Microscopic structure of the mantle and palps in the freshwater mussels *Velesunio ambiguus* and *Hyridella depressa* (Bivalvia: Hyriidae)

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Abstract

There has been increasing interest in freshwater mussels (order Unionoida) in recent years because their numbers are declining in many parts of the world and also because they have potential as monitors of pollution. Most studies have been performed on the families Unionidae and Margaritiferidae from North America and Europe, and comparatively little is known of the Hyriidae from Australasia. The present study describes the microscopic structure of tissues in the mantle and palps of two hyrid mussels, namely *Velesunio ambiguus* and *Hyridella depressa*, as viewed by light and electron microscopy. The two mussels show similarities with the unionoids and margaritiferids, particularly the presence of extracellular mineralised granules. The mantle and palps of *V. ambiguus* and *H. depressa* consist of flaps of tissue bordered on the inner and outer surfaces by simple epithelia. The intervening tissue is dominated by connective tissue containing vesicular cells, muscle, nerves and blood spaces with haemocytes. Orange–yellow extracellular calcified granules are a prominent feature of the interstitial tissues. The abundance of calcified granules in the mantle of *H. depressa* is greater than that in *V. ambiguus* and there are differences in the appearance of the apical vesicles in epithelial cells.

Additional keywords: Australia, connective tissue, epithelium, granules, ultrastructure.

Introduction

The Australian freshwater mussels *Velesunio ambiguus* (Philippi, 1847) and *Hyridella depressa* (Lamarck, 1819) belong to the family Hyriidae, order Unionoida (Smith 1996; Walker et al. 2001). The Hyriidae are generally included in the superfamily Unionoida (which includes the Hyriidae, Margaritiferidae and Unionidae), although recent studies suggest that it should be assigned to the superfamily Etherioidea (Graf 2000; Walker et al. 2001).

Recently, there has been increasing interest in freshwater mussels because populations have shown major declines in many parts of the world, including parts of Australia (Byrne 1998; Walker \textit{et al.} 2001) and basic knowledge is required to develop appropriate conservation strategies. Studies of ultrastructure can also reveal effects of pollutants and environmental stress (Seiler and Morse 1988; Triebskorn \textit{et al.} 1991).

Whereas the ultrastructure of the unionoids and margaritiferids has been examined in a number of papers, there have been few studies on the hyriids. Most studies have concentrated on the structure and composition of the extracellular granules because these granules can accumulate pollutant metals and, therefore, are of interest in pollution studies (Jeffree and Simpson 1984; Adams \textit{et al.} 1997; Adams and Shorey 1998; Vesk and Byrne 1999; Byrne 2000). The present study makes a more general examination of the tissues of the mantle and palps by light microscopy (LM) and electron microscopy (EM).
Materials and methods

Specimens of *Velestio unio* ambiguus and *Hyridella depressa* were collected from the banks of the Nepean River near the town of Menangle, NSW, Australia.

Mussels were opened by cutting the adductor muscles with a scalpel and the palps and pieces of mantle (approximately 5 × 10 mm) were dissected. For scanning EM (SEM), the tissue was placed into a modified Karnovsky fixative (3% formaldehyde, 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) for 24 h (Jeffree and Simpson 1984). Tissues were then washed in 0.1 M cacodylate buffer, dehydrated and critical-point dried. Pieces of tissue were mounted on SEM stubs, sputter coated with gold and examined in a Jeol JSM T-20 scanning electron microscope.

For transmission electron microscopy (TEM) and LM, small pieces (approximately 2 mm²) of palp and mantle tissue were placed in fixative. Two fixatives were used: either 3% formaldehyde, 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 (cacodylate-buffered fixative; CBF) for 24 h or 3% glutaraldehyde in 0.02 M HEPES buffer, pH 7.2 (HEPES-buffered fixative; HBF) for 1 h. The latter method is preferred for freshwater organisms because of the low osmotic potential of the buffer. Tissues were post-fixed with 0.1 M osmium tetroxide buffered with cacodylate or HEPES according to the fixative used, washed and dehydrated in an ethanol series, then infiltrated with propylene oxide and embedded in Spurr’s resin. Sections were stained with uranyl acetate and lead citrate and examined in a Jeol 100S transmission electron microscope.

For LM, semithin resin sections (0.5 µm) were stained with toluidine blue or with methylene blue–Azure II–Basic Fuchsin (Hayat 1989).

Results

General anatomy

The macroscopic anatomy of *Velestio unio* ambiguus and *Hyridella depressa* was similar to the better-known unionid mussels (Pearse et al. 1987). The two mantle lobes lined the inner surfaces of the shell and were elaborated at the posterior end to form pigmented inhalant and exhalant siphons. The mantle and palp tissues showed varying amounts of cream–orange pigmentation that was associated with mineralised granules. *Hyridella depressa* showed marked orange pigmentation in the palps and the mantle central zone (the region within the pallial line; terminology after Bubel (1973)) and margins. *Velestio unio* ambiguus showed orange pigmentation in the palps, gills and along the mantle margin, but the central zone of the mantle was generally translucent, except for a cream or yellow patch towards the posterior in some specimens.

In LM sections, the central zone of the mantle in both species (Fig. 1a,c) consists of a sheet of tissue with thin outer and inner epithelia separated by a loose interstitial tissue containing haemolymph spaces, haemocytes, muscles, nerves and variable numbers of large vesicular cells (Fig. 1a,b,c). The two species differed in the appearance of the interstitial tissue. In the central zone of the mantle of *V. ambiguus* (Fig. 1a), there were generally loosely packed vesicular cells and large extracellular spaces, often traversed by thin muscle fibres. Clumps of calcified granules were rare, except in the patch towards the posterior of the animal. In *H. depressa* (Fig. 1c,d), the interstitial tissue was similar to *V. ambiguus* in Fig. 1a, but with numerous clusters of granules among the vesicular cells. In some *H. depressa* specimens, the tissue was much denser, as shown in Fig. 1c. Larger granules were also often found scattered through the tissue. Near the mantle margin in both species, the tissue became more densely packed with muscles, clusters of granules, vesicular cells and collagen fibres and the extracellular spaces were smaller.

The distal edge of the mantle margin was thickened and formed into three major folds (Fig. 1b), which are generally considered to be secretory (outer fold), sensory (middle fold) and muscular (inner fold) (Morse and Zardus 1997); the periostracum emerged from the groove between the outer and middle folds. The outer fold was often further divided into
Fig. 1. Mantle. (a) *Vesunio ambiguus* mantle, central zone (HEPES-buffered fixation (HBF), toluidine (tol.) blue, light microscopy (LM)). The outer and inner epithelia (oe and ie, respectively) enclose loosely packed interstitial tissue containing blood spaces (bs), haemocytes (h) and vesicular cells (v). Thin bands of muscle (M) traverse the mantle and lie against the epithelial layers. (b) *Vesunio ambiguus* mantle margin, radial section (cacodylate-buffered fixation (CBF), tol. blue, LM). The edge of the mantle forms three main folds (if, inner fold; mf, middle fold; of, outer fold), with the periostracum emerging between the middle and outer folds (arrowhead). The tissue is more densely packed than in the central zone. M, Muscle; v, vesicular cells; n, nerve bundles; g, clusters of granules. (c) *Hyridella depressa* mantle, central zone (HBF, tol. blue, LM). Example of a mantle with densely packed interstitial tissues. oe, Outer epithelium; ie, inner epithelium; g, granules; h, haemocytes; v, vesicular cells. (d) *Hyridella depressa* mantle, central zone, montage of interstitial tissue (CBF, transmission electron microscopy). A vesicular cell (v) with a mass of storage material and thin peripheral cytoplasm is visible at the top left of the picture. A large haemocyte (h) contains several lysosomes. Laminar extracellular granules (g) lie scattered among the filamentous processes from haemocytes and muscle cells (M).
two or more lobes; examination of a series of previously prepared wax sections showed two or three lobes in nine of 10 *H. depressa* and four of 10 *V. ambiguus* (A. E. Colville, personal observation). The edge of the mantle margin was crossed by several bands of dense muscle. The largest of these ran from the inner surface at the base of the inner fold across to the pallial line. Muscle fibres also ran across from the base of the inner fold to the base of the outer fold. Between the muscle bands, there were clusters of vesicular cells and usually several clumps of calcified granules (Fig. 1h).

The oral (apposed) surfaces of each pair of labial palps were deeply ridged and densely ciliated (Fig. 2a–c). The ridges led down to the ciliated oral groove. The outer surfaces were comparatively smooth with scattered patches of cilia, although, towards the anterior, there were irregular protuberances (Fig. 2d,e).

The interstitial tissue in the palps was also dominated by vesicular cells and muscle (Fig. 2b,c). Clusters of granules occurred in both species, sometimes in large quantities (Fig. 2b,c).

**Interstitial tissues**

**Vesicular cells**

The vesicular cells (Figs 1a,b, 3) were large, with a central region filled with fine granular storage material and a thin peripheral layer of cytoplasm containing the nucleus. Near the nucleus, the cytoplasmic layer was thicker and contained many vesicles of varying electron density (Fig. 3). Fine cytoplasmic extensions often projected from the cell surface.

**Nerves and glio-interstitial cells**

Nerve trunks in the mantle and palp contained a number of axons of different sizes and usually some glio-interstitial cells containing large, electron-dense granules (Fig. 4a). Some axons contained neurotubules approximately 20–25 nm in diameter. Nerves containing small dense-core vesicles were common in large nerve trunks and in the finer subepithelial tracts. Electron-lucent vesicles were less common. The two types were sometimes mixed within one fibre. Specialisations of the nerve membranes were occasionally observed, presumably representing synaptic connections (Fig. 4b–d). The glio-interstitial cells usually lay on the periphery of the bundle, but did not form sheaths around the axons (Fig. 4a,b,d). Both glio-interstitial cells and nerves formed connections with the lateral processes on muscle cells. Glio–muscle connections were particularly prominent on the lateral projections from muscle cells in arterial walls (Fig. 4e).

**Muscle**

Muscle cells contained thick and thin filaments and dense bodies (Fig. 5a,b,d), with no cross striations. Mitochondria lay peripherally, often in large lateral cytoplasmic projections. When fixed with CBF, the lateral projections often contained large amounts of granular material that resembled glycogen rosettes (not shown). With HBF, this material usually appeared to be leached out (Fig. 5d, spaces in lateral projections).

Muscle cells varied in size, from large thick cells (up to 8 µm in diameter) in the muscle bands traversing the distal margin of the mantle to thin fibres (1–2 µm in diameter) lying under the epithelia and associated with nerve tracts. The thick fibres (Fig. 5a) generally had short lateral processes with subsarcolemmal cisternae and few visible mitochondria. Thinner fibres, such as those traversing the mantle (Fig. 5b) or forming the walls of arteries (Fig. 5c,d), often had many lateral processes containing mitochondria. The lateral processes
Fig. 2. Labial palps: general anatomy. (a) *Velestoio ambiguus* labial palps (scanning electron microscopy (SEM)). Ridges in the oral epithelium lead down to the oral groove (og). (b) *Hyridella depressa* labial palps, oblique section (cacodylate-buffered fixation (CBF), toluidine (tol.) blue, light microscopy (LM)). The interstitial tissue is packed with clusters of granules (g), interspersed with a few vesicular cells (v) and blood spaces (bs). Ridges of the inner (oral) epithelium are covered with columnar ciliated cells and some glandular cells. (c) *Velestoio ambiguus* labial palp (CBF, tol. blue, LM). Columnar ciliated cells (cc) and glandular cells (gc) cover the ridged oral epithelium (ore). The cells of the outer epithelium (oe) are shorter and rest on a thick basement membrane (bm). In the interstitial tissue, large clusters of granules (g), haemocytes (h), blood spaces (bs) and a small artery (a) can be identified. (d,e) *Hyridella depressa* labial palps (SEM). The outer surface shows considerable variation in the surface structure. (d) The surface is irregular with protrusions (p); (e) the surface is relatively smooth and covered with microvilli (mv) and clumps of cilia (c).
of the muscles in the artery walls made contact with many glio-interstitial cells and nerve endings (Fig. 5c,d). Myomuscular junctions were generally convoluted, but did not appear to involve any membrane specialisation.

**Haemocytes**

The most common type of haemocyte observed in these mussels was a large granulocyte with vesicles 1–2 µm in diameter, containing amorphous material. (These vesicles would commonly be termed ‘granules’ in descriptions of haemocytes because of their granular appearance by LM. However, in this description they will be termed ‘vesicles’ to avoid confusion with the electron-dense ‘granules’ described below.) In semithin sections, these vesicles stained blue with toluidine blue or bright turquoise with methylene blue–Azure II–Basic Fuchsin (Figs 1a,b, 2c). Using TEM, large medium-density vesicles were visible (Figs 6a,b, 7b). The appearance of other organelles varied slightly, depending on the fixative used. With 0.02 M HBF, large numbers of small electron-lucent tubules were present (Fig. 6a), whereas in 0.1 M CBF they appeared much less distended (possibly because of the higher osmotic potential of the buffer) and contrast was poorer (Fig. 6b). These cells also contained small amounts of endoplasmic reticulum, glycogen, scattered mitochondria and, occasionally, a Golgi body.

There were also smaller numbers of haemocytes without large vesicles. These had variable nucleus/cytoplasm ratios and variable numbers (from none to many) of small 0.2 µm-diameter medium-density vesicles (Figs 6c,d, 7b). Strands of rough endoplasmic
Fig. 4. Nerve and muscle. (a) *Hyridella depressa* mantle margin (HEPES-buffered fixation (HBF), transmission electron microscopy (TEM)). A transverse section through a nerve bundle shows a range of axon profiles, neurotubules (nt) and small dense-core vesicles (dcv). Glio-interstitial cells with large electron-dense vesicles (G) lie on the periphery, but do not enclose the fibre. Muscle cells (M) are seen in cross-section (right) and oblique section (left). (b) *Veleunio ambiguus* mantle (HBF, TEM). A neuromuscular connection with membrane specialisation and post-synaptic cisterna is shown (arrowhead). The nerves (n) contain dense-cored vesicles (dcv) and small electron-lucent vesicles (elv). (c) *Hyridella depressa* palp (HBF, TEM). A putative synapse in a nerve trunk is shown (arrowhead). The presynaptic membrane shows increased density and small electron-lucent vesicles are accumulated along the membrane. (d) *Hyridella depressa* mantle central zone (HBF, TEM). A connection between a nerve (n) and muscle (M), showing increased density of the membrane (arrowhead). G, Glio-interstitial cell. (e) *Hyridella depressa* palp (HBF, TEM). A muscle fibre in an artery wall is pictured, showing a connection between a glio-interstitial cell (G) and a lateral projection from the muscle (M).
Fig. 5. Muscle. (a) *Hyridella depressa* mantle margin (HEPES-buffered fixation (HBF), transmission electron microscopy (TEM)). The dense muscle bands at the margin of the mantle contain large muscle fibres (M) with short lateral cytoplasmic projections (lp) and few mitochondria (m). Subsarcolemmal cisternae (arrowheads) lie parallel to the plasma membrane in the lateral projections. Thick and thin filaments are irregularly arranged and dense attachment plates (att) connect the filaments to the plasma membrane. The extracellular spaces contain many collagen fibres. (b) *Veleusunio ambiguus* mantle central zone (HBF, TEM). The muscle fibres traversing the mantle (M) have large lateral cytoplasmic projections containing many mitochondria (m) and often form connections with nerves (n) and glio-interstitial cells. Collagen fibres and many small granules are present in the extracellular space (ecs). (c) *Veleusunio ambiguus* palp (HBF, TEM). Montage of artery wall (aw). The numerous lateral processes on the muscle cells make connections with nerves (n) and glio-interstitial cells (G). h, Haemocytes. (d) *Veleusunio ambiguus* palp (HBF, TEM). A nucleus (N) in an artery wall shows deep convolutions in the nuclear membrane (arrowhead), presumably to allow stretching and contraction. The large lateral cytoplasmic processes (lp) probably contained glycogen. G, Glio-interstitial cell.
Fig. 6. Haemocytes. (a) Hyridella depressa mantle central zone (HEPES-buffered fixation (HBF), transmission electron microscopy (TEM)). Haemocyte with large amorphous vesicles (av). The cytoplasm contains a prominent electron-lucent tubule system (black arrowheads). The white arrow indicates an electron-dense granule within an amorphous vesicle; these were very rarely observed. N, Nucleus; m, mitochondria. (b) Hyridella depressa palp (cacodylate-buffered fixation, TEM). When fixed with cacodylate buffer, the haemocytes with large amorphous vesicles (av) had denser cytoplasm and the electron-lucent tubules (white arrowheads) were not as prominent. (c) Veselusunio ambiguus palp (HBF, TEM). A haemocyte with a number of small medium-density vesicles (sv) in the cytoplasm. Some rough endoplasmic reticulum is present (RER), but there are few electron-lucent vesicles. N, Nucleus; m, mitochondria. (d) Veselusunio ambiguus palp (HBF, TEM). A haemocyte with some rough endoplasmic reticulum (RER), mitochondria (m) and electron-lucent vesicles and very few small medium-density vesicles. N, Nucleus.
reticulum were usually present, often lying parallel to the nuclear membrane. These cells sometimes contained residual bodies and sometimes intracellular electron-dense granules, either free in the cytoplasm or within residual bodies (Fig. 7a,c,d). Haemocytes with
residual bodies and intracellular granules were more common in *H. depressa* than in *V. ambiguus*.

**Calcified granules**

As noted above, the mantle and palps contained varying quantities of orange-pigmented electron-dense granules. Most granules were extracellular, 0.1–1 µm in diameter and with one or two laminae and occurred in large clumps, usually among vesicular cells (Figs 1b, 2b,c). Granules were also scattered in the interstitial tissue (Fig. 7). Only the smaller groups of granules could be photographed using TEM; larger clumps generally disintegrated during sectioning or in the electron beam.

There were some differences between the species in the appearance of the granules. In *H. depressa* mantle, in which the granules were generally very numerous, the majority of granules occurred as large clusters of small granules, but there were also many multilamellar granules (Figs 1d, 7a). In *V. ambiguus* mantle, in which few granules were visible macroscopically, the granules were generally small and scattered among the lateral processes of muscle cells (Fig. 5b).

The smaller granules did not stain with toluidine blue in LM sections, but appeared pale yellow and refractile. Some of the larger granules sectioned in the mantle central zone of *H. depressa* stained pale pink or blue with toluidine blue. Using SEM, the granules in the large clumps appeared as spheres, 0.5–3 µm in diameter. Often filopodia could be seen wrapped around the clumps (Fig. 7b).

Intracellular electron-dense granules of various sizes and shapes were often observed in both species, either free in the cytoplasm (Fig. 7c,d) or in structures resembling tertiary lysosomes (Fig. 7a). In EM, some of these granules closely resembled the extracellular granules in shape and laminar structure (Fig. 7a,c). However, in the toluidine blue-stained LM sections, many of the smaller intracellular granules stained dark blue, so their composition was probably different from the extracellular granules. Large laminar intracellular granules were occasionally observed in TEM sections of *H. depressa* mantle (Fig. 7d), but they could not be positively identified in the LM sections.

**Epithelia**

**Inner epithelium of mantle**

The inner epithelium facing the mantle cavity had three main types of cells: epidermal cells (cells with microvilli and no cilia); ciliated cells (with cilia and microvilli); and glandular cells (terminology after Simkiss (1988)). Cell heights were very variable, ranging from 10 to 30 µm, and the cells rested on a basement membrane ranging in thickness from 3 to 8 µm.

The epidermal cells (Fig. 8a,b,d) generally had pale-staining nuclei, often lobed, with scattered patches of darker chromatin and a large nucleolus. The microvilli ranged from 0.4 to 1.0 µm in length. In the apical cytoplasm, there were often many vesicles containing material of variable electron density. These vesicles were usually elongated (Fig. 8a,b), but they were much more rounded in the central zone of the mantle in *V. ambiguus* (Fig. 8d). The vesicles sometimes appeared to make contact with the plasma membrane, but it was not possible to determine whether they were taking up or releasing material. In both species, there were also numerous small clear vesicles, mitochondria, short lengths of rough endoplasmic reticulum and multivesicular bodies (Fig. 8a,b,d).
The cytoplasm and nucleus of the ciliated cells generally stained more densely than in the epidermal cells, in both ultrathin and semithin sections (Fig. 8c,d). In addition, the microvilli were slightly longer (0.6–1.4 µm) and mitochondria were more common in ciliated cells. In both species, the apical vesicles were generally elongated (Fig. 8c,d). The cilia showed the usual 9+2 microtubular structure (Fig. 8e).
The glandular cells of the inner epithelium contained many vesicles filled with finely textured secretory material (Fig. 9a). In semithin sections stained with Methylene blue–Azure II–Basic Fuchsin, this material varied from large, dark pink-staining globules to paler pink, flocculent material. In some cells, both types of material were present. In the basal region of the glandular cells, there were often large dilated cisternae of rough endoplasmic reticulum filled with fine granular material (Fig. 9b).

In the basal portion of the inner epithelium of the mantle, there were many large extracellular spaces that appeared to communicate with the extracellular fluids below the basement membrane (Figs 8a,d, 9a). These often contained degenerating cellular components or blood cells with large amorphous vesicles in the cytoplasm.

**Outer epithelium of mantle**

In both *V. ambiguus* and *H. depressa*, most cells in this epithelium were cuboidal epidermal cells 10–15 µm high and 8–10 µm wide, lying on a basement membrane (Fig. 9c). The apical surface of the cells was covered by microvilli, 0.5–1.0 µm long. Nuclei were large and usually central or apical, with prominent nucleoli. Much of the cell was filled with fine granular glycogen-like storage material, with thin layers of darker cytoplasm around the periphery, surrounding the nucleus and in strands through the granular material. There were scattered mitochondria in the cytoplasmic strands and occasional lipid-like droplets.

Glandular cells were occasionally observed in the outer epithelium (Fig. 9d), but they were relatively rare. There were no ciliated cells.

**Epithelia of the mantle folds**

The outer mantle fold and the outer surface of the middle fold had no ciliated or glandular cells. The epidermal cells of the outer mantle fold were short with deeply infolded basal membranes. The lateral membranes between adjoining cells were often deeply convoluted. The epidermal cells of the periostracal groove contained large numbers of vesicles, often containing material with a dense core, which appear to have a secretory function (not shown).

Cells in the middle and inner mantle folds were predominantly epidermal cells and glandular cells, with scattered ciliated cells. Glandular cells in this region often extended through the basement membrane into the subepidermal region. Spherical or irregular dark-staining pigment granules were present in the mantle margin epithelium, particularly in the region of the mantle near the inhalant and exhalant openings, where the mantle shows dark pigmentation (Fig. 10a).

**Palps**

The outer epithelium of the palps (Fig. 10b) was similar to the inner epithelium of the mantle, with which it is continuous. In areas where the cells formed protuberances (Fig. 2d), the apical bulges of cytoplasmic material contained fine granular material, probably glycogen, and few organelles (Fig. 10c). The ratio of ciliated cells to epidermal cells varied in different areas, from a complete cover of cilia to scattered tufts.

The ridged surfaces of the palps were covered with ciliated cells up to 50 µm tall, interspersed with glandular cells.

**Discussion**

The mantle and palps in these hyrid mussels resemble those of the unionids in general structure and in the presence of large quantities of extracellular calcified granules in the
Fig. 9. Mantle epithelia. (a) *Hyridella depressa* mantle central zone, inner epithelium (HEPES-buffered fixation (HBF), transmission electron microscopy (TEM)). Glandular cell (gc) showing vesicles of finely textured secretory material (s), basal nucleus (N). cc, ec. Neighbouring ciliated and epidermal cells, respectively; bm, basement membrane; sp, basal space. (b) *Velesunio ambiguus* mantle margin (HBF, TEM). Detail of the basal region of a subepidermal glandular cell showing dilated rough endoplasmic reticulum (RER) cisternae (arrows) and a vesicle of secretory material (S). N, Nucleus. (c) *Velesunio ambiguus* mantle (HBF, TEM). Montage of the outer mantle epithelium. The epidermal cells are almost completely filled with fine granular storage material (st; probably glycogen), with thin strands of cytoplasm around the periphery and an occasional lipid-like droplet (L). Microvilli (mv) cover the apical surface, which lies against the shell. bm, Basement membrane; N, nucleus. (d) *Velesunio ambiguus* mantle central zone, outer epithelium (HBF, TEM). A glandular cell (gc) filled with secretory material. ec, Epidermal cells; bm, basement membrane.
Fig. 10. Mantle and palp epithelia. (a) *Hyridella depressa* mantle (HEPES-buffered fixation (HBF), transmission electron microscopy (TEM)). Pigmented epithelium near inhalant opening. Numerous round, electron-dense pigment granules with no limiting membrane (**) lie in the cytoplasm. White arrowheads, apical vesicles; bm, basement membrane; gc, glandular cell; N, nucleus. (b) *Hyridella depressa* outer epithelium of palp (HBF, TEM). The epidermal and ciliated cells (ec and cc, respectively) are similar in general appearance to the inner mantle epithelium (see Fig. 8). bm, Basement membrane; d, cell debris; N, nucleus, sp, basal space. (c) *Veleunio ambiguus* outer epithelium of palp in a region comparable with that shown in Fig. 2d (HBF, TEM). Apical protuberances (p) contain mainly granular glycogen-like material and are largely free of organelles. ap, Apical vesicles; bm, basement membrane; m, mitochondria.
connective tissue (Beedham 1958; Istin and Masoni 1973; Petit et al. 1978; Pynnönen et al. 1987; Machado et al. 1988; A. E. Colville and E. B. Andrews, unpublished data). However, the majority of granules occurred in clusters or among lateral processes of muscle cells rather than enmeshed in collagen fibres as in the unionid Anodonta.

The vesicular cells in H. depressa and V. ambiguus appear similar to the vesicular, or Leydig, cells described in many molluscs (Sminia 1972; Gabott 1983; Pipe 1987; Beninger et al. 1995; Eckelbarger and Davis 1996; Berthelin et al. 2000). These cells store large amounts of glycogen and act as a nutrient reserve (Lowe et al. 1982; Pipe 1987).

The structure of the nerves and glio-interstitial cells was similar to that described for Anodonta (Gupta et al. 1969; Nakao 1975), although the glial fibres described by Gupta et al. (1969) were not apparent in the cells examined here. The muscles in the mantle and palp were the classic smooth muscle described by Chantler (1983), with no apparent cross or oblique striations. They are similar to the type B cells of the smooth muscle classification system of Matsuno (Paniagua et al. 1996) with regard to the thick filament size and positioning of cell organelles, but the nuclei are peripherally placed rather than central.

There have been numerous attempts to classify bivalve haemocytes (Cheng 1981; Auffret 1988; Hine 1999). Hine (1999) concluded that there is evidence for a number of forms of granular and agranular haemocytes and that the types and numbers of haemocytes present may differ between bivalve families and even between individual animals.

In these hyrid mussels, the granulocytes with large amorphous vesicles formed a well-defined population and were the most common haemocytes observed. Similar cells are present in Anodonta grandis (Silverman et al. 1989) and Anodonta cygnea (Machado et al. 1988).

The remaining haemocytes were rather variable in form. The presence of tertiary lysosomes suggests that these cells are more phagocytic than the haemocytes with large amorphous vesicles, but it is possible that phagocytic ability varies with the nature of the material to be phagocytosed (Hine 1999). Further study is needed to characterise the different forms.

Mineralised granules are commonly observed in invertebrate tissues, but most are intracellular or are expelled as a form of excretion, so extracellular calcium- and phosphorus-rich granules in freshwater mussels are unusual (Brown 1982). When prepared by standard aqueous techniques, most of the granules in H. depressa and V. ambiguus resemble those found in other freshwater mussel species in appearance and chemical composition, with large amounts of Ca and P and lesser amounts of Fe and Mn and other trace constituents detected (Roinel et al. 1973; Davis et al. 1982; Silverman et al. 1983; Jeffree and Simpson 1984; Steffens et al. 1985; Pynnönen et al. 1987; Colville 1994). Some studies of unionids have reported the presence of carbonate in some granules (Moura et al. 1999), but Jeffree et al. (1993) considered that the granules in H. depressa and V. ambiguus were probably mainly phosphate.

However, recent studies indicate that, in cryoprepared tissues, the annular structure of the granules is not visible and the proportion of Fe is markedly increased, so aqueous preparation probably results in artefacts caused by loss and redistribution of elements (Vesk and Byrne 1999; Byrne 2000). Some intracellular granules that could be cut relatively easily in thin sections and that stained blue with toluidine blue in the thick sections are probably not calcium phosphate, but may have a sulfur-based composition (Adams et al. 1997; Vesk and Byrne 1999).

Phosphate-rich granules tend to be associated with so-called ‘hard acid’ or ‘class A’ metals, such as calcium, magnesium and barium, whereas granules rich in sulfur (such as
are common in oyster haemocytes) exhibit a different chemistry and tend to be associated with ‘soft acid’ or ‘class B’ metals, such as copper and mercury (Nieboer and Richardson 1980; Brown 1982; Taylor and Simkiss 1989).

The origin of the granules in these mussels has been the subject of several studies. Silverman et al. (1989) considered that the granules in Anodonta grandis are formed within the amorphous vesicles of haemocytes, which they termed ‘concretion-forming cells’ or CFCs. The granulocytes in the hyrid mussels in the present study look very similar to the CFCs but, although they also occasionally contained small electron-dense bodies, there was no evidence of a sequence of granule formation comparable with that reported in Anodonta (Silverman et al. 1989). In H. depressa and V. ambiguus, most of the intracellular electron-dense granules were in haemocytes without large amorphous vesicles (present study and from inspection of the figures in Byrne (2000)), so these cells are a more likely source. It was also notable that the large granule clumps were usually associated with vesicular cells and were surrounded by filopodia that resembled the thin outer layer of cytoplasm around the vesicular cells. This raises the possibility that vesicular cells may also be involved in some way with granule production.

The ultrastructure of the epithelia of the mantle and palps is very similar in the two species of mussel, with minor differences in the appearance of the apical vesicles of the epidermal and ciliated cells in the inner mantle and palps.

The outer mantle epithelium (nearest the shell) is responsible for secretion of the inner layers of the shell (Istin and Masoni 1973). The outer epithelial cells are similar in structure to the outer epidermal cells in Anodonta (Machado et al. 1988; A. E. Colville, personal observation), Cardium edule (Cardiidae), Nucula sulcata (Nuculidae), Mytilus edulis (Mytilidae) (Bubel 1973) and the freshwater Asiatic clam Corbicula fluminea (Corbiculidae) (Lemaire-Gony and Boudou 1997). In these species, the outer epithelial cells appear to function mainly to store glycogen. There was little cellular machinery to suggest active secretion of shell material. In contrast, outer mantle epidermal cells from Pinctada radiata (Pteriidae) and Isognomon alatus (Isognomoniidae), which are both in the superfamily Pteriacea, contain numerous mitochondria and endoplasmic reticulum cisternae (Nakahara and Bevelander 1967). This difference in cellular structure may reflect phylogenetic differences, seasonal variation or functional differences.

Beedham (1958) found that mucous cells occurred in large numbers in the outer epithelium of A. cygnea. Machado et al. (1988) concluded that the secretory cells in Anodonta were probably responsible for secretion of material for the organic matrix of the shell. In contrast, glandular cells in the outer epithelium of the mantle were rare in H. depressa and V. ambiguus.

Growth rates in H. depressa and V. ambiguus of comparable sizes to the animals examined in the present study are very low (Colville 1994), so it is possible that the inactive appearance of the outer epidermal cells and the small number of glandular cells reflect a low rate of shell deposition in these specimens.

One cell type that was not observed in the present study was the rhogocyte or pore cell, which is diagnostic of molluscs (Haszprunar 1996) and which can accumulate large amounts of glycogen and appear similar to vesicular cells (Skelding and Newell 1975; Beltz and Gelperin 1979; Jones and Bowen 1979). However, slit complexes were not evident in any cells examined in H. depressa and V. ambiguus. Rhogocytes may undergo cyclical changes in the percentage of membrane showing grooves (Baleydier et al. 1969), so it is possible that there were no cells in the appropriate phase when these mussels were collected. It is also possible that rhogocytes are present in the mantle or palps in heavily calcified areas
where the specimens disintegrated in the electron beam before they could be examined; further studies of decalcified tissue would be required to investigate these regions.

The freshwater mussels *V. ambiguus* and *H. depressa* were, by and large, very similar in terms of general histology and ultrastructure of the mantle and palps. The major difference was the greater abundance of granules in the central zone of the mantle of *H. depressa*, which was sometimes associated with an increased density of connective tissue cells and fibres. There were slight differences in the apical vesicles in the cells of the inner mantle and palp epithelia.

Byrne (2000) speculated that the distribution of granules in the interstitial tissues of mussels may be a useful character in phylogenetic analyses. She contrasted the granule distribution in *H. depressa* (Hyriidae) and *Margaritifera margaritifera* (Margaritiferidae), which have extensive aggregations in the mantle and few in the gills, with unionids such as *Anodonta* and *Ligumia*, where the granules occur predominantly in the gills. Unfortunately, this division is not so clear-cut because, within the subfamilies of the Hyriidae, there is variation in the abundance of granules in mantle and gills (Ch’ng-Tan 1968; Colville 1994). However, it would be of considerable interest to determine whether granules are present in the Etherioidea, because this may provide more information about the phylogenetic relationships among the superfamilies.

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