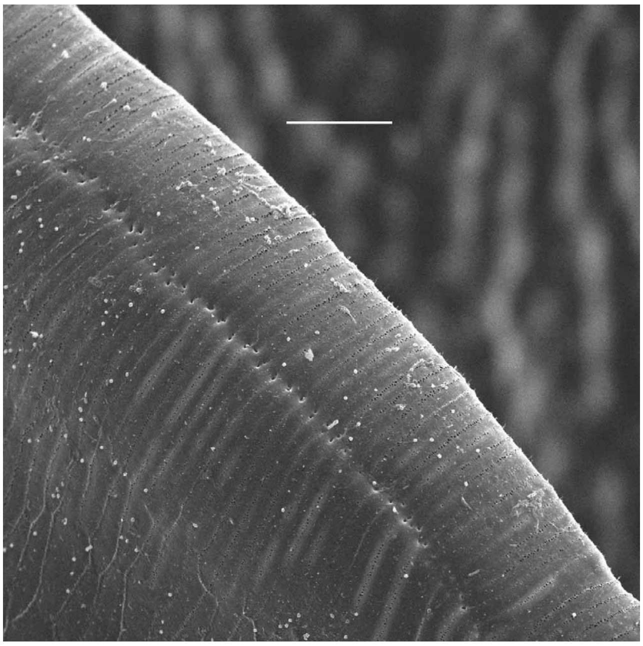

FIGURE 3A. Thoracic gill of *Lipsothrix errans* (Walker), outer rim (x500); from Reserve Naturelle de la Massane, Banuyls-sur-Mer, Pyrenées-Orientales, France, 5-9 May 2003, E.G. Hancock. Scale bar: 20 mu.

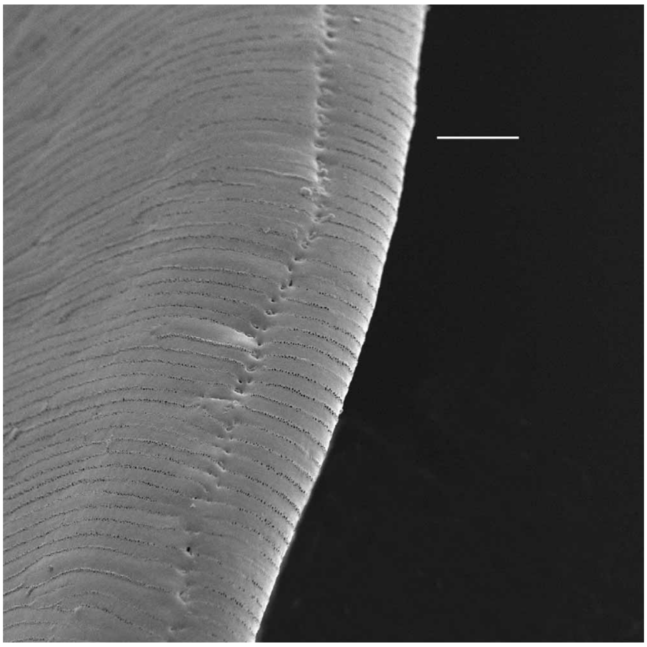

FIGURE 3B. Thoracic gill of *Lipsothrix errans* (Walker) outer rim; from Old Close Wood, Cumbria, England, 1 April 2004, S. Hewitt. Scale bar: 20 mu.

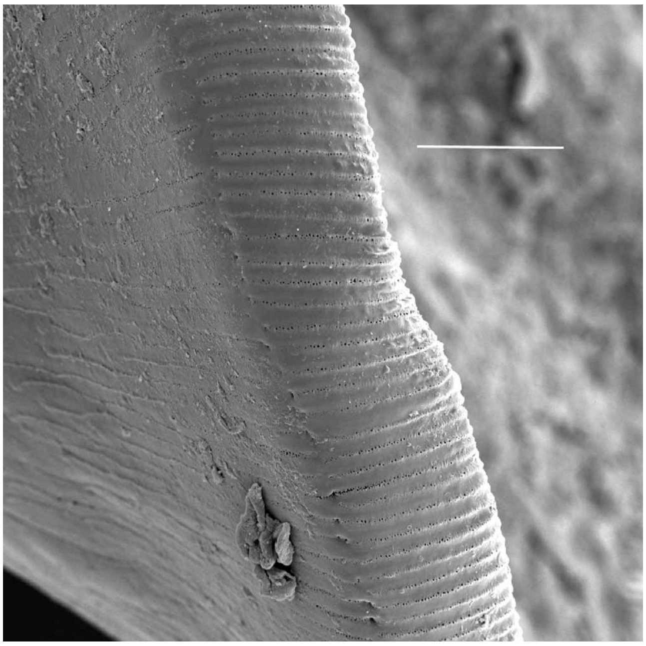

FIGURE 4. Thoracic gill of *Lipsothrix nervosa* Edwards, outer rim (x500); from Millyford Bridge, New Forest, Hants, England, May 2005, A. Godfrey. Scale bar: 20 mu.

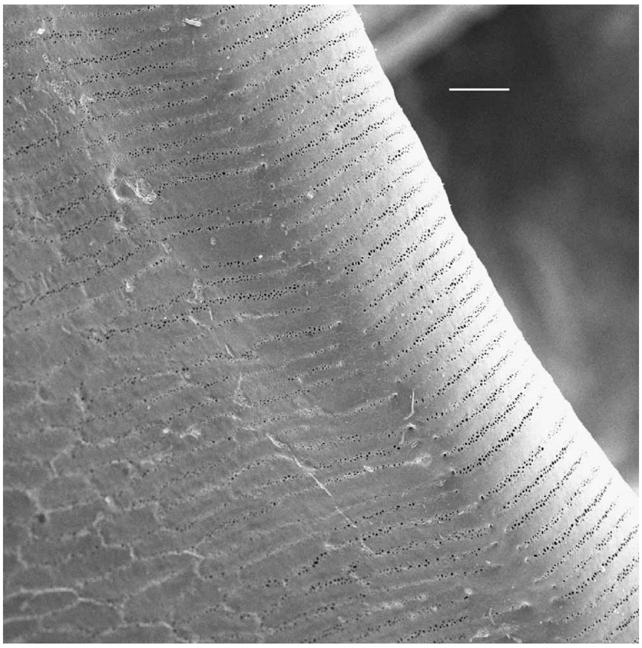

FIGURE 5. Thoracic gill of *Lipsothrix nobilis* Loew, outer rim (x500); from Lyn Woods, Cumbria, England, 11 April 2004, S.Hewitt. Scale bar: 20 mu.

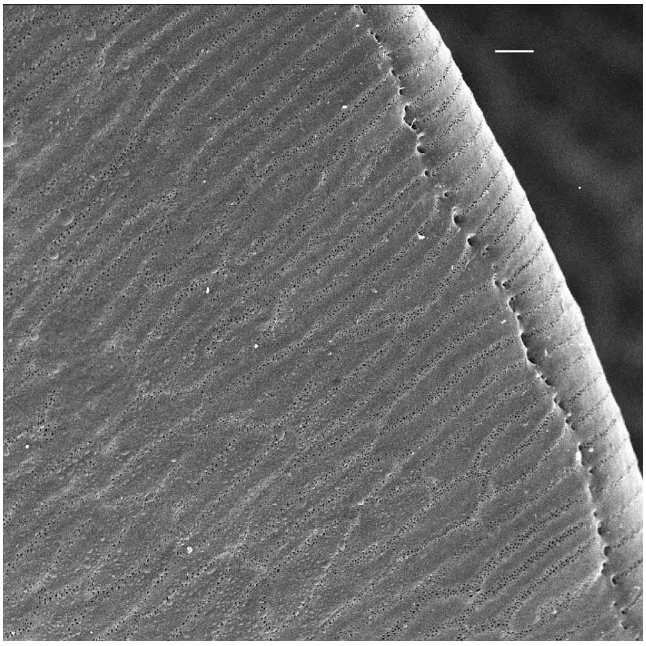

FIGURE 6. Thoracic gill of *Lipsothrix remota* (Walker), outer rim (x500); from Old Close Wood, Cumbria, England, 1 April 2004, S. Hewitt. Scale bar: 20 mu.

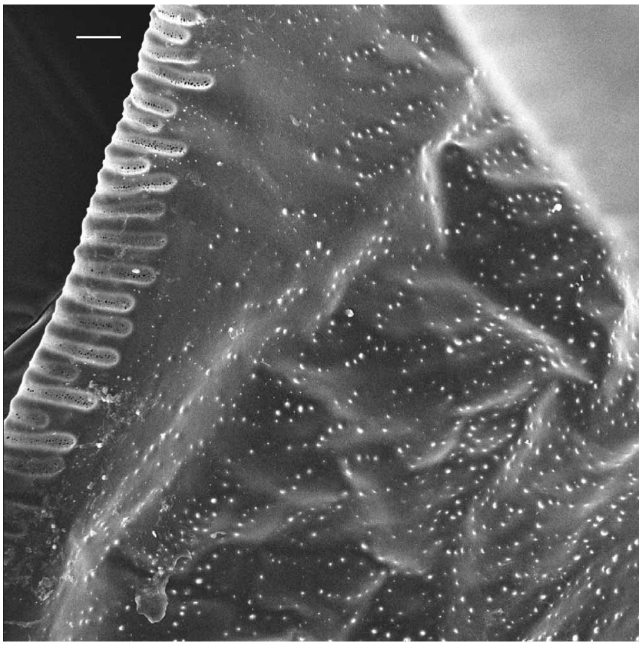

FIGURE 7. Thoracic gill of *Lipsothrix ecucullata* Edwards, inner rim (x500); from Invershin, Sutherland, Scotland, 14 April 2000, E.G. Hancock. Scale bar: 20 mu.

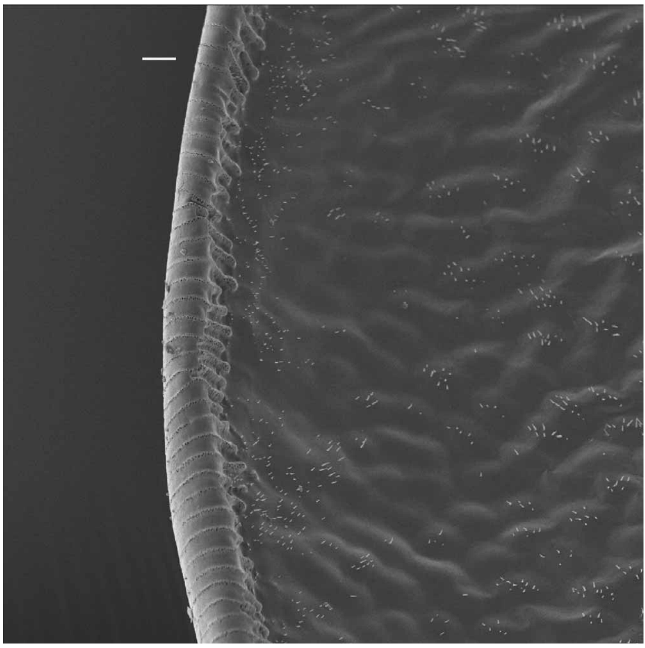

FIGURE 8. Thoracic gill of *Lipsothrix errans* (Walker), inner rim (x500); from Reserve Naturelle de la Massane, Banuyls-sur-Mer, Pyrenées-Orientales, France, 5-9 May 2003, E.G. Hancock. Scale bar: 20 mu.

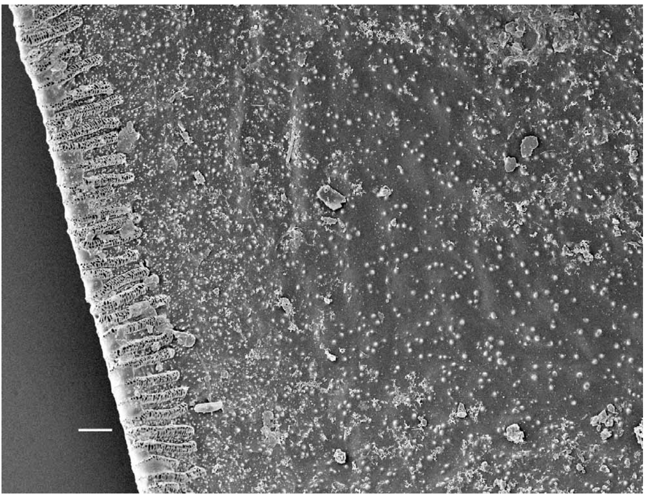

FIGURE 9. Thoracic gill of *Lipsothrix nervosa* Edwards, inner rim (x400); from from Millyford Bridge, New Forest, Hants, England, May 2005, A. Godfrey. Scale bar: 20 mu.

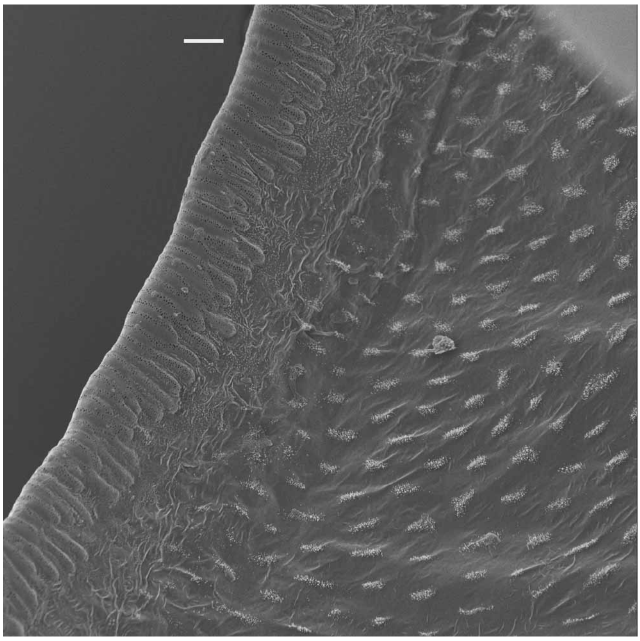

FIGURE 10A. Thoracic gill of *Lipsothrix nobilis* Loew, inner rim (x500); from Lyn Woods, Cumbria, England, 11 April 2004, S. Hewitt. Scale bar: 20 mu.

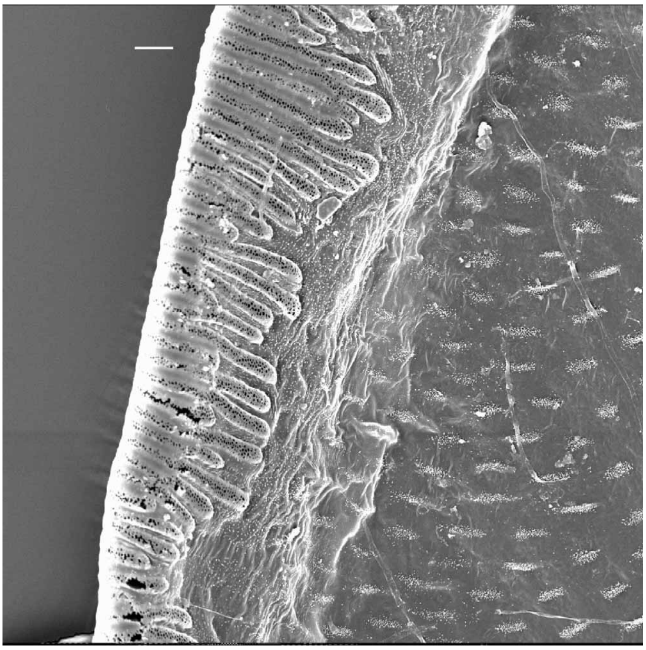

FIGURE 10B. Thoracic gill of *Lipsothrix nobilis* Loew, inner rim (x500); from Highland Water, Millyford Bridge, New Forest, Hants, England, 22 June 2004, N. Mott. Scale bar: 20 mu.

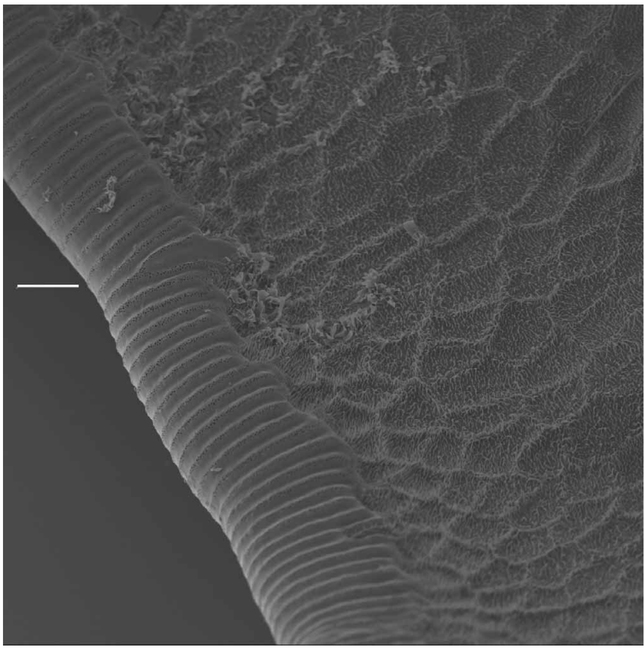

FIGURE 11. Thoracic gill of *Lipsothrix remota* (Walker), inner rim (x500); from Old Close Wood, Cumbria, England, 1 April 2004, S.Hewitt. Scale bar: 20 mu.

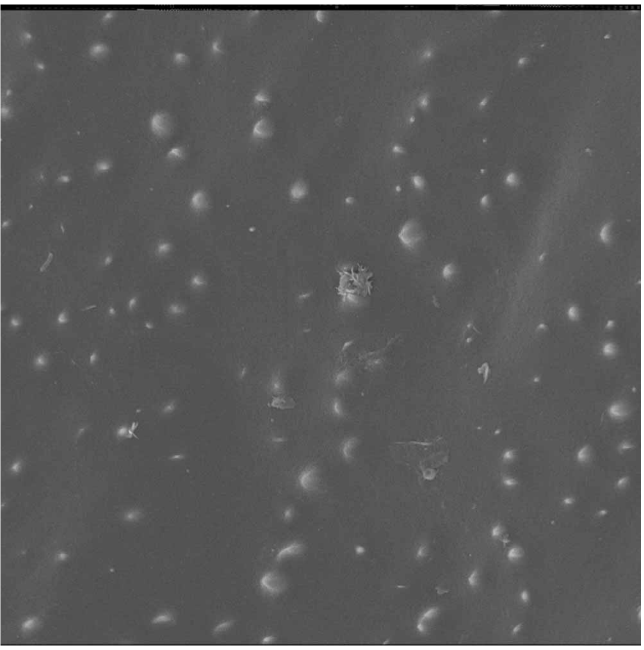

FIGURE 12. Thoracic gill of *Lipsothrix ecucullata* Edwards, inner surface (x2500); from Invershin, Sutherland, Scotland, 14 April 2000, E.G. Hancock.

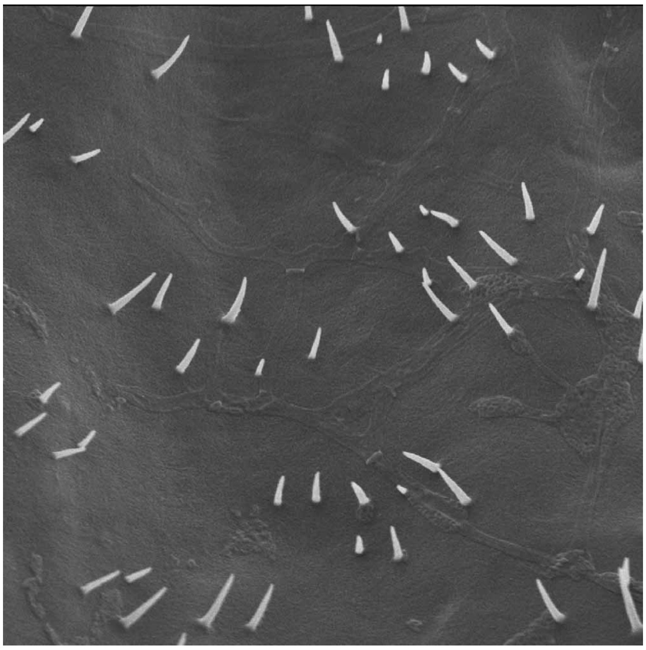

FIGURE 13. Thoracic gill of *Lipsothrix errans* (Walker), inner surface (x2500); from Old Close Wood, Cumbria, England, 1 April 2004, S. Hewitt.

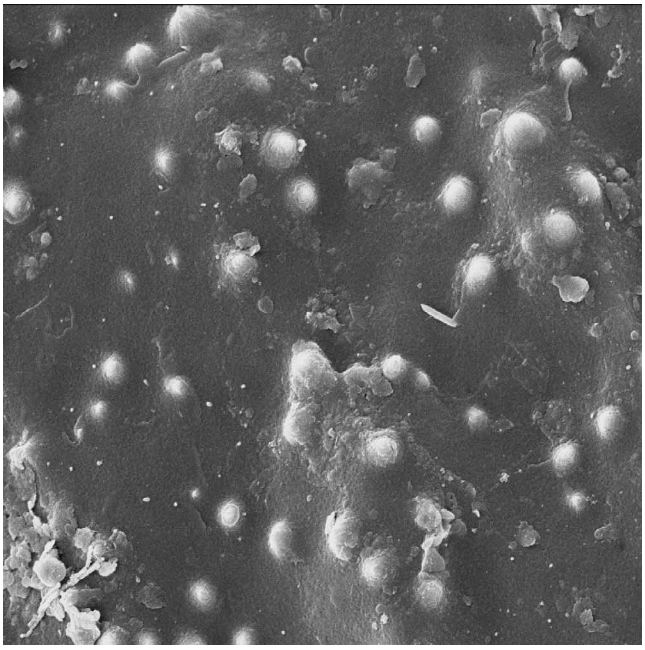

FIGURE 14. Thoracic gill of *Lipsothrix nervosa* Edwards, inner surface (x3000); from from Millyford Bridge, New Forest, Hants, England, May 2005, A. Godfrey.

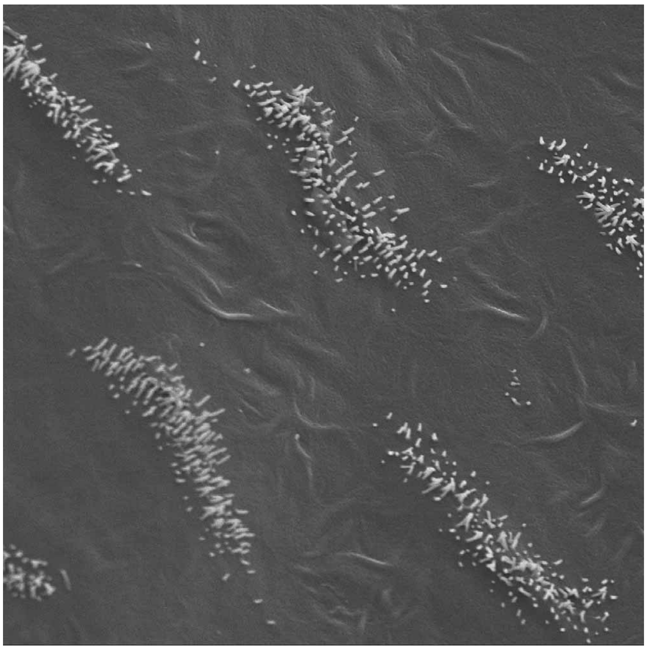

FIGURE 15. Thoracic gill of *Lipsothrix nobilis* Loew, inner surface (x2500); from Lyn Woods, Cumbria, England, 11 April 2004, S. Hewitt.

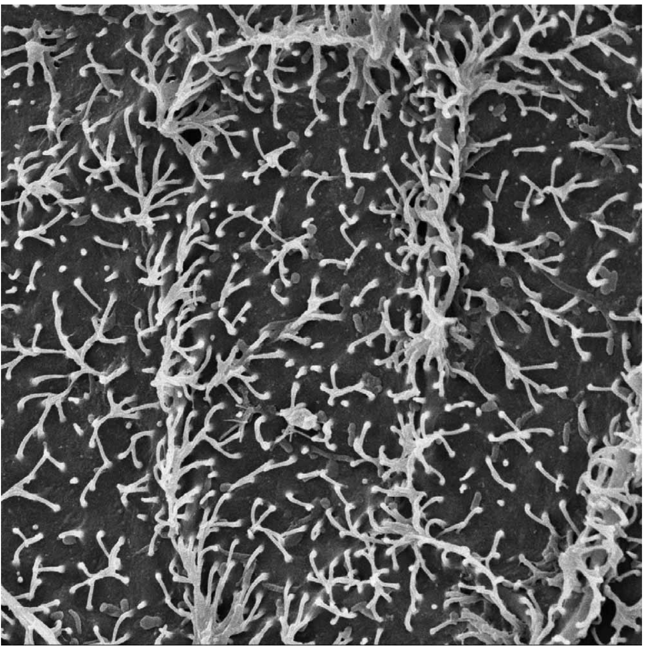

FIGURE 16. Thoracic gill of *Lipsothrix remota* (Walker), inner surface (x3000); from Invershin, Sutherland, Scotland, 14 April 2000, E.G. Hancock.

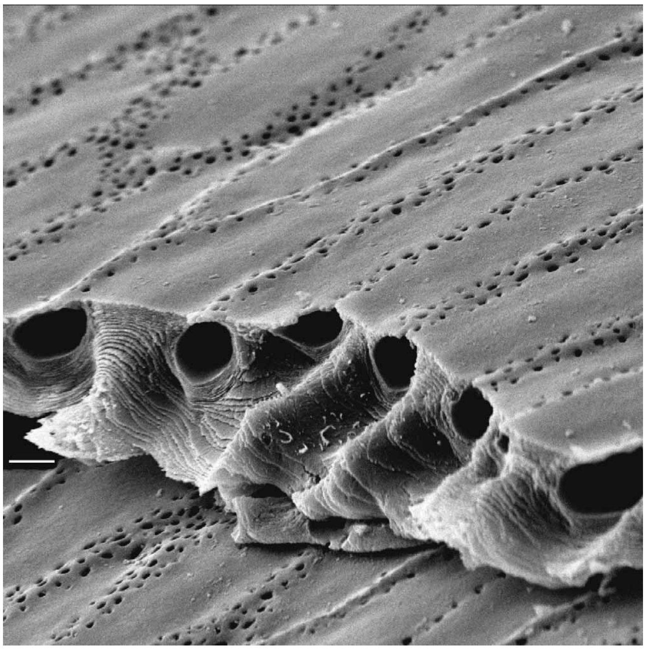

FIGURE 17. Thoracic gill of *Lipsothrix remota* (Walker), broken surface showing plastron lines, (x 3000); from Mugdock Country Park, Glasgow, Scotland, 25 May 2003, E.G. Hancock. Scale bar: 5 mu.

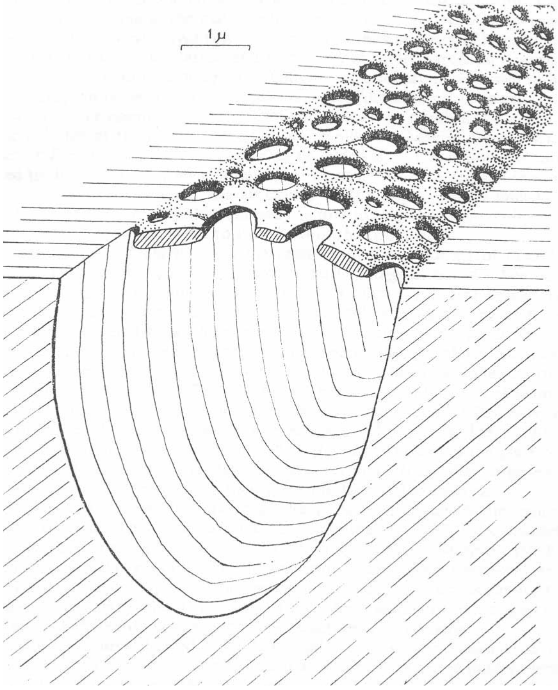

FIGURE 18. Diagram of section through plastron line of *Lipsothrix remota* (Walker) (from Hinton, 1967; figure 1, page 36).